A Study of Human Lymphoblastoid Cell Lines in Tissue Culture

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TABLE OF CONTENTS

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SUMMARY

CHAPTER		PAGES
I	General Introduction and Backround Review	1-24
II	Spontaneous Growth of New Cell Lines from Peripheral Blood	25-47
III	Establishment of New Cell Lines by a Co-cultivation Technique	48-71
IV	The Role of EB Virus in Cultured Cell Lines	72-82
V	Morphology of Cultured Lymphoblastoid Cells By Light and Electron Microscopy	83-97
VI	Cytogenetic Studies on Established Cell Lines	98-144
VII	Antigenic Properties of Cells from Established Lines	145-213
VIII	An Assessment of the Potential Value of Human Lymphoblastoid Cell Lines in Biomedical Research	214-231
IX	General Conclusions	232-235

References

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Summary

The properties and potential applications of human lymphoblastoid cell lines have been investigated. Such lines have been established by others from Burkitt's lymphoma tissue, from leukaemic patients, from those with infectious mononucleosis and latterly from healthy subjects. There is an association between EB virus (Herpes-like virus of Epstein and Barr) and the growth of human lymphoblastoid cell lines *in vitro*. The same virus is linked sero-epidemiologically with Burkitt's lymphoma, nasopharyngeal carcinoma, infectious mononucleosis and a number of other diseases of man. The exact role of the virus both *in vivo* and *in vitro* is a matter of some controversy.

Using a simple culture technique, 40 cell lines were established from the peripheral blood leukocytes of a diagnostically mixed group of patients, from normal cord blood and from one healthy adult. 25 of these cultures were initiated by adding EB virus (in the form of lethally irradiated cells of a virus-positive line) to the fresh blood leukocytes. Evidence from experiments of this type suggest that EB virus has a lymphoproliferative action *in vitro*.

The cell lines are morphologically almost indistinguishable. They resemble primitive cells of the lymphoid series and appear to have the property of indefinite growth *in vitro*. They include some phagocytic cells and immunoglobulins are released into the culture medium.

Few of the lines were chromosomally abnormal when examined shortly after establishment but all of those examined after more than 1 year of continuous growth had abnormal karyotypes. There was no evidence for the recurrence of any specific chromosome aberration in a significant proportion of the lines.

Antigenically the established lines differ from the original donor's fresh lymphocytes. This is shown by measurement of tritiated thymidine uptake by lymphocytes exposed *in vitro* to irradiated cells from an autochthonous line. Together with the activation process, the fresh lymphocytes acquire cytotoxic potential directed specifically against the cell line used in the activation phase. It is suggested that this system constitutes a model for an immunological surveillance mechanism operating *in vivo* to eliminate deviant cells.

Cell lines of this type offer scope for detailed investigation of the induction of proliferation in human tissue (in this case probably by EB virus) and of the immunological processes by which altered autochthonous cells may be recognised and destroyed. Both of these topics are relevant to the basic study of malignant disease.

The prolonged life span and relative stability of these lines *in vitro* also presents opportunities in the field of human genetics, both for the investigation of inherited disorders of man and, experimentally, for the measurement of spontaneous and induced mutation rates, analysis of gene linkage and chromosome mapping.

CHAPTER I

General Introduction and Background Review

In an experimental approach to the study of human disease ethical and practical considerations often dictate that some system must be found as a supplement to, or substitute for, the direct use of human subjects. This need has commonly been met by the use of laboratory animals but as an alternative *in vitro* cell culture can offer a saving of time and money with the added advantage that the living tissue under study can be of human origin.

Within the past decade there has been a growing awareness of the potential value of cell culture, particularly in the fields of cancer research (Hayflick, 1967; Sanders & Burford, 1967; Stoker, 1969) and of human genetics (Hsia, 1970; Krooth & Sell, 1971). Because each culture normally consists of a single cell type growing in isolation, it represents quite a different biological entity from even the simplest laboratory animal. In order, therefore, to make appropriate and effective use of any given culture system it is necessary to have a very clear understanding of its individual properties, including some appreciation of the ways in which the cells have altered during the transition from an *in vivo* to an *in vitro* situation.

The work described in this thesis is concerned with the evaluation of a relatively new type of human tissue culture, namely the long-term lymphoblastoid cell line.

It covers the practical problems of initiation and maintenance of such cultures, an examination of the cellular morphology by light and electron microscopy, some studies on the role of EB virus in the long term growth of human lymphoid cells in vitro and observations on the stability and longevity of the cultured cells particularly in terms of karyotype. The objective has been to assess the potential value and limitations of this system in biomedical research.

One area in which these cultures are proving to have a particularly interesting application is covered in Chapter VII. This deals with experiments to determine the antigenic properties of lymphoblastoid cells in relation to those of the original tissues from which they have been derived.

Lymphoblastoid cell lines are distinct from other cultures of human origin in several respects. First, they can be established from relatively small samples of peripheral blood (see Chapter II) which are usually more readily obtainable than skin or other tissue biopsies. Secondly, they almost invariably grow as a free-floating suspension so that the cells can be dispersed and subcultured without prior trypsinisation. Thirdly, the great majority of such lines appear to have an indefinite lifespan in vitro whereas primary fibroblast or epithelial cultures, even of foetal origin, are limited to a fixed number of cell divisions before inevitable senescence

and death (Hayflick & Moorhead, 1961; Saksela & Moorhead, 1963). A number of permanent non-lymphoblastoid human cell lines already exist but these have been established, mainly from tumour material, with a very low success rate and have sometimes proved difficult to maintain outside the laboratory of origin (Hayflick & Moorhead, 1961; Gey, 1954; Berman, Stulberg & Ruddle, 1957; Fjelde, 1955; Moore & Koike, 1964). By contrast, permanent lymphoblastoid lines can now be established even from the blood of healthy individuals with a success rate approaching (and in some laboratories exceeding) 50% while, once established, they have, as a rule, proved easy to maintain, some of the early lines having now been distributed to a large number of laboratories throughout the world.

On the strength of these properties, it is reasonable to predict that lymphoblastoid cell lines will prove to be valuable research tools. However, their use is currently restricted by the fact that many (and possibly all) of these lines are infected with the herpes-like virus of Epstein and Barr, ("E.B. virus")(Epstein, Achong & Barr, 1964). The role of this virus in human disease and its relationship to the growth of lymphoid cells <u>in vitro</u> are widely debated at present. Both of these issues are discussed below, and, more fully, in Chapter IV. It is clear that in any assessment of this culture system, the question of the EB virus and its properties must occupy a central position.

Historically, the development of interest in human lymphoblastoid cells lines has progressed almost simultaneously on a number of fronts, advances on each of which have had ramifications on the others, producing a highly complex and fluid situation many aspects of which are likely to remain unresolved for some years.

The starting point was a detailed epidemiological survey carried out from 1957 to 1961 by Burkitt, in the course of which he defined the geographical distribution of a particular lymphoid tumour (now known as Burkitt's Lymphoma) within the continent of Africa (Burkitt, 1958-59; Burkitt & O'Conor, 1961; Burkitt, 1962; Burkitt & Wright, 1966). This tumour has several distinctive features. It affects primarily children aged between 2 and 14 years. The jaw and the ovary are the most common primary sites, though any internal organ may be affected. It has a characteristic morphology by light microscopy and is undoubtedly malignant, usually spreading both by local extension and by wide dissemination to a rapidly fatal outcome (Burkitt & O'Conor, 1961; O'Conor, 1961). There are, nevertheless, a number of well documented examples of apparently spontaneous regression or even cure, while the response to relatively small doses of cyclophosphamide or other cytotoxic drugs is often dramatic (Ngu, 1965; Burkitt & Kyalwazi, 1967; Burkitt, 1967). The most striking feature

of all is the fact that within tropical Africa the incidence of Burkitt's Lymphona varies to such an extent that in some areas it outnumbers all other forms of childhood neoplasm, while in others it is virtually unknown. In this survey, Burkitt showed that the tumour is confined to areas in which certain climatic conditions apply (minimum temperature $\geqslant 60^{\circ}F$, annual rainfall $\geqslant 20$ inches) and pointed out that such limitations might apply if an oncogenic agent was carried by a mosquito or other insect vector (Burkitt, 1962; Burkitt & Wright, 1966).

Outwith tropical Africa, Burkitt's lymphona occurs with comparable frequency in parts of New Guinea (Tenseldam, Cooke & Atkinson, 1966; Booth, Burkitt, Bassett et al., 1967), and possibly also in Colombia and Brazil (Beltran, Baez & Corea, 1966; Fagundes, de Oliveira & Amaral, 1968; Dalldorf, Carvalho, Jamra et al., 1969) where its distribution appears to be subject to similar climatic restrictions. Despite some conflicting early reports, it now seems clear that the tumour occurs only occasionally in countries outside the endemic areas. Nevertheless such sporadic cases establish that the disease has a world-wide distribution (Symposium, 1967; Burkitt, 1970).

The simplest explanation for this geographical distribution is that the disease is caused directly by an infectious agent transmitted by an insect vector; but as

early as 1964, Haddow suggested that a more complex situation may obtain, namely that a widely distributed virus of man may have oncogenic potential which can be realised only in very restricted circumstances and that these circumstances may be found with particular frequency in the areas defined by Burkitt (Haddow, 1964). In the light of subsequent discoveries, outlined below, this prediction appears increasingly likely to prove accurate and Burkitt has recently assembled evidence from many quarters which suggests that the component of the oncogenic host-virus interaction which is climatically determined may be the occurrence, from earliest childhood, of heavy and chronic malarial infestation (Burkitt, 1969; Kafuko & Burkitt, 1970).

Attempts to isolate an infectious agent directly from Burkitt's tumour tissue yielded inconclusive results (Simons & Ross, 1965; Woodall, Williams, Simpson & Haddow, 1965; Dalldorf, Bergamini & Frost, 1966; Bell, Massie, Ross, Simpson & Griffin, 1966) and although a number of viruses were found, at least one of which (reovirus 3) was reported to cause lymphoid tumours in laboratory animals (Bell, Manvbe & Wright, 1968; Stanley, Walters, Leak & Keast, 1966), none could be shown to be consistently present in all or even a majority of patients suffering from the disease.

The first tissue culture studies of Burkitt's lymphona were undertaken by Pulvertaft in Ibadan. A number of long term lines were established as a result of this work,

including the widely used ones RAJI and JIYOYE, but the published accounts were principally concerned with the changing morphology of the cells during the early stages of growth in vitro (Pulvertaft, 1964, 1965). In 1963, Epstein and his group at the Bland-Sutton Institute began to culture biopsy specimens from Burkitt's tumour with the specific objective of looking for a virus which might replicate in cultured cells even if undetectable in the fresh material. Their samples were flown from Kenya and Uganda and they noted that, on arrival, the transport medium was often cloudy with free floating, apparently viable, lymphoid cells. Similar floating cells appeared in profusion during the first few days in culture. The culture technique was therefore modified to ensure that floating cells were not discarded whenever the nutrient medium was changed and as a consequence this group was able to establish a number of permanent lines growing in suspension and composed of lymphoblastoid cells (Epstein & Barr, 1964; Epstein, Barr & Achong, 1965, 1965a, 1966). When the first of these lines, EB1, was examined under the electron microscope, 3% of the cells were found to contain virus particles morphologically belonging to the herpes group (Epstein, Achong & Barr, 1964) but serologically and in terms of biological activity, the agent differed from the known

herpes viruses of man or animals, and has become known as the herpes-like virus of Epstein and Barr, or "E.B.virus" (Epstein, Henle, Achong & Barr, 1965; Henle & Henle, 1966, 1966a).

Epstein's original report was followed by accounts of similar cultures being established in a number of centres (Stewart, Lovelace, Whang & Ngu, 1965; O'Conor & Rabson, 1965; Pope, Achong, Epstein & Biddulph, 1967; Osunkoya & Mottram, 1967; Minowada, Klein, Clifford, Klein & Moore, 1967; Nadkarni, Nadkarni, Clifford, Manolov, Penyo & Klein, 1969). These included lines derived from an American and a New Guinean patient with Burkitt's lymphoma in addition to several from different areas of Africa. With the exception of RAJI, all these established lines examined by electron microscopy were shown to carry a virus indistinguishable morphologically from that demonstrated in BBl and within a few months two classes of antibody, directed against this agent, were identified in human sera, both detectable by an indirect immunofluorescence reaction with virus infected lymphoblastoid cells. In one case the antigen is present in the nucleus and cytoplasm of killed fixed cells (Henle & Henle, 1966) and in the other it appears on the cytoplasmic membrane of living cells (Klein, Clifford, Klein & Stjernsward, 1966). Antibody of the first type was found in high titre in the sera of all patients

with Burkitt's lymphoma, but was also present, albeit in much lower titre, in the majority of control sera and this was shown to apply not only to African populations but also to those of the United States, India and Europe (Henle & Henle, 1966; Levy & Henle, 1966; Henle & Henle, 1967; Moore, Grace, Citron, Gerner & Burns, 1966; Goldman, Reisher & Bushar, 1968; Svedmyr & Demissie, 1968; Pereira, Blake & MacRae, 1969). The position with regard to the second class of antibody is evidently more complex but it too is demonstrable in the sera both of Burkitt's lymphona patients and of controls (Klein, Clifford, Klein & Stjernsward, 1966; Klein, Clifford, Klein,& Hamberger, 1967). Further studies in relation to EB virus-associated antigens are discussed in Chapter VII.

The first clue as to the normal biological activity of this widely distributed virus came from the laboratory of Drs. W. and G. Henle in Philadelphia. These workers had been attempting to establish permanent lymphoblastoid lines from the peripheral blood of individuals without malignant disease. Their first success in this respect was achieved with a sample from one of their laboratory technicians who had just recovered from infectious mononucleosis. Previous blood

samples from the same individual had failed to become established in culture so that when EB virus was found in the growing cell line, her sera were tested retrospectively (using indirect immunofluorescence on fixed EB3 cells) for EB antibody. This was shown to be absent from pre-illness samples but present in a titre of 1:80 after recovery (Henle, Henle & Diehl, 1968). The Henles were then able to gain access to a store of sera frozen down (as part of a prospective study of infectious mononucleosis) from Yale University students whose medical histories were known. Further sera had been added to this collection from any students who developed infectious mononucleosis while still at University (Henle, Henle & Diehl, 1968; Niederman, McCollum, Henle & Henle, 1968). From a study of this material it was shown that 38 of 50 students (76%) had indetectable (<1:10 titre) levels of antibody to EB virus in the initial serum sample. Within this group there were eleven recorded cases of infectious mononucleosis over the succeeding five years. All were associated with development of antibody to EB virus. None of the twelve students whose initial serum sample contained antibody against EB virus subsequently developed infectious mononucleosis although two gave a previous history of the disease. On the basis of these and other sero-epidemiological surveys in the United States and in Great Britain (Henle & Henle, 1967; Pereira, Blake & MacRae, 1968; Porter

Wimberly & Benyesh-Melnick, 1969; Dienhardt, Tischendorf, Shramek, Maynard & Noble, 1969; Joint Investigation, 1971) the following facts have become firmly established.

(a) Antibody to EB virus is commonly present in the sera of newborn infants. This is of maternal origin.

(b) Between the age of one and three years very few children have demonstrable levels of EB virus antibody.

(c) From the age of four years, the percentage of EB - and antibody-positive individuals in the community rises rapidly.

(d) In childhood the acquisition of antibody to EB virus is rarely associated with any identified disease.

(e) The lower the social class, the higher the percentage of children with positive titres of antibody to EB virus and the lower the mean age of sero-conversion.

(f) Clinical infectious mononucleosis occurs almost exclusively in individuals (usually young adults) who had no EB antibody in their pre-illness sera. Antibody appears in post-illness sera and persists (with some reduction in titre) for many years, probably for life.

(g) A small number of cases of clinical infectious mononucleosis can be attributed to cytomegalovirus or to some other defined infectious agent.

(h) Within tropical Africa, infectious mononucleosis is rare and is not associated with a change in EB antibody levels. Other infectious agents appear to be responsible in these cases (Diehl, Taylor, Parlin, Henle & Henle, 1969).

Further convincing evidence of a direct causal link between EB virus and infectious mononucleosis has come from a small number of cases of patients with terminal malignant disease and without antibody to EB virus, who have received inoculations of cultured cells bearing the virus or of purified virus extracted from such cultures. In most of these, symptoms of infectious mononucleosis developed within seven weeks. In each case the heterophile antibody (Paul Bunnel) test became positive at the same time (Grace, Blakeslee & Ralph, 1969; Moore & Gerner, 1970). In addition, an agent with the properties of EB virus (as judged by its effect on cultured human lymphocytes) has been isolated from the throat-washings of patients with infectious mononucleosis (Chang & Golden, 1971; Pereira, 1972). Two other reports indicated that the febrile "post perfusion" syndrome, which occasionally affects patients who have undergone major surgery under cardio-pulmonary bypass, may be caused by transmission of EB virus in the fresh blood used to prime the bypass machine (Gerber, Walsh, Rosenblum & Purcell, 1969; Henle,

Henle, Scriba, Joyner, Harrison et al. 1970). This syndrome has several features in common with infectious mononucleosis (Smith, 1964).

These findings did not, in themselves, clarify the relationship of EB virus to Burkitt's lymphoma but recalling Haddow's predictions (Haddow, 1964), a working hypothesis has been widely adopted in which EB virus is seen primarily as a lymphoproliferative agent; the clinical manifestations of this proliferation (including its termination) being influenced by genetic and/or environmental factors in the host. It was also recalled by several commentators that as long ago as 1958 Dameshek had postulated that infectious mononucleosis may be a self-limiting leukaemia (Dameshek & Gunz, 1958).

It became important therefore to determine whether the virus played an active role in promoting the growth of some (or even all) human lymphoblastoid cell lines in vitro. During the period of the sero-epidemiological surveys described above there had been very considerable activity in the tissue culture field. At a conservative estimate more than seventy new Burkitt lymphoma lines had been grown, while even before Henles' had published their evidence for an association between EB virus and infectious mononucleosis, three groups had independently described the successful establishment of lymphoblastoid cell lines from the peripheral blood of

infectious mononucleosis patients (Pope, 1967; Mumford, Trentin, Taylor, Van Hoosier, Sullivan & Sharp, 1967; Glade, Kasel, Moses, Whang-Peng, Hoffman, Kammermeyer & Chessin, 1968). Benyesh-Melnick and her colleagues obtained two long-term suspension cultures, evidently of the same type, from bone marrow aspirates from children with infectious mononucleosis (Benyesh-Melnick, Phillips, Lewis & Seidel, 1968). Others recorded similar success with blood samples from leukaemia patients (Foley, Lazarus, Farber, Uzman, Boone & McCarthy, 1965; Iwakata & Grace, 1964; Moore, Ito, Ulrich & Sandberg, 1966; Armstrong,1966; Clarkson, Strife & de Harven, 1967) or with lymph node material from a variety of conditions including cancer (Jensen, Korol, Dittmar & Medrek, 1967; Nilsson, Ponten & Philipson, 1968). Finally a number of reports were published of lymphoblastoid lines grown from the peripheral blood of healthy individuals (Moore, Gerner & Franklin, 1967; Diehl, Menle, Henle & Kohn, 1968; Moore, 1969). Where the appropriate tests had been undertaken, it appeared that lymphoblastoid lines could be grown only from patients whose serum contained antibody directed against EB virus. Furthermore, herpes-like virus particles could be demonstrated in the majority of lines, regardless of origin (Moore, 1969). Thus the virus did appear to be closely related to the capacity of human lymphoid cells to become established in long-term culture.

The most direct evidence for the lymphoproliferative action of EB virus came again from the Henles' laboratory. In 1967 this group reported that peripheral blood lymphocytes from healthy infants could be induced to grow as long term lymphoblastoid lines after exposure in vitro to lethally irradiated cells from an EB-virus-positive Burkitt lymphoma line (JIJOYE). Killed cells from a virus-negative line (RAJI) were ineffective in this co-cultivation system (Henle, Diehl, Kohn, Zur Hausen & Henle, 1967). Their observations were confirmed and extended in a second paper (Diehl, Henle, Henle & Kohn, 1969) in which they noted that after a period in culture their JIYOYE cells appeared to have become EB-virus-negative and that simultaneously the capacity for irradiated material from this line to promote the growth of new lines was lost. Pope and his colleagues in Queensland made similar observations (Pope, Horne & Scott, 1968, 1969). They used cell-free filtrates from EB-virus-positive and EB virus-negative cell lines in attempts to establish lymphoblastoid lines from foetal bone marrow cells, and were able to show that only those filtrates which bame from virus-producing cells were effective in this system, that the active constituent could pass through a filter of 200 mμ average pore diameter (APD) but was retained by a filter of less than 100 mμ APD and that the growth-promoting effect of an active filtrate could be abolished by pre-incubation with human serum containing antibody to EB virus but was unaffected by pre-incubation with EB antibody-negative serum.

There remained, however, the disconcerting problem of those cell lines in which EB virus could not be detected either by immunofluorescence or by electron microscopy even on persistent and repeated examination (Epstein, Achong, Barr, Zajac, Henle & Henle, 1966). The possibility could not be rigorously excluded that EB virus was simply an almost ubiquitous passenger which replicates preferentially in proliferating lymphoid tissues. Glade and his colleagues (Glade, Hirschhorn & Douglas, 1969) argued that the common factor in all those conditions from which it had proved possible to grow lymphoblastoid lines might not be EB virus infection (recent or persistent) but the presence of lymphoid cells undergoing DNA synthesis and division in vivo. This situation obtains not only in lymphoma, leukaemia and infectious mononucleosis but also in the acute stage of many viral infections and these authors demonstrated that lymphoblastoid lines could be established from the peripheral blood of patients with viral hepatitis, rubella, mumps, herpes zoster and herpes simplex (Glade, Hirschhorn & Douglas, 1969; Glade, Hirshaut, Douglas & Hirschhorn, 1968). Shortly afterwards, a very high success rate for the establishment of lymphoblastoid cell lines from adults with infectious hepatitis was reported by Stevens et al. (Stevens, Barker, Fike, Hoffs & Meyer, 1969). In some of Glade's patients the serum

antibody titre against EB virus was 1:10 or less, a level considered negative by most workers (Henle, 1966; Moore, Grace, Citron, Gerner & Burns, 1966; Goldman, Reisher & Bushar, 1968; Svedmyr & Demissie, 1968; Pereira, Blake & MacRae, 1969), yet abundant EB virus particles were seen in the cell lines grown from these blood samples. The explanation offered by Glade was that previous (possibly congenital) EB virus infection could permit the virus to persist indefinitely in the presence of a low antibody titre. An intercurrent event, such as infection by an unrelated virus could lead to lymphoproliferation in vivo and hence to establishment of a lymphoblastoid line in vitro. The EB virus, replicating as a passenger in dividing lymphoid cells would then be seen in the established line and would also provoke a secondary antibody response in the patient (Glade, Hirschhorn & Douglas, 1969). Support for this view has come from another group which claimed to have detected significant titres of antibody against EB virus in pre-illness sera from patients developing infectious mononucleosis (Stevens, Pry & Manaker, 1970). These observations undoubtedly require further investigation but the view that EB virus is a mere passenger runs counter to the substantial body of evidence cited earlier (Grace, Blakeslee & Ralph, 1969; Moore & Gerner, 1970; Henle, Diehl, Kohn, Zur Hausen & Henle, 1967; Diehl, Henle, Henle & Kohn, 1969; Pope, Horne & Scott, 1968, 1969). Furthermore, a number of extensive

surveys have failed to find any general association between proliferation of lymphoid tissue *in vivo* (either malignant or non-malignant) and a raised or rising level of antibody to EB virus (Evans, Niederman & McCollum, 1968; Henle, Henle, Clifford, Diehl et al. 1969; Blacklow & Kapikian, 1970; Beltran, Northington, Leiderman, Mogabgab & Stuckey, 1971; Goldman & Aisenberg, 1970), but have confirmed the clear association of this agent with Burkitt's lymphoma and infectious mononucleosis.

In surveys of American and Swedish patients with Hodgkin's disease, chronic lymphatic leukaemia and other lymphomas (Levine, Ablashi, Berard et al., 1971; Johansson, Klein, Henle and Henle, 1970, 1971), elevation of EB virus-associated antibodies, above the mean value for control subjects, was found to be common but strikingly high titres were found almost exclusively among patients with the very malignant ("sarcoma") form of Hodgkin's disease which is associated with lymphocyte depletion. There was no correlation between EB antibody levels and the estimated lymphoid tumour mass in each patient (Johansson, Klein, Henle & Henle, 1970).

The surveys have also led to the identification of two other diseases in which high titre antibody against EB virus is regularly found, namely carcinoma of the post-nasal space (Old, Boyse, Oettgen, De Harven et al., 1966; Henle,

Henle, Hung-Chiu, Burtin et al., 1970; De Schryver, Friberg, Klein, Henle et al., 1969) and sarcoidosis (Hirshaut, Glade, Octavio, Vieira et al., 1970; Wahren, Carlens, Espmark et al. 1971). Lymphoblastoid cell lines have been grown from patients with each of these conditions (Hirshaut, Glade, Octavio, Vieira et al., 1970; De The, Ambrosioni, Ho & Kwan, 1969; De The, Ho, Kwan, Desgranges & Favre, 1970; Achong, Mansell, Epstein & Clifford, 1971). Some of the lines contained EB virus though in one, from a nasopharyngeal tumour biopsy, a morphologically distinct virus was present, the identity and significance of which remain to be established (Achong, Mansell, Epstein & Clifford, 1971).

The sero-epidemiological evidence for a wide distribution of EB virus among healthy populations and for its association with several, otherwise unrelated, diseases does not detract from the possibility that it may act as an oncogenic agent in man since comparable situations seem to exist in other species. Examples include the leukaemogenic viruses of mice (Schneider, Hayat & Berumen, 1967; Kajima & Pollard, 1968) and the herpes virus which causes Marek's disease in chickens (Biggs, Churchill, Rootes & Chubb, 1968).

Attempts to define those factors which may permit the development of an EB virus-induced tumour in a given

patient have concentrated on the immunological aspects of the host-virus relationship. In addition to the antigens, mentioned earlier, which can be demonstrated on the cells of lymphoblastoid lines, a large (and growing) list of new ones has been described. Some of these may represent the same antigen detected by a variety of different techniques but this field is now so involved that it cannot be adequately summarised here. Attention is drawn to a recent review (Klein, 1970). The subject is also discussed in detail in Chapter VII. Some of the immunological findings are relevant to the question of the role of EB virus as a lymphoproliferative agent since they reveal the presence in a virus-free cell line (RAJI) of one or more antigens which appear to be specific to the EB virus (Pope, Horne & Wetters, 1969; Gerber & Deal, 1970; Vonka, Benyesh-Melnick & McCombs, 1970).

Evidence is also available from careful cloning experiments which have demonstrated that all the sublines grown from single-cell isolates of a virus-producing line are themselves virus-producing even when only 2% of the cells of the parent line release virus particles and when the cloning efficiency exceeds 50% (Hinuma & Grace, 1968; Maurer, Imamura & Minowada, 1969; Zajac & Khon, 1970; Miller, Stitt & Miller, 1970). The capacity to synthesise virus must therefore be present in every cell of such a line, though remaining occult in 98%.

The direct demonstration of EB virus DNA in RAJI cell line (which does not release virus particles) has been claimed by Zur Hausen and his colleagues (Zur Hausen & Schulte-Holthausen, 1970) using the technique of chemical hybridization between DNA extracted from the test cells and a radio-active DNA homologous with purified EB virus nucleic acid. In a subsequent paper, the same group has produced similar evidence for the presence of EB virus DNA in fresh biopsy material from Burkitt's lymphoma and nasopharyngeal carcinoma (Zur Hausen, Schulte-Holthausen, Klein, Henle et al., 1970). This work is clearly of the greatest importance and the findings have been confirmed by another group using an analogous system, RNA/DNA hybridization (Nonoyama and Pagano, 1971). Nevertheless because the procedures involved are technically very demanding, the conclusion that the EB virus genome is present in all cultured cell lines has yet to become universally accepted. The concept of cell transformation in vitro by the action of an oncogenic virus which is present, but does not replicate to give free virus particles in the transformed cells is now well established for a number of tissue culture systems of animal origin (Marin, 1970). The question inevitably arises, "does E.B. virus-induced proliferation of lymphoblastoid cells in vitro represent malignant transformation in a human system?". Verification by inoculation of the transformed cells into tumour-susceptible syngeneic hosts is obviously impossible. Propagation of several lymphoblastoid cell

lines in newborn or immunosuppressed hamsters, rats and mice has been reported (Adams, Foley, Uzman, Farber, Lazarus & Kleinman, 1967; Chandra, Brown, Aldenderfer et al., 1969; Southam, Burchenal, Clarkson et al., 1969, 1969a; Adams, Hellerstein, Pothier, Foley, Lazarus & Stewart, 1971; Deal, Gerber and Chisari, 1971) but this is by no means invariably successful (Moore & McLimans, 1968; Moore, 1969). Of the other indirect criteria for malignancy in vitro (McPherson, 1970) loss of contact-inhibition cannot be assessed in suspension cultures while cloning efficiencies and serum requirements have received relatively little attention. Moore and his colleagues (Moore, 1969; Moore & McLimans, 1968; Imamura & Moore, 1968) have suggested that suspension cultures should be regarded as "normal" (i.e. non-malignant) if they have diploid karyotypes, low cloning efficiencies and synthesise complete immunoglobins in vitro. Any deviation from these standards they regard as evidence of malignancy and they have claimed that such deviation is particularly common in cell lines derived from patients with malignant disease. This view, based as it is on limited and circumstantial evidence, is not universally accepted and Epstein has recently pointed out that on grounds of morphology, continuous proliferation, ability to grow in suspension, the presence of an apparently 'specific' marker chromosomal abnormality and of surface neo-antigens, virtually all lymphoblastoid cell lines can be regarded as

having undergone malignant transformation by EB virus (Epstein, 1971).

The contribution of cytogenetic studies to an understanding of the properties of these cell lines is discussed in Chapter VI but it should be noted here that Epstein's reference to a specific chromosomal aberration raises a contentious issue (Whang-Peng, Gerber & Knutsen, 1970). Many of the established lines are aneuploid but with relatively stable karyotypes which retain most of the normal human complement. Regardless of the 'malignancy' or 'normality' of the individual cells, this feature in a continuously growing line promises to be of considerable value in the mapping of human chromosomes.

Lymphoblastoid cell lines, because of their permanence, are likely to be of major importance in genetics at the biochemical as well as the chromosomal level. First reports of the establishment of lines from individuals with familial disorders have already been published (Blume, Glade, Grainick, Chessin, Haase & Wolf, 1969; Choi & Bloom, 1970) and one may confidently predict that many more will follow in the next few years.

The work described in the succeeding chapters covers several of the issues discussed above, notably the technical problems encountered in establishing new cell lines, the assessment of similarities and differences between lines from a

variety of sources in terms of morphology and karyotype, the significance of productive and non-productive EB virus infection and the antigenic properties of the cultured cells. It is felt that such a broadly-based analysis of the cell lines is an essential preliminary to their proper exploitation in medical and biological research.

CHAPTER II

Spontaneous Growth of New Cell Lines from Peripheral Blood Leukocytes

Introduction

Since methods for the establishment of lymphoblastoid cell lines from human peripheral blood have been developed almost simultaneously in a number of centres, considerable variation is found in the techniques favoured by different authors and very few comparative studies have been made under controlled conditions. There is, therefore, no agreed optimum procedure. In devising a culture system for routine use, I considered published data on the following variables.

1. Volume of blood sample from which leukocytes were to be obtained.
2. Method of separation of leukocytes.
3. Initial concentration of leukocytes in culture and possible need to maintain concentration.
4. Type of culture medium and supplements.
5. Type of culture vessel and volume of culture.
6. Possible use of feeder-layers.
7. Frequency of feeding.

The following assessment was made of the data available.

Volume of blood sample

Moore and his colleagues at Rosewell Park Memorial Institute have established over one hundred and eightycell lines from healthy individuals (Moore, 1969). This vastly exceeds the total achieved in any other centre and may be accounted for by the fact that the Rosewell Park group use, as their starting material, the leukocytes culled by plasmapheresis from over 1 litre of peripheral blood (Moore, Gerber & Franklin, 1967). Similarly Gerber and Monroe established six cell lines from normal subjects using initial blood samples of 500 ml. (Gerber & Monroe, 1968). The rationale for using such large volumes was that a line may grow from a single 'stem' cell and that, in health, such cells may represent so minute a proportion of the total circulating leukocyte population that only one or two are present per litre of blood (Moore & McLimans, 1968; Chessin, Glade, Kasel et al., 1968; Chang, Hsieh & Blankenship, 1971). It was clear, however, that lymphoblastoid cell lines could be grown from the peripheral blood of patients with leukaemia, infectious mononucleosis or other viral diseases using initial samples of 30 ml. or less (Armstrong, 1966; Diehl, Henle, Henle & Kohn, 1968; Glade, Hirshaut, Douglas & Hirschhorn, 1968; Stevens, Barker, Fike, Hoffs & Meyer, 1969) while the technique of co-cultivation seemed to hold out a good prospect for the establishment of lines from similar blood samples from healthy donors (Henle, Diehl, Kohn, Zur Hausen & Henle, 1967). With

this in mind, and in view of the obvious limitations in the application to patients of a study requiring blood samples of several hundred ml., I decided at the outset to concentrate on developing a technique which could be applied to blood samples of 20 ml. or less.

Methods of Separation of Leukocytes

Although numerous techniques have been devised for the separation of high yields of relatively pure lymphocytes from peripheral blood (Ling, 1968) they do not appear to have been applied in the establishment of lymphoblastoid lines. In virtually all the published methods, leukocytes (including platelets and granulocytes as well as lymphocytes) are separated by gravity sedimentation from heparinised or citrated whole blood, or from leukocyte-rich plasma obtained by plasmapheresis (Iwakata & Grace, 1964; Foley, Lazarus, Farber et al., 1965; Armstrong, 1966; Moore, Grace, Citron et al., 1966; Moore, Ito, Ulrich and Sandberg, 1966; Clarkson, Strife & De Harven, 1967; Moore, Gerner & Franklin, 1967; Mumford, Trentin, Taylor et al., 1967; Pope, 1967; Diehl, Henle, Henle & Kohn, 1968; Gerber & Monroe, 1968; Glade, Hirshaut, Douglas & Hirschhorn, 1968; Glade, Kasel, Moses et al., 1968; Moore, 1969; Stevens, Barker, Fike et al., 1969). Apart from simplicity this approach has the advantage of allowing serum or plasma to be separated from the cells without the addition of any material which might interfere with subsequent antibody titrations.

Initial concentration of leukocytes in culture and possible need to maintain concentration.

Within the range 0.5 x 10^6 to 10^7 cells/ml. the concentration of leukocytes in freshly set-up cultures does not appear to have a critical influence on the prospects of establishing new lymphoblastoid lines (Iwakata & Grace, 1964; Foley, Lazarus, Farber et al., 1965; Armstrong, 1966; Moore, Grace, Citron et al., 1966; Moore, ito, Ulrich & Sandberg, 1966; Clarkson, Strife & DeHarven, 1967; Moore, Gerner & Franklin, 1967; Mumford, Trentin, Taylor et al., 1967; Pope, 1967; Diehl, Henle, Henle & Kohn, 1968; Gerber & Monroe, 1968; Glade, Hirshaut, Douglas & Hirschhorn, 1968; Glade, Kasel, Moses et al., 1968; Moore, 1969; Stevens, Barker, Fike et al., 1969). Thereafter, some authors have made particular efforts to maintain a minimum concentration of 5 x 10^5 cells/ml by reducing the volume of medium or by combining the contents of two or more vessels (Moore, Grace, Citron et al., 1966; Moore, Gerner & Franklin, 1967; Gerber & Monroe, 1968; Stevens, Barker, Fike, Hoffs & Meyer, 1969), but in other centres, particularly where relatively small quantities of blood were used as source material, the cultures, once initiated, were left undisturbed apart from weekly or twice-weekly change of the top 50% of the medium (Pope, 1967; Glade, Kasel, Moses et al., 1968; Diehl, Henle, Henle & Kohn, 1968; Glade, Hirshaut, Douglas & Hirschhorn, 1968). Armstrong (1966)

compared these two approaches on leukaemic cells in culture (in at least one case) and found that a sample which was fed once weekly, but otherwise left untouched, gave rise to a cell line, whereas five other bottles of the same cells carefully maintained at fixed concentrations, failed to grow. Glade (personal communication) has also stressed the importance of avoiding disturbance to the cells during the first few weeks in culture. It was decided therefore not to alter the volume of medium or to add extra cells once the cultures were set up.

Type of Culture Medium and Supplements

The most popular medium in current use for the initiation and maintenance of lymphoblastoid cell lines is undoubtedly RPMI 1640, (Moore, Grace, Citron et al., 1966; Armstrong, 1966; Moore, Ito, Ulrich & Sandberg, 1966; Moore, Gerner & Franklin, 1967; Diehl, Henle, Henle & Kohn, 1968; Glade, Kasel, Moses et al., 1968; Gerber & Monroe, 1968; Glade, Hirshaut, Douglas & Hirschhorn, 1968; Stevens, Barker, Fike, Hoffs & Meyer, 1969; Moore, 1969) a derivative of McCoy's 5A which was used by Iwakata and Grace (1964) and by Clarkson (Clarkson, Strife & DeHarven, 1967). In almost every case, however, 20-30% foetal calf serum or a mixture of 10% foetal calf serum and 10% human serum (Moore, Ito, Ulrich & Sandberg, 1966; Chandra, Buscheck, Garon & Manaker, 1969) has been added which is likely to compensate for any deficiencies in the nutrient properties of the medium. Eagle's Basal or

Minimal Essential medium have been used with success (Foley, Lazarus, Farber et al., 1965; Armstrong, 1966; Pope, 1967; Clarkson, Strife & De Harven, 1967; Chandra, Buscheck, Garon & Manaker, 1969; Epstein, personal communication) as have RPMI 1629 (Armstrong, 1966) NCTC 109 (Clarkson, Strife & De Harven, 1967), TC 199 and Ham's F10 (Chandra, Buscheck, Garon & Manaker, 1969). Moore, Ito, Ulrich & Sandberg (1966) compared a number of media for the capacity to prolong the viability of human leukaemic leukocytes in vitro and concluded that RPMI 1630, RPMI 1640 and McCoy's 5A gave the best results; but new lines were initiated, in the course of these studies, from only one patient and the authors comment on the apparent variability in the nutritional requirements of leukaemic cells from different patients. There is no convincing evidence that the prospect of establishing a new line from a given blood sample is materially influenced by the choice of culture medium, provided that an adequate serum supplement is added. It was therefore decided that initial studies should be carried out with the medium which had proved most successful, in this laboratory, for the culture of epithelial cells and fibroblasts of human and hamster origin, namely Ham's F10 (Ham, 1963) supplemented with 10% Tryptose phosphate broth and 20% Human or Foetal Calf serum. In addition to the reference already cited, this particular medium has recently been used by Nilsson (1971) to establish and maintain a very

demanding cell line derived from a patient with an Ig E myeloma. A careful comparison with RPMI 1640 was reported in his paper and no significant difference was found between the nutrient properties of the two media. The same author has reported a high success rate for the establishment of human lymphoblastoid lines from foetal lymphod tissue infected *in vitro* with EB virus, using Ham's F10 as the culture medium (Nilsson, Klein, Henle & Henle, 1971).

Osunkoya and Mottram (1967) obtained better results with human serum than with foetal calf serum, as medium supplements, in the culture of lymphoblastoid lines from Burkitt lymphoma biopsies. They reported that chick embryo extract was a useful adjuvant but this has not been found necessary by others. Moore and his colleagues, in contrast, preferred foetal calf to human serum (Moore, Ito, Ulrich & Sandberg, 1966) and assessed the value of a variety of adjuvants including insulin, folinic acid, asparagine, oxalic acid and phyto-haemagglutinin. Of these, only oxalic acid appeared to improve the nutrient properties of the medium and even this finding was applicable only to particular cell lines grown in McCoy's 5A or RPMI 1630 medium. Broder, Glade and Hirschhorn (1970) have claimed that purified phyto-haemagglutinin added to the culture medium will significantly improve the chance of establishing a long-term line from the leukocytes of a healthy

donor but their work was published some time after the techniques described here and in Chapter III had been found successful. I have therefore not used phyto-haemagglutinin in any of my cultures.

Type of Culture Vessel and Volume of Culture

These two factors are clearly related. At one extreme, Moore and his colleagues, starting with huge inocula of cells obtained by plasmapheresis, have set up cultures in Roux bottles, litre flasks or a "trophocell unit" in which 25 litres of medium are continuously recycled. Some of their lines have been maintained in 200 litre stainless steel tanks for the purpose of growing up a large bulk of cells from a single line (Moore, Ito, Ulrich & Sandberg, 1966; Moore, Gerner & Franklin, 1967; Moore, Hasenpusch, Gerner & Burns, 1968; Moore, 1969). At the other end of the range Stevens et al. have reduced the volume of some of their cultures to 2 ml. (Stevens, Barker, Pike et al., 1969) while recently, Chang, Hsieh and Blankenship (1971) have obtained good results with an initial volume of only 1 ml.; 10 ml in 2 oz screw-cap bottles (Glade, Kasel, Moses et al., 1968; Glade, Hirshaut, Douglas & Hirschhorn, 1968), 25 ml. flasks or 60 mm. plastic petri dishes (Pope, 1967) is clearly an adequate initial volume for cells from patients with infectious mononucleosis, and Epstein (personal communication) has found 15 ml insulin bottles containing 5 ml. of medium very satisfactory for such

cultures. Epstein expressed a preference for "pyrex" or other "hard" glassware rather than vessels made from soda glass, but other groups who have tested a variety of culture vessels (Foley, Lazarus, Farber, Uzman, Boone & McCarthy, 1965; Clarkson, Strife & De Harven, 1967; Diehl, Henle, Henle & Kohn, 1968) have reported no clear superiority for any one type.

The system which I have developed was based on the one in use in Professor Epstein's department at Bristol. Since 'insulin bottles' are no longer commercially available, some modification was necessary. At the same time, the provision of gassed incubators obviated the need for sealing the culture vessel and allowed the adoption of a loose-fitting lid which greatly reduces the work involved in feeding large numbers of cultures. Practical details of the culture system are given under "Methods".

Feeder Layers

Although the biochemical explanation is obscure, there is good evidence that a feeder layer, usually of irradiated WI38 foetal lung fibroblasts, will help to sustain the growth of other cells *in vitro*, particularly when the latter are seeded at low density (Stoker & Sussman, 1965). Such feeder layers have been used in some culture systems for human leukocytes (Iwakata & Grace, 1964; Clarkson, Strife & De Harven, 1967).

Diehl, Henle, Henle and Kohn (1969) reported that they were not required for the establishment of a cell line from samples obtained, during the course of the illness, from infectious mononucleosis patients but that they were essential for success with blood samples from healthy subjects with a past history of infectious nononucleosis. If the donor had no history of the disease, these authors were unable to establish a cell line from his leukocytes even when plated over WI38 fibroblasts. The use of feeder layers would restrict the number of cultures which could be maintained by a single worker. Care would be required to exclude the possibility that a cell line growing in suspension might be derived from transformed 'feeder' fibroblasts rather than from the inoculated fresh leukocytes and this consideration would apply particularly in a co-cultivation system where there is a third possible source for any growing cells. I therefore decided not to use a feeder layer in developing a culture technique while recognising that the difficulties are not insuperable and that an optimum system for the establishment of new cell lines may well require one.

Frequency of feeding

A priori one might expect optimum culture conditions to include a continuous flow of fresh medium, a situation which is virtually achieved by Moore's "trophocell unit" (Moore, Hasenpusch, Gerner & Burns, 1968). In simple culture systems

however this is impracticable and in the case of human leukocyte cultures it may even be undersirable. The feeding schedules adopted in different centres have varied considerable. Stevens et al. (Stevens, Barker, Fike, Hoffs & Meyer, 1969) for example, fed their cells twice weekly by centrifugation and resuspension in complete fresh medium, while Pope (1967) and Glade and his group (Glade, Kasel, Moses et al., 1968) simply changed the top 30-50% of the medium at the same intervals. Latterly, Glade has found that best results are obtained by a 50% medium change only once weekly (personal communication). Both he and Moore (Moore, Ito, Ulrich & Sandberg, 1966) have found that a newly-growing culture must be allowed to become well established (i.e. signs of growth must have been present for some weeks, rapid growth and a high density of viable cells must be attained) before subculture is attempted, otherwise the line is liable to die out. This may imply that the cells are initially dependent on a threshold concentration being exceeded for some metabolite which they are themselves producing and would add weight to the view that infrequent feeding is advisable during the early period in culture before a cell line has become established.

Materials and Methods

Blood samples

Peripheral blood (5-35 ml) was collected from patients and healthy subjects by venepuncture, into a sterile plastic syringe. Samples (30-65 ml) were also obtained from fresh placentae, within 10 minutes of delivery, by aspiration from a large tributary of the umbilical vein close to the insertion of the cord on the foetal surface of the placenta. The blood was transferred immediately to sterile containers, either plastic tubes containing dry lithium-heparin (Staynes) or universal containers with wet Heparin B.P.(Evans), 10 units/ml of blood.

The heparinised blood was allowed to sediment under gravity at room temperature until a buffy coat had just begun to form. In the case of placental blood samples (which have a very low sedimentation rate) centrifugation at 70 x g for 20 minutes was necessary to produce adequate separation of erythrocytes and plasma.

The leukocyte-rich plasma (including buffy coat) was aspirated and spun down at 150 x g for 15 minutes. The plasma was stored at -70^{o}C and the cell pellet resuspended in 5ml, or multiples of 5 ml. of growth medium.

A total of 198 samples from 180 individuals was treated in this way. Details of the sources are given in table I.

TABLE I

Source of Blood Samples set-up in Culture

Diagnosis	No. of Patients	No. of Samples
Placental Blood	31	31
Neonate (exchange transfusion)	2	2
Healthy Adults	13	13
*Children with 'minor' illness	7	7
Infectious mononucleosis	28	28
Post-perfusion syndrome	1	1
Sarcoidosis	12	12
Leukaemia		
Chronic myeloid	14	18
Chronic lymphatic	7	9
Monocytic	1	1
Acute	9	11
Polycythaemia	5	5
Myelofibrosis	2	4
Fanconi's Anaemia	4	4
Macroglobulinaemia	2	2
Lymphomas	3	3
Ca.Breast	2	2
Mycosis Fungoides	2	2
Lymphadenopathy (? cause)	4	5
Renal Failure		
On haemodialysis	11	14
Transplant recipients	13	16
≠ Other	7	8
Total	180	198

* Includes: Hypoglycaemic attacks, hydronephrosis, otitis media, periodic syndrome, eneuresis and recurring urinary tract infection. The children were in-patients and blood was obtained only when samples were being taken in the course of clinical investigation.

≠ includes: Letterer-Siwe disease, Batten's disease, Lesch-Nyham syndrome, cystic fibrosis, XYY syndrome (one patient each) and ankylosing spondylitis post irradiation (2 patients).

Setting-up of cultures

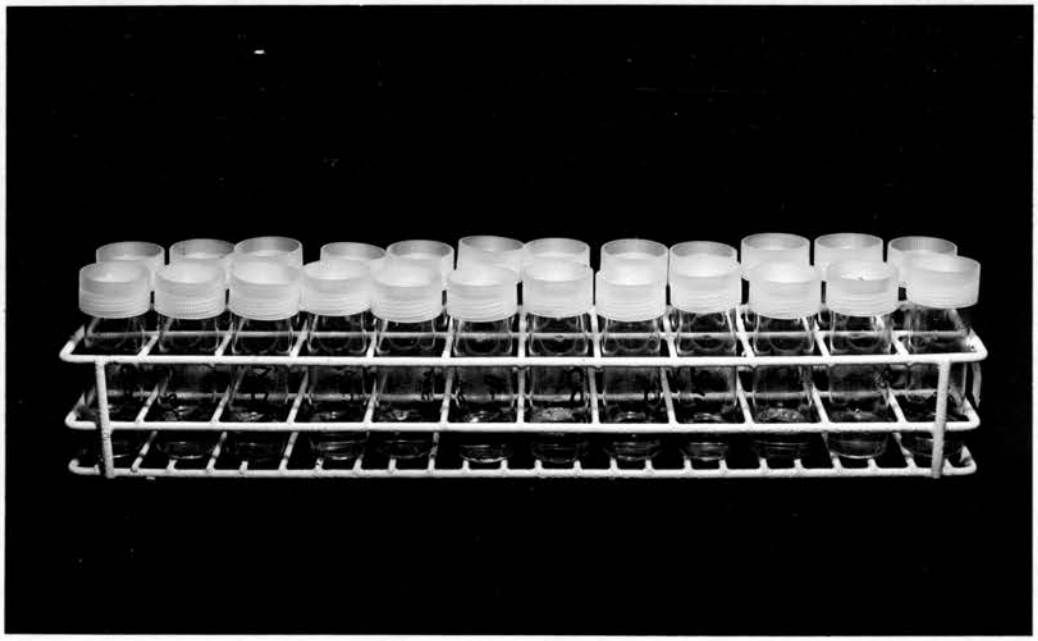
All manipulations involving living cells were carried out under sterile conditions. Growth medium consisted of Ham's F10 with 10% tryptose phasphate broth (Oxoid or Difco) and 2% Foetal calf serum (Flow or Bio-cult), except in the case of twenty-five cultures which were initiated and maintained in medium supplemented with 20% unpooled allogenic human serum (obtained from healthy blood donors) as a substitute for foetal calf serum, Penicillin (100,000µ/litre) and streptomycin (100 mg/litre) were added routinely to the medium.

The initial concentration of leukocytes in growth medium was measured by counting an aliquot (0.05 ml) in a Neubauer haemocytometer after dilution 1:20 with 1% glacial acetic acid. From the majority of blood samples a single 5 ml culture was obtained, containing 5×10^5 to 2×10^7 leukocytes per ml. plus a large number of red cells. Each was set up in a one-inch diameter flat-bottomed tube of thin hard glass (Samco) over which the clear plastic screw cap from a disposable universal container (Sterilin) fitted loosely, allowing direct observation of the culture with an inverted microscope. The tubes were set vertically in racks (fig.1) and incubated in a humidified atmosphere of 7% CO_2 in air at 37°C.

Maintenance of cultures

During the first week after setting up a culture, the pH of the medium tended to fall rapidly and a 50% medium change was required every second or third day. Thereafter feeding was under-

1a) 1" x 3" 'Samco' tube with clear plastic lid from 'Sterilin' disposable universal container

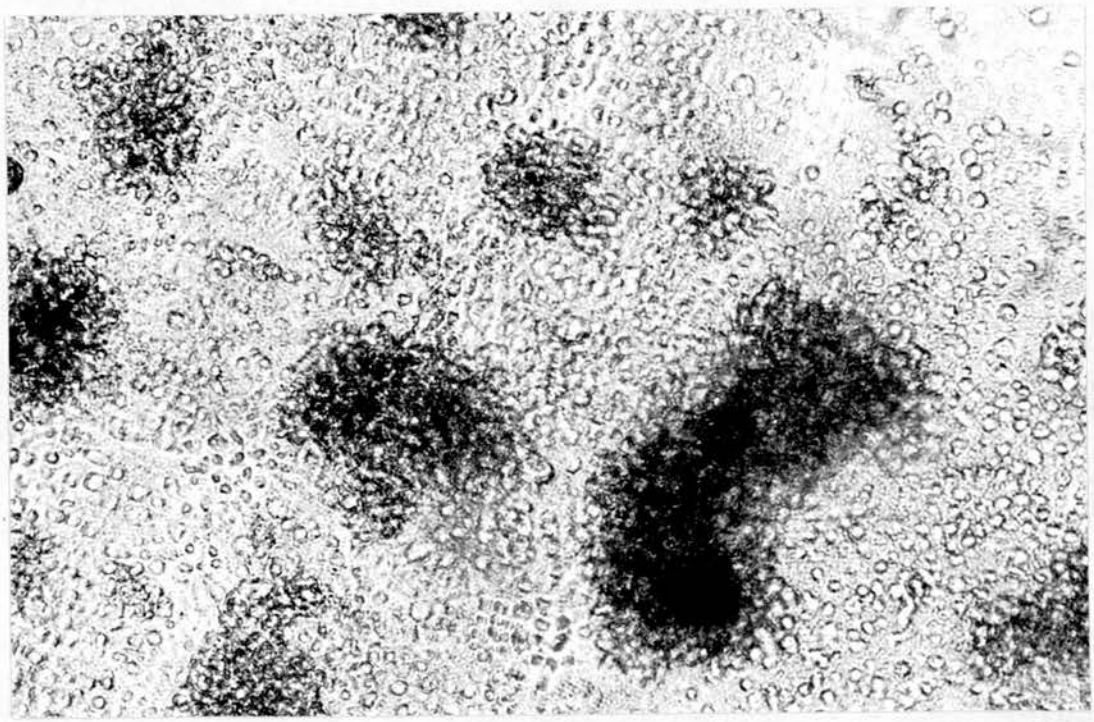


1b) Rack of tubes, each containing 5 ml. of growing cell suspension.

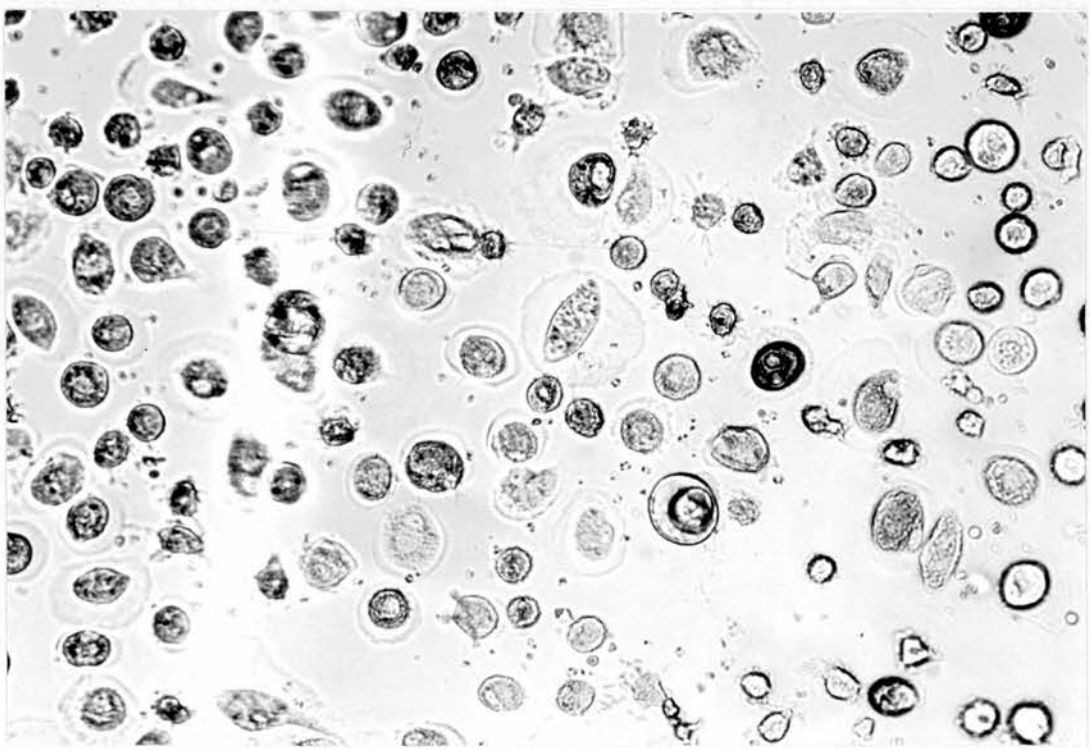
taken once weekly and consisted of gentle aspiration of the top half of the fluid, replacing it with fresh medium without disturbing the cells which had settled on the bottom of the tube. Because of the possible hazards associated with EB virus, all discarded medium was transferred immediately to 5% Hypochlorite solution ("Chloros" ICI).

The cultures were inspected at least twice weekly via an inverted microscope. Evidence of growth, in the form of small floating clumps of rounded, highly refractile cells, appeared in a proportion of cultures after an interval of 20 to 80 days. This invariably coincided with a drop in the pH of the medium and twice-weekly feeding was again instituted. The clumps of viable cells enlarged and coalesced (fig.2) but subculture was not attempted until there were large aggregates readily visible to the naked eye and the pH of the culture was persistently acid. In most cases this stage was reached within three weeks of the first signs of growth, but in a few cultures the delay was as long as six weeks.

The first subculture was carried out by increasing the volume of medium in the tube to 10 ml, dispersing the cells by repeated aspiration with a pasteur pipette and transferring half of the suspension to a second (identical) tube. When growth was well established in two tubes, successive subcultures were made into 25 ml., 50 ml., 100 ml. and 250 ml. conical flasks. In most cases, the growing cell lines required a 50% medium change twice



2. Coalescing clumps of cells in a culture which has just become established as a growing line (MON_1) x 200.



3. Pleomorphic cells adherent to the bottom of the tube in a 6-week-old culture from a patient with renal failure on maintenance haemodialysis. No cell line grew from this culture. x 300.

Both figures from living preparations photographed in diffuse light

weekly and a 1:2 or 1:3 subculture once weekly.

Cultures which failed to show evidence of growth were maintained for at least three months. An aliquot of 0.1 ml. was then removed from the bottom of the tube, added to 3 ml fixative (3:1 methanol:glacial acetic acid) and spun down at 1000 x g for 15 minutes. The deposit was dropped on a slide, dried and stained for 5 minutes in 10% Giemsa. If no viable cells were seen the culture was then discarded. If apparently viable cells were present, the culture was retained and the sampling process repeated at two-weekly intervals.

Storage in liquid nitrogen

As soon as any line had become established in culture, samples were frozen down in liquid nitrogen. For this purpose, cells were separated from growth medium by centrifugation at 400 x g for 5 minutes followed by aspiration of the supernatant. The cell pellet was resuspended in freezing medium consisting of Eagles minimum essential medium with 15% Bovine serum and 10% glycerol. The volume was adjusted to give a concentration of approximately 5 x 10^6 cells/ml. 1 ml aliquots of this suspension were sealed in glass ampoules, cooled to -50^{o}C at 1-2^{o}C per minute and stored in liquid nitrogen tanks.

More than 50 such ampoules have been recovered from storage, thawed rapidly, and the contents added to 4 ml of fresh growth medium. In almost every case, the culture has continued

to grow, even after periods of two years in liquid nitrogen, but a few failures have occurred, some due to bacterial or fungal contamination of the culture during the freezing or the thawing process and others for unexplained reasons.

Observations and Results

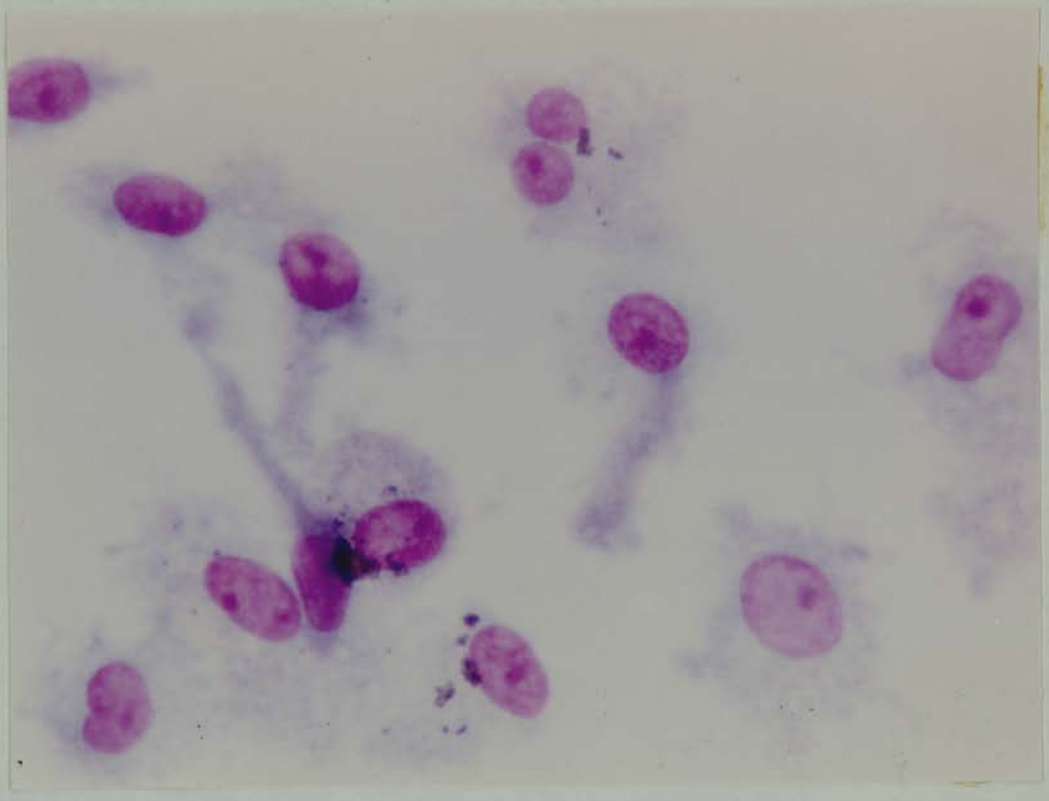
Morphological changes in culture

In more than half the cultures, small numbers of pleomorphic cells, adherent to the bottom of the tube, appeared within the first two weeks in culture (Fig.3). The appearance of such cells in leukocyte cultures has been well documented (Bloom, 1938; Prempree & Merz, 1966; Diehl, Henle, Henle & Kohn, 1968) and they are presumed to be macrophage-derived since they have phagocytic properties. They sometimes increased rapidly in number to form a near-confluent monolayer, particularly in cultures from leukaemia patients, from patients with renal failure on dialysis and from placental blood samples, In such cases, they remained intact and sufficiently active to produce a distinct fall in the pH of the medium for up to 100 days. Where cells of this type were present in small number they may have contributed to the development of a lymphoblastoid line since growing aggregates of round cells sometimes appeared to be attached to them. This feature has also been described by Diehl, Henle, Henle and Kohn (1968). Nevertheless no culture in which large adherent cells were prominent ever gave rise to an established

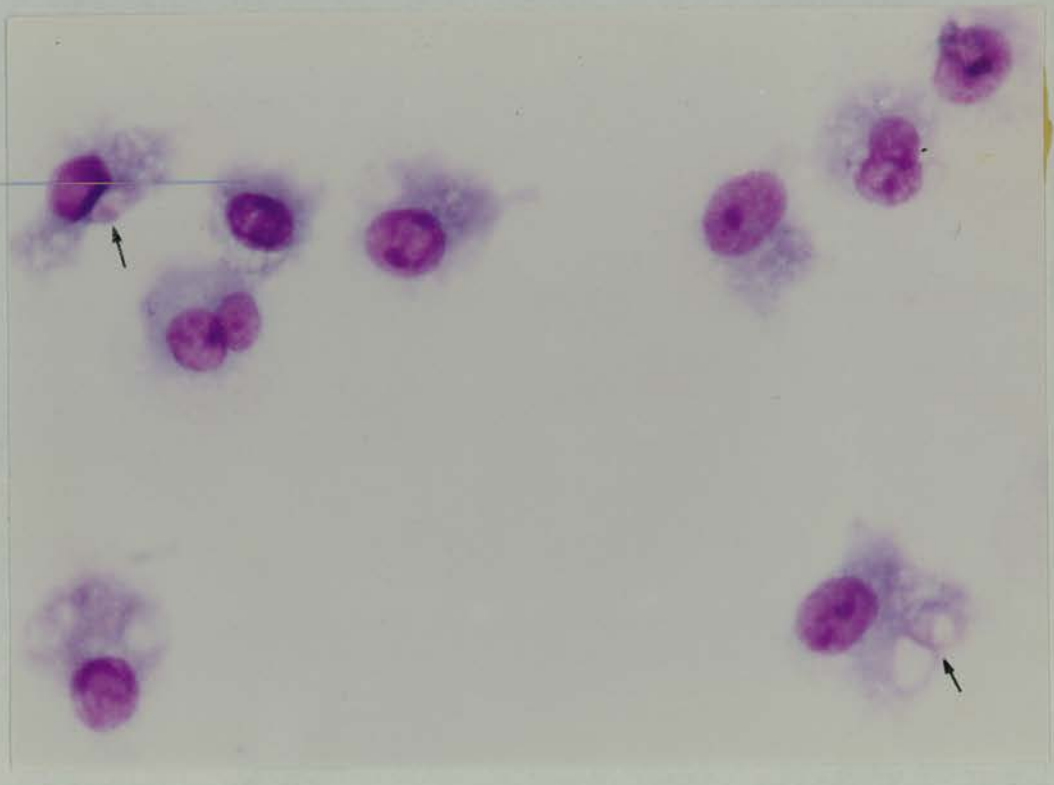
line and while many cultures died out without forming an obvious monolayer, it seems that, under the conditions described here, the adherent pleomorphic and the floating lymphoblastoid cell types may represent mutually incompatible paths of development from peripheral blood cells.

In order to study the morphology of adherent cells, ten blood samples (3 from infectious mononucleosis, 2 leukaemias, 2 healthy adults and 3 from renal failure) from which large numbers of leukocytes had been obtained, were set up in replicate tubes (6 each) with a 22 mm diameter circular coverslip in the bottom. The cultures were maintained for up to 100 days and the coverslips, removed at intervals during this period, were washed in saline, fixed in absolute alcohol and stained for 5 minutes in 10% Giemsa.

The cells were found to vary in outline from round to spindle shaped. The nucleus was oval with one or more prominent nucleoli; the cytoplasm stained pale blue and was sometimes slightly granular. Some contained vacuoles with ingested material, usually identifiable as red-cell debris. Giant cells were common (Figs.4-5). The number of adherent cells reached a peak between 3 and 6 weeks, declining slowly thereafter. Unlike Prempree and Merz (1966) I found no mitotic figures in over 50 coverslips examined.

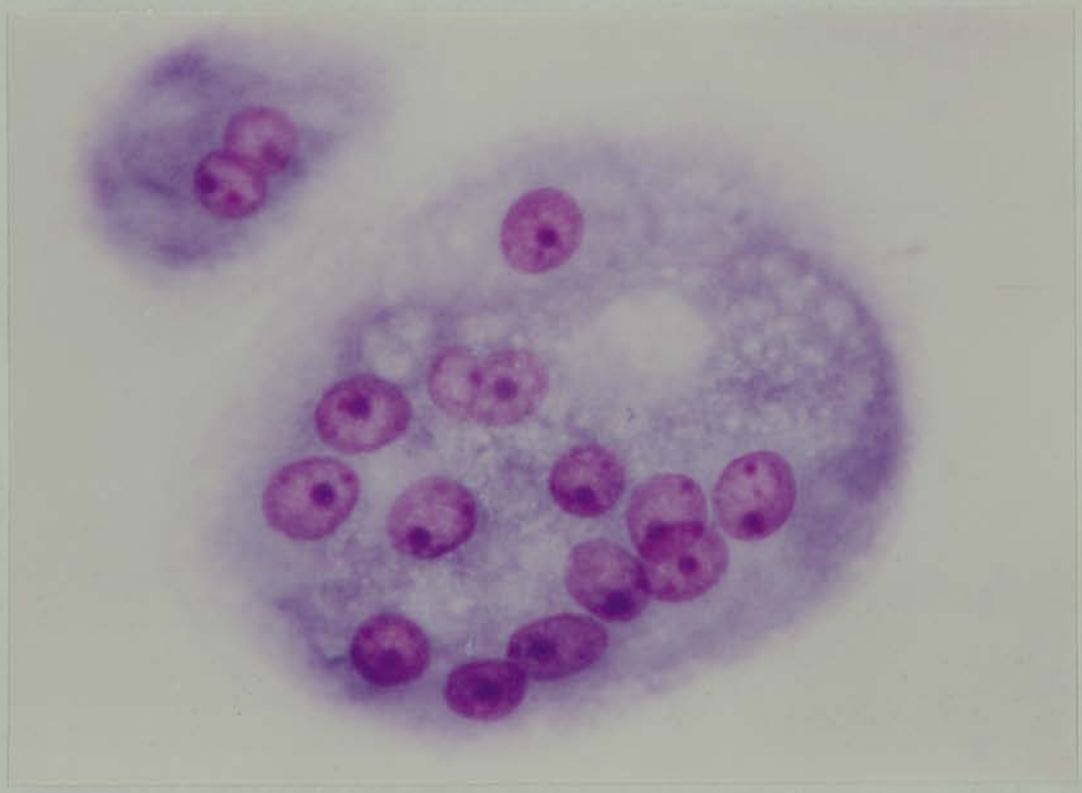


4a. Cells adherent to coverslip. 2 week-old culture of leukocytes from infectious mononucleosis patient. Coverslip was sterilised by flaming and residual carbon particles have been ingested by several cells.



4b. Cells adherent to coverslip. 1 week-old culture of leukocytes from healthy adult. 2 cells (arrowed) contain engulfed material (? red cell debris).

May-Grünwald-Giemsa. x 1,100



5. A binucleate and a multinucleate giant cell in a coverslip preparation from a 5-week-old culture of leukocytes from a child with monocytic leukaemia.

May-Grünwald-Giemsa. x 1,100

Lymphoblastoid lines established

Of the 198 blood samples set up in culture, 26 were lost through contamination or laboratory accidents before they had been maintained for three months. In at least two of these, there were clear signs of growth (on examination via the inverted microscope) before the culture was lost. Since the growing cells could not be characterized, however, all twenty-six cultures were excluded from further consideration.

Of the remaining 172 blood samples (from 16 patients) 17/gave rise to lymphoblastoid cell lines. The details of the successful cultures are given in table II and descriptions of the morphology and karyotypes of the cell lines are covered in chapters V and VI. One line (SAN_1) became infected with yeasts at the time of first subculture. This was controlled by the addition of amphotericin-B (2-5 μg/ml) to the medium and the line continued to grow but as soon as cytogenetic and electron microscopic studies were completed the entire culture was frozen down to prevent spread of the yeasts. The other lines (with one exception) have been maintained as described above though, for practical reasons, it was not possible to handle very large numbers of cultures indefinitely and three of the lines (BER_1, SAD_1 and COA_2) have therefore been consigned to storage after periods of six to

TABLE II

Cultures from which Lymphoblastoid cell lines were grown.

Age Yrs	Sex	Diagnosis	Initial Leukocyte concentration (x 10^6 ml)	Serum / Supplement	Interval from setting up to signs of growth	Cell line Designation
22	F	Infectious mononucleosis * (convalescent)	20	HS	92	GOL_1
29	M	Infectious mononucleosis (convalescent)	4.5	FCS	33	MAR_1
18	F	Infectious mononucleosis (convalescent)	3.9	FCS	47	SAN_1
19	M	Infectious mononucleosis (acute)	5.6	FCS	50	DUN_1
18	F	Infectious mononucleosis (convalescent)	2.9	FCS	60	DEW_1
30	M	Infectious mononucleosis (acute)	1.3	FCS	58	FLE_1
17	M	Infectious mononucleosis (convalescent)	0.5	FCS	49	BER_1
13	M	Infectious mononucleosis (acute)	5.6	FCS	48	SAD_1
27	M	Infectious mononucleosis (acute)	9.0	FCS	60	GRA_1
21	F	Infectious mononucleosis (acute)	2.5	FCS	41	LIZ_1

18/

TABLE II - cont'd

18	F	Infectious mononucleosis (acute)	1.5	FCS	48	KAT_1
86	F	Chronic Lymphatic Leukaemia (treated)	3.0	HS	80	$G\text{-}S_1$
67	F	?Myelofibrosis	1.0	FCS	20	COA_1
			0.5	FCS	49	COA_2
11	M	Acute Lymphatic Leukaemia	2.5	FCS	60	HUN_1
57	F	Acute Myeloid Leukaemia	0.8	FCS	62	MIT_1
	M	Cystic Fibrosis	2.9	FCS	100	MON_1

* 'Acute' = within 10 days of onset, symptoms still present
'Convalescent' = 14-60 days from onset, symptoms improved or absent

H.S. = allogenic human serum 20%
F.C.S.= foetal calf serum 20%

twelve months of growth *in vitro*. The exception was line GRA_1 (from a patient with infectious mononucleosis) which grew rapidly from the 60th to the 80th day in culture, allowing chromosome preparations to be made; thereafter viability and growth rate declined dramatically and the line died out one month later.

Patients from whom lymphoblastoid cell lines were derived

Eleven of the seventeen lines were grown from patients with the clinical and haematological features of infectious mononucleosis. In all but one, the heterophile antibody (Paul-Bunnel) test was positive also. The exception was a twenty-nine year old farm labourer who was admitted to hospital with a two month history of fever, headache, lassitude and cervical lymphadenopathy. There were atypical lymphocytes in his peripheral blood, but the heterophile antibody test was negative on four occasions. On testing by Dr. Elizabeth Edmond at the Wellcome Virus Laboratory, Edinburgh City Hospital, his serum gave an EB virus antibody titre of 1:80 (Steel & Edmond, 1971) and his condition improved steadily without specific therapy. When followed-up one year later he was entirely healthy. His blood give rise to the MAR_1 cell line.

One line (MON_1) was grown from the peripheral blood of a 4 year old boy with a clinical diagnosis of cystic fibrosis (mucoviscidosis) confirmed by the finding of a grossly elevated sweat sodium concentration (Dr. W.M. McCrae, personal communication).

He suffered from recurrent chest infections, but was not known to have had infectious mononucleosis.

The remaining five lines were derived from patients with severe haematological disease.

$G\text{-}S_1$ was obtained from an 86 year old female patient with chronic lymphatic leukaemia diagnosed two years previously. Treatment with chlorambucil and vinicristine had reduced the white cell count to 5000/mm^3 at the time of culture.

COA_1 and COA_2 were from a 67 year old female patient with a hypoplastic anaemia which progressed rapidly to a fatal outcome. An iliac crest trephine biopsy suggested a diagnosis of myelofibrosis, but this remains unconfirmed as permission for postmortem examination was refused. The sample which gave rise to the COA_1 line was obtained three months before death. COA_2 grew from a second specimen taken one week before death. The white cell count in both samples was 1400/mm^3.

HUN_1 was derived from an 11 year old boy with acute lymphoblastic leukaemia diagnosed eight weeks before the sample was obtained. Treatment had included blood transfusion, 6-mercapto purine and steroids. He was in remission when blood was taken for culture, his white cell count being 7,700/mm^3 with a normal differential and no circulating blast cells.

MIT_1 grew from the peripheral blood leukocytes of a 57 year old female patient who presented with a refractory anaemia.

The peripheral white cell count was low (3000/mm^3) and the differential count was abnormal though not diagnostic (10% Neutrophils; 45% lymphocytes; 41% Monocytes; 2% Eosinophils; 2% blast cells). At this stage she had received no cytotoxic drugs. The bone marrow contained numerous primitive cells and within a few weeks she had developed all the haematological features of acute myeloid leukaemia.

Discussion

The culture method described here has the merit of almost irreducible simplicity and appears to give a satisfactory success rate for blood samples from patients with infectious mononucleosis (lines grown from 11 of 28 patients) although it has so far failed when applied to thirteen samples from patients with post-perfusion syndrome or sarcoidosis from which one might have expected comparable results (Gerber, Walsh, Rosenblum & Purcell, 1969; Henle, Henle, Scriba et al., 1970; Hirshaut, Glade, Octavio et al., 1970).

The five cultures from patients with a diagnosis of leukaemia or atypical myelofibrosis are of interest since in none of these cases were there large numbers of blast cells in the circulation at the time of sampling. Of the nine other samples from patients with acute leukaemia, blast cells were prominent in six (reports from clinical haematology laboratories at time of obtaining blood samples for culture). The fact that these failed to grow argues against Glade's suggestion (Glade, Hirschhorn & Douglas, 1969) that the common factor in samples which give rise to lymphoblastoid lines is not EB virus infection

but the presence of circulating lymphoid cells undergoing active DNA synthesis.

Moore and his colleagues have stressed the importance of setting up cultures within 4 hours of drawing the blood sample, and of avoiding cooling of the leukocytes at any stage (Moore, Grace, Citron et al., 1966). Almost all of my successful cultures, however, were derived from blood samples obtained in other hospitals or in patients' homes and in many of these cases the blood samples had been kept at ambient temperature for more than four hours before being set-up in culture; indeed the samples which gave rise to lines HUN_1, SAD_1 and MON_1 had all been in transit for approximately twenty-four hours. This point is of practical importance when efforts are being made to establish cell lines from patients with rare disorders, since samples may have to be obtained from hospitals at considerable distances from the tissue culture laboratory.

As mentioned under "Methods" a total of twenty-five cultures (including four from patients with infectious mononucleosis and five from patients with leukaemia) were set up in medium supplemented with human rather than foetal calf serum. Two of these gave rise to lymphoblastoid lines (GOL_1 and $G\text{-}S_1$) but in both cases, the interval between setting-up the culture and the appearance of clumps of growing cells was much longer (92 and 80 days) than the mean for all seventeen lines (56.3 days). Furthermore, in two cases duplicate cultures were set up from infectious mono-

nucleosis blood samples, one supplemented with foetal calf serum, the other with human serum. In both cases, only the sample in foetal calf serum gave rise to a growing line (MAR_1 and GRA_1). Following this observation, foetal calf serum has been used exclusively.

Although a single line has been established by the above method from one patient with a genetically determined disorder (MON_1), it is clearly not sufficiently reliable to offer a good prospect of success with any given sample. Of the possible variations in the technique indicated in Chapter I and in the introduction to this chapter, the most promising was felt to be co-cultivation with killed cells from an EB virus-positive line. Using the above system as a basis, I have therefore developed the co-cultivation method which is described in Chapter III.

CHAPTER III

Establishment of new cell lines by a co-cultivation technique

Introduction

A co-cultivation system for the establishment of lymphoblastoid cell lines from human peripheral blood was first described by the Henles and their colleagues (Henle, Diehl, Kohn, Zur Hausen & Henle, 1967). These authors mixed lethally irradiated (3000-6000 rads) cells from a male Burkitt lymphoma line (either JIYOYE, which released EB virus, or RAJI, which did not) with freshly separated leukocytes from the peripheral blood of healthy female infants. In order to promote maximum cell-cell contact, the mixtures, containing 1 to 2 x 10^6/ml of each cell type, were incubated for 24 hours in conical centrifuge tubes before being seeded onto monolayers of W138 (female) foetal lung fibroblasts. Control cultures of irradiated cells or of fresh leukocytes on their own failed to grow as did all the mixtures with irradiated RAJI cells. All seven experiments involving mixtures of irradiated JIYOYE cells with fresh leukocytes however gave rise to growing lymphoblastoid lines. Five of these were examined cytogenetically and each was found to have a diploid female karyotype. In a second paper (Diehl, Henle, Henle & Kohn, 1969) the same group reported that in their system, virus-positive lines other than JIYOYE also had the capacity to promote the growth of fresh leukocytes, that JIYOYE lost this property when it ceased to release virus and that the leukocytes of infants were more readily induced to grow as established lines than those of healthy adult donors.

As mentioned in chapter I, Pope, Horne and Scott (1968, 1969) were able to show very convincingly that long term lymphoproliferation *in vitro* is a direct effect of EB virus infection of the fresh leukocytes. Using viruses isolated by ultrafiltration from lysates of established leukaemic cell lines, they showed that this material would promote the growth of new lymphoblastoid lines from foetal bone marrow, thymus and spleen. The 'transforming factor' in their ultrafiltrate was destroyed by ether or by heating and was neutralised by pre-incubation with human serum containing antibody against EB virus. The latter point has been confirmed in a large series of similar experiments recently reported by Miller, Lisco, Kohn and Stitt (1971).

These authors did not use a feeder layer in their culture system but the foetal lymphoid cells were almost certainly admixed with fibroblasts which may have fulfilled a feeder role in the early stages of culture.

Miller, Enders, Lisco and Kohn (1969) established nine new lines from the peripheral blood of a healthy adult male by co-cultivation with irradiated (4500 rads) cells of an EB virus positive female cell line derived from the bone marrow of a 3 year old child with acute leukaemia. Their techniques was similar to that of Diehl and the Henle's but they omitted the period of mixing in a centrifuge tube and, instead, introduced inactivated Sendai virus, in some experiments, to promote cell

fusion. They concluded that this may have been of some value but was clearly not essential since four of the nine successful cultures did not have Sendai virus added. In all cases they pre-stimulated the fresh leukocytes with phyto-haemagglutinin (PHA), before introducing irradiated cell line cells, on the grounds that stimulated lymphocytes might be more susceptible than unstimulated cells to infection by EB virus. There is no direct evidence for this but even if it should prove to be true, it should not be necessary to add PHA in a co-cultivation system using intact irradiate lymphoid cell line cells since Hardy, Ling and Knight (1969) have shown that in such a mixture, a very intense activation of the fresh lymphocytes occurs within the first week. This is comparable to a one-way mixed lymphocyte reaction (Bain, Vas & Lowenstein, 1964) and is independent of the presence or absence of EB virus in the irradiated line. Baumal, Bloom and Scharff (1971) reported that stimulation of lymphocytes by exposure in vitro to an antigen to which the donor shows a delayed hypersensitivity reaction, will permit these cells to become established as a long-term lymphoblastoid line provided that exposure to the antigen is followed by infection in vitro with isolated EB virus particles. In their system, neither antigen alone nor EB virus alone was sufficient to initiate the growth of a long term line from fresh blood leukocytes. Gerber, Whang-Peng and Monroe (1969) established two lines from the

peripheral blood leukocytes of a healthy man using concentrated filtrates from a virus positive cell line (P3HR1-k, a subline of JIYOYE). No feeder layer was used, nor was PHA, but the initial blood samples were very large (500 ml and 100 ml). Control cultures, inoculated with material derived from RAJI, failed to grow. It is of interest that, in this report, a very heavy inoculum of EB virus did not promote the growth of a new cell line, while a four-fold lower dilution of the material was effective. Taken in conjunction with the observation of Diehl et al. (Diehl, Henle, Henle & Kohn, 1969) that irradiated cells of the HR1-k clone of JIYOYE (which produces a very high yield of EB virus particles) proved ineffective in co-cultivation experiments while material from the parent line (releasing fewer virus particles) was successful, this could imply that an excess of infective EB virus may have a cytopathic effect on human lymphoid cells. Horoszewicz has demonstrated that an apparently virus-free lymphoblastoid line (64-10) is rapidly killed when infected with EB virus (from HR1k cells) at high input multiplicities while lower multiplicities of virus allow the 64-10 cells to survive indefinitely with EB virus replicating in a small proportion (Horoszewicz, Dunkel, Avila & Grace, 1970). Similar findings have been reported by Durr, Monroe, Schmitter, Traul and Hirshaut (1970).

Choi and Bloom (1970, 1970a) have described two methods for transferring growth potential from an established line to

fresh lymphocytes. In the first, they simply added a "small inoculum" of live cells from a female, EB-virus positive line to a culture of freshly isolated peripheral blood leukocytes from a healthy male. After a period of four weeks, the culture consisted of rapidly growing lymphoblastoid cells all of which had a male karyotype. The authors record only a single experiment of this type and despite its success they considered the method unsatisfactory in view of the possibility that the inoculum of viable lymphoblastoid cells might outgrow any emergent line or that hybrid cells might be formed. Accordingly they investigated the effect of lysates produced by repeated freezing and thawing of 10^7 cells from an EB virus-positive line. This material was added to seven fresh leukocyte cultures (from four donors) set up from 10 ml aliquots of peripheral blood. Two new cell lines from healthy donors were established as a result and the method has been applied successfully to establish lines from the peripheral blood of a child with Lesch-Nyhan syndrome (Choi & Bloom, 1970). Chang, Hsieh and Blankenship (1971) established nineteen new cell lines from umbilical cord leukocytes to which they had added filtrates from freeze-thawed cells of an infectious mononucleosis line. EB virus could not be detected by indirect immunofluorescence in the cells of the donor line, but the agent was found in some of the newly initiated cultures.

The claim has been made that new lymphoblastoid lines have been established by the transformation of two human embryo fibroblast cultures inoculated *in vitro* with supernatants from short-term cultures of human leukocytes (Osato, Ito, 1967; Kurita, Osato & Ito, 1968). However the authors do not appear to have excluded the possibility that small numbers of viable leukocytes were carried over in the supernatant fluids and thus seeded onto a fibroblast feeder layer. Since the blood cultures were both derived from patients with acute leukaemia and since the chromosomal sex of the cell lines corresponded in each case with that of the leukocyte donor from whom the "transforming principle" was obtained, it seems probable that the lymphoblastoid lines were in fact grown from the peripheral blood cells.

As in the case of the methods permitting 'spontaneous' growth of lymphoblastoid lines from fresh leukocytes, the information summarized above offered a wide choice of co-cultivation techniques, between which no valid comparisons had been made. For the reasons given in Chapter II I wished to dispense with a feeder layer, at least initially, and in view of the work of Hardy, Ling and Knight (1969) I decided not to use PHA. I therefore adopted the following system.

Blood samples

Venous and placental blood samples were obtained and the leukocytes separated from them as described in chapter II.

All manipulations involving live or irradiated cells were carried out under sterile conditions.

Established lines

Killed cells from a total of seven established lymphoblastoid cell lines were used in co-cultivation experiments. Two of these, JIYOYE and EB_2 had 'lost' EB virus during culture in another laboratory before being sent to me (Dr. B. Hampar and Professor M.A. Epstein, personal communication) and on repeated electron microscopic examination in this laboratory, no virus particles were found in either . Two, GOL_1 and $G\text{-}S_1$ grown by me, from the peripheral blood of patients with infectious mononucleosis and chronic lymphatic leukaemia respectively were initially considered not to release virus, but after some months in culture, EB virus particles were found in both (see Chapter IV). The other three MAR, SWB-2D and F137 were all demonstrably virus positive when first used in these experiments. Virus was subsequently lost from the F137 line. Details of the seven lines are given in table III.

Irradiation Procedure and storage of killed cells

The seven established lines were maintained by the methods described in Chapter I. Before irradiation, the cells of a given line were allowed to grow to a density of about 10^6/ml in a 100 ml or 250 ml conical flask, counts being made, without dilution of the suspension, in a Neubauer haemocytometer. An aliquot containing 4×10^7 cells was then removed and spun down at 100 x g for 5 minutes. Sufficient supernatant was discarded to leave a volume of 20 ml containing 2×10^6 cells/ml and the suspension was

TABLE III

Details of seven cell lines used as irradiated 'donors' in co-cultivation experiments

Cell line	Diagnosis of Donor	Karyotype /	EB virus present * or absent on E/M Examination	Reference and laboratory from which sample was obtained
JIYOYE	Burkitt's lymphoma	Male Aneuploid	-	Pulvertaft, 1964. Via Dr.B.Hampar Nat.Cancer Inst. USA
EB_2	Burkitt's lymphoma	Female Near-tetraploid	-	Epstein, Barr & Achong, 1965.From Prof.M.A. Epstein, Bristol.
MAR_1	Infectious mononucleosis	Male Diploid	+	Grown in this laboratory
GOL_1	Infectious mononucleosis	Female Diploid Later Aneuploid	+	Grown in this laboratory
$G\text{-}S_1$	Chronic Lymphatic Leukaemia	Female Aneuploid	+	Grown in this laboratory
F137	Chronic Lymphatic Leukaemia	Male Aneuploid	+, later -	Jensen,Korol,Dittmar & Medrek, 1967. From Dr.E.M.Jensen, Chas. Pfizer Inc.Maywood, N.J.
SWB-2D	Viral gastroenteritis	Male Diploid	+	From Dr.P.R. Glade, New York

/ Chromosome studies carried out in this laboratory during period of co-cultivation experiments. For details of findings in each cell line, see Chapter VI.

* Results of E/M studies in this laboratory during period of co-cultivation experiments. See Chapter IV for details.

transferred to a sterile universal container which fitted into a prepared recess in the centre of a 12 cm beeswax cube. The cube, enclosing the cells, was placed 50 cms from a 250 kv x-ray source using a Thoraeus I filter, and an 8 x 6 cm applicator without accessory filter. The dose rate to the cell suspension was 33 rads/min and a total dose of 3000 rads was given over a period of 90 minutes. At intervals of fifteen minutes throughout the procedure, the universal container was gently agitated to disperse the cells, thus ensuring an even exposure to the radiation.

At the end of the irradiation period the cells were either frozen down in medium containing 10% glycerol and stored, in aliquots of 5×10^6, in liquid nitrogen or were returned to a conical flask and maintained in culture as before, aliquots being removed over the succeeding hours or days for use in co-cultivation experiments.

Setting-up co-cultivation experiments.

All experiments were set up in 1" diameter flat-bottomed glass-tubes of the type used for the initiation of spontaneously-growing cell lines. The growth medium was Ham's F10 supplemented with 10% tryptose phosphate broth, 20% foetal calf serum and antibiotics.

Freshly separated peripheral blood leukocytes suspended in growth medium were counted as previously described (Chapter II) and separated into two or more aliquots, depending on the numbers

available. One 5 ml culture, containing at least 0.5×10^6 cells per ml. was maintained as a control, without the addition of any irradiated cells. To each of the remaining cultures was added a known number of irradiated cells from a single established line. At the same time, at least one aliquot of irradiated cells alone (not less than 10^6 cells) was set up as a separate control. The volume of each mixture was adjusted to 5 ml by the addition of growth medium.

Mixed and control cultures were maintained at $37^{\circ}C$ in a humidified atmosphere of 7% CO_2 in air. They were fed, by 50% medium change, twice or thrice during the first week, thereafter at weekly intervals. They were examined regularly by the inverted microscope and even in the absence of any evidence of growth, were not discarded until at least three months after being set up.

Within the framework of the above plan trials were undertaken to test the importance of the following variables:

1) Delay between collecting blood samples and setting up the leukocytes in culture. ("Fresh cell delay").
2) Delay between irradiation of cell line and use of the killed cells in co-cultivation experiments ("Post-irradiation delay") discounting time spent in liquid nitrogen.
3) The absolute and relative numbers of fresh and of irradiated cells in a given culture.

The design and results of these trials will now be described before the total series of co-cultivation experiments is discussed as a whole.

Experiments testing the effects of 'fresh cell delay'

Experiment 1

40 ml of blood (Sample A, table IV) was obtained from a freshly delivered placenta (the infant was normal full-term female). The sample was split between two identical tubes containing heparin 200 u. One of these was allowed to stand at room temperature for 24 hours. From the other, the leukocytes were separated and suspended in growth medium within 3 hours of collection (3 hour leukocytes), one 5 ml sample of which containing 3.3×10^6 leukocytes/ml, was maintained as a control. The remaining '3 hour' leukocytes were divided between six tubes, three of which contained 10^7 cells, the others 10^6 cells each. F137 cells, lethally irradiated 24 hours earlier, were added to the six tubes in amounts of 5×10^6 cells, 2×10^6 cells or 5×10^5 cells according to the scheme illustrated in table IV. 3×10^6 irradiated cells on their own were set up and maintained in a separate 5 ml culture as a further control.

Twenty-four hours later, six mixed cultures were set up in identical fashion from the second half of the blood sample ('24 hour leukocytes'). The F137 cells were now 48 hours post-irradiation and the control culture of placental blood contained 8.8×10^6 leukocytes/ml but in other respects the procedure exactly

TABLE IV

2 Experiments testing the effect of "fresh cell delay".

Experiment 1

	Number of Cells/Tube	Irradiated F137 5×10^6	2×10^6	5×10^5
Placental Blood 'A'	10^7	0	0	+
'3 hr leukocytes	10^6	0	0	0
	10^7	0	0	0
24 hr leukocytes	10^6	0	0	+

Experiment 2

		Irradiated MAR_1 3×10^6 cells/tube				
Post-irradiation delay (days)		0	4	8	12	16
Placental Blood 'B' 10^7 cells/tube	'3 hr leukocytes	+	+	0	0	0
	'24 hr leukocytes'	+	+	0	0	0

Each grid square represents a single mixed culture containing placental blood leukocytes as indicated by the left hand scale and irradiated cells as indicated by the scale vertically above.

+ = New cell line grew from this culture
- = No long-term growth

duplicated that applied to the first half of the sample.

All the tubes were maintained in identical fashion. Two new cell lines ultimately grew from them (BAT_1 and BAT_2), one each from the '3 hour' and the '24 hour' leukocytes. Neither the control cultures of placental blood leukocytes nor that of irradiated F137 cells showed any long term growth.

Experiment 2

65 ml of blood (sample B, table IV) was obtained from a freshly delivered placenta (the infant was a normal full-term female) and divided equally between two heparinised containers, one of which was allowed to stand at room temperature for 24 hours. From the other, leukocytes were separated and suspended in growth medium within three hours of collection ('3 hour leukocytes'). A single 5 ml culture containing 6×10^6 cells/ml was maintained as a control. The remainder was distributed between five tubes each containing 10^7 leukocytes. MAR_1 cells had been lethally irradiated four weeks earlier. An aliquot had been stored in liquid nitrogen immediately after irradiation and the rest returned to the 37° incubator. On days 4, 8, 12 and 16 post irradiation, further aliquots had been frozen down.

As soon as the '3 hour' leukocytes from sample B had been distributed between the five culture tubes, five ampoules of frozen MAR_1 cells, one from each post-irradiation interval, were recovered from storage and 3×10^6 cells from a single ampoule added to each tube according to the plan illustrated in table IV. Twenty-four hours

later, the leukocytes from the second half of sample B were treated in an identical fashion ('24 hour leukocytes'). The control culture from this fraction contained 1.8×10^6 cells/ml but all the mixed cultures contained the same numbers of fresh and irradiated cells as in the first half of the experiment. Surplus irradiated MAR_1 cells (2×10^6 from each ampoule) were pooled and distributed between two 5 ml control cultures. None of the control cultures showed any signs of long-term growth but four new lines (CLA_1, CLA_2, CLA_3, CLA_5) were established from the ten mixed cultures. As shown in table IV, these successful cultures came from the four mixtures which included MAR_1 cells frozen down on days 0 and 4 post-irradiation, i.e. two each from the '3 hour' and the '24 hour' leukocytes.

It was concluded from these results that within the limits of 3 to 24 hours, the delay between collection of a blood sample and setting up its leukocytes in culture ('fresh cell delay') was not an important factor in determining the success or failure of a co-cultivation experiment. I have not studied the effect of a longer fresh cell delay since it has been possible to set up all co-cultivation experiments within 24 hours of the blood sample being withdrawn, even when the blood has been sent from another centre.

Experiments testing effect of 'post-irradiation delay'

The second of the two experiments just described tested the importance not only of "fresh cell delay", but also of "post-irradiation" delay. Three further experiments were carried out using the same design. Blood samples C and D were obtained from fresh placentae (in each case the infant was a healthy full-term female). Sample E was obtained from a healthy 23 year-old female laboratory technician. One control culture of fresh cells alone was set up from each, containing 4.4×10^6/ml, 2×10^6/ml and 1.5×10^6/ml respectively. 3×10^6 irradiated MAR_1 cells from different post-irradiation periods were added to the tubes as indicated in table V. Control cultures of pooled irradiated cells on their own were set up and maintained in parallel.

None of the control cultures showed signs of long-term growth. Nor did any of the mixed cultures including fresh cells of samples C or E. Of the five mixed cultures from sample D, the two which included MAR_1 cells from days 0 and 4 post-irradiation both gave rise to new cell lines (WEB_1 and WEB_2). The combined results (table V) indicated that a post-irradiation delay of 8 days or more was associated with a significant decline in the potency of MAR_1 cells for co-cultivation purposes.

This interpretation is confirmed by a review of the entire series of co-cultivation experiments. A total of 136 mixed cultures was set up in which, with hindsight, the irradiated cells could be regarded as potentially capable of inducing long-term growth in

TABLE V

Experiments testing the effect of 'post-irradiation delay'

Source of Fresh Leukocytes	No. of Fresh leukocytes per tube	Irradiated MAR_1 cells 3×10^6/tube					Post-irradiation delay (days)
		0	4	8	12	16	
Placental Blood 'B' '3' hour'	10^7	+	+	0	0	0	
'24' hour	10^7	+	+	0	0	0	
Placental Blood 'C'	4.4×10^6	0	0	0	0	0	
Placental Blood 'D'	5×10^6	+	+	0	0	0	
Healthy Adult 'E'	5×10^6	0	0	0	0	0	

Each grid square represents a single mixed culture containing fresh blood leukocytes as indicated by the left hand scale and 3×10^6 irradiated MAR_1 with post-irradiation delay indicated by the scale vertically above.

+ = Lymphoblastoid line grew from this tube
O = No long-term growth

fresh leukocytes (i.e. excluding these experiments in which the irradiated material came from EB_2, JIYOYE or SWB-2D or from F137b which had 'lost' EB virus. See table VIII). In 112 of these, the post irradiation delay was 1-7 days and in 24 it was 8-18 days. Twenty-four of the former were successful (i.e. new cell lines grew from them) but only one of the latter, the success rates being 21.5% and 4.2% respectively, by considering the confidence intervals for these parameters, there is sufficient evidence to say that these two values are different, with 99% confidence. The single success in the latter group came from a mixed culture of fresh placental blood leukocytes with killed GOL_1 cells (which had not been frozen) used on the ninth post-irradiation day.

The possibility that there might be an optimum post-irradiation delay for killed cells was suggested by the observations of Moses et al. (Moses, Glade, Kasel et al. 1968) that a cell line, which under normal culture conditions appeared to be virus-negative, began to release complete EB virus particles some days after lethal irradiation. No clear optimum interval is apparent from my results, but because of variations in the source of fresh cells and in the numbers of cells included in each experiment, detailed comparisons of different post-irradiation delays within the range 1-7 days would be invalid. Since it is clear that killed cells can be used successfully in co-cultivation experiments immediately after irradiation my current practice is to freeze down the

entire batch of cells on the day of irradiation, thawing out aliquots as fresh cells become available for co-cultivation. This ensures that a reasonably large number of experiments can be set up in which one can compare the susceptibility of leukocytes from different blood samples to the growth promoting effects of identical irradiated cells.

Experiments testing the importance of absolute and relative numbers of fresh and of irradiated cells in a given culture.

The first of the two experiments designed to test the effect of 'fresh-cell delay' (table IV) also examined the importance of the absolute and relative numbers of fresh and of irradiated cells in a given culture. A further ten sets of mixed cultures were set up using fresh leukocytes from a variety of donors and irradiated cells from the MAR_1 or the F137 lines (I have again excluded those experiments in which the irradiated cells came from an "ineffective" line or in which the post-irradiation delay was greater than seven days). In most of these, only two tubes could be set up in addition to the control cultures of fresh leukocytes and of irradiated cells on their own. The details of these experiments are set out in table VI.

The results obtained from blood samples A (table IV) and F (table VI) suggest that an increase in the numbers of either irradiated or fresh cells may actually have an adverse effect but overall there is little indication that cell numbers have an important influence on the outcome of co-cultivation experiments

TABLE VI

10 Experiments testing the effect of relative and absolute numbers of irradiated and of fresh cells in a given culture

Source	Fresh Leukocytes Number per tube	Irradiated MAR_1 cells 5×10^6	2×10^6
Sample F (adult female, Chronic Myeloid Leukaemia)	10^7	+	+
	10^8	0	0
		Irradiated F137 cells 2×10^6	5×10^5
Sample G (12 year old boy with hypoglycaemic attacks)	5×10^6	0	0
Sample H (9 year old boy with otitis media)	3×10^6	0	0
Sample J (8 year old boy with Fanconi's Anaemia)	3×10^6	0	0
Sample K (5 year old boy with Fanconi's Anaemia)	3×10^6	0	0
Sample L (10 year old boy with Fanconi's Anaemia)	3×10^6	0	0
Sample M (6 year old boy with hydronephrosis)	3×10^6	+	+
Sample N (26 year old healthy female)	5×10^6	+	+

Sample P/

TABLE VI - cont'd

		Irradiated MAR_1 cells	
		3×10^6	10^6
Sample P (Fresh placental blood)	4×10^6	+	+

		Irradiated MAR_1 cells	
		2×10^6	5×10^5
Sample Q (68 year old female with Macroglobulinaemia	1.8×10^6	0	0

Each grid square represents a single mixed culture containing fresh leukocytes as indicated by the left hand scale and irradiated cells are indicated by the scale vertically above.

+ = new cell line grew from this culture

- = no long term growth observed

within the range 10^6 to 10^7 per tube for fresh cells and 0.5×10^6 to 5×10^6 per tube for irradiated cells.

When all 113 experiments, in which the irradiated material was from an 'effective' line, as defined in table VIII, are reviewed in terms of cell numbers, the results can be plotted as shown in Table VII. The figures indicate that the prospects of success were poor when there were fewer than 3.1×10^6 fresh leukocytes per tube (5 new lines from 57 cultures, compared with 20 new lines from the 56 cultures with more than 3×10^6 fresh leukocytes $\chi^2 = 10.4$ $p < 0.01$). The success rate does not appear to improve as the number of irradiated cells per tube is increased. Caution must be exercised in interpreting the above data since, as is shown in tables IX and X, there are probably factors associated with the origin of a given blood sample which influence susceptibility to the growth promoting influence of irradiated lymphoblastoid cells independently of the cell numbers involved. Hence, with reference to the grid square (table VII) in which five of seven cultures resulted in emergence of a new cell line, it is relevant that four of these were from a single blood sample.

Effectiveness of irradiated cells from different lines in co-cultivation experiments

When experiments in which there was a post-irradiation delay greater than seven days are excluded (except in the case of irradiated GOL_1 cells), some impression of the relative efficiencies of the seven cell lines used in co-cultivation experiments can be gained.

These data are set out in table VIII. The capacity to promote the growth of a new line in co-cultivation does not appear to be related to the origin (i.e. the diagnosis of the patient from whom it was derived) of the irradiated cell line since MAR_1 and GOL_1 are from infectious mononucleosis patients, $G\text{-}S_1$ and F137 from chronic lymphatic leukaemia. As expected from the work quoted earlier, however, the release of complete EB virus particles appears to be an important criterion (For details of electron microscopy methods and results, see Chapter IV). This is particularly clearly illustrated by the findings in relation to F137. This line has been grown continuously in this laboratory from August, 1969, until the end of 1971 and samples used at intervals for co-cultivation experiments. It has also been examined on several occasions by electron microscopy for the presence of EB virus particles. Between August 1969 and April, 1970, irradiated cells from this line were used in a total of 34 co-cultivation experiments (excluding those with post-irradiation delay $>$ 7 days). Nine new lines were established, successes being relatively evenly distributed throughout the period. Two samples were examined by electron microscopy during this phase; both contained EB virus particles. From April to September 1970 the line was used in 52 similar co-cultivation experiments, none of which resulted in the establishment of a new line. By contrast irradiated MAR_1 cells were used in only five experiments over the

TABLE VIII

Performance of irradiated material from different cell lines in co-cultivation experiments.

Cell line	*No. of experiments	No. of ⁄ Successes	% Success
JIYOYE	6	0	0
EB_2	7	0	0
MAR_1	44	11	25
GOL_1	3	1	33
$G\text{-}S_1$	9	1	11
SWB-2D	7	0	0
^{xx}F137(a)	34	9	26
^{xx}F137(b)	52	0	0
^{xx}F137(c)	23	3	13

*Post-irradiation delay<8 days except for GOL_1 when post-irradiation delay 10 days.

⁄Success = new cell line established

^{xx}F137(a) = Cells of this line growing 22/8/1969 to 30/3/1970. EB virus seen on electron microscopy.

F137 (b)= Cells of this line growing 30/3/1970 to 9 /9/1970 EB virus notseen on electron microscopy.

F137(c) = Cells of this line regrown from sample frozen in September 1969.

The phrase "effective cell lines" used in the text refers to lines MAR_1, GOL_1, $G\text{-}S_1$, F137(a) and F137 (c) since, as shown above, these are capable of inducing growth of new lines in co-cultivation.

same period, two of which were successful. A further sample of F137 cells was processed for electron microscopy in August, 1970 and examined in October 1970. Despite a very thorough search, no virus particles could be found. This is an exact parallel with the findings of Diehl and his colleagues in relation to the JIYOYE line (Diehl, Henle, Henle & Kohn, 1969). A fresh supply of F137 cells was provided by thawing out a sample frozen down in September, 1969, at a time when virus was still demonstrable on electron microscopy. EB virus particles were seen again in fresh preparations from this material and cells from this source were found to be effective once more in co-cultivation. The success rate with this latter material, F137(c) is lower than with F137(a) or with MAR_1 but many of the recent experiments have involved rather small blood samples yielding less than 3.1×10^6 fresh leukocytes per tube.

Importance of Source of fresh leukocytes

When the 113 co-cultivation experiments with "effective" irradiated cells are analysed with respect to the source of fresh leukocytes, (table IX) it is apparent that placental blood samples and those from patients with leukaemia are more likely to give rise to new lymphoblastoid cell lines in co-cultivation than those from patients with non-malignant disease. Allowance must be made for the fact that larger numbers of leukocytes are usually obtained from placental or leukaemic blood than from other samples, but as shown in table IXa, if experiments with fewer than 3.1×10^6 fresh leukocytes per tube are excluded,

TABLE IX

113 Co-cultivation experiments analysed with respect to source of fresh lymphocytes.

Source of Fresh leukocytes	No.of Patients	No.of Experiments	No. of new lines ##	% Success
*Placental Blood	17	41	14(7)	34
Children with non-malignant disease	14	23	2(1)	9
/Adults with non-malignant disease	9	23	2(1)	9
**Leukaemia etc (Adults & Children)	15	26	7(5)	27

* Includes 2 neonates with rhesus iso-immunisation. Samples obtained at start of exchange transfusion.

/ Includes 4 healthy adults

** Includes 1 patient with Waldenströms Macroglobulinaemia and one with Letterer-Siwe disease

Figures in brackets indicate number of patients from whom new lines were grown.

TABLE IXa

Data as above but including only experiments with more than 3×10^6 fresh leukocytes/tube.

Source of Fresh Leukocytes	No. of Patients	No.of Experiments	No.of New lines	% success
Placental Blood	8	23	12(6)	52
Children with non-malignant diseases	3	3	0	0
Adults with non-malignant diseases	4	8	2(1)	25
Leukaemia etc. (Adults & Children)	11	22	6(5)	27

TABLE X

Results obtained when two or more co-cultivation experiments were set up with leukocytes from a single placental blood sample.

Placental Blood Sample	*No. of Experiments	No. of successes
MEE	2	0
DUN	2	0
CLA	5	5
WEB	2	2
LAM	2	2
YUN	2	1
WOO	3	0
BAT	12	2
MAC	2	1

*Including only those in which "effective" irradiated cells were used.

new lines were obtained from six out of eight placental blood samples, from five out of eleven patients with leukaemia but from only one out of seven adults or children without malignant disease. Considering the confidence intervals for these parameters, the figures indicate a clear difference (99% probability) between placental blood samples and those from patients with non-malignant disease. There is insufficient evidence in the case of leukaemic samples to establish a statistically valid distinction from the leukocytes of children and adults with nonmalignant disease. The point will be re-examined as data are accumulated from more culture experiments. Within the group of placental bloods, evidence can be found of differences between individual samples in their susceptibility to the growth-promoting influence of co-cultivation. Table X illustrates the results obtained from nine such samples, each of which was used in two or more experiments. In six cases the results were concordant (i.e. all successes or all failures) and in only three were they discordant (i.e. some experiments successful, others failed). This suggests that the likelihood of a given co-cultivation experiment resulting in the growth of a new lymphoblastoid cell line is influenced by some property of the individual blood sample from which the leukocytes were derived.

Discussion

Details of all twenty-five lines initiated by co-cultivation are given in table XI. It should be noted that none of the corresponding

TABLE XI

Details of 25 cell lines established by co-cultivation

Cell line	Origin of fresh leukocytes	Irradiated cells used in co-cultivation	∠ Karyotype of new line
YUN_1	Placenta ♂	F137	Diploid
LAW_1	C.M.L.* ♀	MAR_1	Diploid
LAW_2	"	F137	Diploid
MAC_1	Placenta ♀	GOL_1	Diploid
BAT_1	Placenta ♀	F137	Diploid
BAT_2	"	F137	Diploid
SHA_1	C.M.L. ♀	MAR_1	Diploid
SHA_2	"	MAR_1	Diploid
CLA_1	Placenta ♀	MAR_1	Diploid
CLA_2	"	MAR_1	Diploid
CLA_3	"	MAR_1	Diploid
CLA_4	"	F137	Diploid
**CLA_5	"	MAR_1	N.E.
WEB_1	"	MAR_1	Diploid
WEB_2	"	MAR_1	Diploid
LEO_1	6 yr.old ♂ Hydronephrosis	F137	Diploid
LEO_2	"	F137	Diploid
ORI_1	Healthy adult ♀	F137	Diploid
** ORI_2	"	F137	N.E.
LAM_1	Placenta ♀	MAR_1	Diploid
LAM_2	"	MAR_1	Diploid
ART_1	C.M.L. ♀	$G\text{-}S_1$	Diploid
WHE_1	Placenta ♂	F137	Diploid
BLA_1	Adult acute ♂ Leukaemia	F137	Aneuploid
ODY_1	Adult Waldenstrom's Macroglobulinaemia ♂	F137	Diploid

*C.M.L. = Chronic myeloid leukaemia.

In all these cases, short term blood cultures without PHA revealed presence of Ph^1 chromosome in 90% of cells. Ph^1 chromosome was not seen in any established lymphoblastoid line.

** These 2 lines were lost shortly after becoming established.
∠ For details, see chapter VI.

TABLE VII

Effect of cell numbers on outcome of 113 co-cultivation experiments

Number of Fresh Blood Leukocytes per tube (x 10^6)	No. of Irradiated cells/tube(x 10^6)						
	0.5-100	1.1-2.0	2.1-3.0	3.1-4.0	4.1-5.0	Sub-Totals	
31-100		0/1	1/3	1/3	0/1	2/8	
7.6-30	3/11	1/3	5/7	2/6	1/3	12/30	
5.1-7.6	0/1					0/1	20/56
3.1-5.0	2/8	1/1	3/8			6/17	
1.6-3.0	3/39	1/8				4/47	
0.5-1.5	1/8	0/2				1/10	5/57
Sub-totals	9/67	3/15	9/18	3/9	1/4		

Each grid square represents mixed cultures composed of fresh blood leukocytes as indicated by the left hand scale and irradiated cells as indicated by the scale vertically above. Each set of figures represents $\frac{\text{Number of established lines}}{\text{Number of cultures set up}}$.

control cultures of fresh leukocytes alone gave rise to new cell lines. Two control cultures of leukocyte samples which failed in co-cultivation did become established as lymphoblastoid lines, MIT_1, from a female patient with acute myeloid leukaemia and MON_1 from a boy with cystic fibrosis; details of these are given in Chapter II.

None of the control cultures of irradiated cells showed signs of continued growth. In this respect, the irradiation technique described, though much slower, appears to be more satisfactory than that used by Diehl, Henle, Henle & Kohn (1969).

From the data presented in this chapter, the following requirements for a simple but effective co-cultivation system have been defined.

Irradiated cells. These should be from alline demonstrably EB-virus-positive at the time of irradiation. As large a batch as possible should be irradiated at one time and frozen down within one week in aliquots of a few million cells. The 'effectiveness' of the batch should be proved as soon as possible by setting up co-cultivation experiments with leukocytes from three or four placental blood samples. The number of irradiated cells used in a single experiment does not appear to be critical within the range 0.5×10^6 to 5×10^6.

Fresh leukocytes.

'Fresh cell delay', up to 24 hours after withdrawal of the blood sample is of little consequence, but it is important to use more than 3×10^6 cells per ml. culture.

When these conditions are satisfied an overall success rate of about 30% is likely. The observation that placental blood samples grow more readily than others may be exploited both when cell lines from healthy individuals are required and when genetically determined disorders are being studied since in the latter case it is often possible, from family studies, to anticipate the birth of an affected infant.

Further improvement in the success rate may be possible by the use of a fibroblast feeder layer. It may be significant that in contrast to the observations on spontaneously growing cell lines, successful growth in co-cultivation often began as growing clumps of small round cells in close physical association with aggregates of large glass-adherent cells, as if the latter were fulfilling a feeder role. It is not known whether these large cells belonged to the irradiated line or were derived from the fresh blood leukocytes.

If a feeder layer is to be used, it will be particularly important to have a means of identifying the origin of any emergent cell line. The most convenient marker is undoubtedly the karyotype and f137 is a particularly useful line in this respect since it

has a number of consistent and distinctive chromosomal abnormalities (see chapter VI) and can therefore be used as the irradiated component in mixed cultures with fresh cells from a donor of either sex. In addition to the karyotype, means of identification can include the pattern of iso-enzymes or of HL-A surface antigens on the cell line. Fialkow et al. have recently claimed that there is a high incidence of disparity between the iso-enzyme pattern of fresh tissue of Burkitt lymphoma patients and that of the lymphoblastoid lines subsequently grown from their tumours (Fialkow, Klein, Giblett et al., 1971). This has not, however, been the experience of the group at the Galton laboratories, London (Prof. Harry Harris, Dr. S. Povey) who have examined almost all of the cell lines described in this and the preceding chapter. In nine cases, fresh blood from the donor of the cell line has also been screened. Up to 20 different loci have been analysed and discrepancies between fresh and cultured tissue have proved extremely rare. The few that have been observed have been attributable to the effect of recent blood transfusion on the enzyme complement of the fresh blood sample.

Rogentine and Gerber (1969) have reported that the HL-A patterns of lymphoblastoid cell lines are relatively stable in culture though the variation, on retesting, was somewhat greater than they had found on repeated samples of fresh blood from the same donor. Using a different technique, Dr. Heather Dick has

examined my cell lines and has shown that considerable change in the apparent HL-A phenotype of a given line can occur as the period of culture increases (Dick & Steel, 1971; Dick, Steel & Crichton, 1971, and unpublished observations). Nevertheless, the method remains useful for distinguishing one line from another and when applied within the first few months of growth in vitro, the HL-A pattern of an established line is usually identical with, or very close to that of the donor's fresh lymphocytes.

Establishment of a new lymphoblastoid line by co-cultivation may, in some respects, be a similar phenomenon to the 'transformation' of human fibroblasts in vitro by SV 40 virus (Koprowski, Ponten, Jensen et al., 1962). In the latter system it has been shown that cells from patients with Down's syndrome (Trisomy 21) or Fanconi's anaemia are more susceptible to transformation than those from control subjects (Todaro, Green & Swift, 1966; Todaro & Martin, 1967; Young, 1971) a finding which is of interest in view of the high incidence of malignant disease that accompanies both of these conditions. It will be possible in the near future to compare success rates, in co-cultivation experiments, of peripheral blood leukocytes from patients with a variety of disorders, including Down's syndrome and Fanconi's anaemia. To

date, blood has been obtained from three boys with Fanconi's anaemia who are included under "children with non-malignant disease" in table IX. No lymphoblastoid line grew from these samples while two lines (LEO_1 and LEO_2) were established from the leukocytes of one "control" child without haematological disease. Finally, an efficient co-cultivation system may ultimately permit investigation of the precise mechanism whereby the EB virus confers the property of prolonged, and possibly indefinite, growth upon cells which otherwise would have a very limited life-span *in vitro*. A serious attempt to solve this problem, however, is unlikely to be made until substantial progress has been reported in studies of cell transformation by the very much smaller DNA viruses of the polyoma-SV40 group (MacPherson, 1970).

CHAPTER IV

The role of EB virus in cultured cell lines

As pointed out in Chapter I, there exist a number of lymphoblastoid cell lines in which EB virus cannot be detected either by electron microscopy or by immunofluorescence. In addition, there are several lines, originally virus-positive, from which virus has subsequently disappeared. These include JIYOYE (Diehl, Henle, Henle & Kohn, 1969), EB_2 (Epstein, personal communication) AL_1 (Rabson, O'Conor, Baron et al., 1966) and F137 (see Chapter III). On the basis of molecular hybridization experiments, first of the DNA/DNA type (Zur Hausen & Schulte-Holthausen, 1970; Zur Hausen, Schulte-Holthausen, Klein et al., 1970) and more recently DNA/RNA (Nonoyama & Pagano, 1971) it has been proposed that the EB virus genome, or a substantial part of it, is present within the cells of apparently virus-negative lines. This could reconcile the apparent conflict between the evidence, quoted in Chapters II and III, that EB virus is necessary for the continued *in vitro* proliferation of human lymphoid cells and the failure to detect virus particles in many of the established lines.

By analogy with the observations on animal cells 'transformed' *in vitro* by oncogenic viruses (for review see Winocour, 1971) it seems probable that only a part of the EB viral genome is essential for the maintenance of lymphoproliferation and it is therefore possible that

consistently virus-free cell lines, such as RAJI, contain incomplete viral DNA. On the other hand, cloning experiments (Hinuma & Grace, 1969; Maurer, Imamura & Minowada, 1969; Zajac & Kohn, 1970; Miller, Stitt & Miller, 1970) have shown conclusively that the complete viral genome can be present within every cell of a lymphoblastoid line, even though fewer than 3% of the cells release complete virus particles. Thus, some "virus-free" cell lines may harbour the complete EB virus genome, with the potential for virus replication, in an occult form. Indeed there have been two recent claims that such virus-free lines (including RAJI) become virus-positive, by immunofluorescence, electron microscopy and by co-cultivation studies, following treatment of the cultures with 5-Bromodeoxyuridine, the assumption being that the drug disturbs the integration of viral DNA with the host cell genome (Hampar, Derge, Martos and Walker, 1972; Gerber, 1972). Little is known of the factors which determine the proportion of cells within a culture which support the replication of EB virus at any given time, but it is clear, regardless of the precise mechanism, that this proportion can be altered by environmental influences. The Henles have shown that the percentage of virus-releasing cells is significantly increased by removal of Arginine from the culture medium (Henle & Henle, 1968) and similar claims have been made for

delayed feeding and/or low temperature cycling of the cultures (Hinuma, Konn, Yamaguchi et al., 1967; Grace, 1967; Hinuma, Konn, Yamaguchi & Grace, 1967; Maurer, Glick & Minowada, 1968), and for co-cultivation of lymphoblastoid cells with a continuous line of African green monkey (Vero) cells (Hampar, Martos & Walker, 1971). Differences in culture technique may therefore explain the striking variation between different centres in the proportion of cell lines in which EB virus can be detected. This has ranged from 100% (Jensen, Korol, Dittmar & Medrek, 1967; Diehl, Henle, Henle & Kohn, 1968) to 0% (De Harven, 1967). At the same time allowance must be made for differences in the means used for identification of the virus for while the two most widely used techniques, electron microscopy and indirect immunofluorescence, have been shown to give very comparable results (Henle & Henle, 1966; Hinuma, Konn, Yamaguchi et al., 1967; Steel & Edmond, 1971), some workers find wide discrepancies between them (Moses, Glade, Kasel et al., 1968).

With the exception of lines GRA_1, CLA_5 and ORI_2 which were lost at an early stage, all the cell lines established by the methods described in chapters II and III have been examined for the presence of EB virus particles by electron microscopy usually within four months of setting up the culture and invariably within three months of first subculture. Several of the lines have been re-examined, at intervals, after periods of up to 33 months continuous growth <u>in vitro</u>.

Methods

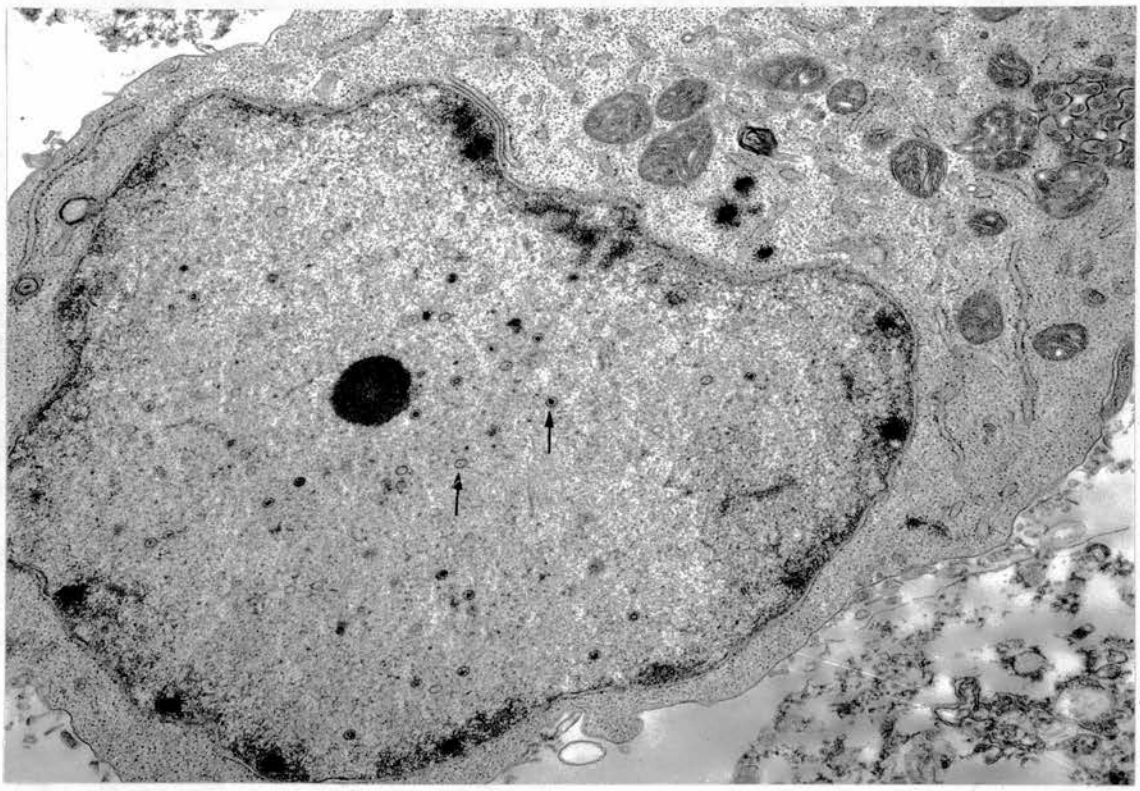
A single 5 ml culture of each growing line has been left unfed for 4 to 7 days. The cells have been separated by centrifugation at 200xg for 5 minutes, washed twice with 0.1 molar cacodylate buffer (pH 7.3), fixed by resuspension in 5% glutaraldehyde in cacodylate buffer at room temperature and spun down to form a pellet. After 2 hours in fixative the cell pellet was washed in 10% buffered sucrose for 2 x 15 minutes, post-fixed in 2% Osmium tetroxide for 1 hour, dehydrated in alcohol then cleared in 1:2 epoxy-propane and embedded in araldite. Sections 50 mμ thick were cut on an LKB ultramicrotome, mounted on uncoated copper grids, stained with saturated aqueous uranyl acetate for 15 minutes followed by Reynold's lead citrate for 10 minutes and examined on an AEI EM 6B or 6M electron microscope. All processing of the cell pellets from the stage of first fixation has been undertaken by the staff of the electron microscopy suite in the M.R.C. Clinical and Population Cytogenetics Unit, under Mr. Alan Ross. Examination and photography of the prepared material has been carried out by myself.

Results

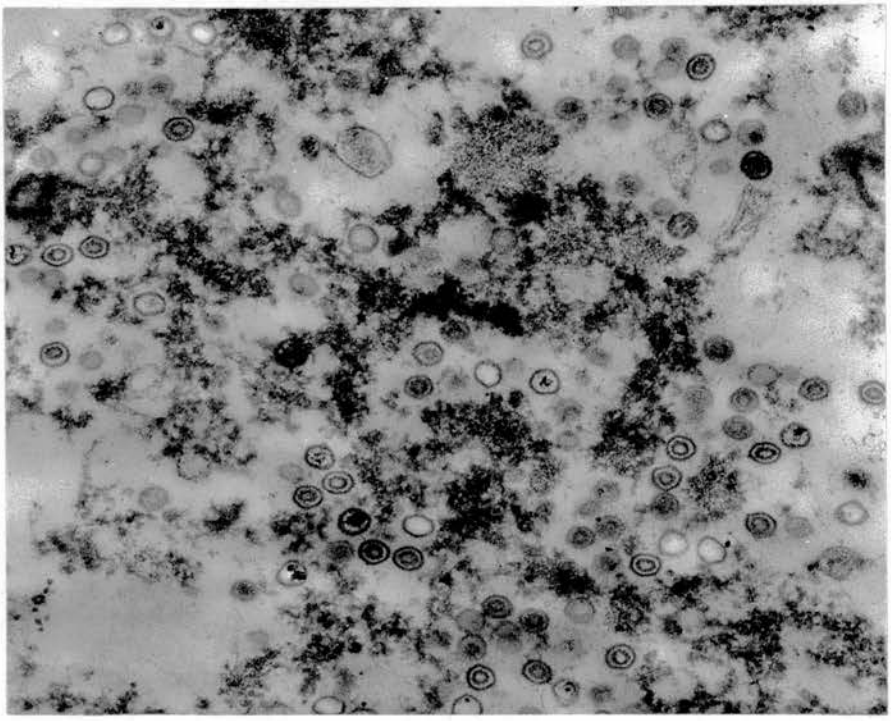
Appearance of EB virus

The EB virus, as originally described by Epstein and his colleagues (Epstein, Achong & Barr, 1964; Epstein, Henle, Achong & Barr, 1965) consists of a hexagonal capsid 75 to 80 mμ in diameter,

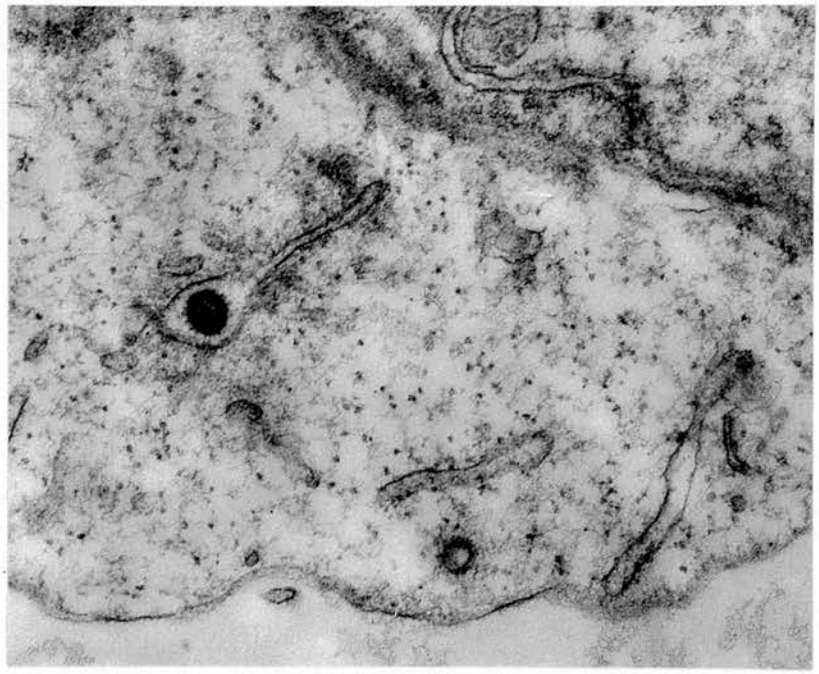
with a dense or ring-shaped centrally placed nucleoid. Such particles were present in large numbers within the disintegrating nuclei of EB_1 cells and the same essential features have been reported in many other lines (Henle,& Henle, 1966; Stewart, Lovelace, Whang & Ngu, 1965; O'Conor & Rabson, 1965; Pope, Achong, Epstein & Biddulph, 1967; Osunkoya & Mottram, 1967; Minowada, Klein, Gerber & Monroe, 1968). This appearance has occasionally been noted in my cell lines (fig.6) but much more commonly I have seen the virus in its enveloped form within cytoplasmic vacuoles (fig.7) or closely applied to the external surface of intact cells (fig.8). This distribution of the virus has also been well documents (Epstein, Henle, Achong & Barr, 1965; De Harven, 1967; Epstein, Achong & Pope, 1967; Dalton & Zeve, 1967). In common with other herpes viruses (Darlington & Moss, 1969) it appears that EB virus acquires its envelope by budding through the nuclear membrane or through some part of the endoplasmic reticulum (Epstein and Achong, 1970a). In my experience, the envelope has been poorly defined because of the aggregation on its outer surface of granular electron-dense material (fig.8) the composition of which is uncertain but which resembles the coating of immunoglobulin seen on the surface of EB virus particles exposed to EB antibody-positive human serum (Henle, Hummeler & Henle, 1966; Durr, Monroe, Schmitter et al., 1970; Horoszewicz, Dunkel, Avila & Grace, 1970). It therefore seems possible that the material is



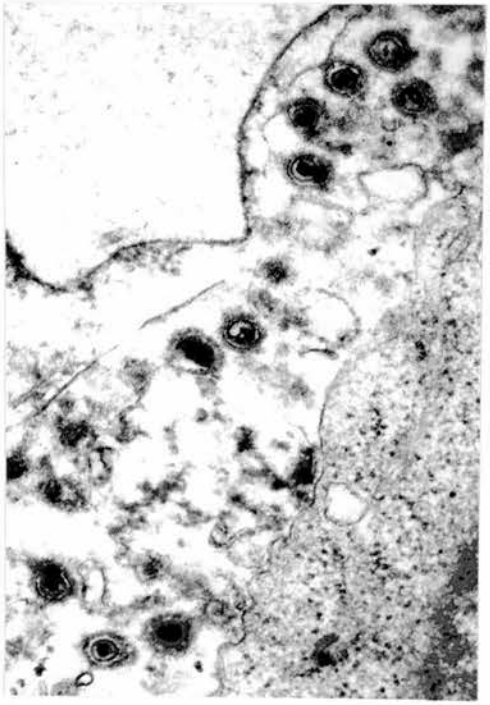
6a. Cell from line CLA_4 with many intranuclear EB virus particles (arrowed). Cytopathic changes are evident. The nuclear membrane has disappeared at several points and there is gross distortion of the mitochondria. x 15,000



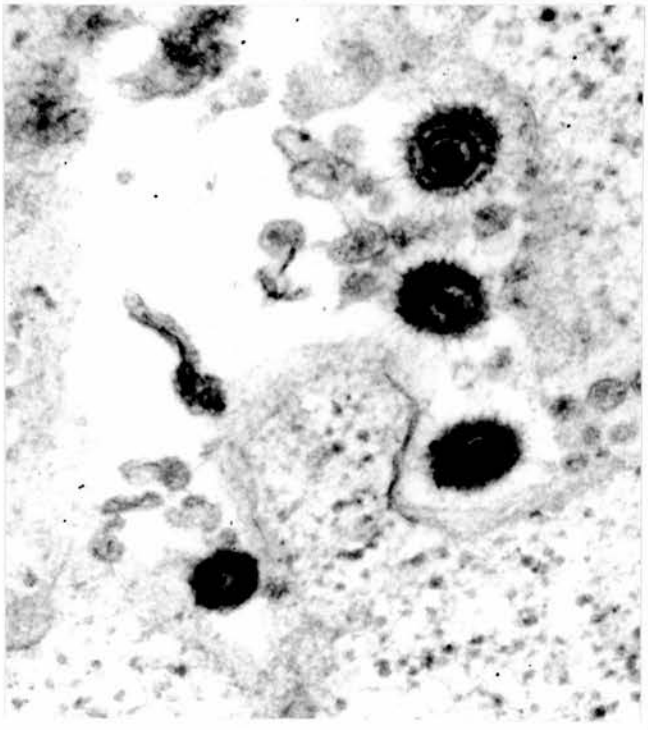
6b. EB virus particles among debris of a cell from line EB_3. Some particles are empty, others have a circular or a dense solid nucleoid. x 41,500



7. EB virus particle within a cytoplasmic vacuole EB_3 cell x 64,800



8a. From line F137 x 40,000

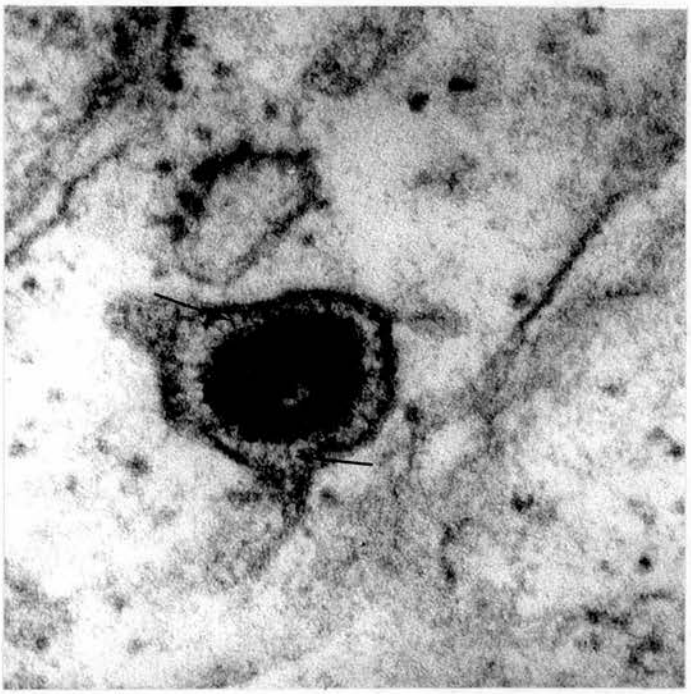


8b. From line MAR_1 x 83,000

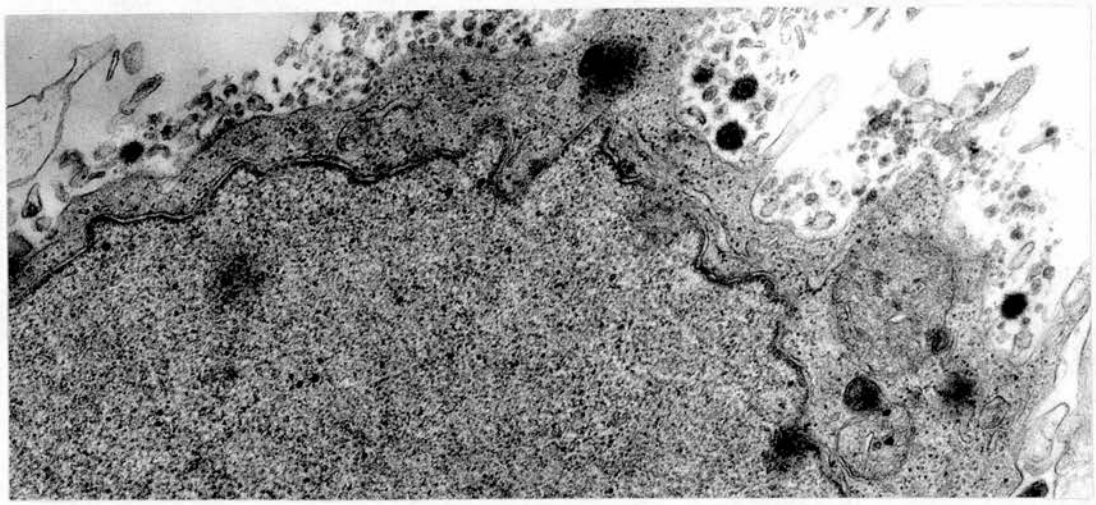
Figs 8a and b show enveloped extracellular EB virus particles (arrowed in 8a) with a granular or fibrillary electron-dense outer coating.

immunoglobulin synthesized by the lymphoblastoid cells, a view which is supported by the observation that strands of the electron dense counting can sometimes be seen running between the surface of a virus particle and the adjacent endoplasmic reticulum (fig.9) and by a recent report that fluorescence staining provides evidence for the presence of an EB virus-related antigen-antibody complex within, and on the surface of virus-positive lymphoblastoid cells in culture (Floyd, Vonka & Benyesh-Melnick, 1971).

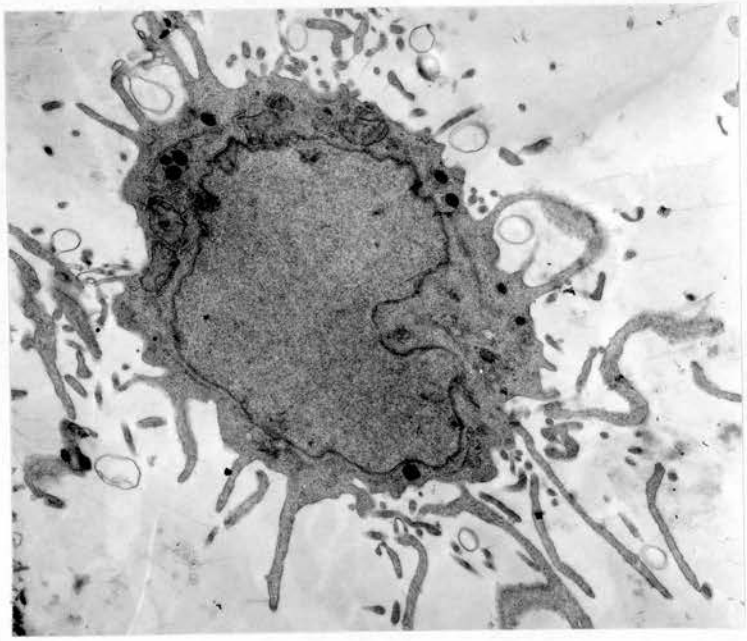
In many cases, virus particles at the periphery of the cell have been associated with aggregates of small, irregular, membrane-bound particles (fig.10). Attention has been drawn to these by Moses et al, and by Chandra and his colleagues (Moses, Glade, Kasel, et al., 1968; Chandra, Liszczak & Monroe, 1970). Their significance is unknown but in almost every instance, the material within the membrane is structurally similar to that of the adjacent cytoplasm which suggests that they may not be isolated particles but micro-pseudopodia seen in cross section. Many lymphoblastoid cells show multiple finger-like projections from their surface (fig.11) localised concentrations of such processes may be involved either in the discharge of virus particles from within the cell or in the adsorption of the cell surface of virus particles liberated from other cells.



9. EB virus particle within cytoplasmic vacuole. Note strands of electron-dense material (arrowed) connecting viral capsid to vacuole wall. Cell line SHA_1. x 143,000.



10. Particulate debris associated with EB virus particles close to cell periphery. Line MAR_1. x 27,500.



11. Multiple finger-like projections from surface of a cultured lymphoblastoid cell. Line WHE_1. x 7,300.

Presence or absence of virus in individual cell lines

Table XII records the findings in the 39 cell lines examined for EB virus-production within three months of becoming established in culture. It is clear that virus particles could be found in only a minority of lines although the examination involved a thorough search of 200 to 1000 well-fixed cell profiles from each line. In DEW_1 a single virus particle, and in SAD_1 and LAW_2 two particles were found after a careful search of more than 500 cell sections in each case. When the results of electron microscopy were compared with those of indirect immunofluorescence on thirteen of these lines (Steel & Edmond, 1971), electron microscopy was shown to be at least as sensitive as the latter technique for the detection of EB virus (Indirect immunofluorescence was carried out by Dr. E. Edmond at the Wellcome Virus Laboratories, City Hospital, Edinburgh).

Since virus particles have been detected in such small numbers in some cases, it is possible that other lines have been scored as virus-negative when, in fact, they do liberate a few complete particles. This view is supported by the findings on re-examination of some lines after periods of 4 to 33 months in culture (Table XIII). $G\text{-}S_1$, GOL_1 and HUN_1 all became demonstrably virus-positive following initial negative findings. While it is difficult absolutely to exclude the possibility of cross-contamination of cultures as an

TABLE XII

EB virus in cell lines on initial examination (~3 months growth since line became established)

Cell Line	Diagnosis of Donor	Irradiated cells used in co-cultivation	EB virus on E/M	Notes
$G\text{-}S_1$	Chronic lymphatic leukaemia	None	0	
COA_1	Myelofibrosis (Atypical)	"	0	
COA_2	" "	"	0	
HUN_1	Acute Leukaemia	"	0	
GOL_1	Infectious Mononucleosis	"	0	
MAR_1	"	"	2-3	
DEW_1	"	"	1	
DUN_1	"	"	2-3	
FLE_1	"	"	2	
SAD_1	"	"	1	
SAN_1	"	"	5	
BER_1	"	"	0	
LIZ_1	"	"	1-2	
KAT_1	"	"	0	
MIT_1	Acute Leukaemia	"	0	
MON_1	Cystic Fibrosis	"	0	
YUN_1	Placental Blood	F137	1	
MAC_1	"	GOL_1	0	
BAT_1	"	F137	0	
BAT_2	"	F137	0	
WEB_1	"	MAR_1	0	
WEB_2	"	MAR_1	0	
CLA_1	"	MAR_1	0	
CLA_2	"	MAR_1	0	
CLA_3	"	MAR_1	0	
CLA_4	"	F137	3-4	
LAM_1	"	MAR_1	0	
LAM_2	"	MAR_1	0	
WHE_1	"	F137	0	
LAW_1	Chronic Myeloid Leukaemia	MAR_1	0	
LAW_2	"	F137	1	
SHA_1	"	MAR_1	1	
SHA_2	"	MAR_1	1-2	

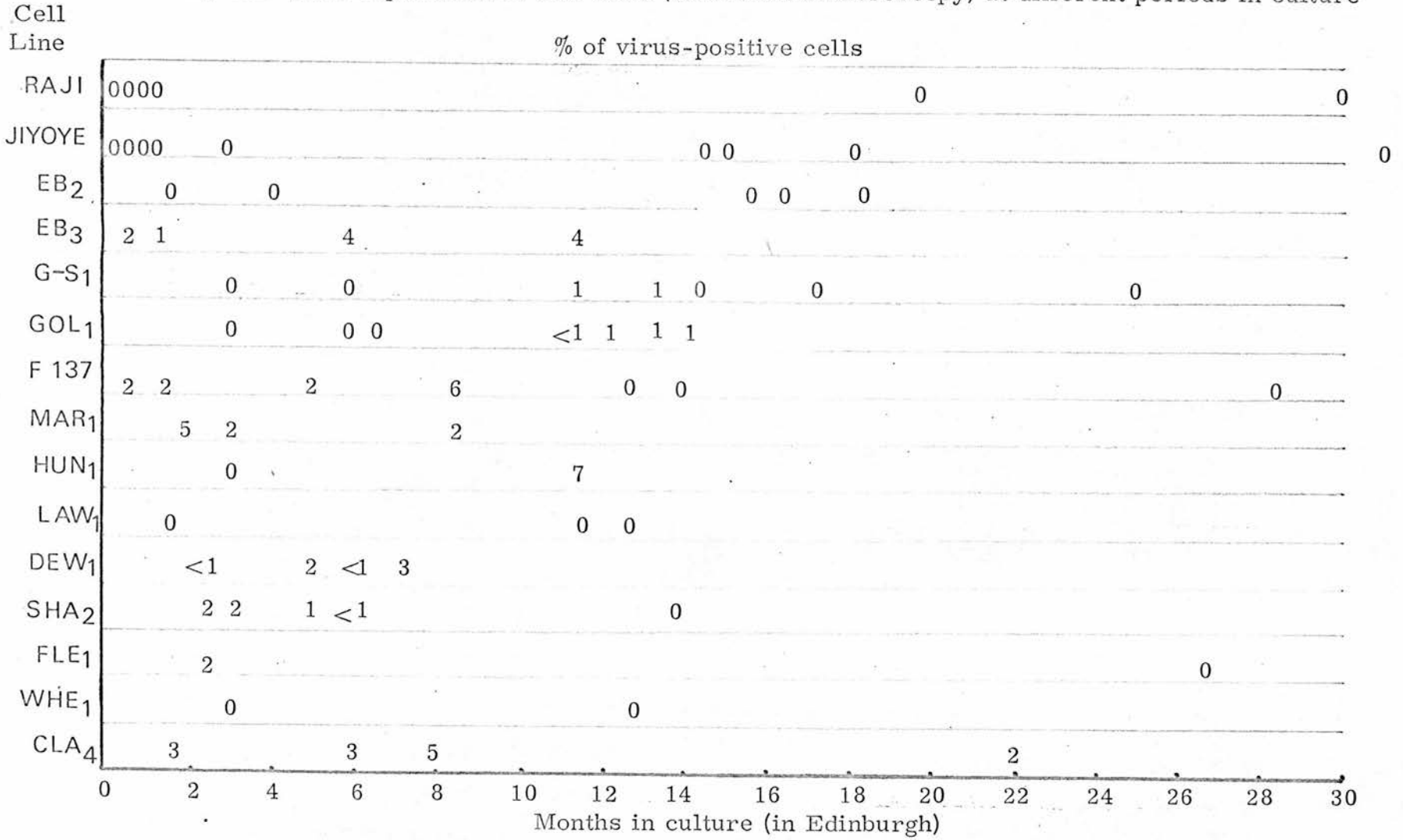
TABLE XII - cont'd

ART_1	Chronic Myeloid Leukaemia	$G\text{-}S_1$	0
ORI_1	Healthy adult	F137	1-2
LEO_1	Child, Hydronephrosis	F137	1-2
LEO_2	"	F137	3-5
BLA_1	Acute Leukaemia	F137	0
ODY_1	Waldenstroms Macroglobulinaemia	F137	0

/ In Amphotericin ·· Yeast infection.

explanation, it should be noted that the three very long-established lines reported to be virus-negative when first introduced into this laboratory (RAJI, JIYOYE and EB_2) remained consistently negative on repeated electron microscopic examination over periods of 19 to 33 months (table XIII). Furthermore, the findings in relation to the $G\text{-}S_1$ line suggest that the appearance of virus in this culture was influenced by the type of serum used as medium supplement. The line was grown initially in medium with 20% allogeneic human serum and was apparently virus-negative at 3 and at 5 months in culture. From the parent line, two sublines were subcultured into medium with 20% foetal calf serum in place of human serum, the first after seven months, the second after nine months *in vitro*. Virus particles were seen in the first subline two months after separation from the parent line and a similar finding was made in the case of the second subline. A virus-positive sample from the first subline was returned to medium supplemented with human serum and within one month it was found to be virus-negative by immunofluorescence and by electron microscopy. After a total of 24 months in culture, the second subline was also scored as virus-negative on electron microscopy although foetal calf serum had been employed continuously as medium supplement from the ninth month.

Table XIII. EB virus replication in cell lines (on electron microscopy) at different periods in culture



In GOL_1, HUN_1 and in lines reported by other authors (Miller, Enders, Lisco and Kohn, 1969; McCormick, Mumford, Stenback and Trentin, 1971) the appearance of EB virus particles among cells previously regarded as virus-negative has not been related to any obvious change in culture conditions. Moses, Glade, Kasel et al. (1968) have reported the appearance of virus particles in one line following lethal irradiation of the cells. I have therefore examined, by electron microscopy, samples from JIYOYE, RAJI, EB_2 and F137 (following the apparent 'loss' of virus), taken on days 0, 4, 8, 12 and 16 post-irradiation (3000 rads delivered as described in Chapter III). In no case did I find any virus particles, though the material from days 12 and 16 post irradiation was in such an advanced state of degeneration that identification of virus, if present, among the cellular debris would have been extremely difficult.

Taken as a whole, the findings in the cultures which I have examined are consistent with the view that inability to detect EB virus particles does not mean absence of the viral genome from that cell line, not only because some apparently virus-free lines were found to be virus-positive at a later date but, more significantly, because many of the lines grown by co-cultivation did not release virus particles though the evidence presented in Chapter III indicates strongly that infection with EB virus was necessary before they could become established in culture.

Discussion

Although nothing is known of the mechanisms whereby the EB virus confers on human lymphoblastoid cells the capacity for long-term growth and replication in vitro, it is clear that this function is not dependent on the persistance in culture of cycles of virus replication. This situation is probably analogous to the phenomenon of infection of non-permissive hamster kidney cells by SV40 virus, in which morphological transformation of the cells is induced by non-replicating virus, the entire viral genome being present in the transformed cells (Sabin & Koch, 1963; Black, 1966).

In the case of EB virus in human lymphoblastoid cells, a delicate balance seems to be achieved between promotion of cellular growth and virus replication. Where a cell line begins to release increasing quantities of virus, the viability of the line may be prejudiced (Henle, Diehl, Kohn et al. 1967) and if excessive quantities of virus are used to infect human leukocytes or established lymphoblastoid lines in vitro, the result is total lysis of the cells (Durr, Monroe, Schmitter et al., 1970; Horoszewicz, Dunkel, Avila & Grace, 1970). The consistent failure to find any virus particles in the long-established RAJI line (Epstein, Achong, Barr et al., 1966) suggests that replication of EB virus serves no useful purpose once a line has begun to grow. In a sense, therefore, the tendency for the percentage of virus-producing

cells in a given line to decline with age of the line in culture (exemplified by the observations in EB_2, JIYOYE, F137 and probably also $G\text{-}S_1$), may be an indication of increasingly successful adaptation of the cells to the conditions of growth *in vitro*.

CHAPTER V

Morphology of cultured lymphoblastoid cells by light and electron microscopy

In spite of the apparent diversity of lymphoblastoid cell lines, in terms of diagnosis of donor, tissue of origin and technique used to initiate each culture, there is general agreement that, morphologically at least, the cells of one line cannot readily be distinguished from those of any other (Chandra, Moore & Brandt, 1968; De Harven, 1967; Gerber & Monroe, 1968; Moore, Kitamura & Toshima, 1968). This has been my experience also. In this chapter, therefore, I shall not attempt to describe the morphological features of the cells line by line but rather to illustrate the main characteristics of all the lines, referring to individual cultures only when they show some deviation from the general picture. All the cell lines described in chapters II and III have been examined both by light and electron microscopy. In addition I have examined cells from line YUD_1, grown by Miss Karin Buckton from the peripheral blood of a 21 year old female patient with infectious mononucleosis.

Methods

Light microscopy

Two methods of fixation and staining of lymphoblastoid cells for light microscopy have been employed.

a) Air dried smears

In this technique, 1-5 ml of a growing suspension culture containing 2-8 x 10^5 viable cells/ml, was transferred to a centrifuge tube and spun down at 200 x g for 5 minutes. The supernatant medium was discarded and the cell button resuspended in 1 drop of bovine serum. A small drop of the suspension was placed on one end of a clean glass slide and a thin smear made by means of a glass spreader. Smears were dried rapidly by waving the slides in the air, fixed for 6 minutes in absolute methanol then stained as follows:

6 mins in May Grünwald (Gurr) stock solution freshly diluted 1 + 3 with buffered distilled water, pH 6.8.

8 mins in Giemsa (Gurr) stock solution freshly diluted 1 + 9 with buffered distilled water, pH 6.8.

The stain was rapidly washed off with distilled water and the slides allowed to stand in fresh buffered distilled water (pH 6.8) for 2 minutes before being blotted dry and dipped in Xylol. A coverslip was then applied on De-pex mountant (E.Gurr Ltd.) and the slides dried on a horizontal heated surface.

b) Cells fixed in suspension

This method was suitable for rapid examination of very small quantities of cells.

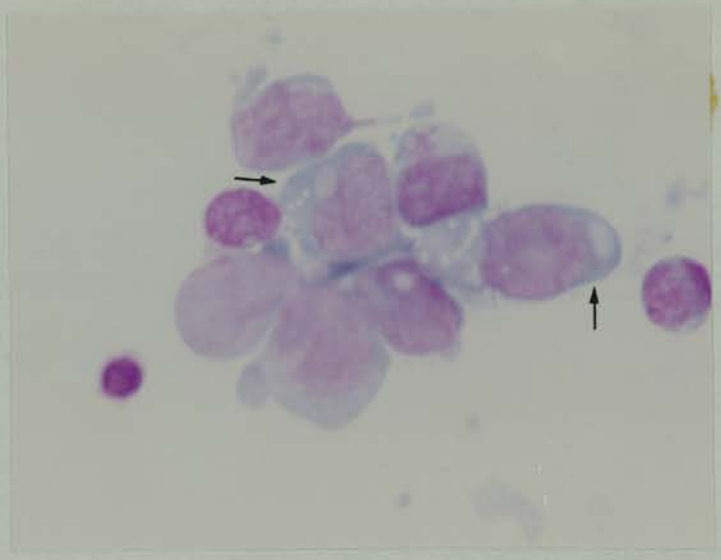
A single drop was removed by means of a Pasteur pipette, from among the sedimented cells at the bottom of a growing

culture. This was transferred immediately to a centrifuge tube containing 5 ml of fixative (3 parts absolute methanol + 1 part glacial acetic acid) and the cells dispersed by repeated aspiration into the pipette. The suspension was spun down at 200 x g for 5 minutes in a bench centrifuge and the supernatant fixative discarded, leaving only 1 drop in which the cells were resuspended. If the suspension appeared very cloudy (i.e. contained very many cells) further drops of fixative were added until it was opalescent. Drops were then placed on clean slides and allowed to spread out and dry.

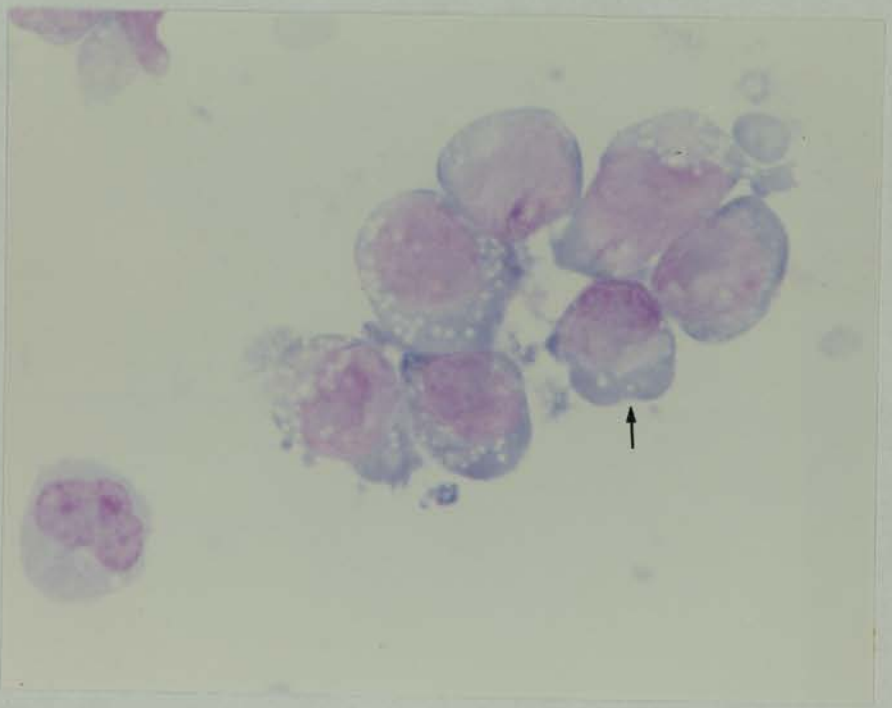
The slides were stained and mounted as described above for 'air-dried smears'.

Findings

The appearance of stained cells was strongly influenced by the method of fixation adopted. In air-dried smears (see fig.12) the nucleus was usually oval, but sometimes kidney shaped or indented. It contained one to four readily visible nucleoli. The cytoplasm stained pale blue in most cells though in a few cases it appeared to be a deeper blue, particularly in cells which were smaller than average with a round nucleus and which therefore resembled plasmablasts (see fig.12 and compare McDonald, Dodds & Cruikshank, 1965). Small numbers of such cells were present in every line. The clear areas scattered

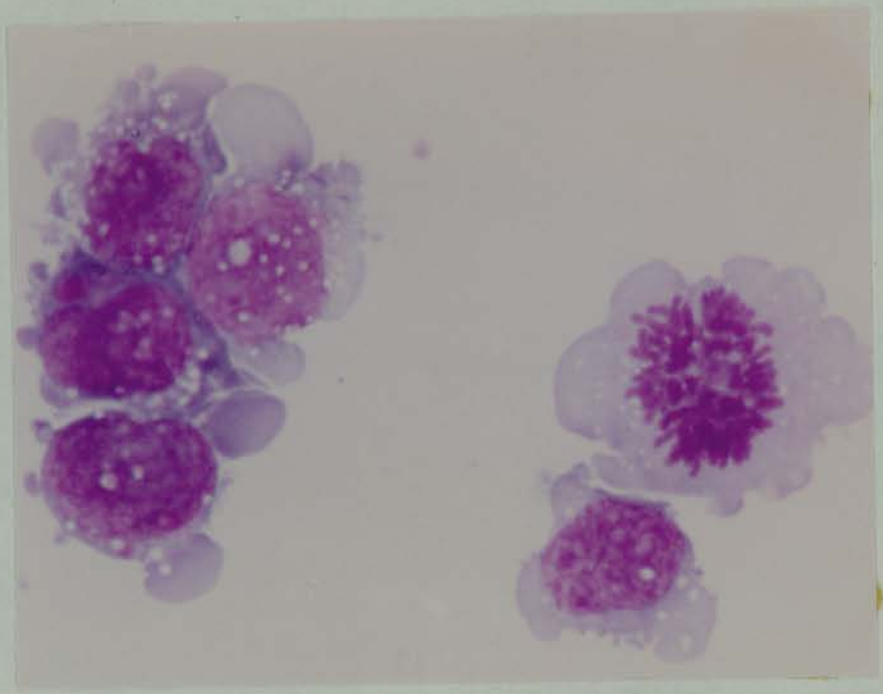


12a. WEB_1 cells. Air-dried smear. May-Grunwald-Giemsa. x 1100

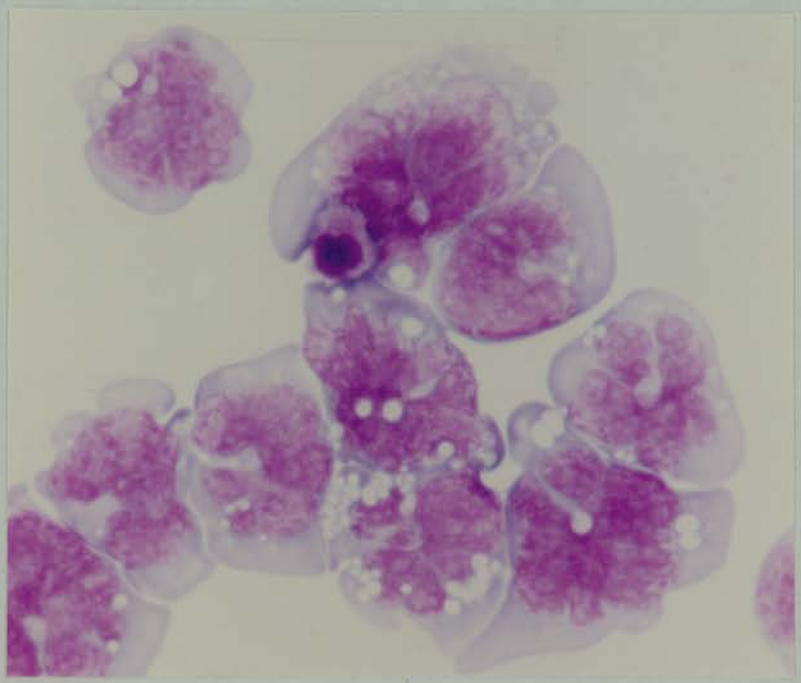


12b. $G\text{-}S_1$ cells. Air-dried smear. May-Grunwald-Giemsa. x 1,100

Note plasmablast-like cells (arrowed) in both figs.



12c. FLE_1 cells (near-tetraploid). Air-dried smear
One cell is in mitosis.
May-Grunwald-Giemsa. x 1100



12d. EB_2 cells (near-tetraploid). Air-dried smear.
Note bizarre nuclear outlines.
May-Grunwald-Giemsa x 1100

throughout the cytoplasm and even over the nucleus of many cells are thought to represent lipid droplets whose contents have been leached out by the fixative (Epstein, 1970). In general the cytoplasm appeared to be most abundant in those cells with multiple indentations of the nucleus and least abundant in those with round or oval nuclei. All lines contained a range of cell types from small (10 μ diameter) with round nuclei up to very large (20 μ diameter) with indented nuclei, but it is uncertain whether this represents a truly heterogeneous population. Evidence from cytogenetic studies (see Chapter VI) suggests that at least some of the lines are monoclonal, and this view was supported by observations on immunoglobulin production by the cultures (Nilsson, 1971) so that much of the variation in morphology is probably due to the different stages of the cell cycle represented at any one time in a non-synchronous mass culture. However, the possibility is not entirely excluded that a very small proportion of the total population is comprised of cells of a different type and with a different origin from the majority, or that morphological variants may arise regularly from the cells of the parent line, such variants having a limited life-span in vitro.

It is noteworthy that cells of the EB_2 line had much more prominently indented nuclei and were, on average, considerably larger than those of other lines. In part, this may be attributable to the fact that the EB_2 line has become polyploid after many years of growth in vitro (chapter VI) but lines GOL_1 (2) and FLE_1, which

have also become polyploid, did not show particularly prominent nuclear indentations (figure 12). Electron micrographs of the EB_2 line made shortly after it was established (Epstein, Barr and Achong, 1965) suggest that the unusual nuclear outline has been a characteristic of these cells from a very early stage in culture.

The cytoplasm of cells fixed in suspension is very poorly preserved but the nuclear shape is well displayed and probably less distorted than in smear preparations (Fig.13). It is apparent that few cells have a perfectly spherical or ovoid nucleus and within most lines there is a range from the slightly indented to the multilobed form. The nuclei of virtually all EB_2 cells present a bizarre polymorph-like appearance. When samples from the same cell line were compared after different periods of growth in vitro, there was no indication that lobulation of the nucleus tended to increase with time within the range of three months to two years.

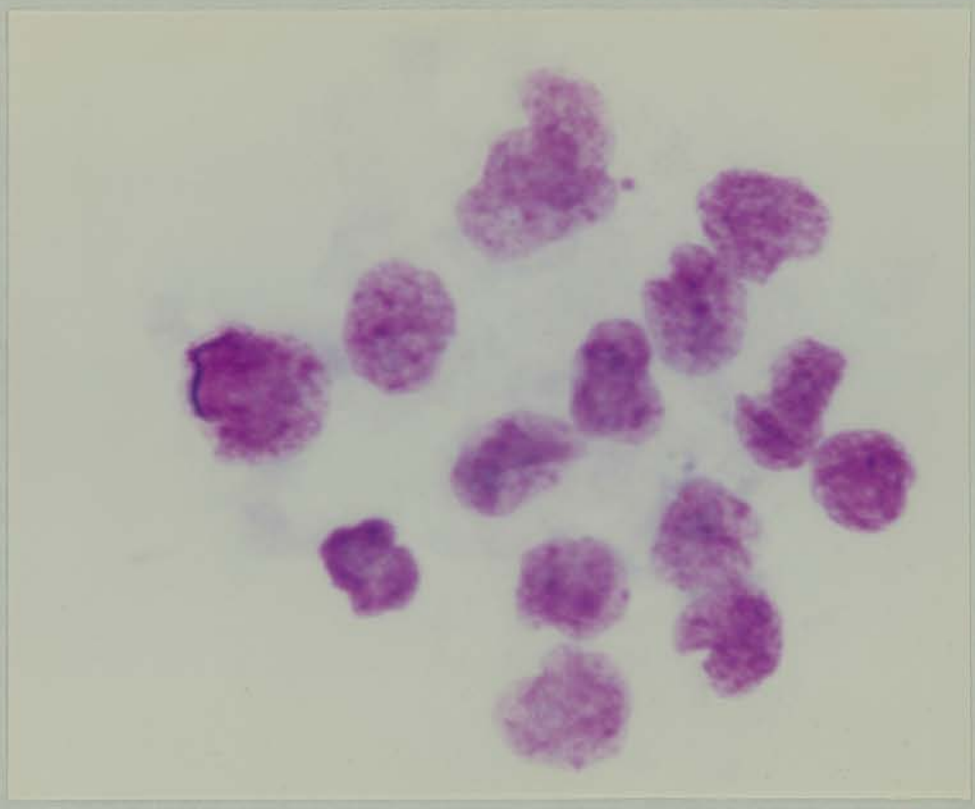
Electron Microscopy

Methods

The preparation of cell pellets for E.M. examination has been described in Chapter IV.

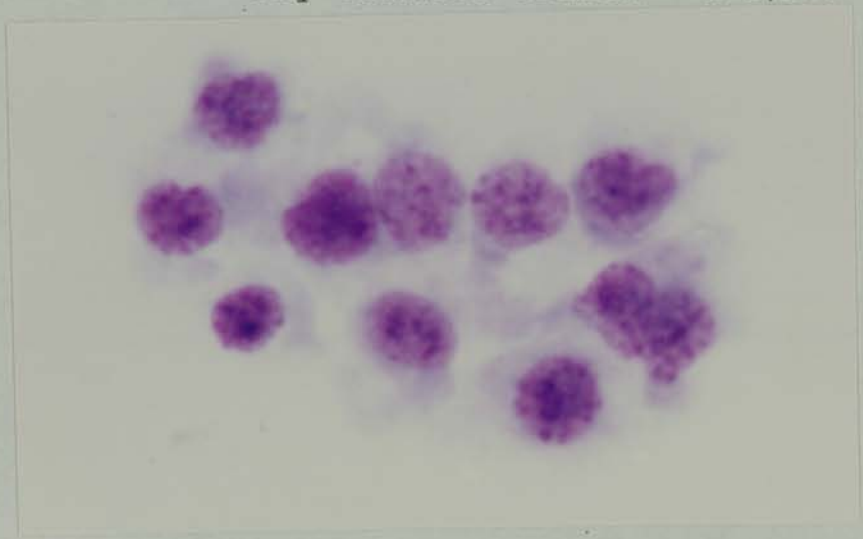
Findings

As in the case of light microscopy, electron microscopy reveals a range of appearances within the cells of any



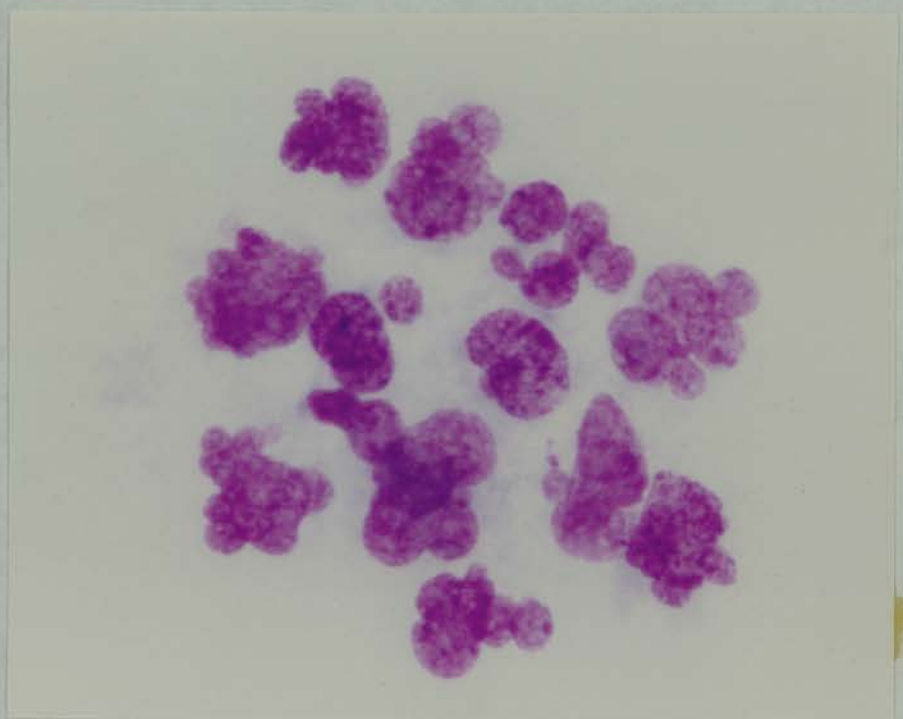
13a. Cells from line GOL_1 (polyploid) fixed in suspension.

May-Grünwald-Giemsa. x 1,100



13b. Cells from line GOL_1-4299 (near-diploid) fixed in suspension. Note that nuclei are smaller and less indented than in GOL_1 (polyploid).

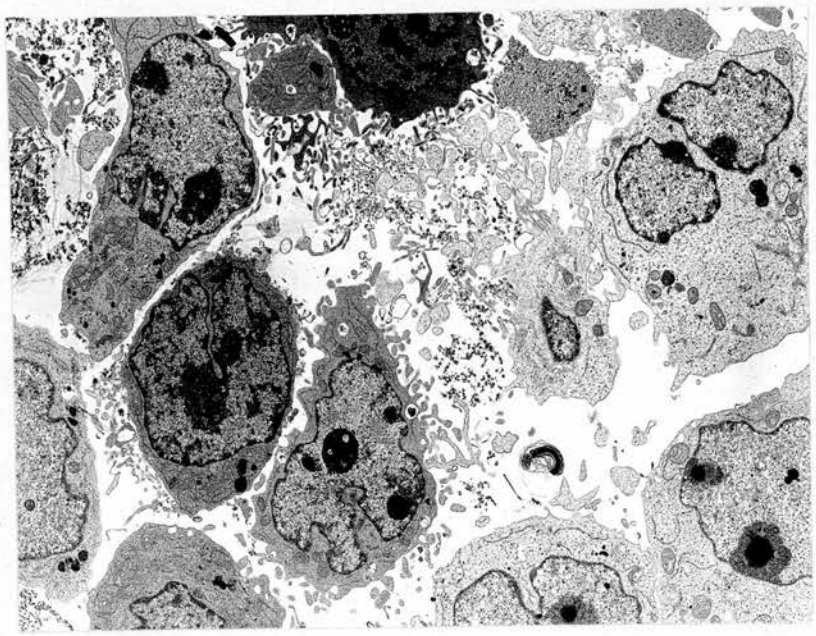
May-Grünwald-Giemsa. x 1,100



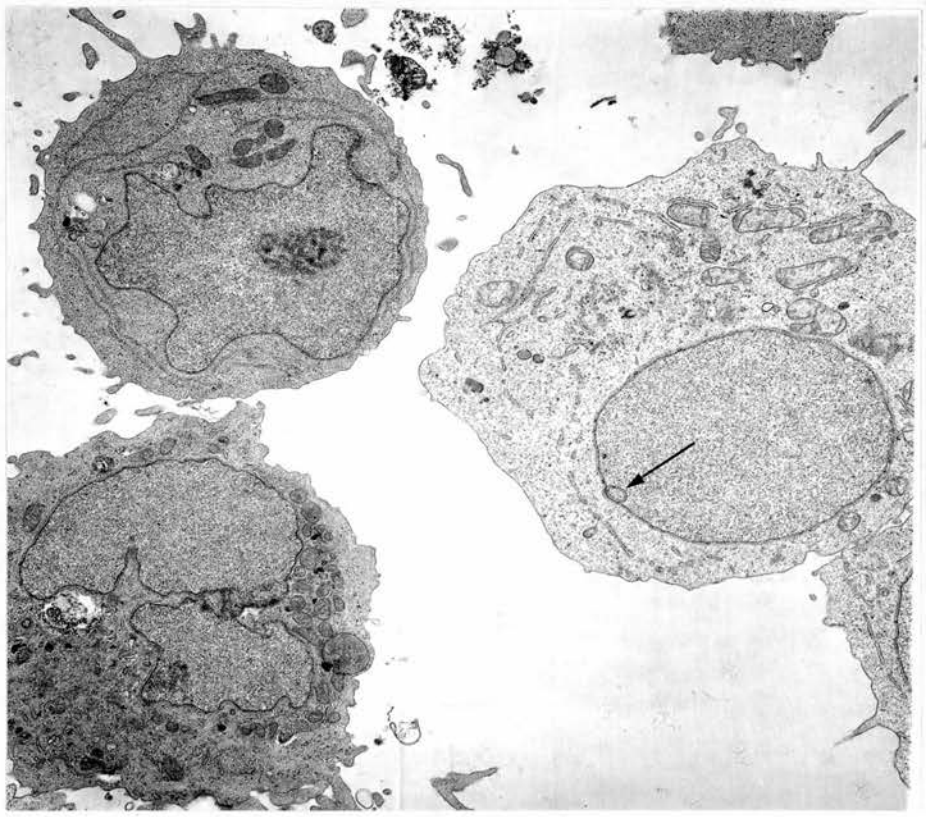
13c. Cells from line EB_2 (near-tetraploid) fixed in suspension. Note bizarre nuclear shapes.

May-Grünwald-Giemsa. x 1,100

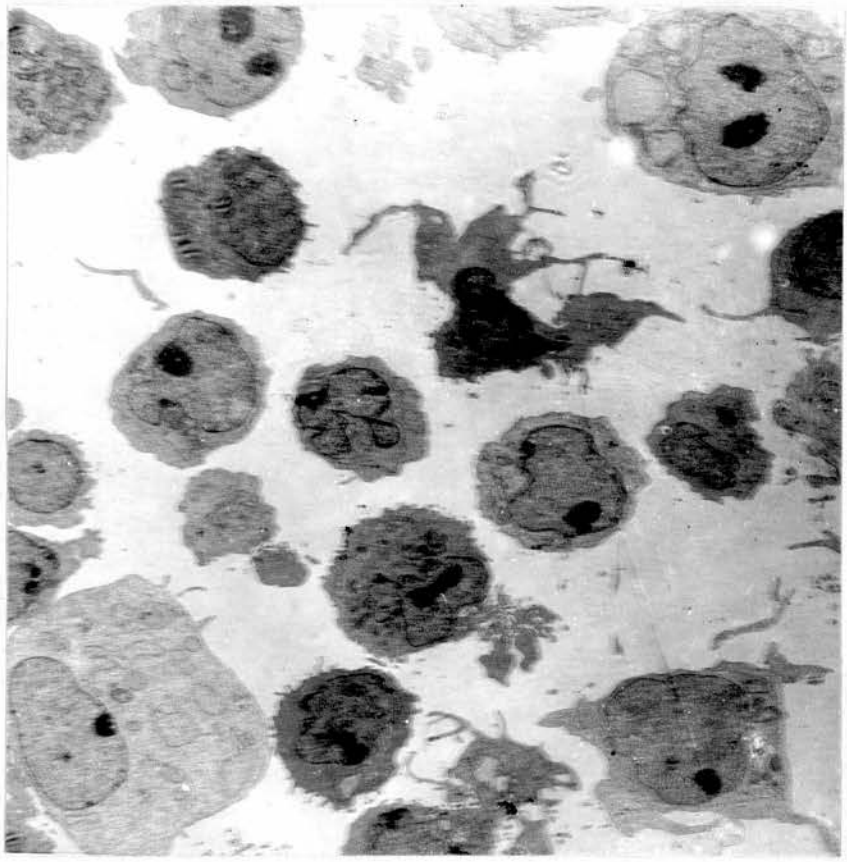
one line (figs.14-16). Some of this variation is clearly due to the presence of cells which are beginning to degenerate and some presumably reflects the differing stages of the growth cycle at which the cells have been fixed. The most striking difference between cells is in the overall degree of electron density in both nucleus and cytoplasm. The shape of the nucleus varies considerably, as anticipated from the light microscopy findings though the E.M. preparations which consist of thin slices through individual cells, are not ideal for demonstrating this phenomenon. Within the nucleus, the chromatin often shows one or more areas of nucleolar condensation and in a few cell very dense patchy consolidation of the chromatin is observed, particularly at the periphery of the nucleus (fig.17). Such cells usually show other evidence of degeneration, but unlike Epstein, Henle, Achong & Barr (1965) I have found that they rarely contain visible EB virus particles. Epstein and his colleagues (Epstein, Achong, 1965; Achong & Epstein, 1966) have described a characteristic appearance of the nuclear membrane in cultured lymphoblastoid cells from Burkitt's lymphoma biopsies. The membrane is thrown into projections forming a simple loop or a more complex laminated structure, within the folds of which cytoplasm is enclosed. An identical appearance has been noted in cell lines from patients with leukaemia (Uzman, Foley, Farber & Lazarus, 1966) and in those from the peripheral blood of healthy adults



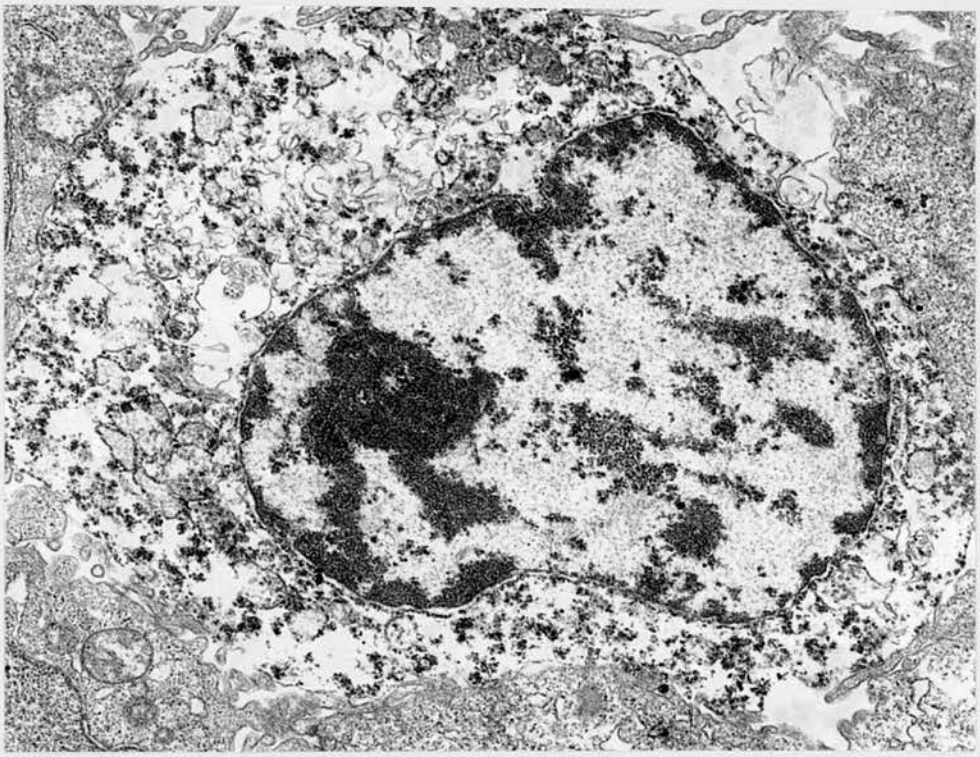
14. Cells from line BAT_2. x 3,400.
Note variation in cell size, nuclear/cytoplasmic ratio and overall electron density.



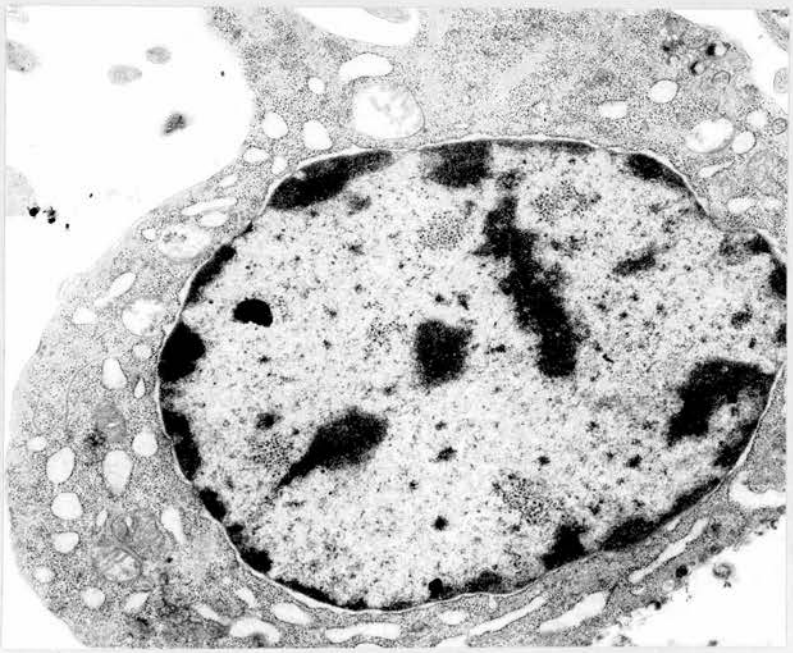
15. Cells from line LAM_1. x 4,700.
Note variation in nuclear outline and overall electron density. A nuclear 'bleb' is arrowed.



16. Low-power view of cells from line SHA_2 showing variation in size, cell outline and overall electron density. x 2,400



17 a) Cell from line SHA_2. x 11,000.

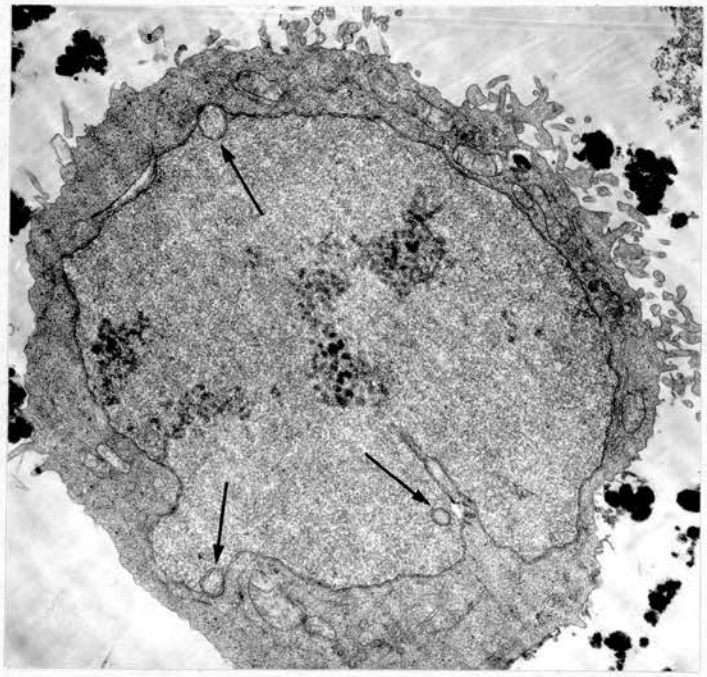


17 b) Cell from line $G\text{-}S_1$. x 10,500.

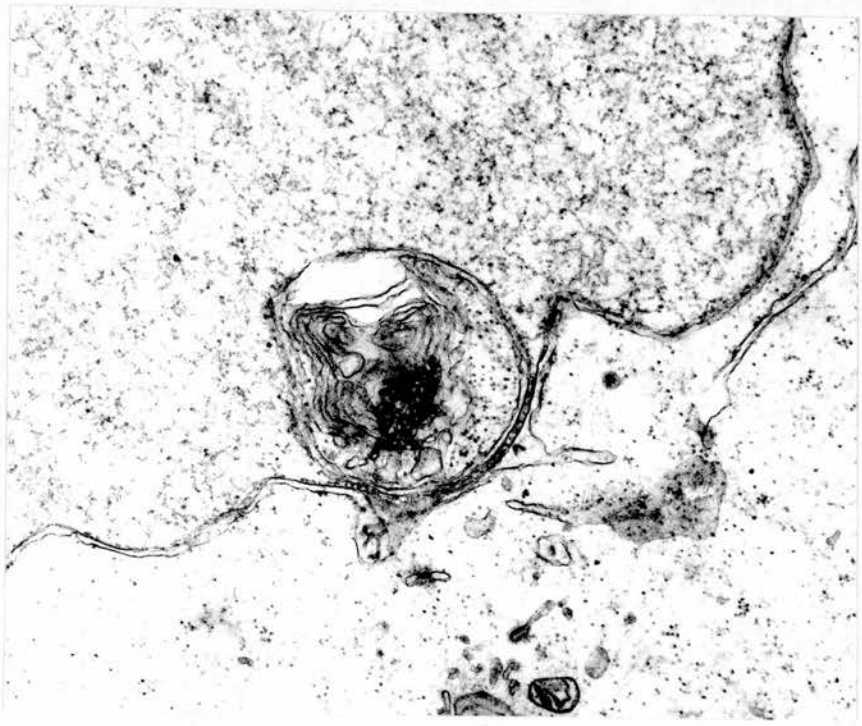
Both cells show evidence of impending disintegration. There is peripheral condensation of the nuclear chromatin but no EB virus particles are present.

(Gerber and Monroe, 1968). These nuclear projections are also present in directly prepared material from Burkitt's tumour biopsies and in a recent review Epstein and Achong (1970) record numerous examples of their occurrence in a variety of cell types of both normal and malignant origin. I have identified this feature in a few of the cells from the Burkitt lymphoma lines obtained from other Centres, but it is exceptionally rare in my own cell lines (fig.18). Doubling of a part of the nuclear membrane does occur in a small percentage of cells from most lines, however (fig.19) and occasionally this process leads on to the formation of a bizarre "onion slice" structure (fig.20). On rare occasions, I observed the formation of dense rings or partial rings by the nuclear membrane of a degenerating cell (fig.21). This was almost always associated with the presence of EB virus particles in that cell and sometimes the thickening of the nuclear membrane appeared to form a type of envelope around a virus particle. The structure was, however, quite distinct from the typical envelope of mature virus particles as illustrated in Figs. 7-9. The appearance has previously been noted by Moore, Kitamura and Toshima (1968) and by Chandra, Korol, Ames et al. (1971).

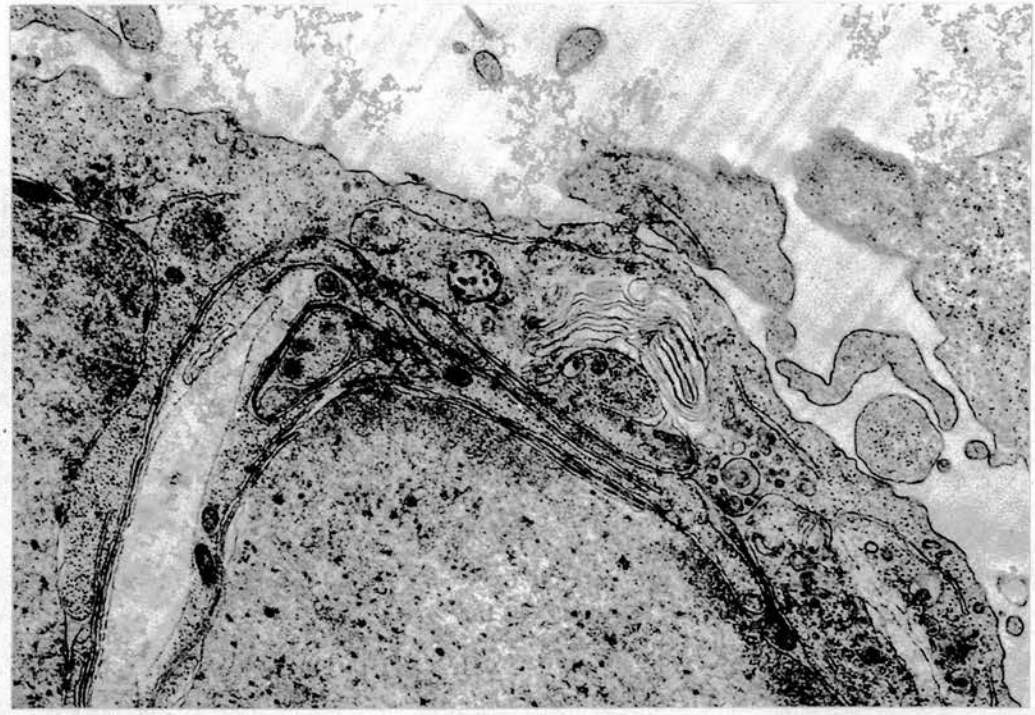
The cytoplasm is filled with free ribosomes and a variety of organelles, including in almost every case, a Golgi apparatus which is often very prominent (fig.22). Rough endo-



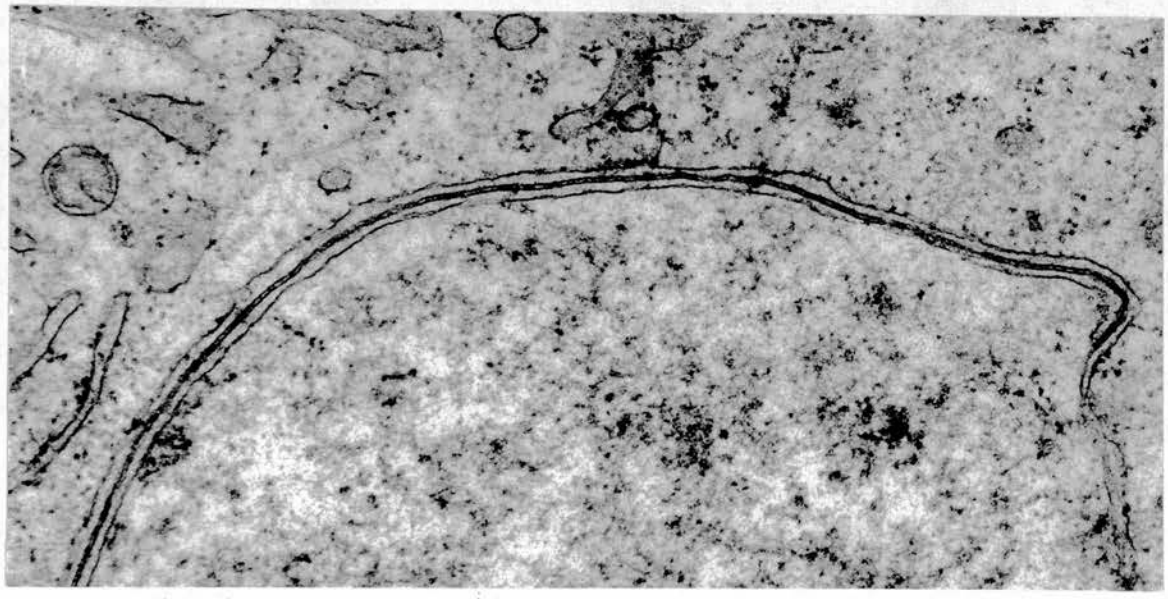
18 a) 3 nuclear 'blebs' (arrowed) in a cell from line EB_2. x 7,100.



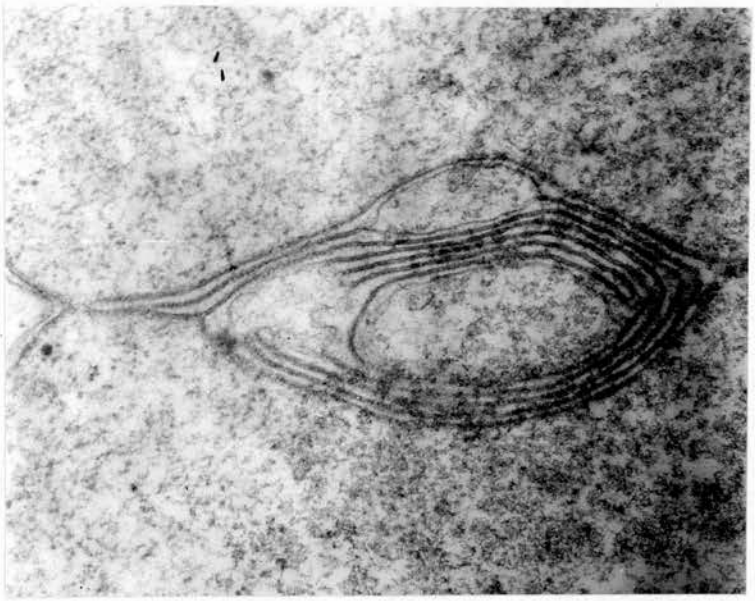
18 b) Nuclear bleb formed by invagination of nucleus by cytoplasm. The membrane-bound area within the nucleus contains cytoplasmic organelles (free ribosomes and microtubular aggregate). EB_3 cell. x 23,500.



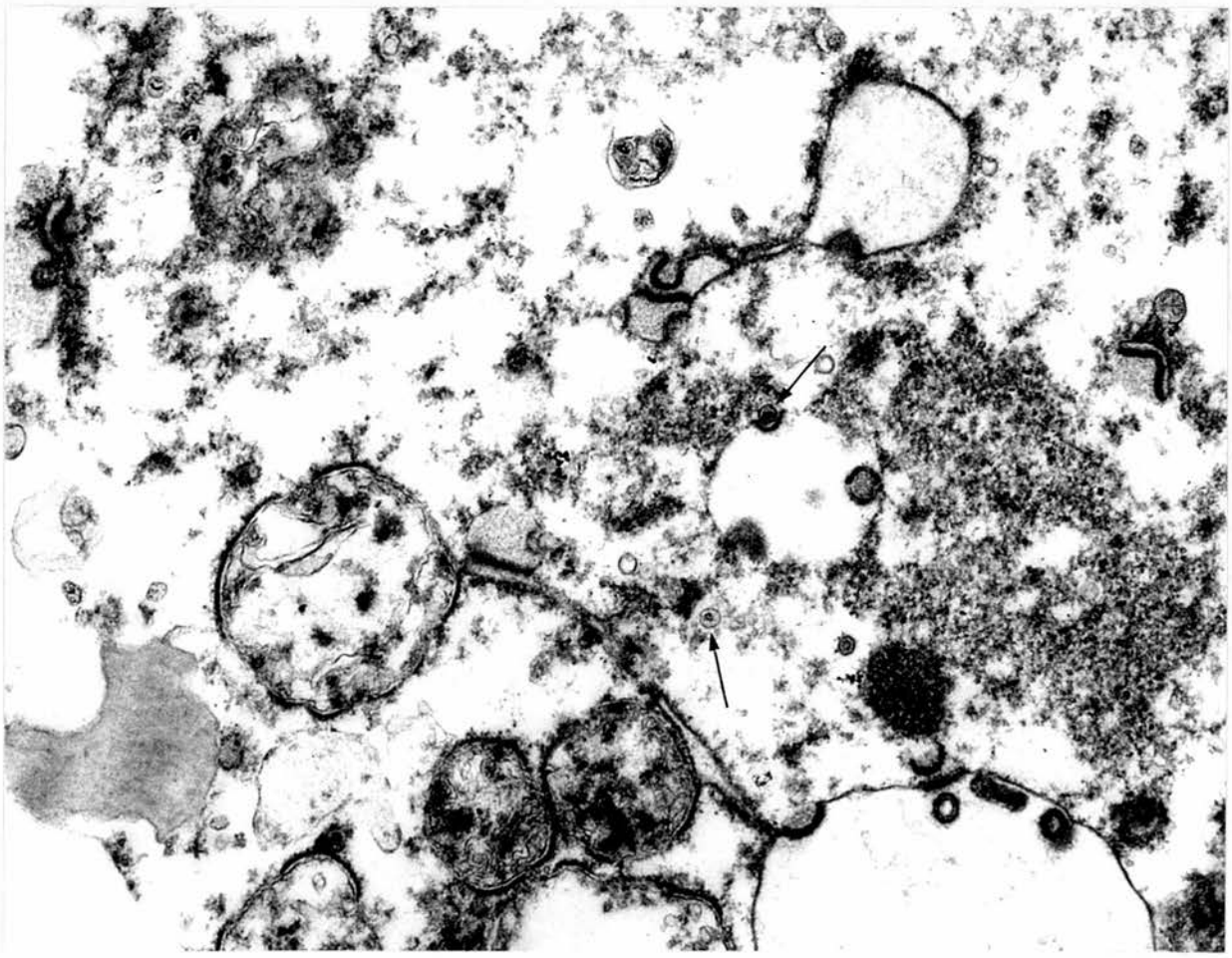
19a. Reduplication of nuclear membrane in a cell from line EB_3. x 13,800



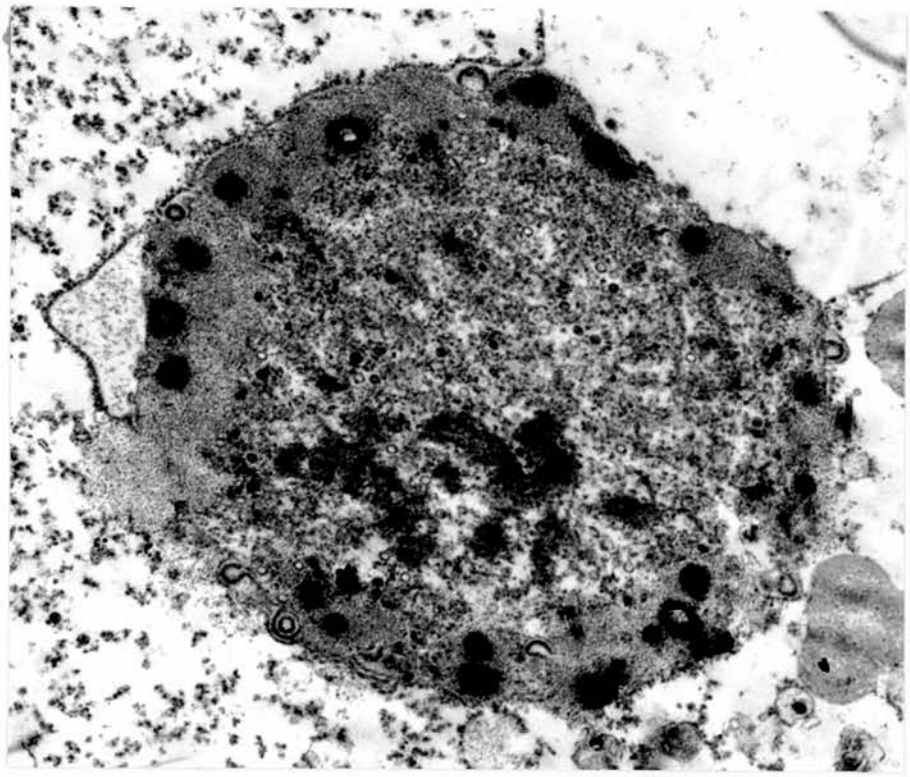
19b. Segmental doubling of nuclear membrane in a cell from line $G\text{-}S_1$. x 44,700.



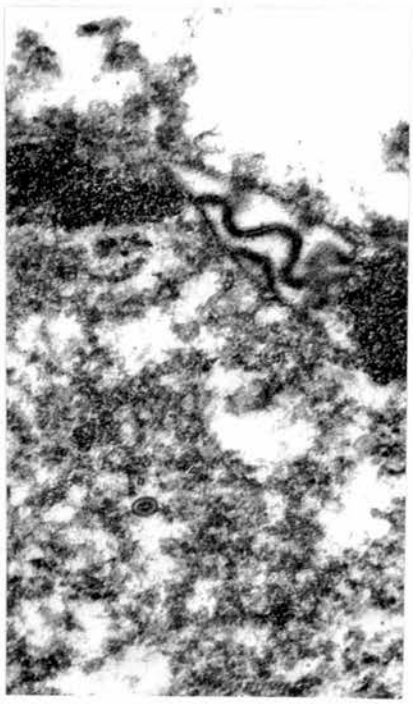
20. Multiple infolding of nuclear membrane forming "onion slice" structure. G-S_1 cell. x 40,000



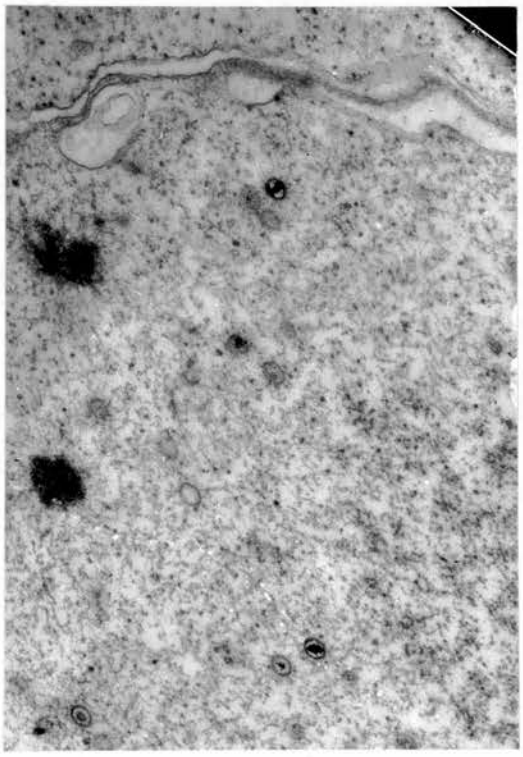
21a Multiple areas of dense membrane thickening within the nucleus of disintegrating SHA_2 cell. EB virus particles are also present (arrowed). x 27,800.



21b. Cell from line SHA_2. x 16,600

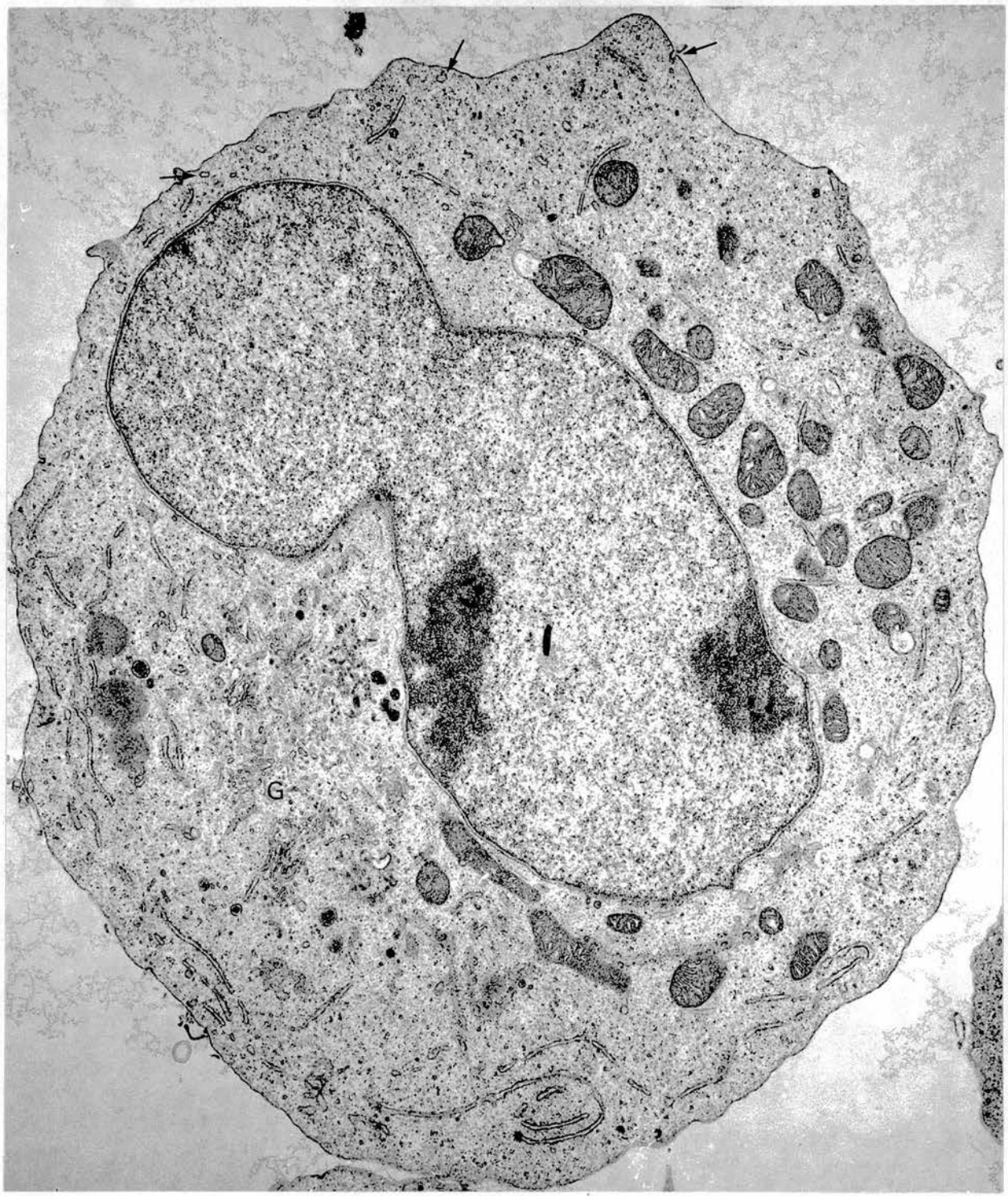


21c. Cell from line F137. x 43,400



21d. Cell from line MAR_1. x 32,000

All three pictures show areas of very dense thickening of the nuclear membrane, associated with the presence of EB virus particles within the nucleus.



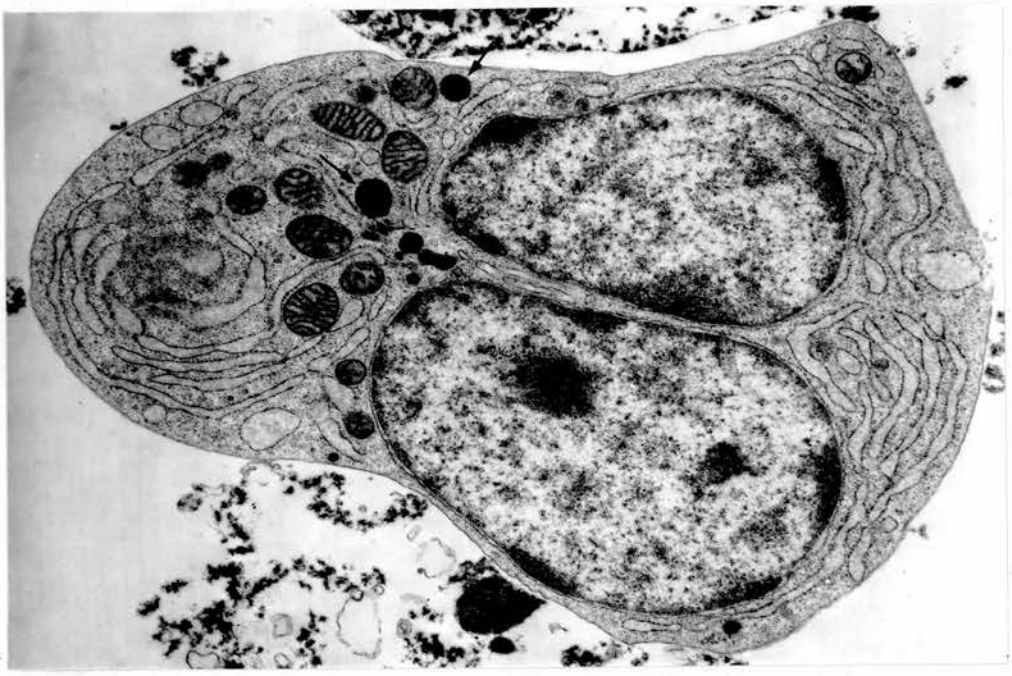
22. Cell from line CLA_4 showing prominent Golgi apparatus (G) and a number of pinocytotic vesicles (arrowed) x 15,000.

plasmic reticulum is always present, usually in scanty or moderate amounts, but occasionally is very abundant, concentrically arranged around the nucleus in a manner reminiscent of the mature plasma cell (fig.2?). Mitochondria are rounded or oval, rarely elongated. They are usually found in large numbers (up to 20 in a single cell section) and sometimes, though not invariably, grouped at one pole of the cell (see figs.14-16). Round osmiophilic bodies, presumably composed of lipid, are frequently seen (fig.24). Their membrane is impossible to define because of the electron density of their contents. Most cells display small pinocytotic vesicles near the external surface (fig.22) and in all lines a few cells are demonstrably phagocytic, having engulfed one or more dead cells from the culture (fig.25).

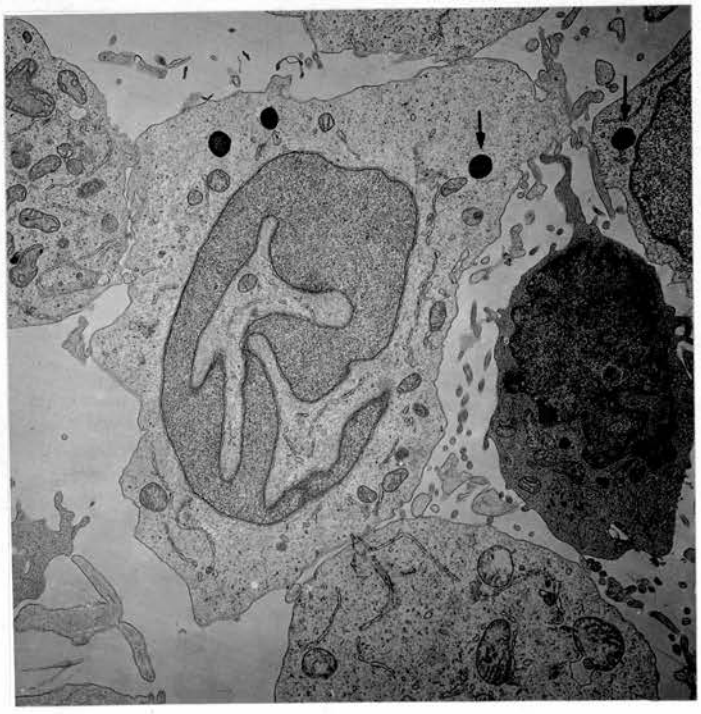
A number of other intracytoplasmic structures have aroused comment and controversy in the published literature because of their striking appearance, sometimes eliciting suggestions that they represent viruses or virus-associated material (Moses, Glade, Kasel et al. 1968). These include the following.

Annulate lamellae

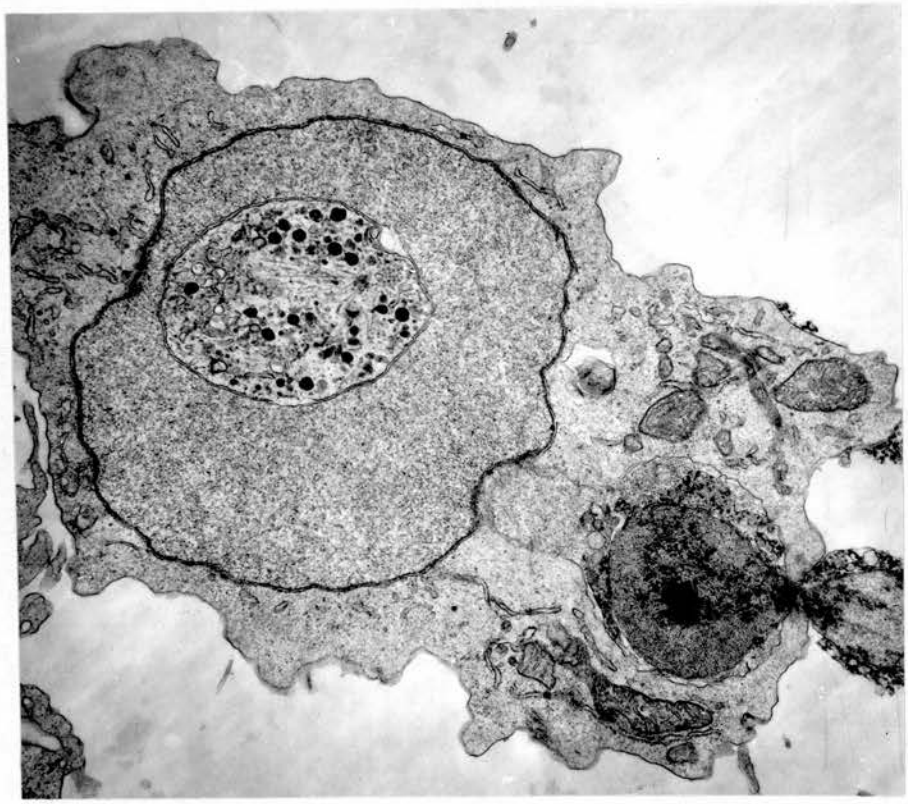
The common appearance of this structure (fig.26) is a stack formed of parallel layers of smooth endoplasmic reticulum arranged in pairs, the membrane being continuous at



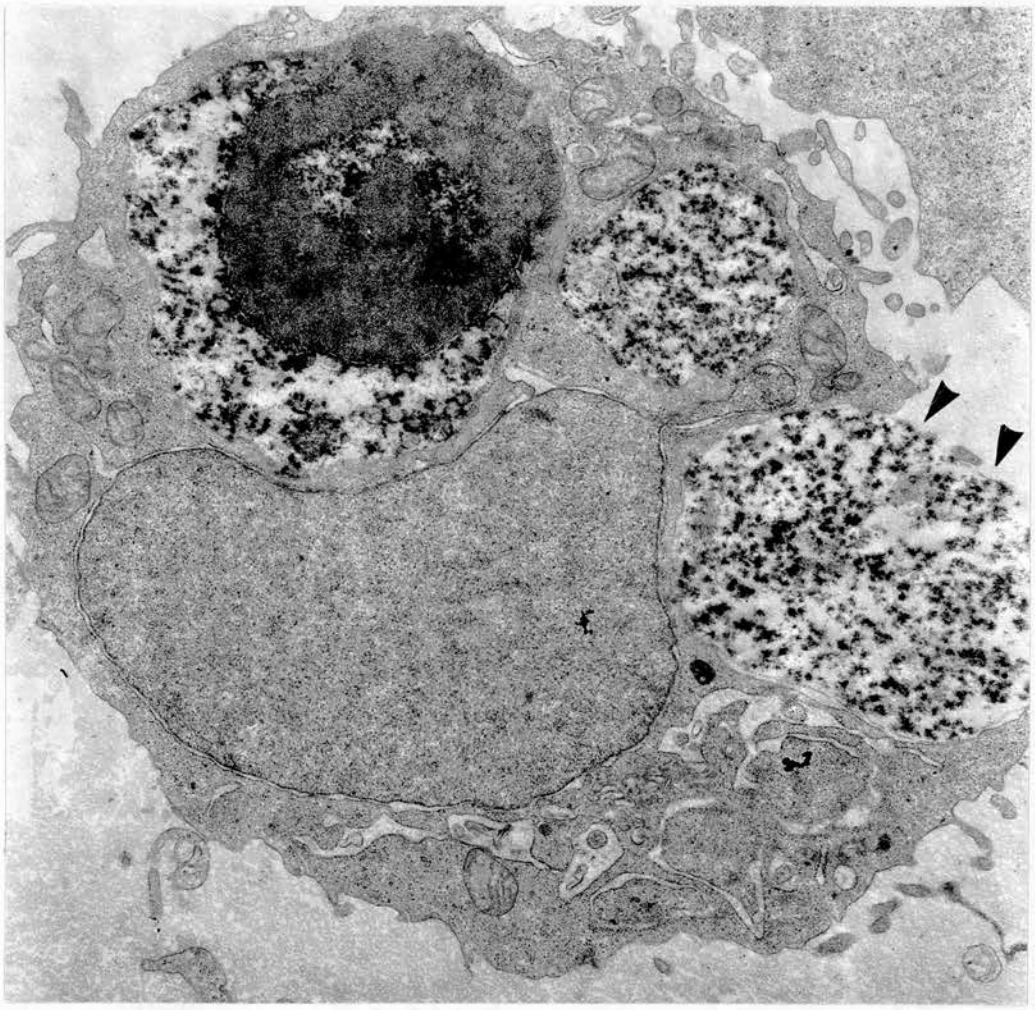
23. Cell from line SAD_1 showing highly developed rough endoplasmic reticulum arranged in layers parallel to the cell surface. The cell also contains lipid bodies (arrowed) x 9000.



24. Cells from line ORI_1 showing variation in size, nuclear shape and overall electron density. Lipid bodies in 3 cells are arrowed x 4,300.



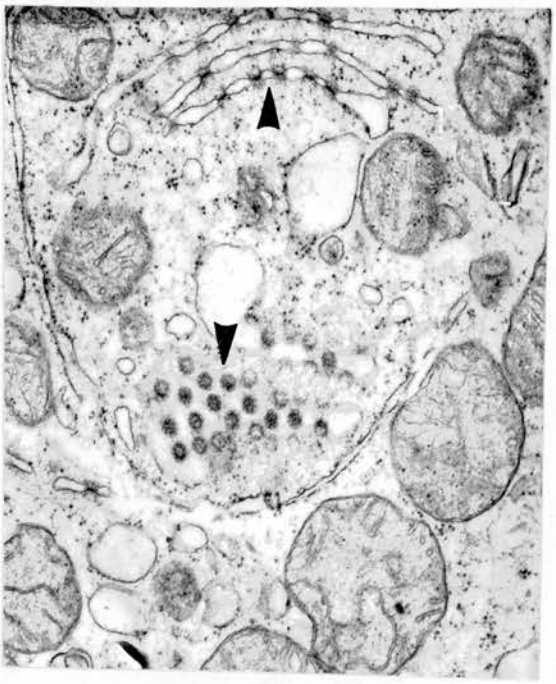
25a. Cell from line LAM_2 showing active phagocytosis. One cell is partially engulfed and the nucleus of the phagocytic cell appears to be invaginated by a vacuole containing cellular debris, including lipid bodies x 8000.



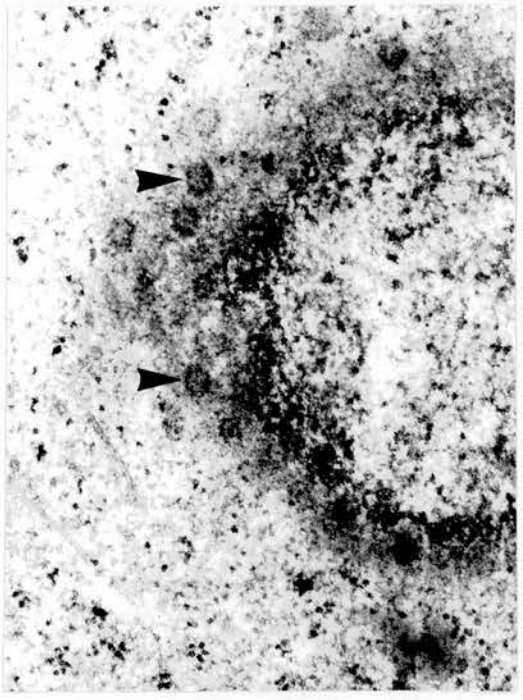
25b. Giant phagocytic cell from line SHA_1. Vacuoles contain the remnants of 3 cells at varying stages of digestion. One vacuole appears to be discharging its contents since it is in direct communication with the exterior (arrows). x 8,800.

the lateral extremity of each pair - i.e. in effect a series of flattened vesicles. The opposed surfaces of each vesicle form adhesions at fairly regular intervals of about 100 mμ, each adhesion also occupying about 100 mμ. There is an increase in electron density at each adhesion though a detailed structure cannot be discerned when cut in this plane. Occasionally however an annulate lamella is sectioned in the plane of one of the flattened vesicles and the adhesions are seen "en face" (fig.26) when they are shown to have a hexagonal or circular outline, sometimes with a small central density. This appearance is very similar to that of a nuclear pore (fig.27). Proof that these aggregates of circular organelles do in fact represent areas of adhesion between the membranes forming an annulate lamella is obtained on the rare occasions when (possibly due to distortion of the structure during fixation) a transition can be seen from one plane to the other in a single section (fig.28).

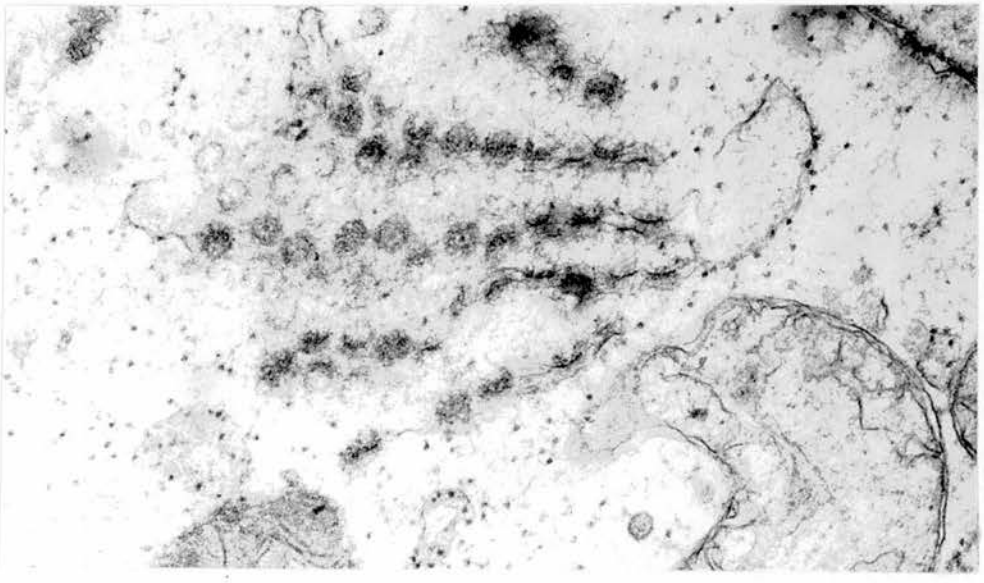
Epstein and Achong describe annulate lamellae in the EB_1 line of Burkitt tumour-derived lymphoblasts (Epstein & Achong, 1965) and stressed the association of this structure in other situations with primitive or malignant cells and with developing germ cells (Palade, 1956; Swift, 1956; Rebhun, 1956; Epstein, 1957; Epstein, 1961; Ross, 1962; Chambers & Weiser, 1964; Goldstein, 1971). In a more recent review (Epstein and Achong, 1970),



26. Annulate lamellae. 2 views of the structure (arrowed) in JIYOYE cell x 24,000



27. Nuclear pores (arrowed) in F137 cell x 40,000 Note similarity to structure of annulate lamellae



28. Annulate lamella in MAR_1 cell x 40,000 Plane of section appears to change, in continuity, through 90°.

N.B. For comprehensive review of fine structure and significance of annulate lamellae see Wischnitzer (1970).

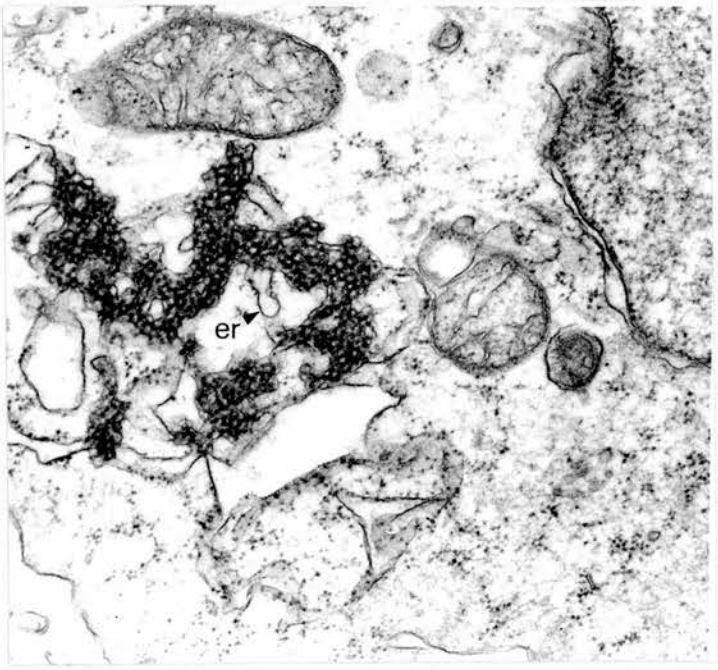
the same authors point out that annulate lamellae have now been found in the cells of the adult human apocrine sweat gland (Gross, 1966) but they nevertheless affirm that they are most characteristic of cells dividing rapidly or with a potential to divide rapidly.

Since annulate lamellae have not been described as a feature of the normal haemopoietic cells of any species, their presence in cultured human lymphoblastoid cells may therefore be taken as an indication of the relatively primitive state of these cells. Gerber and Monroe (1968) regularly found annulate lamellae in lymphoblastoid cell lines derived from healthy adults. I have also found them in every cell line examined, regardless of origin though they are rarely present in more than 2% of the cells and are often less prominent than those illustrated in fig.26.

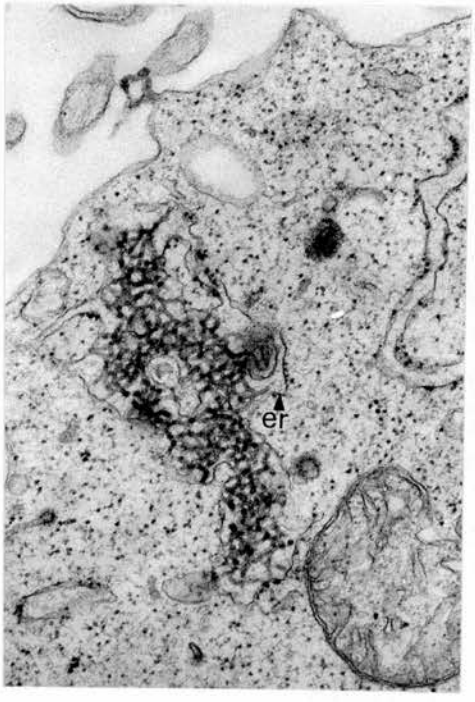
Microtubular aggregates

The appearance of microtubular aggregates is illustrated in figs. 29 to 31. The microtubules form a complex three-dimensional "Gordian knot" and when densely packed, the true nature of the subunit may not be readily apparent (fig.29), hence some of the confusion which has arisen in the literature and the misleading descriptions of "reticular aggregates of small cytoplasmic particles" (Moses, Glade, Kasel

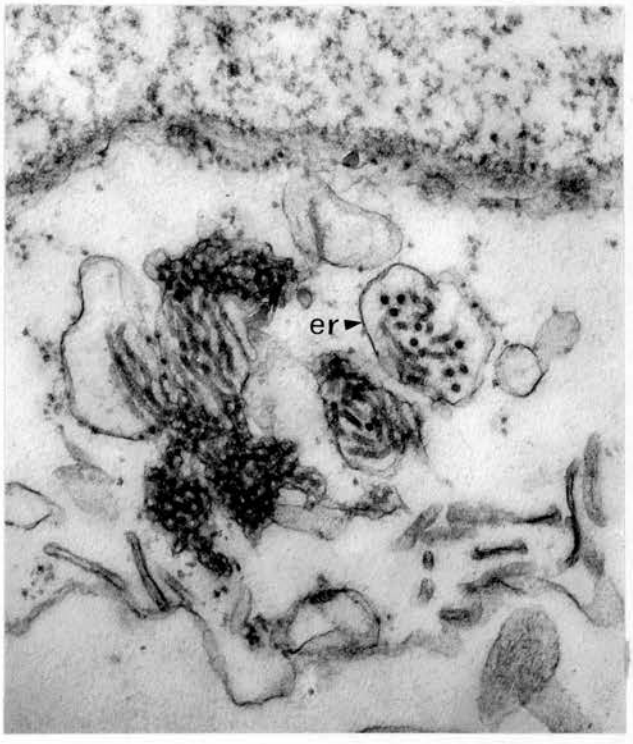
et al. 1968) and "aggregates of dense bodies" (Moore, Kitamura and Toshima, 1968). The aggregate is invariably surrounded by a membrane continuous with the endoplasmic reticulum. In cells which appear to have swollen during fixation, the convolutions of the microtubules may be straightened and some detail of the structure revealed (Figs.30,31). The microtubules have a diameter of 20-22 mμ. In my own preparations I have seen no evidence of regularity in the arran ement of coiling, but a regular "crystalline array" or "lattice" pattern formation by these microtubules has occasionally been seen by others (Pope, Achong, Epstein and Biddulph, 1967; Chandra, Brown, Aldenderfer et al., 1969; Chandra, 1968). Microtubular aggregates could be found in a small proportion of the cells (1-2%) of the majority of my lines, including some from infectious mononucleosis patients, from leukaemia patients, and from placental blood samples. Their significance is unknown and they are by no means an exclusive feature of cultured lymphoblastoid cells since an identical structure has been found in the luminous cells of the polynoid worm elytra (Bassot, 1966), in cultured mouse liver cells infected with mouse hepatitis virus (David-Ferreira & Manaker, 1965), in a cultured human epidermoid cancer cell line, Hep-2, infected with Ilheus virus (Erlandson & Tandler, quoted by Chandra, 1968), in normal primate thymoblasts (Sebuwufu, 1968), in the vascular-endothial cells from a



29. SHA_2 cell. x 29,000



30. MAR_1 cell. x 28,000



31. HUN_1 cell. x 46,000

All three figures show areas of microtubular aggregate bounded by membrane which is part of the endoplasmic reticulum (arrowed "er"). Fig 29 shows the common appearance in which the tubular structure of the subunits is not obvious. The microtubules are clearly seen in Figs 30 and 31.

cutaneous lesion in a patient with Degos disease (Niskida & Howard, 1968) in similar cells from the glomerular and peritubular capillaries of patients with systemic lupus erythematosus (Grausz, Harley, Stephens et al., 1970) and in circulating lymphoid cells in the same disorder (Gyorkey & Sinkovics, 1971). Recently Helder, Blomjous, Feltkamp-Vroom and Van Laghem (1971) have described them in a variety of affected tissues from patients with "auto-immune" disorders, usually in endothelial cells*. The wide distribution of this structure provides no clue as to its identity or function, nor does it support the contention of some authors (see Gyorkey & Sinkovics, 1971) that it represents the undeveloped nucleocapsids of myxovirus.

Straight tubules

Sheaves of straight tubules, lying free in the cytoplasm, have been described by Epstein in numerous Burkitt lymphoma cell lines (for review see Epstein & Achong, 1970) and by Moore, Kitamura and Toshima (1968) in lines from healthy patients and from those with a variety of haematological disorders. Structurally they resemble spindle tubules and in some cases they are seen to radiate from the centriole region. However, they are usually found in the presence of an interphase nucleus so that the term "altered spindle tubules" as used by Epstein contains an assumption which may not be fully justified.

* Baring (1971) has also identified this structure in brain tissue from patient with Herpes simplex encephalitis.

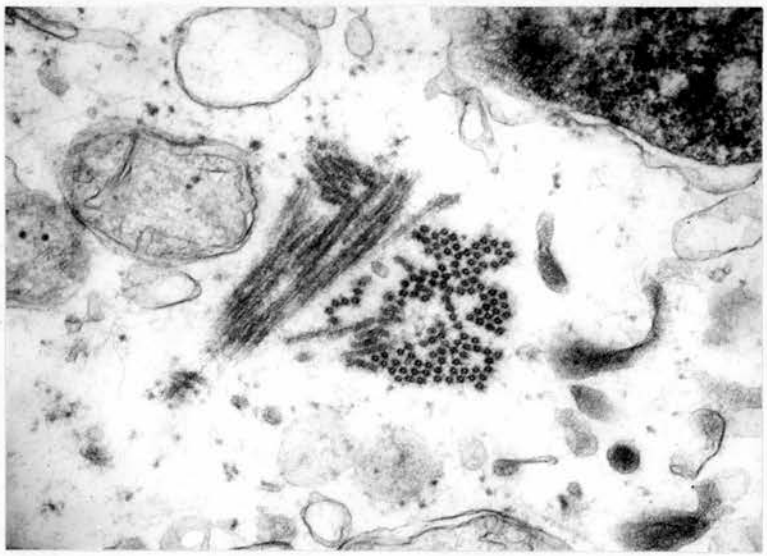
Structures of this type have been found in only two cell lines examined by me, YUD_1 and DUN_1 (both from infectious mononucleosis patients), and in each case in only a single cell (figs. 32,33).

Other tubule structures

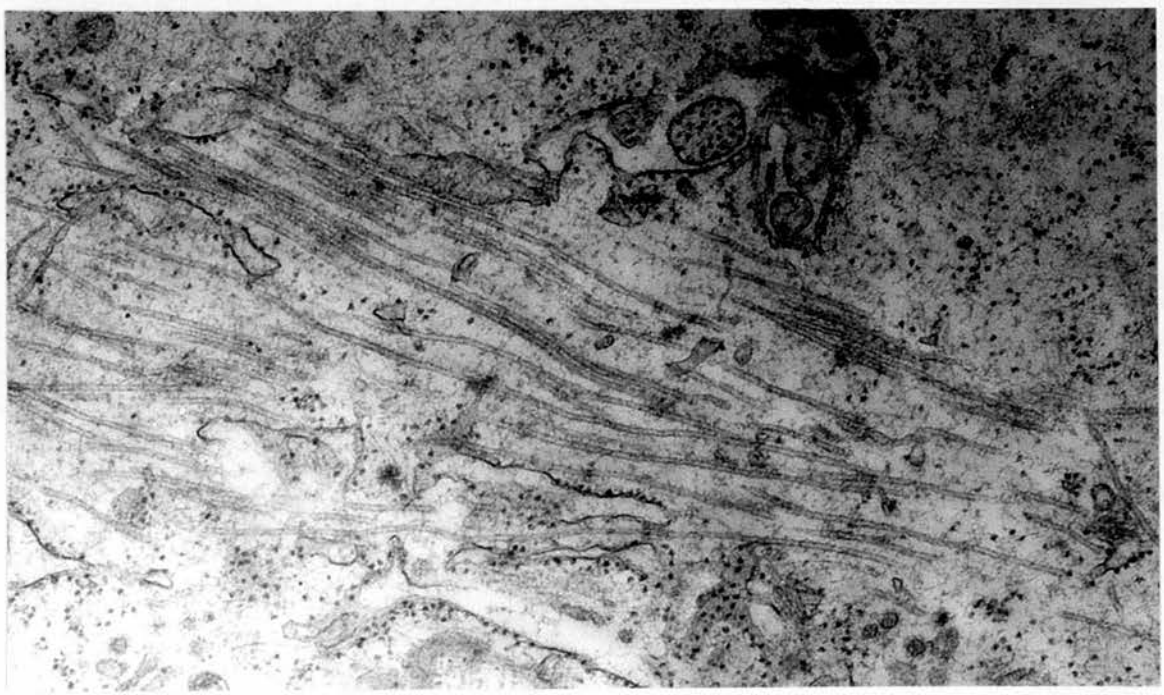
On rare occasions I have found aggregates of smooth-surfaced undulating tubules or elongated vesicles within the cytoplasm of apparently healthy cells (figs.34,35). They are not enclosed by any membrane and are invariably found close to the periphery of the cell, suggesting that they may be formed from invaginations of the plasma membrane. They do not appear to be associated with the presence of EB virus particles either within the cell or in close association with the cell surface. Such structures do not appear to have been described by other authors.

Cell outline

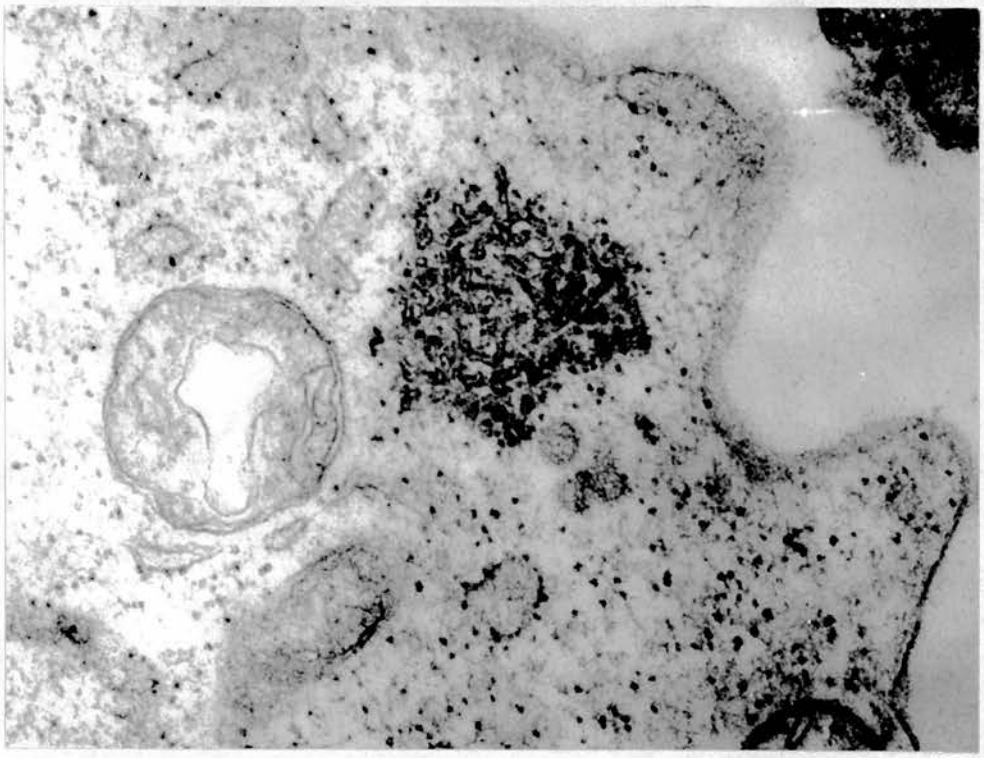
Epstein has compared the smoothness of cell surface, as judged by electron microscopy, for a number of Burkitt lymphoma lines and concludes that "the plasmalemma of cells of the more differentiated strains (EB_1, EB_3, EB_4 and EB_5) was usually smooth 'whereas' in cells from less differentiated strains (EB_2, RAJI, GOR and AMB) there was a marked tendency for the surface membrane to be thrown up to give irregular microvilli" (Epstein, Achong, 1970). In this context, the criteria for differentiation were a relatively small mean cell diameter ($\sim$10 mμ), a high nuclear/cytoplasmic ratio and mainly rounded or oval nuclei. I have not found it



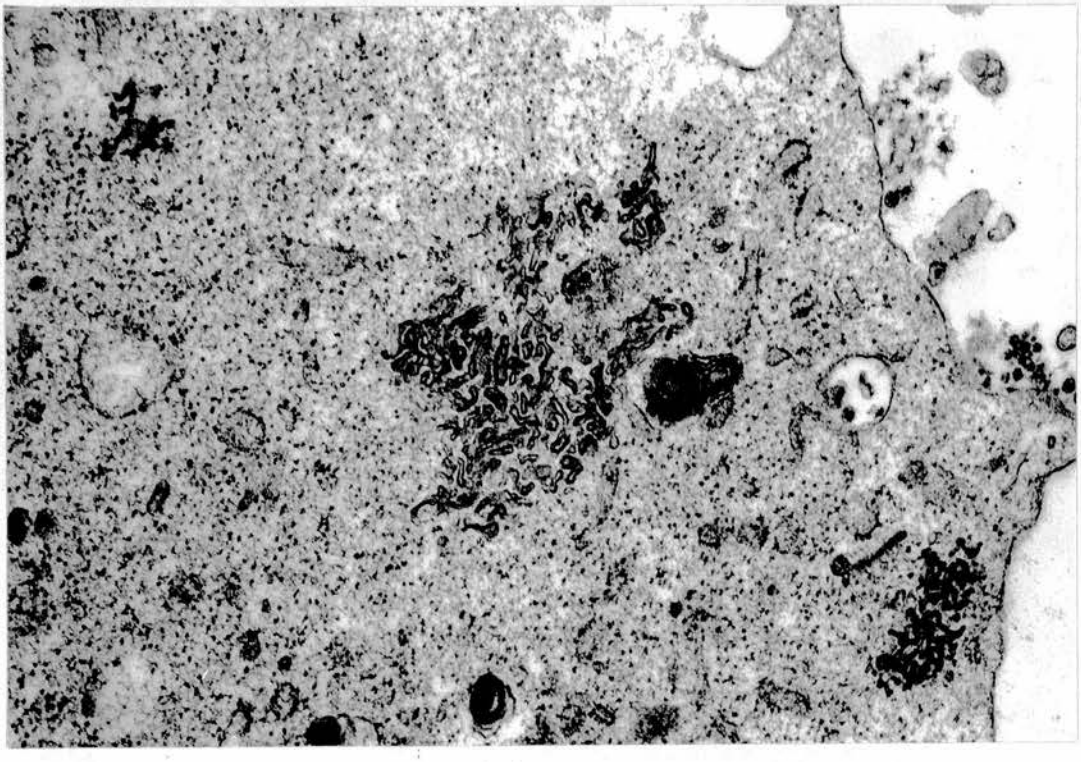
32. Straight tubules in cytoplasm of YUD_1 cell, sectioned in 2 planes.
x 41,300



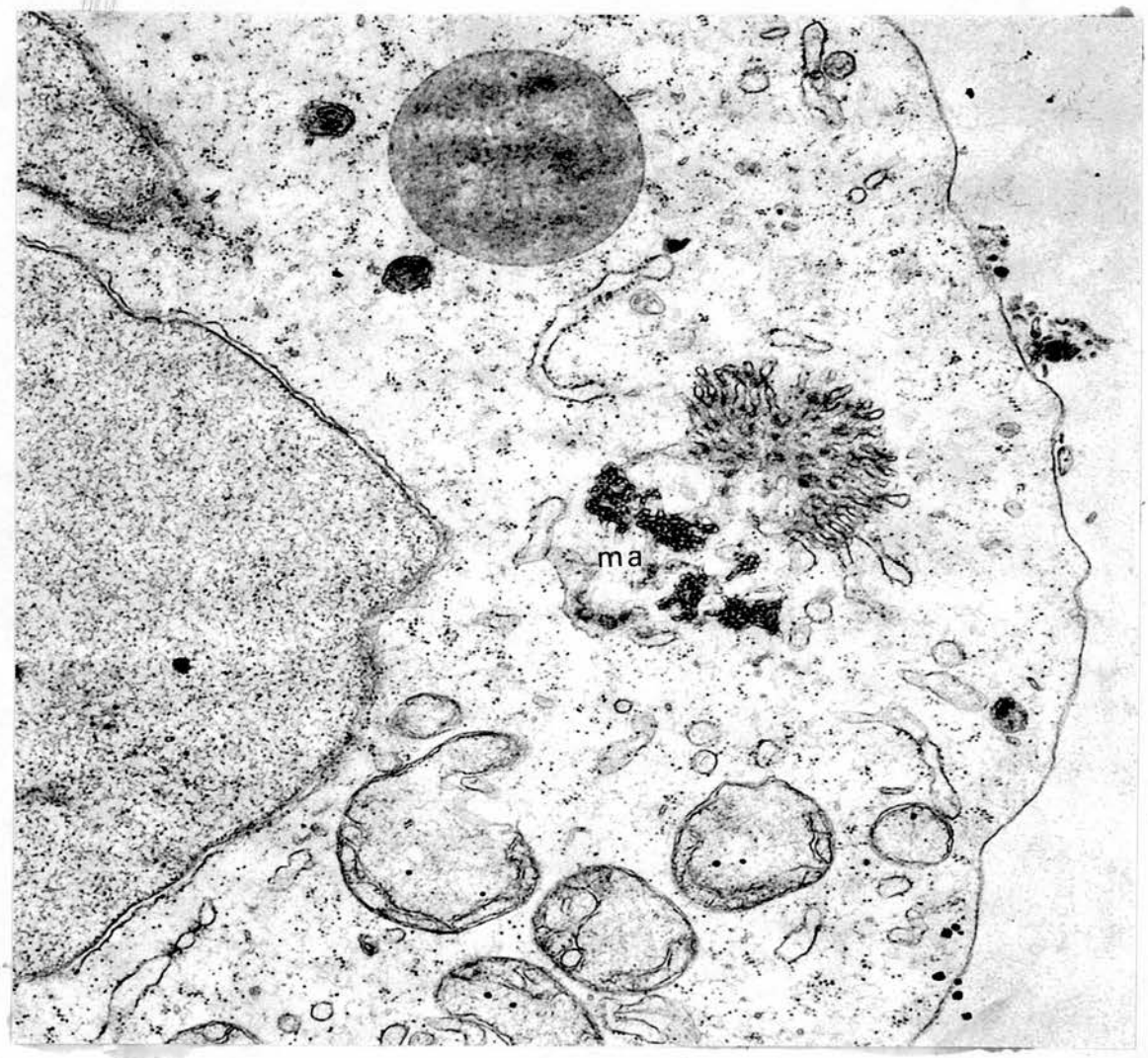
33. Straight tubules in cytoplasm of DUN_1 cell.
x 41,000



34a. Undulating tubules within the cytoplasm of a G-S_1 cell. They are not surrounded by any membrane. x 54,400.



34b 2 areas of undulating tubules in the cytoplasm of a MAR_1 cell which had been lethally irradiated (3000 rads) 5 days earlier x33,000

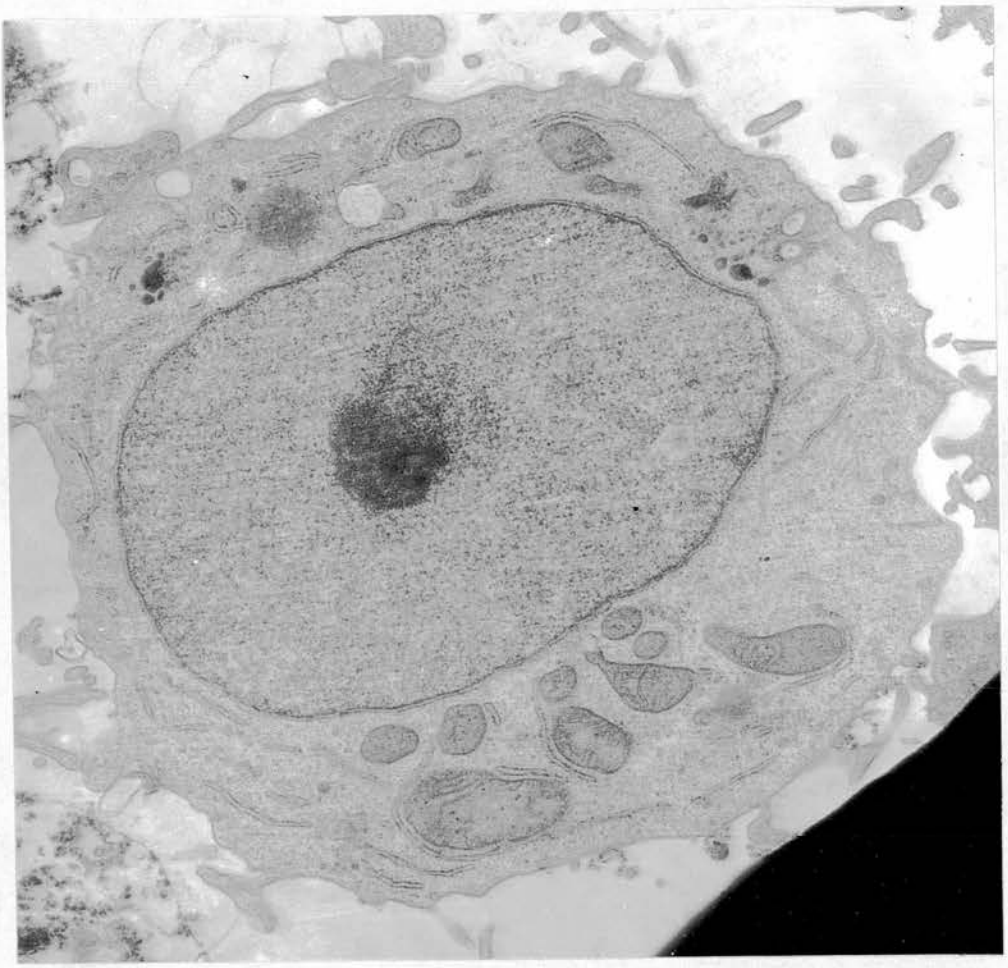


35. SHA_2 cell showing an area of microtubular aggregate ("ma") and an adjacent structure which seems to be formed by multiple folding of the endoplasmic reticulum. This structure resembles the undulating tubules shown in fig 34.
x 19,000

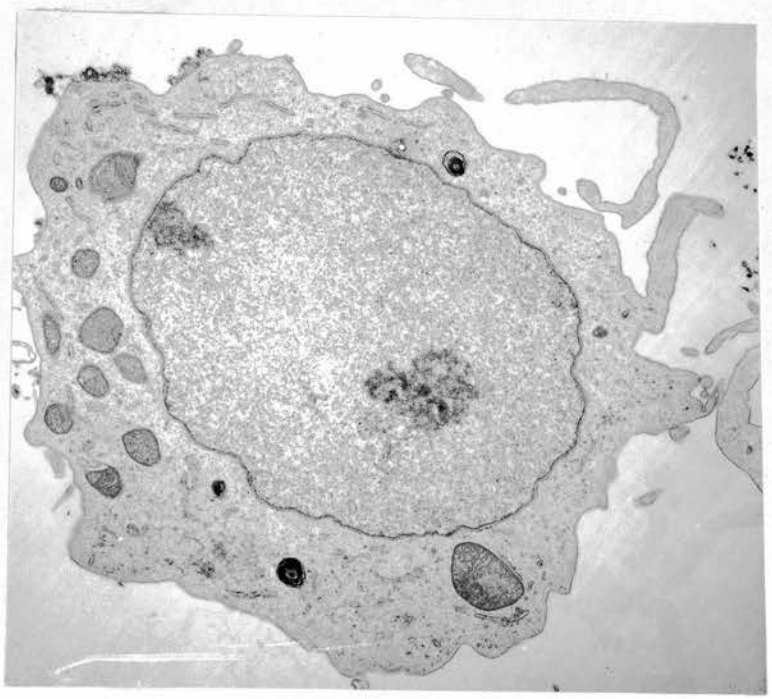
possible to classify my cell lines in this way as the range of appearances within each line is too great. Furthermore, surface projections have frequently been very prominent even in the presence of a rounded or only slightly-indented nucleus and a high nuclear/cytoplasmic ratio (figs. 36,37). Surface projections have not become noticeably more prominent or more abundant as the cell line has aged in culture.

Discussion

In the absence of identifiable virus particles, it is highly doubtful whether any features of the gross or fine structure of the lymphoblastoid cells included in this study can be taken to indicate that EB virus is present in the line. Peripheral condensation of the nuclear chromatin, which in the material examined by Epstein, Henle, Achong and Barr (1965), was a useful marker for virus-infected cells on low-power scanning, has not proved to be so in my own cell lines. Nuclear projections, annulate lamellae and microtubular aggregates have all been identified in a variety of tissues and in circumstances in which there has been no suggestion of EB virus involvement. I have not found these structures to be correlated with the presence of virus particles, either in individual cells or in other cells of the same line.



36. Cell from Line BUR_2 x 12000



37. Cell from line SHA_2 x 8000

Both cells have a very irregular outline although the nucleus is non-indented and the nuclear/cytoplasmic ratio high.

The position with regard to sheaves of straight (?spindle) tubules is uncertain since I have observed these on only two occasions. In both lines EB virus particles were present though not in the actual cells which contained the tubules.

Taken as a whole, my observations confirm the similarity in appearance and fine structure of the cells from different human lymphoblastoid lines, regardless of their origin. The development of rough endoplasmic reticulum, sometimes very abundant, is in keeping with the observations on immunoglobulin production in these cultures (Finegold, Fahey & Granger, 1967; Glade & Chessin, 1968; Nilsson, Ponten & Philipson, 1968; Takahashi, Takagi, Yagi et al., 1966; Nilsson, 1971). Most, if not all of the lines included in this study synthesise immunoglobulins (Miss J. Evans, unpublished observations) and on testing in a mixed lymphocyte culture system they appear to lack any cytotoxic capacity (see chapter VII). This strongly suggests that the cells of human lymphoblastoid lines are more closely related to marrow-derived 'B' lymphocytes than to thymus derived 'T cells'. (Makela, Cross and Kosunen, 1971). However, the finding of phagocytic activity in the cultures implies a need for caution in attempts to identify an *in vivo* counterpart for the cultured lymphoblastoid cell since it is a matter of considerable controversy whether any member of the lymphoid series *in vivo* can display phagocytosis (Yoffey & Constice, 1970).

CHAPTER VI

Cytogenetic studies on established cell lines

The karyotypes of each cell line grown by me has been determined from orcein-stained preparations as soon as possible after the line has become established in culture (usually within four months of obtaining the original blood sample). In a number of cases, the chromosome constitution of the donor's fresh blood cells has also been studied after short term (72-120 hour) culture with or without added phyto-haemagglutinin (PHA). These studies have been carried out in collaboration with Mrs. S.McBeath and Miss M.L. O'Riordan. All chromosome preparations from established lines and most of those from short-term blood cultures, have been made by me and I have analysed ten to twenty metaphase spreads from each. The slides have then been passed to Mrs. McBeath who has independently analysed thirty metaphase spreads. Where two or more cells carried the same abnormality, a further twenty cells were analysed by Mrs. McBeath. Cell lines bearing chromosome abnormalities were further checked by Miss O'Riordan.

Lines introduced into the laboratory from other centres have been examined in the same way and many of the lines, particularly those bearing chromosome abnormalities, have been re-examined at intervals in order to follow the changes in the predominant karyotype with time in culture. Most of the analyses for this purpose have been undertaken by me.

During the last year of this study it has been possible to identify individual chromosomes in the karyotype of cultured lymphoblastoid cells by staining the prepared slides with 0.5% quinacrine dihydrochloride and examining the material under ultra violet light. (Caspersson, Zech and Johansson, 1970; O'Riordan, Robinson, Buckton & Evans, 1971). I have applied this technique to all those cell lines recognised, on orcein staining, as carrying chromosome aberrations and to several lines regarded, on the same basis, as having a diploid karyotype. The results, presented at the end of this chapter, are incomplete as yet, since the prodedure and the analysis is very much more time consuming than with standard staining methods, and only a small number of cells has been fully analysed from each line. The fluorescence technique is extremely powerful in permitting the precise interpretation of breakage and recombination events which have resulted in an abnormal karyotype. It is therefore particularly well suited to cytogenetic studies on lymphoblastoid cell lines, since they offer a higher frequency and a wider variety of abnormal karyotypes than any human population.

The preparation and analysis of quinacrine-stained metaphase spreads of lymphoblastoid cells has been undertaken by myself, but I am indebted to Miss M.L. O'Riordan, Miss J.A. Robinson and Miss K.E. Buckton for valuabe advice and discussion.

Materials and Methods

Preparation and fixation of metaphase spreads (Modified from Hungerford, 1965).

The best chromosome preparations, in terms of the number of dividing cells and the morphology of individual chromsomes, are made from cell lines which have been growing rapidly before examination and which contain fewer than 20% dead cells. A sample (2.5 ml) from such a culture, which has been left unfed for at least 48 hours, is transferred to a sterile 1" x 3" flat-bottomed culture tube, and a further 2.5 ml of fresh culture medium is added. The medium used throughout has been Ham's F_{10} with 10% tryptose phosphate broth, 20% foetal calf serum and antibiotics.

Dimethylcolchicine (Colcemid, Ciba) 0.05 µg is added immediately to this 5 ml culture, which is loosely capped and incubated at 37°C, in a humidified atmosphere of 7% CO_2 in air, for 1-2 hours.

The cells are then separated by centrifugation at 800 x g for 3 minutes and resuspended in 0.075 M KCl for six minutes at room temperature. The cells are again separated by centrifugation and fixed by the addition of absolute methanol:glacial acetic acid 3:1, the fixative being added dropwise with continuous agitation of the cells. After two changes of methanol: acetic acid, the cells are resuspended in a sufficient volume of the same fixative to give an opalescent appearance (usually about 1 ml).

Two drops of the suspension are placed on dry glass slides which have been steeped in absolute alcohol/conc. hydrochloric acid mixture to remove grease and other surface impurities. The slides are dried and hand-polished before use.

The drops of cell suspension are allowed to spread out and dry at room temperature and the slides are then examined microscopically using diffuse light (condenser racked down) to assess the density of the suspension and the extent of spreading of the individual chromosomes from each metaphase. Since acetic acid increases, and methanol decreases, the tendency of the chromosomes to spread out, satisfactory preparations can usually be made by varying the proportions of the two constituents in the fixative. Suitable slides are then stained by one of the following methods.

Orcein Staining

Stain is prepared by dissolving crystalline orcein (Gurr) 2 grammes in 100 ml of lactic acid (Reagent grade). The solution is filtered through Whatman grade 1 paper. Slides are immersed in the stain and left overnight at room temperature. Excess stain is then removed by washing in 45% Acetic acid and the slides are taken through two changes of Cellosolve (5 minutes each) and two changes of Euparal essence (5 mins. each) before mounting in euparal and drying on a horizontal heated surface. The preparations have been examined on a Leitz orthoplan or ortholux microscope using a Balzar's orange filter, (peak transmission 550nm).

Quinacrine Staining

Stain is prepared by dissolving quinacrine dihydrochloride ("Atabrine", Sigma) 0.5 grammes in 100 ml of deionised water. The stain is stored at 4°C when not in use.

Slides are stained by immersion in this solution for 8 minutes followed by washing in running tap water (cold) for two minutes and rapid rinsing in deionised water. They are blotted dry and a single drop of deionised water is placed on each. A clean dry coverslip (Chance, Grade I) is floated on this drop and excess water removed by gently pressing a clean filter paper on top of the coverslip. The edge between the coverslip and the slide is then sealed with rubber solution.

The slides have been examined on a Leitz ortholux microscope using an HbO-200 W/4 mercury vapour light source and Ploem's vertical illumination. The Ploem was used at setting number 3 with a BG 12 exciting filter and a 510 nm barrier filter. Photographs were taken with a Leitz orthomat automatic camera on Kodak panatomic-X 35 mm film.

Chromosome preparations from short-term leukocyte cultures

Cytogenetic studies have been undertaken on short term cultures of many of the blood samples from which established lines have subsequently been grown. The method has been modified from Hungerford (1965) and is as follows.

Leukocytes are separated from heparinised whole blood as described in Chapter II. They are suspended in growth medium (Ham's F_{10} with 10% tryptose phosphate broth, 20% Foetal calf serum and antibiotics) and counted, as described, in a Neubauer haemocytometer. For short term culture, aliquots of 2-5 x 10^6 cells are transferred to separate sterile 1" diameter flat-bottomed glass tubes ('Samco'). The volume in each tube is made up to 5 ml by adding fresh medium. From two to six such cultures are set up, depending on the number of leukocytes available. To half the tubes 0.05 ml of sterile reagent grade phytohaemagglutinin (PHA) (Burrough's Wellcome Ltd.) is added. All the tubes are then loosely capped and incubated at 37°C in a humidified atmosphere of 7% CO_2 in air. Cultures are harvested in pairs (one with added PHA, one without) at 72, 96 and 120 hours. 2-3 hours before harvesting, demethylcolchicine (Colcemid, Ciba) 0.1 µg is added to each tube.

Chromosome preparations are then made from these cells exactly as described above for long-term cultures.

Findings

Orcein-stained preparations.

The results obtained on examining orcein-stained preparations from most of the cell lines described in Chapter II

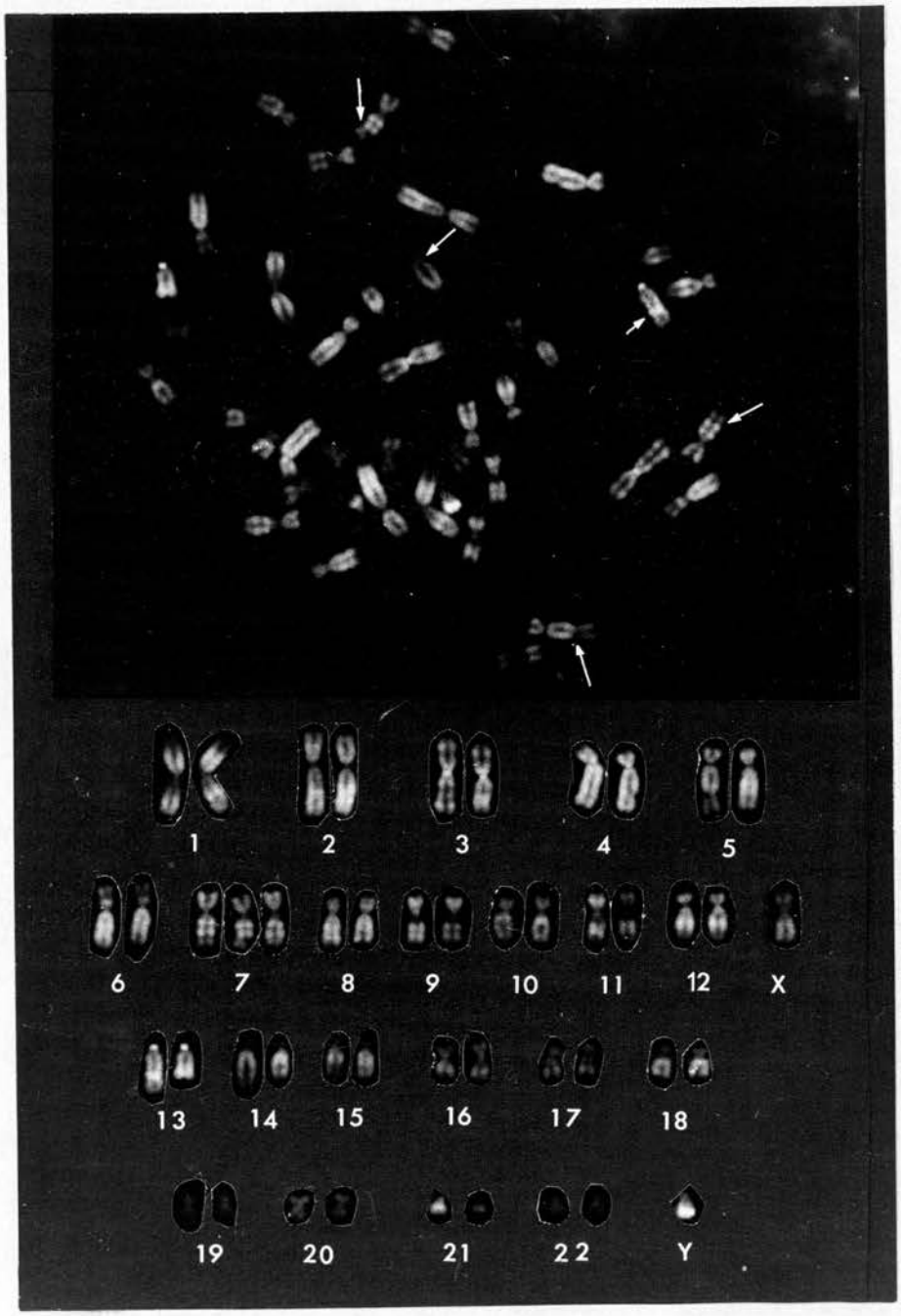
have been reported in the accompanying paper, reprinted from the Journal of the National Cancer Institute, 47, pp11203-1214, 1971. Since the submission of this paper, similar studies have been completed on all the cell lines described in Chapters II and III. Only two more lines have been found to be aneuploid on first examination; MON_1, grown spontaneously from the peripheral blood leukocytes of 4 year old boy with cystic fibrosis and BLA_1, derived from a 57 year old man with undifferentiated leukaemia, by co-cultivation of his peripheral blood leukocytes with irradiated F137 cells. In both cases, short term cultures of the donor's peripheral blood cells revealed a normal male karyotype. The chromosome abnormalities in the two lines are discussed later.

Quinacrine-stained preparations

On examining quinacrine-stained preparations from aneuploid cell lines, two conclusions were very rapidly established; first that karyotypes constructed from orcein-stained material tended to underestimate the extent of chromosomal breakage, re-combination and non-disjunction which had taken place, and secondly that abnormal chromosomes with similar morphology are rarely formed in precisely the same way. These points are made in the accompanying paper reprinted from Nature 233, pp.555-556 (1971).

Preliminary quinacrine fluorescence studies have now been undertaken on all but three cell lines which have been cultured for one year or more (discounting time spent in liquid nitrogen storage). In many cases it has been possible to examine two or more samples of the same line which have spent different periods in deep freeze and which therefore differ widely in their effective 'age' in vitro. The results, with photographs, are presented in the succeeding section.

Fig 38a



38a. F137 cell representative of the line after 2-4 months growth in Edinburgh. For description of abnormalities (arrowed) see text opposite.

Cell line F137

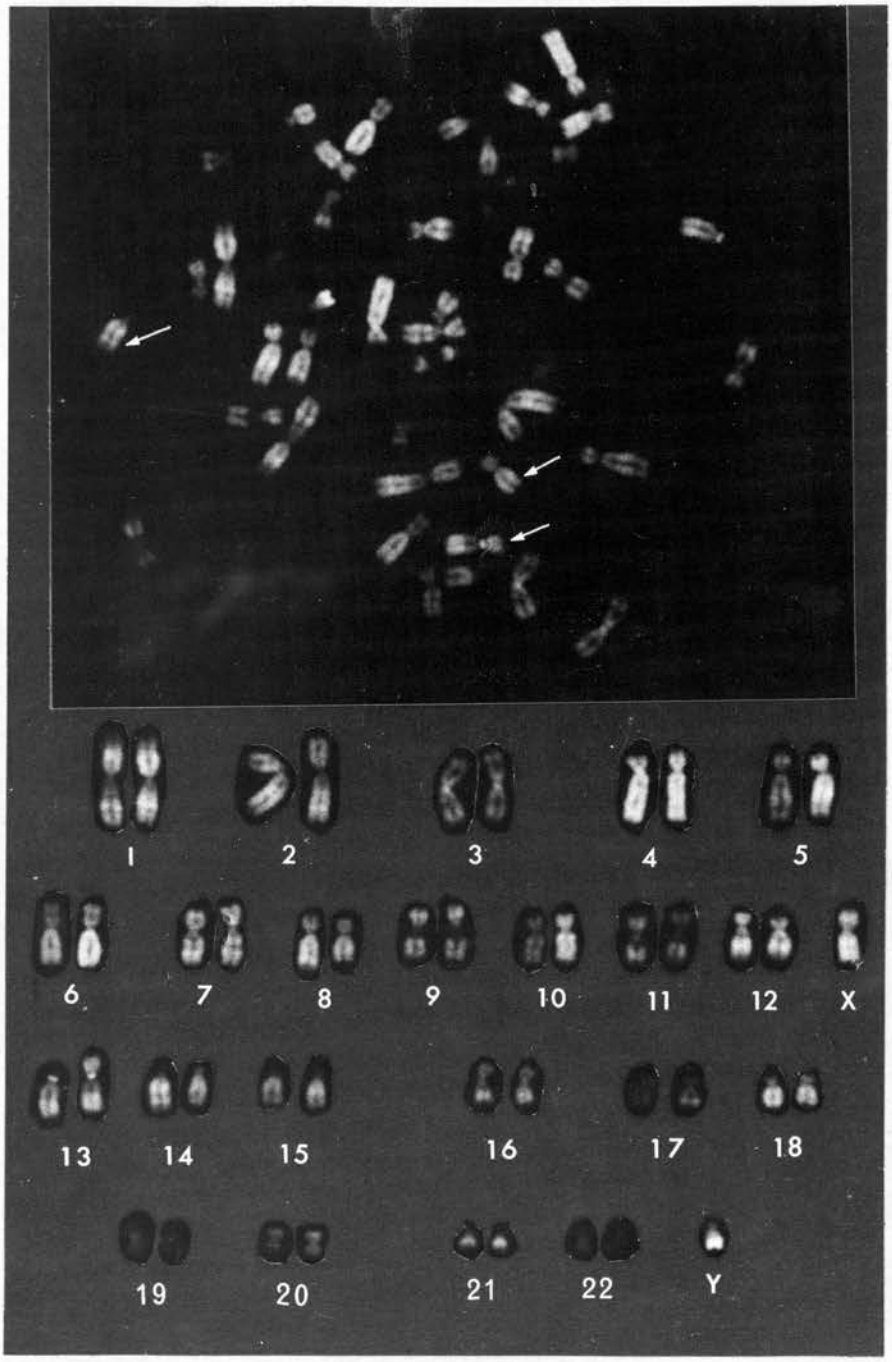
Origin Grown by Dr. E.M. Jensen from lymph node biopsy material of a male patient with chronic lymphatic leukaemia (Jensen, Korol, Dittmar & Medrek, 1967). Sent to me in August, 1969, having been grown for 18 months to 2 years in Dr. Jensen's laboratory (plus approximately 2 years storage in liquid nitrogen).

Examination of orcein-stained material is reported in the preceding paper (Steel, McBeath & O'Riordan, 1971).

Quinacrine-stained preparations were made from four samples growing simultaneously but representing periods of culture (in this laboratory) of 2 to 30 months. Within this time span, the modal karyotype changed twice as detailed below:

2-4 months in culture (in Edinburgh)

11 cells photographed. All have 47 chromosomes including and additional C7. A subterminal secondary constriction is present on the long arms of one or two C7 chromosomes in every cell (never on all three) and there is additional material on the long arms of one D13 and one D14 in every cell. A typical cell is shown in fig.38a. The consistent chromosome aberrations are arrowed. In addition this cell includes a prominent secondary constriction or gap on the long arms of one B5 chromosome.



38b. F137 cell representative of the line after 7-13 months growth in Edinburgh. For description of abnormalities (arrowed) see text opposite.

7-13 months in culture (in Edinburgh)

20 cells photographed. The modal karyotype (14 cells) comprised 46 chromosomes. In every cell, one D13 had additional material on the short arms. One of the D14 pair had additional material on the long arms, and one of the C8 pair appeared to have lost material from the long arms. A subterminal secondary constriction on the long arms of one of the C7 pair could be identified in about half the cells.

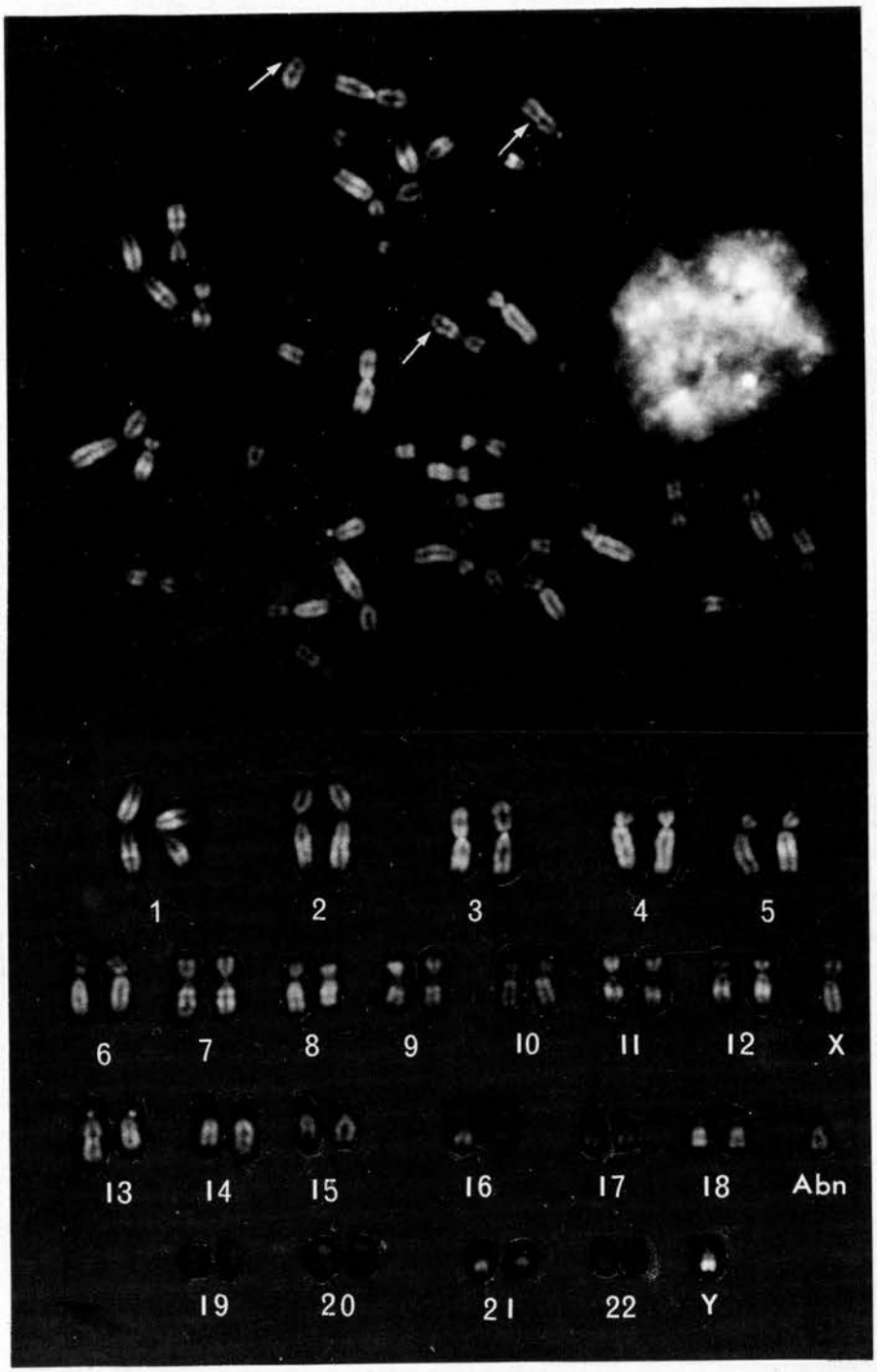
A typical modal cell is shown in fig.38b. The abnormalities are arrowed.

Of the remaining six cells, three appeared to belong to the modal clone, differing from it only by apparently random chromosome loss (two had 45 chromosomes and one 43). The other three appeared closer to the earlier clone (fig.38a) having 47 chromosomes and additional material on the long arms of a D13 and a D14. In each case, however, the extra chromosome was a C9, not a C7.

18-30 months in culture (in Edinburgh)

17 cells photographed. The modal karyotype (14 cells) comprised 47 chromosomes, the additional one being a small acrocentric, about the size of the long arms of a 17/18 chromosome. The abnormal chromosome showed moderately bright fluorescence with increased brightness at the centromere. Additional material was present on the long arms of one D13 and one D14 chromosome in

Fig 38c



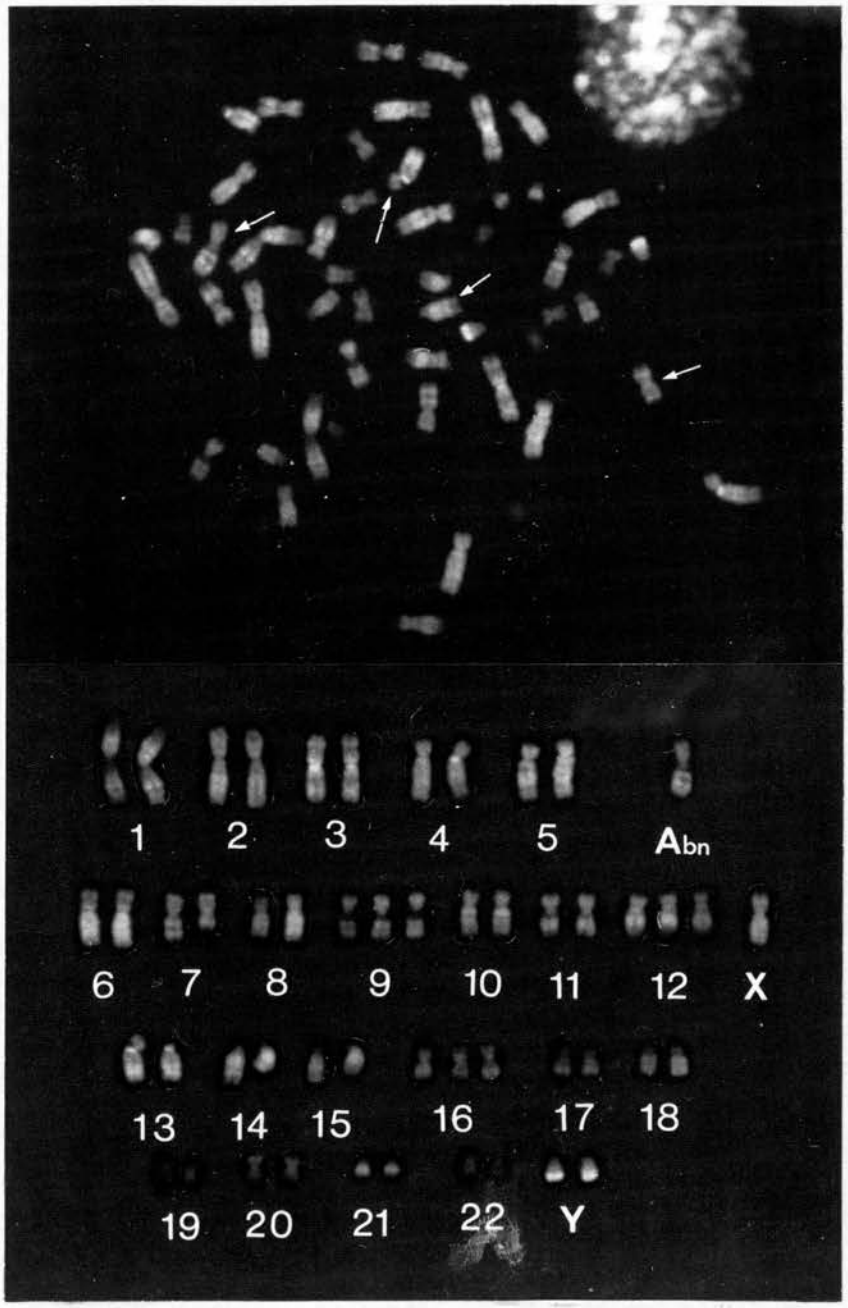
38c. F137 cell representative of the line after 18-30 months growth in Edinburgh. For description of the abnormalities (arrowed) see text opposite.

every cell. A subterminal secondary constriction on the long arms of one C7 chromosome was present in the majority of cells and in every cell one of the C8 pair appeared to have lost a small amount of material from the long arms.

A typical modal cell is shown in fig.38c. The abnormalities are arrowed.

Of the three remaining cells, two had 46 chromosomes and differed from the modal karyotype only by the loss of a number 1 and the abnormal chromosome respectively. The other had 47 chromosomes. Neither of the D13 chromosomes had enlarged long arms but one had additional material on the short arms, as in the cell shown in fig.38b. The small acrocentric marker shown in fig.38c was also present in this cell.

The D13 chromosome with additional material on the short arms shown in fig.38b was previously reported as being probably derived from an A3 chromosome with partial deletion of one arm (see fig.1c of preceding paper, Nature 233, 555-556, 1971). Similarly, in the last cell described above, the small acrocentric extra chromosome was thought to be partly deleted D13. The enlargement of the long arms of a D14 was particularly obvious in this cell and the possibility of a 13/14 translocation was raised (see fig. 1 m of the same paper). As more cells have been examined it has become necessary to revise both of these opinions.



39a. Jiyoye cell representative of the line after 3-6 months growth in Edinburgh. For description of the abnormalities (arrowed) see text opposite.

Cell line JIYOYE

Originally grown from ascitic fluid of a Nigerian boy with Burkitt's lymphoma (Pulvertaft, 1965). A sample was sent to the National Cancer Institute (USA) in August, 1965 and sublines were maintained in a number of American centres from that date onwards. Material was received in this laboratory in March, 1969 (from Dr. B. Hampar, National Cancer Institue, USA). It had spent at least 6 months in liquid nitrogen storage before dispatch. I have examined samples from this material representing three different periods of growth in Edinburgh.

3-6 months in culture (in Edinburgh)

13 cells photographed. Five had an identical karyotype comprising 51 chromosomes, the extra ones being a C9, a C12, an E16, a Y and an abnormal submetacentric marker, about the size of a C6, the long arms of which had a banding pattern corresponding to the long arms of a C7, but the centromere and short arms showing only weak fluorescence. One of the C7 pair was missing and a metacentric chromosome corresponding in size and banding pattern to the short arms, centromere and proximal part of the long arms of a C7 was present in every cell. In addition, one member of the D13 pair had extra material on the short arms and one of the D14 pair had enlarged long arms. These features are shown in a typical modal cell (fig.39a). The abnormalities are arrowed.

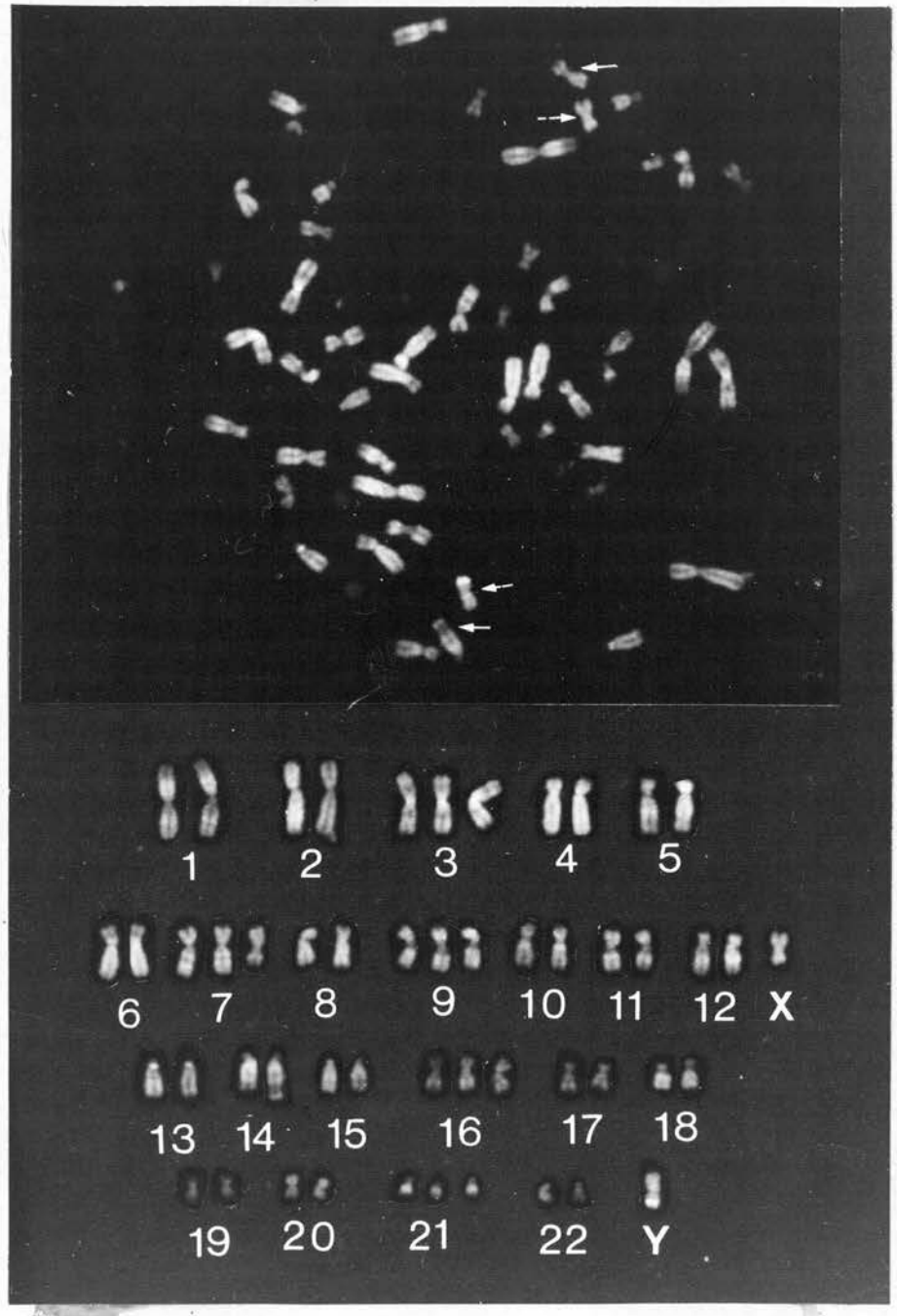
Two of the remaining eight cells were clearly related to the modal clone. Each had 51 chromosomes; in one the extra C12 was replaced by an isochromosome for the long arms of a number 12 (in addition to the normal C12 pair) and in the other one of the A1 pair was missing, extra material (probably derived from the missing A1) being present in the form of a large acrocentric marker and as elongation of the short arms of the submetacentric marker described in the modal cell.

The other six cells were less closely related to the modal clone. None had an additional Y chromosome. All had the extra metacentric chromosome derived from the short arms, centromere and proximal part of the long arms of a C7, but in five of them there was a normal C7 pair. An extra C9 was present in five cells, an extra C12 in three and an extra isochromosome for the long arms of C12 in two. An extra E16 was present in three cells and every cell showed one of the D14 pair with enlarged long arms. Two cells had 48 chromosome, one 49 and two 50. The remaining cell had 52 chromosomes including the above extra material plus trisomy F20 and G21.

20-25 months in culture (in Edinburgh)

7 cells examined. The modal karyotype (5 cells) comprised 50 chromosomes, the additional ones being a C9, an E16, a G21 and a

Fig 39b



39b. Jiyoye cell representative of the line after 32-35 months growth in Edinburgh. For description of the abnormalities (arrowed) see text opposite.

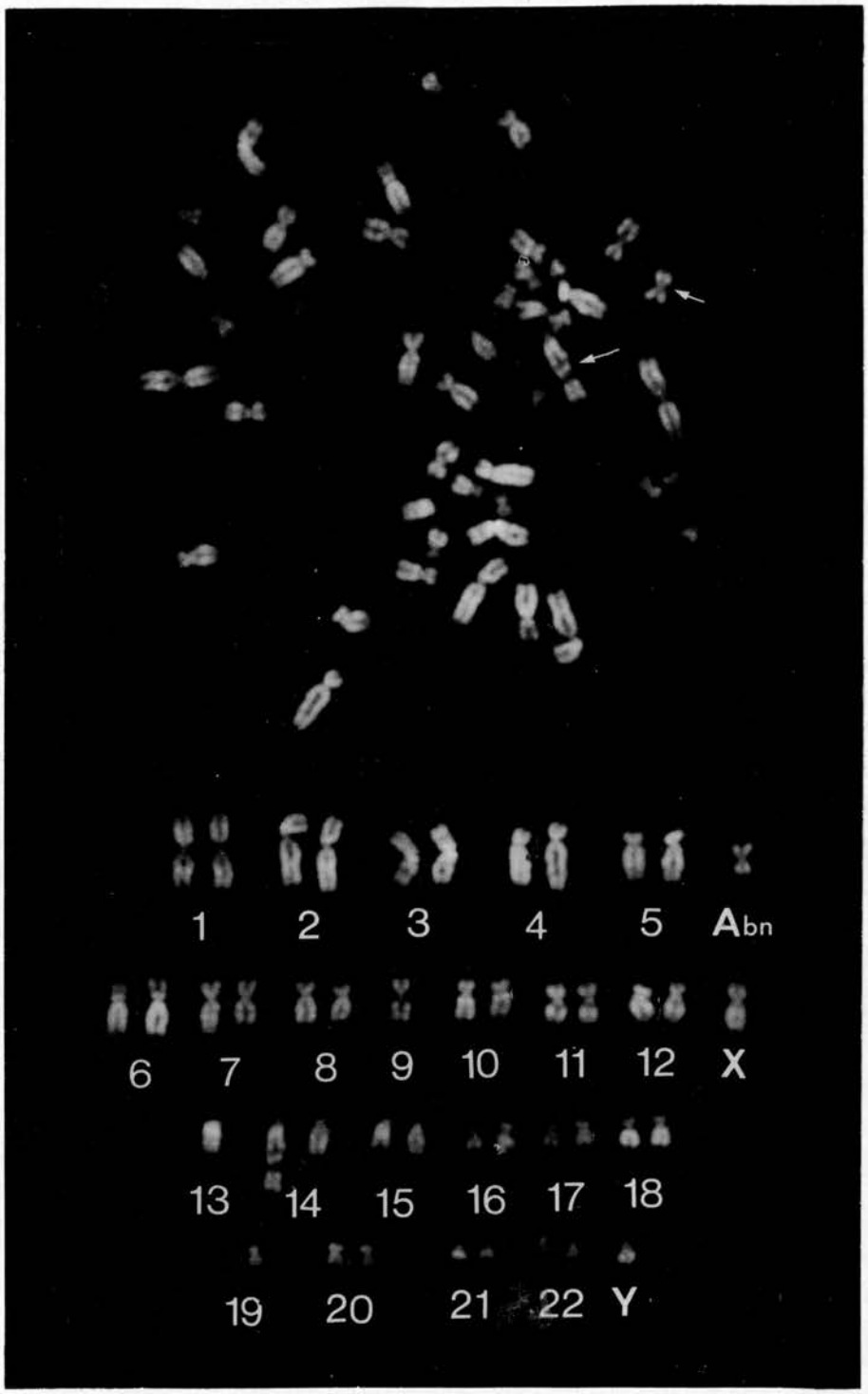
metacentric chromosome derived from the short arms, centromere and proximal part of the long arms of a C7. One of the D14 pair had extra material on the long arms which consisted of a dark band and a relatively bright tip. There was partial deletion of the long arms of the X in every cell and the material appeared to have been translocated onto the short arms of the Y. Two cells had 49 chromosomes, differing from the mode only by the loss of a D13 and an F20 respectively.

32-35 months in culture (in Edinburgh).

12 cells examined. The mode (9 cells) comprised 51 chromosomes, the karyotype being identical to that observed at 20-25 months, but with the addition of an extra A3 chromosome. A typical modal cell is shown in fig.39b. The abnormalities are arrowed.

Two of the remaining cells had 50 chromosomes. One lacked the additional A3 and the other lacked the extra C9. The third cell had 49 chromosomes, lacking the extra C9 and one of the F19 pair.

Fig 39c



39c. Jiyoye ("Flow") cell representative of the line after 1-3 months growth in Edinburgh. For description of the abnormalities (arrowed) see text opposite.

JIYOYE ('Flow')

A separate subline of JIYOYE was obtained in November 1971 from Flow labs. Ltd. This material was also derived from stocks held at the National Cancer Institute (USA) though its precise history is obscure. It is understood have been stored in deep freeze for most of the time since March, 1969 and has therefore spent less time in culture than the samples described above.

Samples of this material have been examined after 1-3 months growth in Edinburgh.

13 cells examined. The modal karyotype (7 cells) comprised 45 chromosomes. In each cells one of the C9 pair and one D13 and one D14 were missing. There was a large dicentric chromosome evidently formed from a fusion between the short arms of a C9 and the long arms of a D14 and there was an extra metacentric marker about the size of an E16 but with a different fluorescence pattern. A typical modal cell is shown in fig.39c. The abnormalities are arrowed.

Of the remaining six cells, four had 46 chromosomes and included all the features of the modal type but with a

normal D13 pair. One was identical to these four but had an additional C7 chromosome (47 in all) and two had 44 chromosomes, differing from the mode by the loss of a C8 and a D15 respectively.

While there is little obvious relationship between the karyotype of JIYOYE (Flow) cells and those of the sublines grown for longer periods in this laboratory, the possibility arises that the enlarged D14 seen in the latter material (figs. 39a and b) may be derived from the 14/9 fusion shown in fig. 39c if the dicentric marker has subsequently broken just proximal to the C9 centromere.

Cell line F89

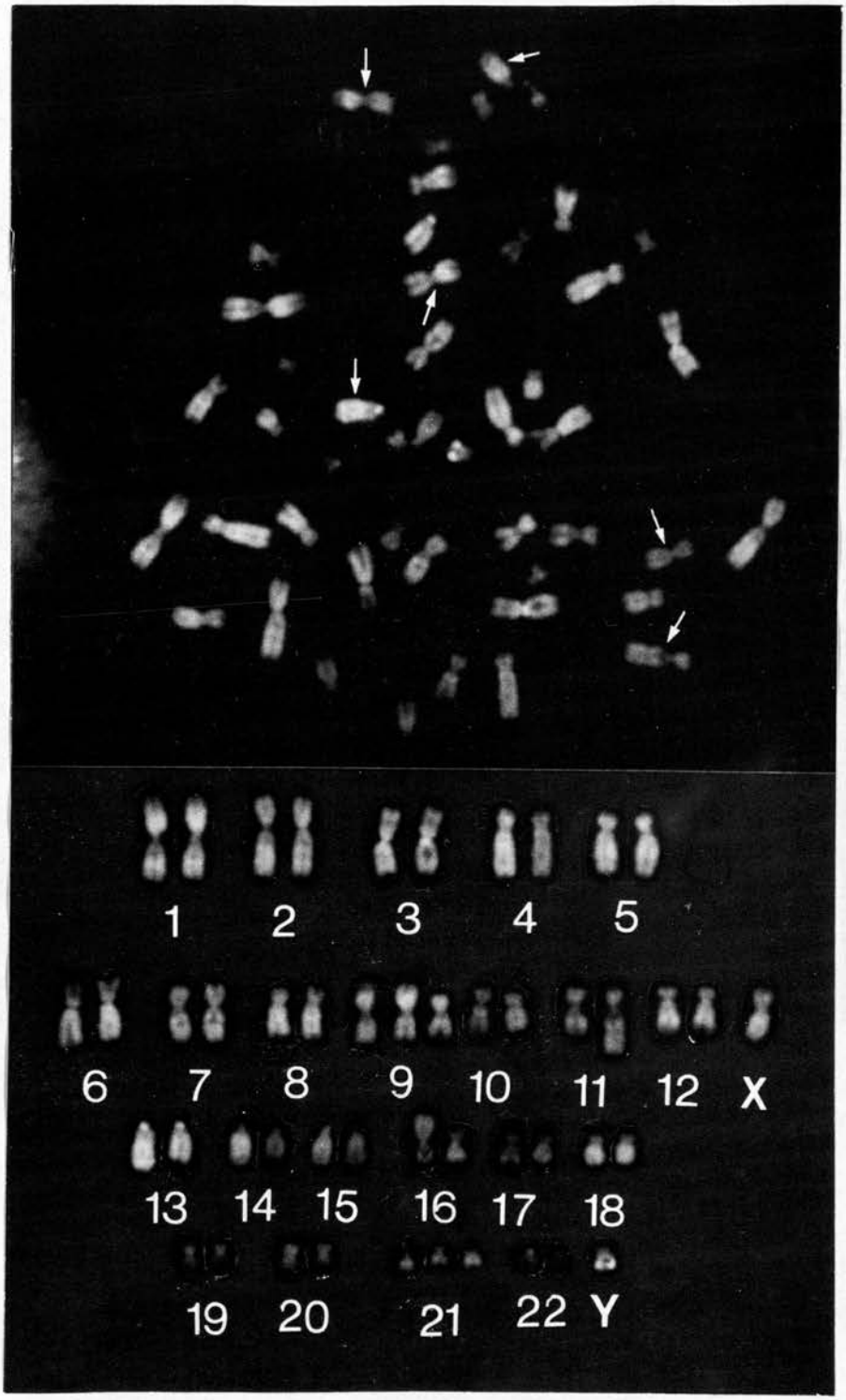
This line was grown from lymph node biopsy material from an elderly male patient with subacute lymphatic leukaemia. (Jensen, Korol, Dittmar and Medrek, 1967). It was received in Edinburgh inAugust, 1969, having been grown for some 18 months to 2 years in Dr. Jensen's laboratory. The findings on examination of orcein stained material from this line are reported in a preceding paper (Steel, McBeath and O'Riordan, 1971).

I have examined quinacrine-stained preparations of this line representing two different periods of growth in Edinburgh.

16-18 months in culture (in Edinburgh)

5 cells photographed. The modal karyotype (4 cells) comprised 46 chromosomes. Every cell had additional material on the short arms of one of the C9 pair and on the long arms of one C11, one D13 and one D14. The fifth cell had 45 chromosomes. All the features of the modal clone were present but one of the C8 pair had been lost. This cell had the enlarged A2 marker noted in a minority

Fig. 40



40. F89 cell representative of the line after 30 months growth in Edinburgh. For description of the abnormalities (arrowed) see text opposite.

of cells in orcein preparations. The fluorescent pattern of the extra material appeared to be indistinguishable from the distal part of the normal A2 long arms.

30 months in culture (in Edinburgh)

11 cells examined. The modal karyotype (9 cells) comprised 48 chromosomes. All the abnormalities of the modal clone described above were present but the C9 chromosome with additional material on the short arms was now duplicated and there was trisomy G21. Extra material was also present on the short arms of one of the B16 pair. A typical modal cell is shown in fig.40. The abnormalities are arrowed.

Of the remaining two cells, one had 47 chromosomes, only differing from the modal type/by the loss of a C7 and the other had 46 chromosomes, including an enlarged A2 marker. In other respects it corresponded to the modal karyotype in the 16-18 month material described above.

Cell line RAJI

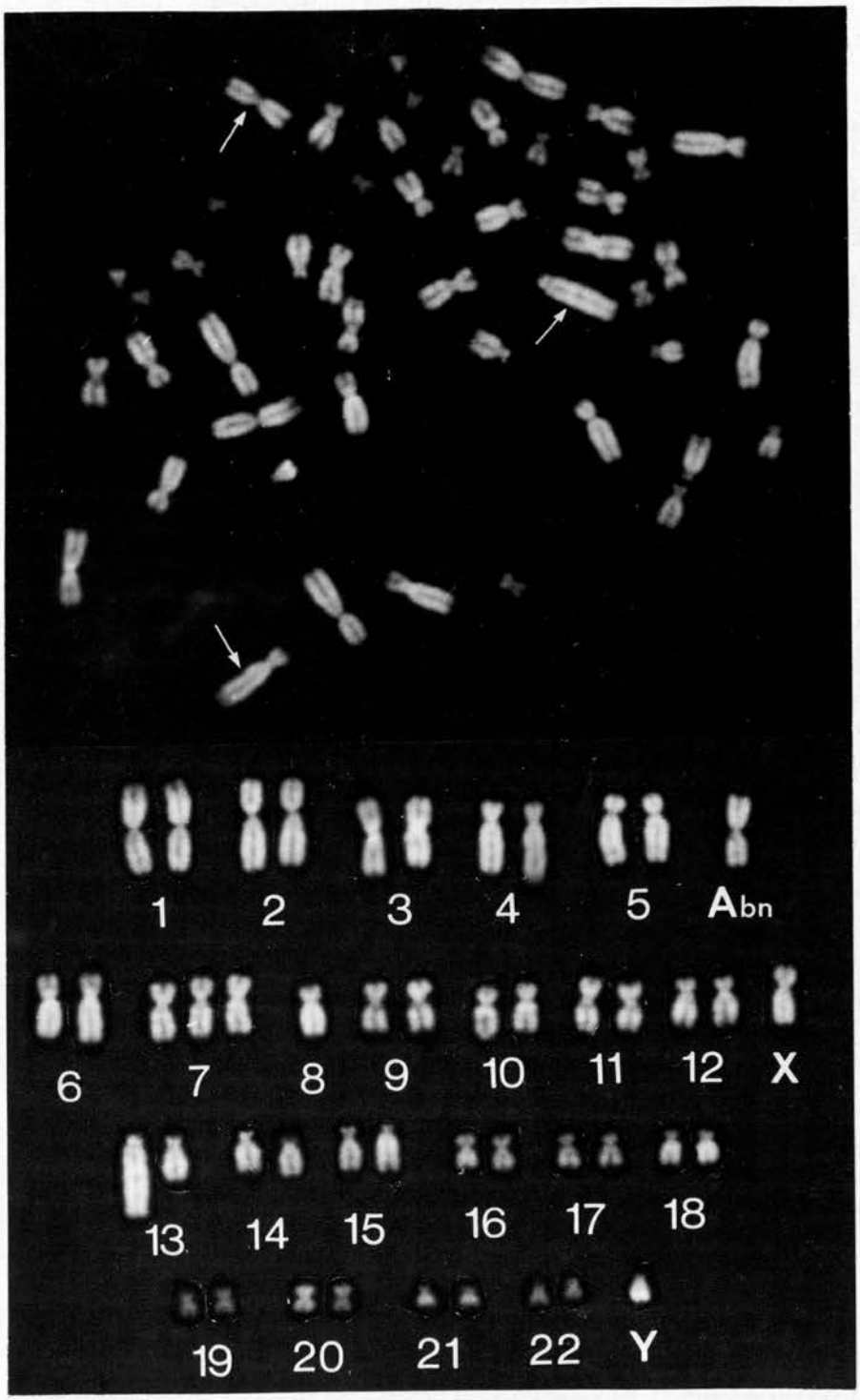
This line was grown from the biopsy of a jaw tumour from an 11 year old Nigerian boy with Burkitt's lymphoma (Pulvertaft, 1965). After some months in culture, material was sent to the National Cancer Institute (USA). From there, samples were distributed to a number of American centres including the laboratory of Dr. Werner Henle in Philadelphia. Material was obtained from Dr. Henle and arrived in Edinburgh in April, 1970. The total period in culture up to this time was estimated at 3 years.

Samples have been examined after three different periods of culture in Edinburgh.

10-13 months in culture (in Edinburgh)

5 cells photographed. The modal karyotype comprised 47 chromosomes. In each cell one C8 was missing and there was an extra C7 plus an abnormal metacentric chromosome slightly smaller than an A3 but with a dark centromere. One of the pairs of arms of this chromosome may have been the long arms of a C8. One of the B4 pair in every cell had additional very weakly fluorescent material on the end of the long arms. Only two of the five cells corresponded exactly to this description. The other three each had 46 chromosomes and included all the above features but lacked an A2, a D14 and an E16 chromosome respectively.

Fig. 41



41. Raji cell representative of the line after 21 months growth in Edinburgh. For description of the abnormalities (arrowed) see text opposite.

19 months in culture (in Edinburgh)

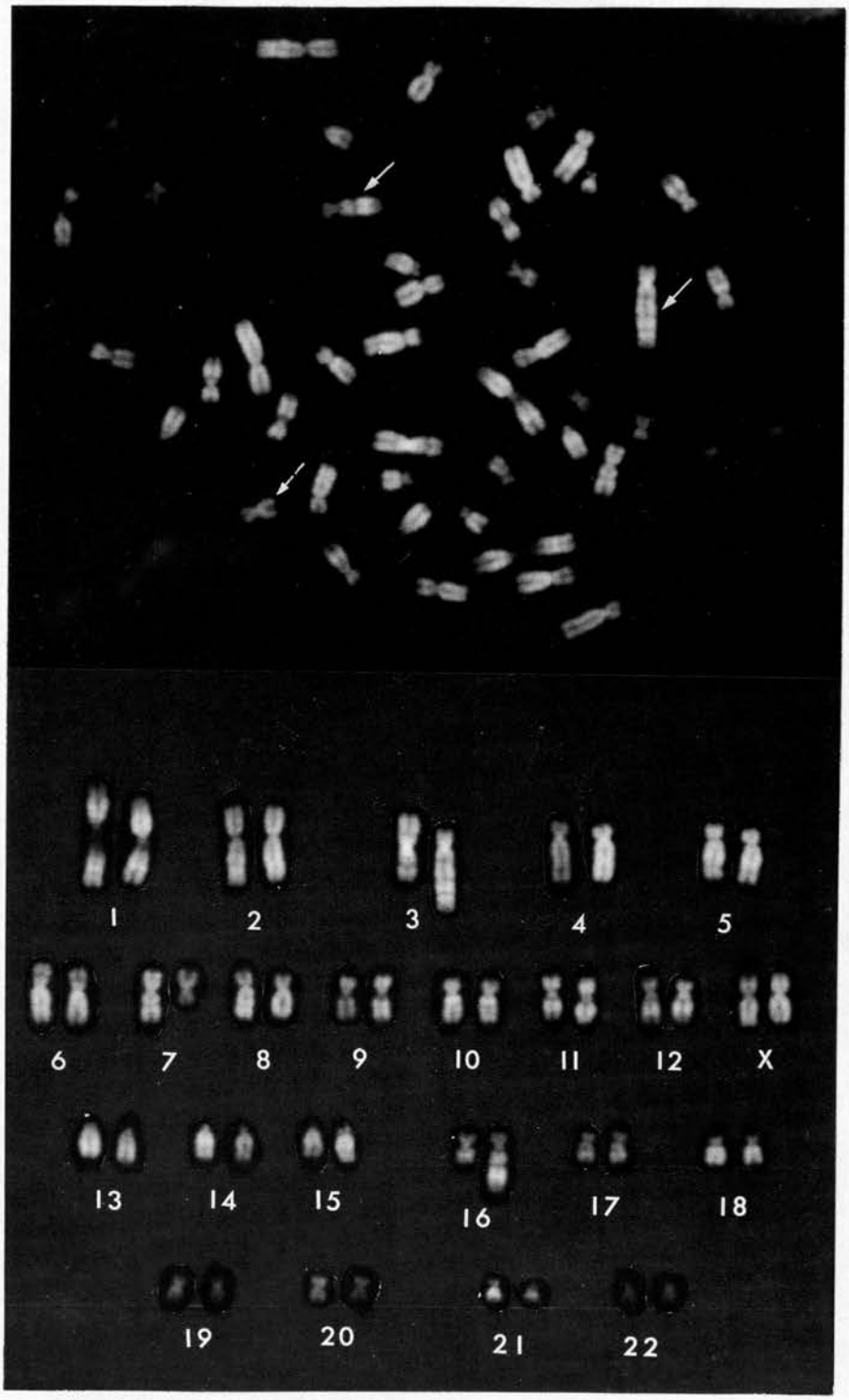
5 cells photographed. No two cells were identical in karyotype but all shared the major abnormalities described above. Two had 46 chromosomes, in one a C6 was missing and in the other, the D14 pair appeared to have fused to form a very large acrocentric marker. Three had 47 chromosomes, one corresponded exactly to the modal karyotype of the 10-13 month cultures, but had a prominent secondary constriction at the mid point of the long arms of a D13. In the other two, an additional small submetacentric marker was present similar in fluorescent pattern to an E18, but with shorter long arms. One of these cells had lost the normal member of the B4 pair, the other had lost an E16 and one of the A1 pair appeared to have undergone pericentric inversion.

21 months in culture (in Edinburgh)

5 cells photographed. The modal karyotype (4 cells) comprised 47 chromosomes, with an extra C7 and a metacentric marker as described above. One of the C8 pair was absent and extra material was present, as before, on the long arms of a B4. In addition one of the D13 pair in every cell was greatly enlarged, apparently by duplication of the long arms. A typical modal cell is shown in figure 41. The abnormalities are arrowed.

The remaining cell also had 47 chromosomes and differed from the modal type only in that two normal D13's were present but extra material, equivalent in size and fluorescent pattern to the long arms of a D13, was present on the short arms of a D15.

Fig. 42a



42a. G-S$_1$ cell representative of the line after 7-8 months growth. For description of the abnormalities (arrowed) see text opposite.

Cell line G-S_1

This line was grown spontaneously from the peripheral blood of an 86 year old female patient with chronic lymphatic leukaemia (see Chapter II). The results of examining orcein-stained preparations are reported in the preceding paper (Steel, McBeath and O'Riordan, 1971).

I have examined quinacrine-stained preparations from samples representing four different periods of growth in vitro.

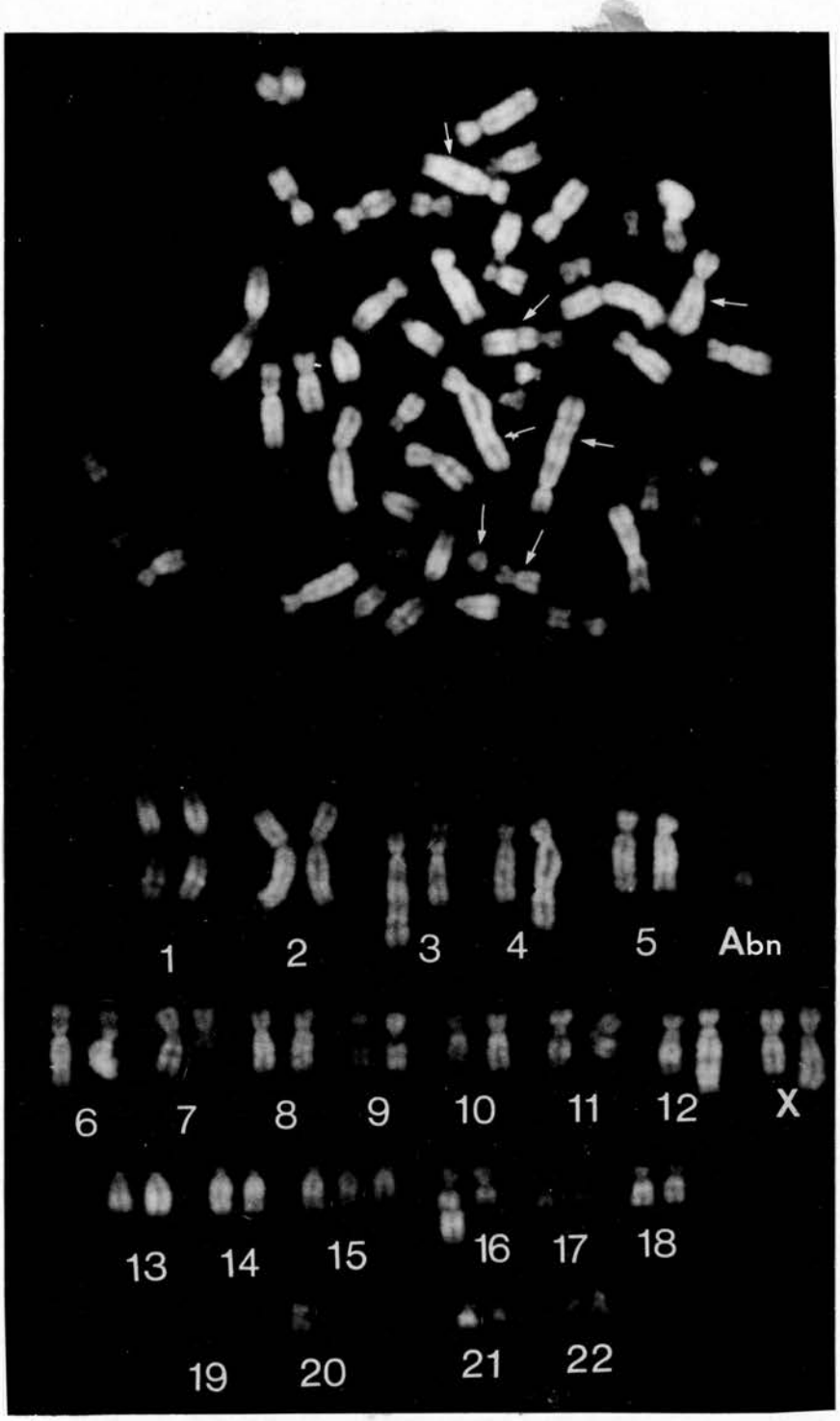
7-8 months in culture

9 cells photographed. Modal karyotype (5 cells) comprised 46 chromosomes. There appear to have been two separate translocations involving chromosomes A3, C7 and E16 so that most of the long arms of one C7 have become attached to one of the arms of an A3. Part of the other arms of the same A3 chromosome has detached and joined itself to the long arms of an E16. The remaining C7 material (short arms, centromere and proximal part of long arms) remains free and in orcein-stained material would be regarded as an E16. In total, therefore, these cells contain all the material of the normal diploid karyotype with the exception of fragments lost at the points of breakage.

A typical modal cell is shown in fig.42a. The abnormalities are arrowed.

Of the remaining four cells, two had 45 chromosomes, both lacking a G21 but showing all the abnormalities described

Fig. 42b



42b. G-S$_1$ cell representative of the line after 32 months growth. For description of the abnormalities (arrowed) see text opposite.

above. Two had 47 chromosomes and differed from the modal type by the addition of a metacentric chromosome slightly smaller than an F19.

21-24 months in culture

5 cells photographed. All had an identical karyotype comprising 47 chromosomes. All the abnormalities of the modal type described above were present. In addition there was an extra D15 and enlargement of the long arms of one of the B4 pair.

30 months in culture

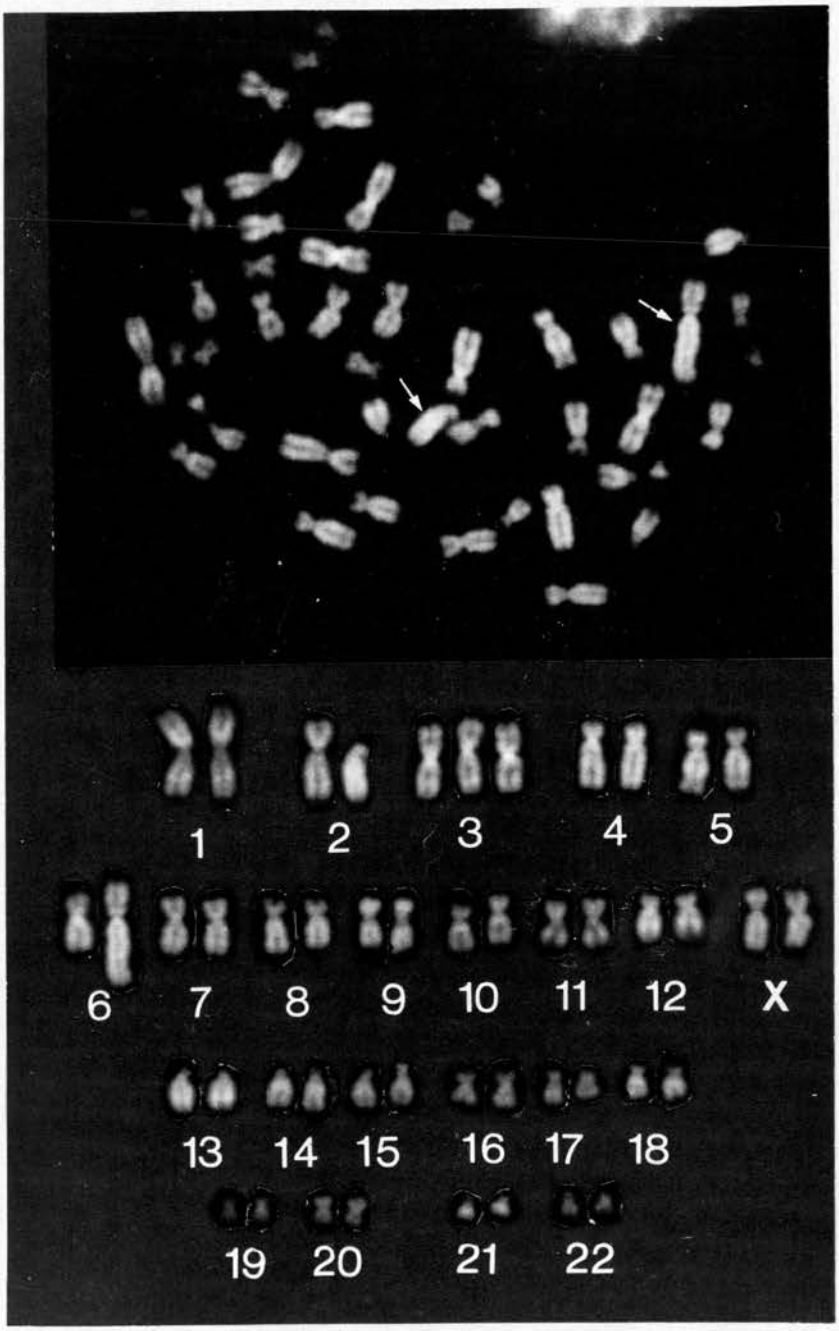
5 cells examined. All had an identical karyotype comprising 47 chromosomes. All the abnormalities of the 21-24 month modal type were present but extra material was now present on the long arms of an X and on the long arms of one of the C12 pair. In the latter case the additional material had a distinctive banding pattern suggesting that it represented partial reduplication of the C12 long arms.

32 months in culture

4 cells photographed. All had an identical karyotype comprising 48 chromosomes. All the abnormalities of the 30 month modal type were still present plus an extra small acrocentric chromosome which was moderately fluorescent and which appeared to lack satellites. It therefore did not appear to represent trisomy of the G21 or G22 group.

A typical modal cell is shown in fig. 42b. The abnormalities are arrowed.

Fig. 43



43. COA_1 cell representative of the line after 13-20 months growth. For description of the abnormalities (arrowed) see text opposite.

Cell line COA$_1$

The origin of this line from the peripheral blood of a female patient with atypical myelofibrosis is described in Chapter II and the findings on analysis of orcein-stained chromosomes are reported in the preceding paper (Steel, McBeath and O'Riordan, 1971).

A total of 20 cells have been photographed after periods of 13 to 21 months growth. There was no evidence of a change in the modal karyotype within this period.

Eight cells had 47 chromosomes including an extra A3. One A2 and one C6 was missing fromeach and two markers were present, one larger than an A2 with a dark band on the short arms close to the centromere, similar to the feature seen on a normal C6. The centromere itself was brighter than in a normal A2. The other marker was about the size of a C group chromosome but with a sub-terminal centromere. It seems likely that the larger marker consists mainly of a C6 with extra material on both arms, some of which is presumably derived from an A2. The smaller marker probably consists mainly of the centromere and long arms of an A2.

A typical modal cell is illustrated in fig.43. The abnormalities are arrowed.

Of the remaining twelve cells, five had 46 chromosomes, there were two each with 45, 44 and 48 and one with 49. They appeared to differ from the modal type only by random gains or losses of normal chromosomes.

Cell line BLA_1

The origin of this line from a male patient with acute leukaemia, by co-cultivation with irradiated F137 cells, is described in chapter III.

Short term cultures of the donors peripheral leukocytes yielded no divisions without phyto-haemagglutinin. From preparations cultured with added phyto-haemagglutinin ten metaphase spreads were analyzed (orcein stained preparations). All had a normal male karyotype.

Fifty cells were analysed from orcein stained preparations made after the line had been in culture for 4 months. The modal karyotype (47 cells) comprised 45 chromosomes, interpreted as +A3, -C, -D, -$\frac{1}{2}$9, + tiny acrocentric marker.

I have examined quinacrine stained preparations from samples representing two different periods of growth in vitro.

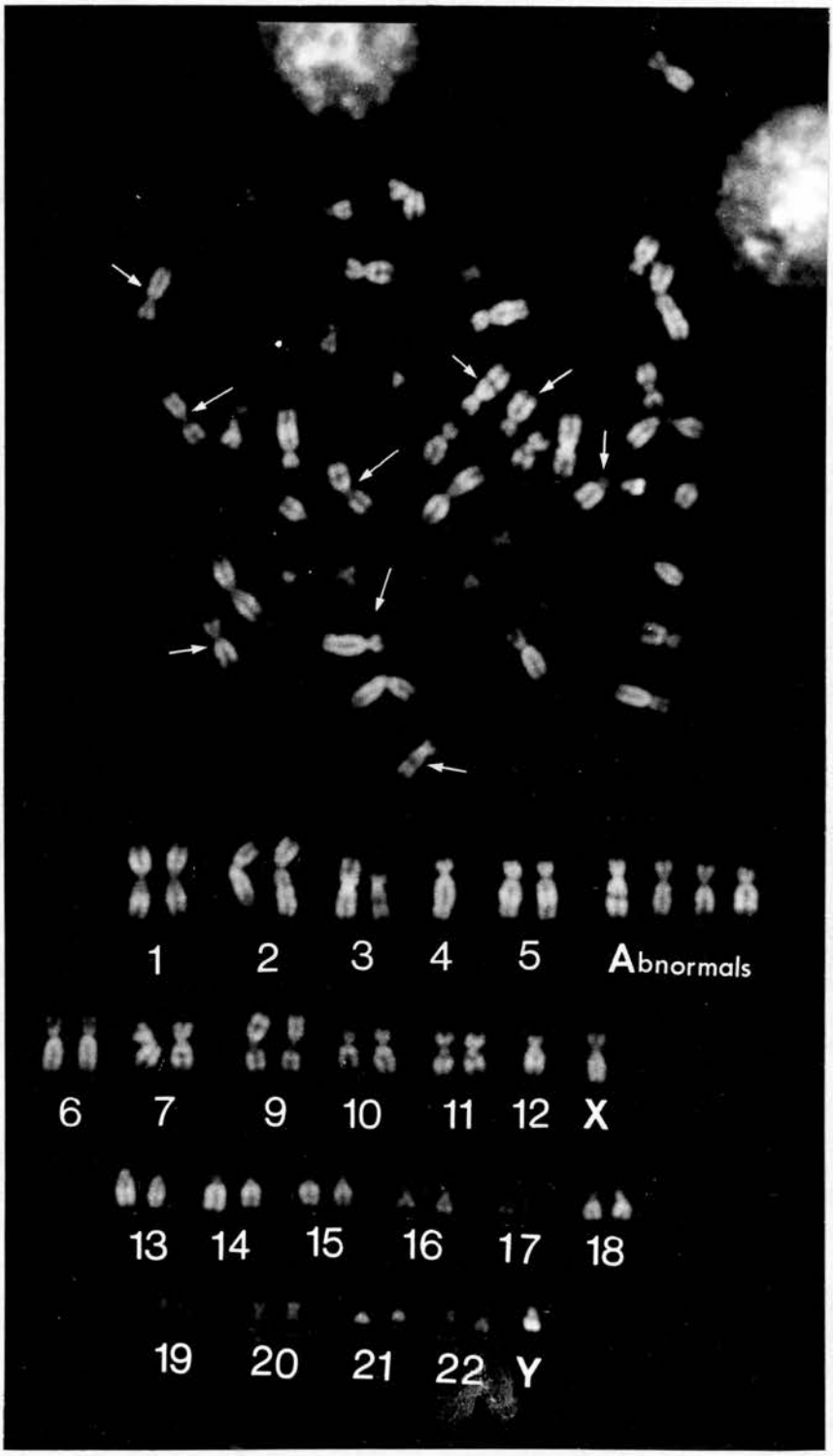
3-7 months in culture

7 cells photographed. The modal karyotype (5 cells) comprised 45 chromosomes. Each lacked a normal A3, a B4, both C8's, both C9's and one C12, one C14 and an F19. In their place was a medium-sized chromosome corresponding to an A3 with partial deletion of one arm, and three other medium sized markers with banding patterns which did not correspond

to any normal C group chromosomes; one had a very prominent dark band at the mid point of the long arms, the second had a relatively weakly fluorescent centromere zone with a bright region near the tip of the short arms, similar to a normal C7 but the long arms were only moderately and uniformly fluorescent. The third had a bright spot at the centromere and very dark tips to the long arms. Two metacentric markers, similar in size to an A3, were present. The centromere and one arm of each could be identified as being derived from a C9 (centromere + long arm) but the other arm was different in the two cases. One had a dark region in the centre and probably represented the deleted portion of the broken A3 chromosome. The other was less prominently banded and may have represented the missing D14. Two other markers were present, one similar to a C8, but with very small dark short arms, the other a centric fragment probably representing a partly deleted F19.

Of the remaining two cells, one had 44 chromosomes, differing from the mode by loss of a G22, the other had 49 chromosomes with all the above abnormalities plus an A3, a B4 and a C12 and a Y.

Fig. 44



44. BLA$_1$ cell representative of the line after 11-13 months growth. For description of the abnormalities (arrowed) see text opposite.

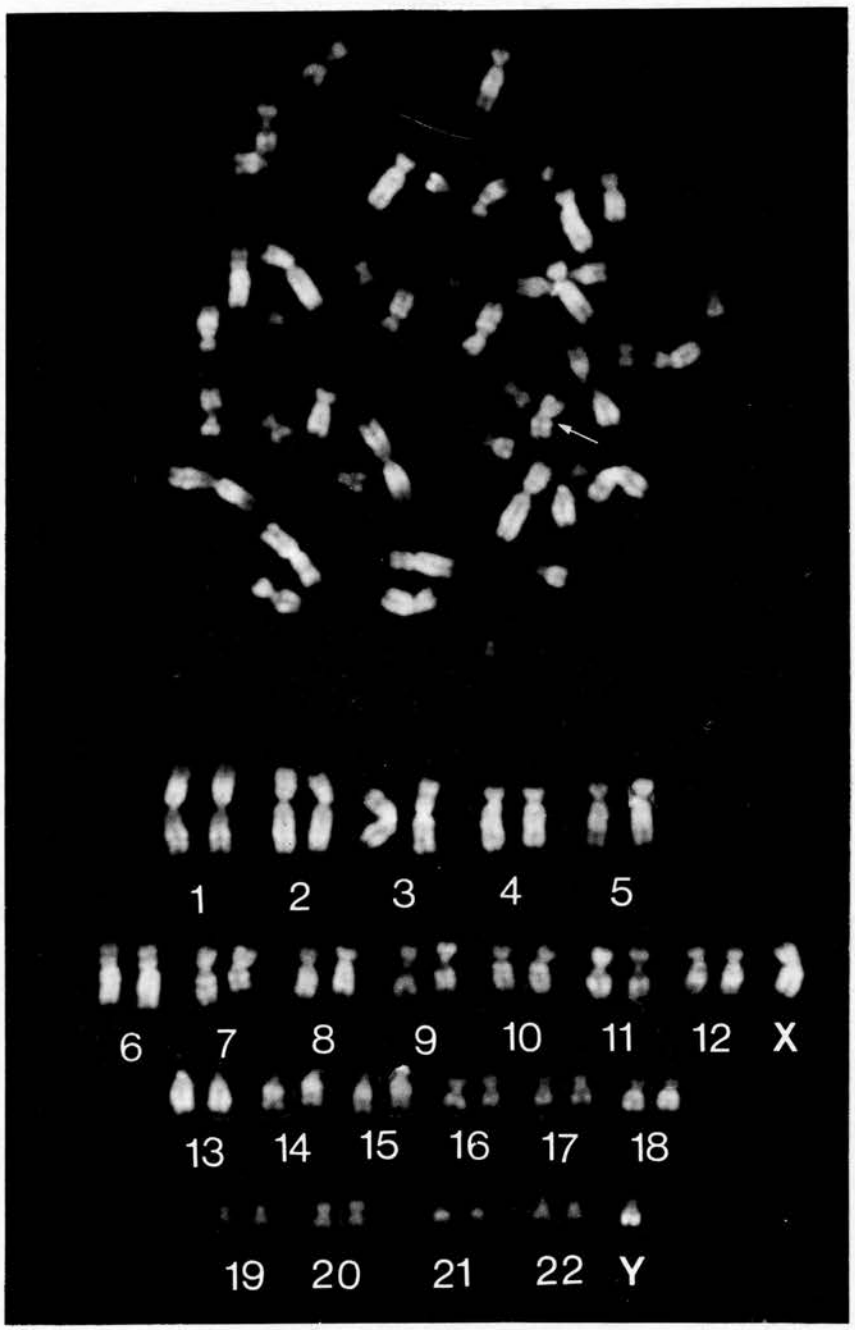
11-13 months in culture

16 cells photographed. The modal karyotype (15 cells) comprised 46 chromosomes including all the abnormalities of the earlier modal type described above, plus an additional abnormal chromosome the size and shape of a member of group C, but with a bright region near the distal end of the long arms and a very dark zone just beyond this.

A typical modal cell is shown in fig.44. The abnormalities are arrowed.

The remaining cell had 45 chromosomes and corresponded exactly to the modal karyotype of the 3-7 month cultures.

Fig. 45



45. MON_1 cell representative of the line after 7-9 months growth. For description of the abnormalities (arrowed) see text opposite.

Cell line MON_1

The origin of this line from the peripheral blood of a 4 year old boy with cystic fibrosis is described in Chapter II. It grew rather slowly at first and chromosome preparations were not made until it had been in culture for almost 5 months. Analysis of 50 orcein-stained metaphase spreads revealed a clear mode (48cells) of 45 chromosomes with one member of group C missing in every case.

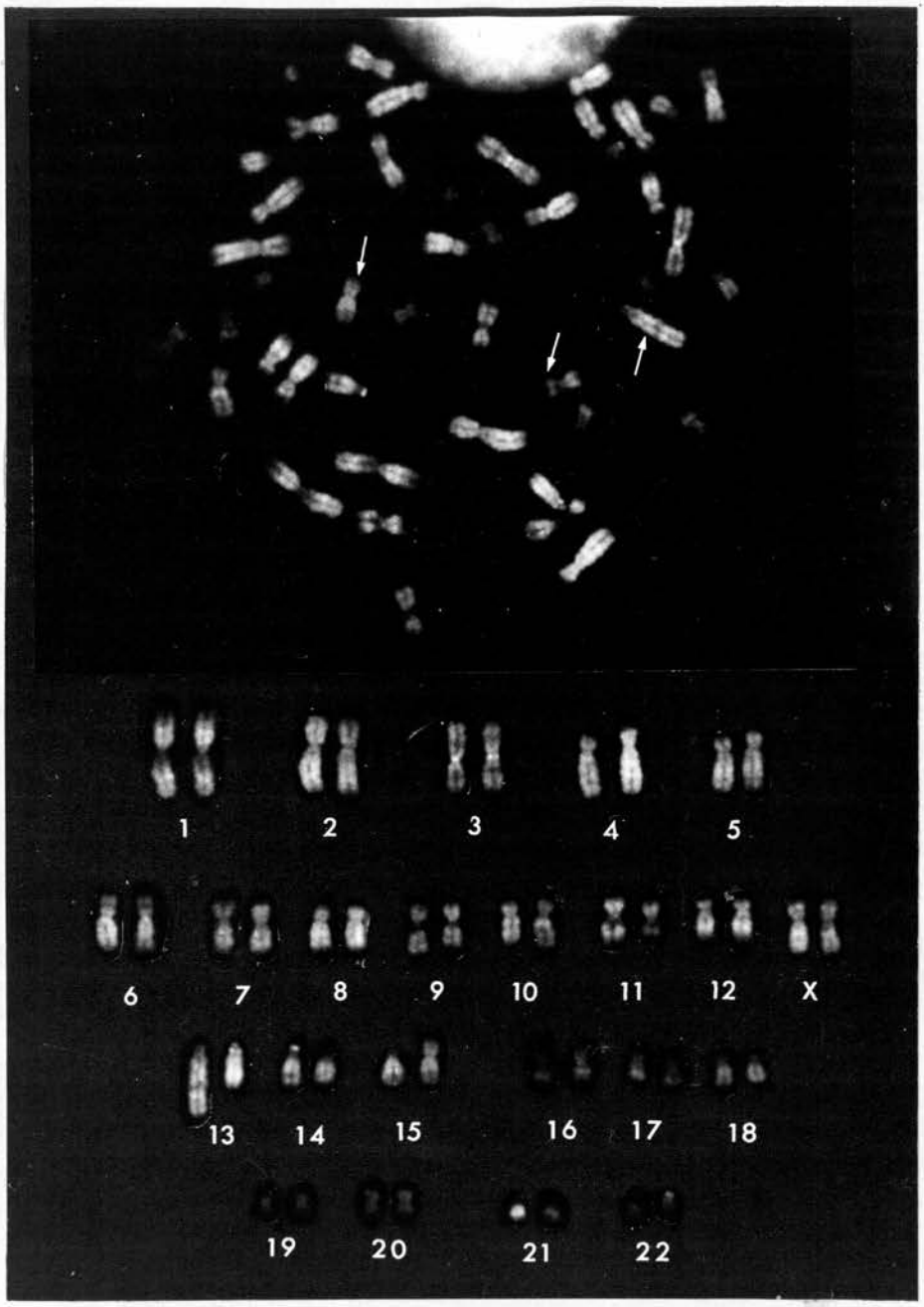
Short term culture of the donor's fresh blood lymphocytes (with added PHA) revealed a normal diploid male karyotype in all of the ten cells examined.

Quinacrine-stained preparations have been analysed from material which has been in culture for 7 to 9 months.

Sixteen cells were photographed. The modal karyotype (11 cells) comprised 46 chromosomes. In every case there was partial deletion of the long arms of one C7. A representative cell is shown in fig. 45. The abnormality is arrowed.

Of the remaining five cells, three had 45 chromosomes, and one each had 47 and 49. All had the partly deleted C7 and the gains or losses from the modal karyotype appeared to be random.

Fig. 46



46. GOL$_1$ (4299) cell representative of the line after 19-27 months growth. For description of the abnormalities (arrowed) see text opposite.

Cell line GOL_1

The establishment of this line from the peripheral blood of a female patient with infectious mononucleosis is described in Chapter II. Orcein stained preparations were initially found to be diploid but after ten months growth *in vitro* the modal karyotype was near-tetraploid. A subline (GOL_1 - 4299) was returned to culture from liquid nitrogen storage. After a total of ten months growth this subline was found to have become aneuploid (see preceding paper, Steel, McBeath and O'Riordan, 1971).

Quinacrine stained preparations have been examined from the 4299 subline after 19-27 months growth *in vitro*. Ten cells were photographed. The modal karyotype (nine cells) comprised 46 chromosomes. There was partial deletion of the long arms of a C11 in every cell and marked elongation of a D13, apparently by reduplication of the long arms. In five of the nine cells one of the D15 pair had extra weakly fluorescent material on the short arms. A typical modal cell is shown in fig. 46. The abnormalities are arrowed.

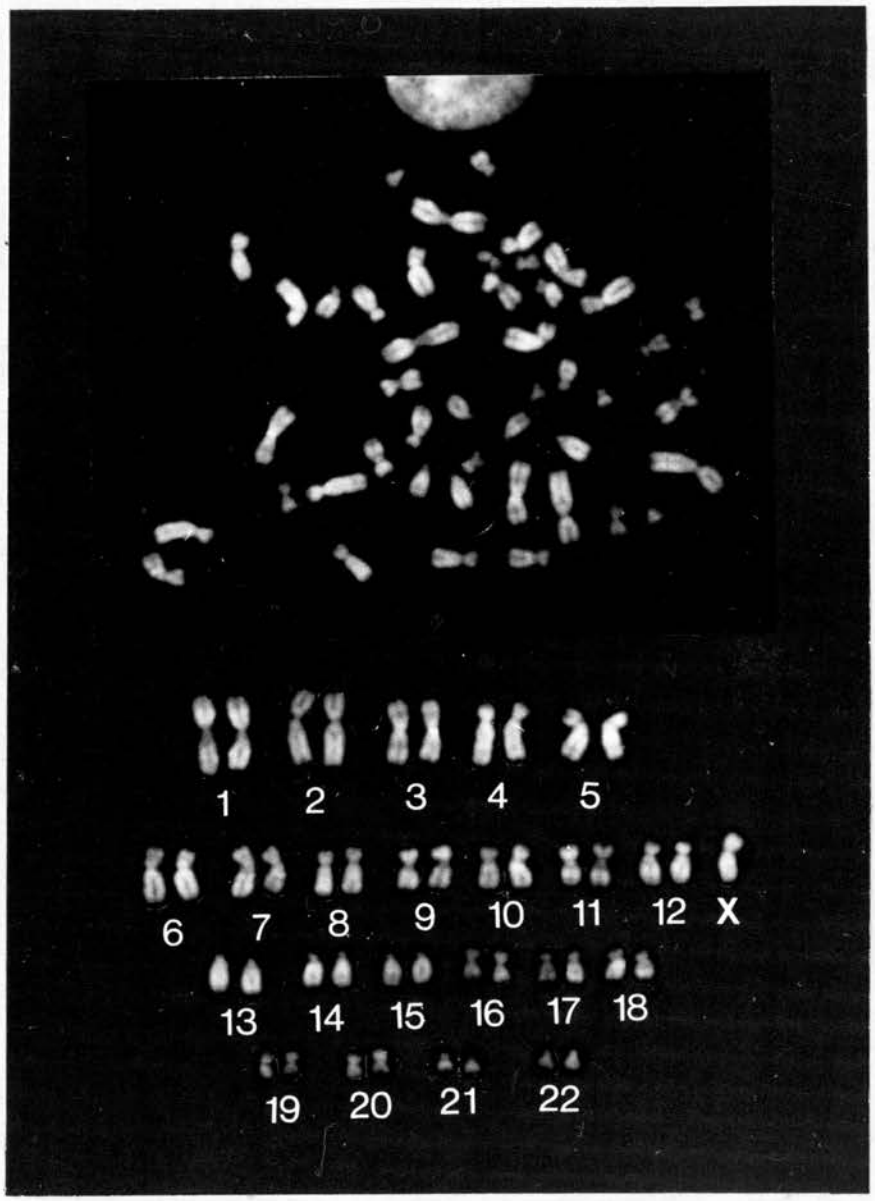
Fig. 47

47. GOL_1 cell (subtetraploid) representative of the line after 31 months growth. The abnormal metacentric chromosome (arrowed) is formed from a C4 with extra material.on the short arms.

The remaining cell had 45 chromosomes and differed from the modal type only by the absence of an A1.

Material from the parent (polyploid) line was examined after 31 months growth. Only three cells were photographed of which two contained 90 chromosomes and the other 89. The Robertsonian D/D translocation, noted earlier in orcein-stained preparations from this line, was not present at this stage, but in each of the three cells photographed there was an abnormal metacentric chromosome formed by the addition of weakly fluorescent material to the short arms of a C11 (see arrow in fig. 47).

Fig. 48



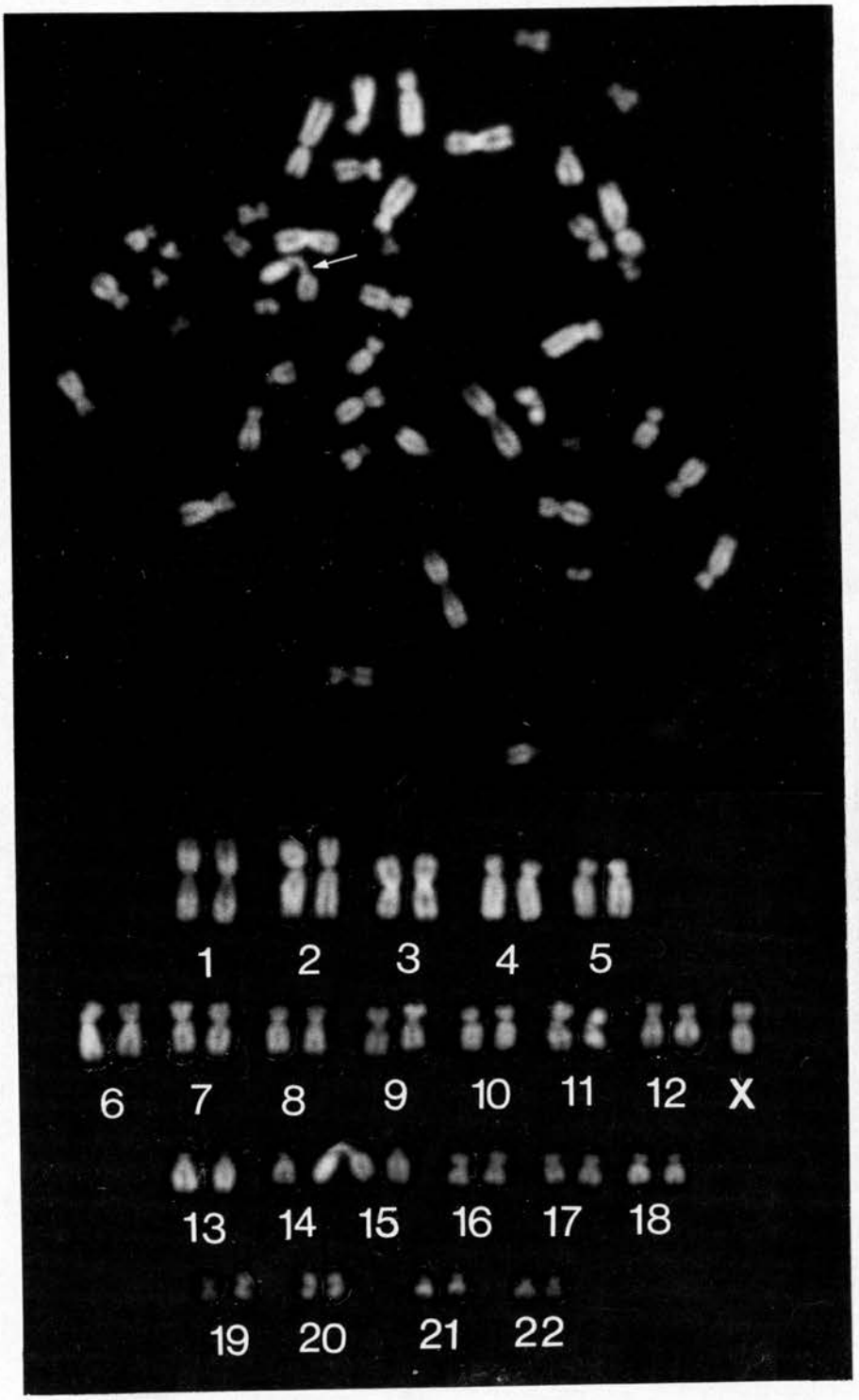
48. HUN$_1$ cell representative of the line after 17-18 months growth. There are 45 normal chromosomes but the Y is absent.

Cell line HUN_1

The establishment of this line from the peripheral blood of an 11 year old boy with acute leukaemia was described in Chapter II. Short term cultures of peripheral blood and orcein-stained preparations from the lymphoblastoid line during the first ten months in culture showed a diploid male karyotype (see preceding paper, Steel, McBeath & O'Riordan, 1971). This was confirmed in quinacrine-stained material prepared from the line after seven months growth (sex cells examined, all 46XY, no abnormality detected).

Futher preparations have been examined after 17-18 months *in vitro*. 15 cells were photographed. Of these, only one had a normal diploid male karyotype. A second had 45 chromosomes including a Y, the only abnormality being a Robertsonian 14/15 translocation. The Y was absent from the other thirteen cells. In five of them there was a 14/15 Robertsonian translocation, but in another four the remaining 45 chromosomes appeared normal. Two had 43 and 42 chromosomes respectively, including the 14/15 translocation. The remaining two cells each carried a dicentric chromosome apparently formed by fusion between

Fig. 49

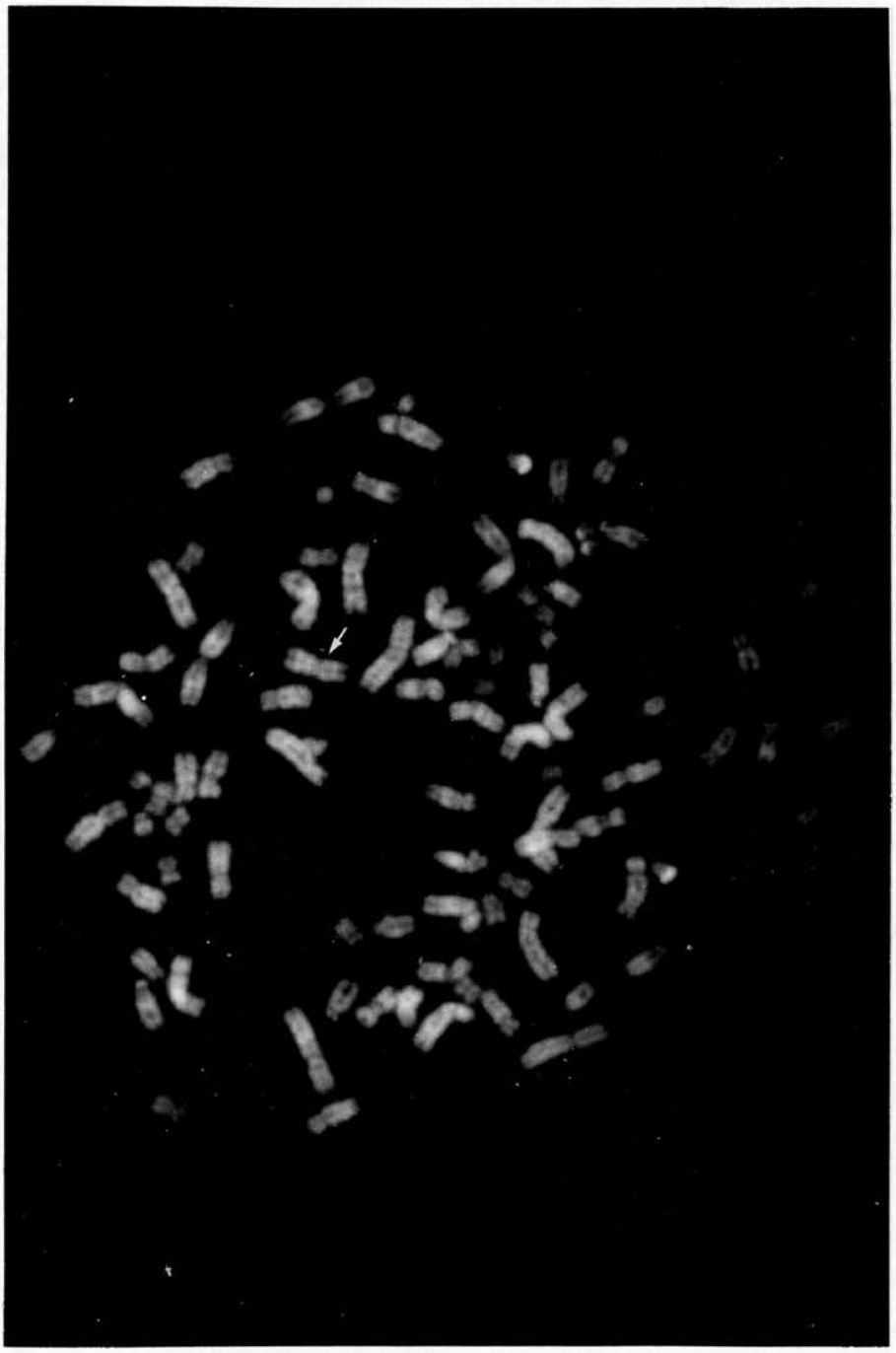


49. HUN_1 cell representative of the line after 17-18 months growth. There are 44 chromosomes, the Y is absent and there is a 14/15 Robertsonian translocation (arrowed).

the short arms of a C7 and a D14, one of these had no other abnormality except the absence of a Y, while the other had an additional submetacentric marker, slightly smaller than a member of group C, resembling an E16 in banding pattern, but with brighter short arms.

Figs. 48 and 49 illustrate two representative cells, one with a 45,XO karyotype, the other 44,XO tr 14/15.

Fig. 50



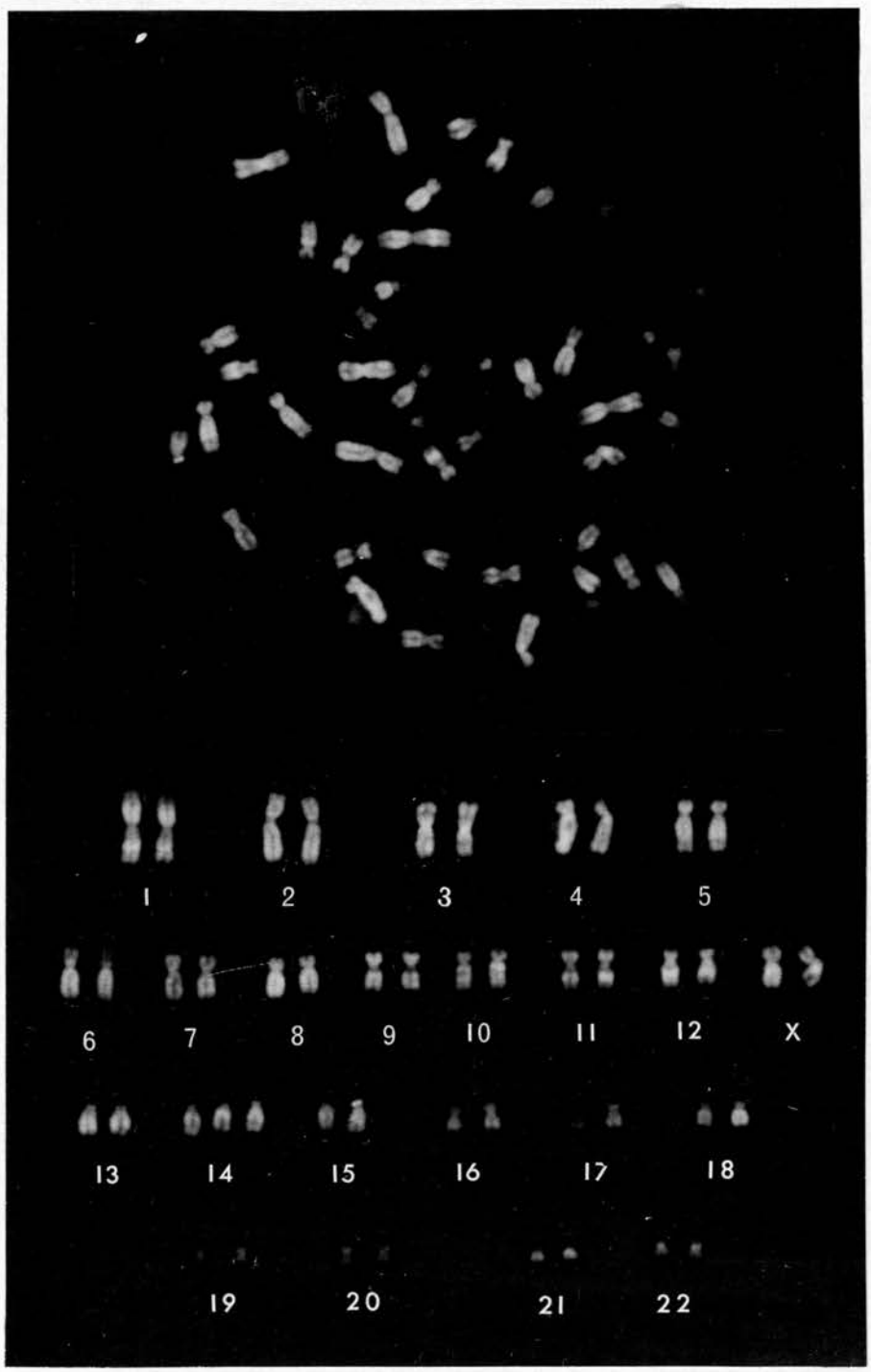
50. FLE_1 cell (subtetraploid) representative of the line after 24 months growth. The abnormal metacentric chromosome (arrowed) is formed from a C7 with additional material on the short arms.

Cell line FLE$_1$

The origin of this line from the peripheral blood of a male patient with infectious mononucleosis is described in Chapter II. Orcein stained preparations made during the first year in culture showed a normal male karyotype. (See preceding paper, Steel, McBeath & O'Riordan, 1971). This was confirmed in quinacrine-stained preparations after 13 months growth (six cells examined, all normal male karyotype).

Further quinacrine-stained preparations were made after 24 months in culture. Over 90% of the dividing cells were noted to be polyploid. Three were photographed. Two had 90 chromosomes, the other 89. All three included one C7 chromosome with extra, weakly fluorescent material on the short arms. This abnormality is arrowed in the cell illustrated in fig.50.

Fig. 51.



51. LAW_1 cell representative of the line after 27-29 months growth. There is trisomy D14.

Cell line LAW_1

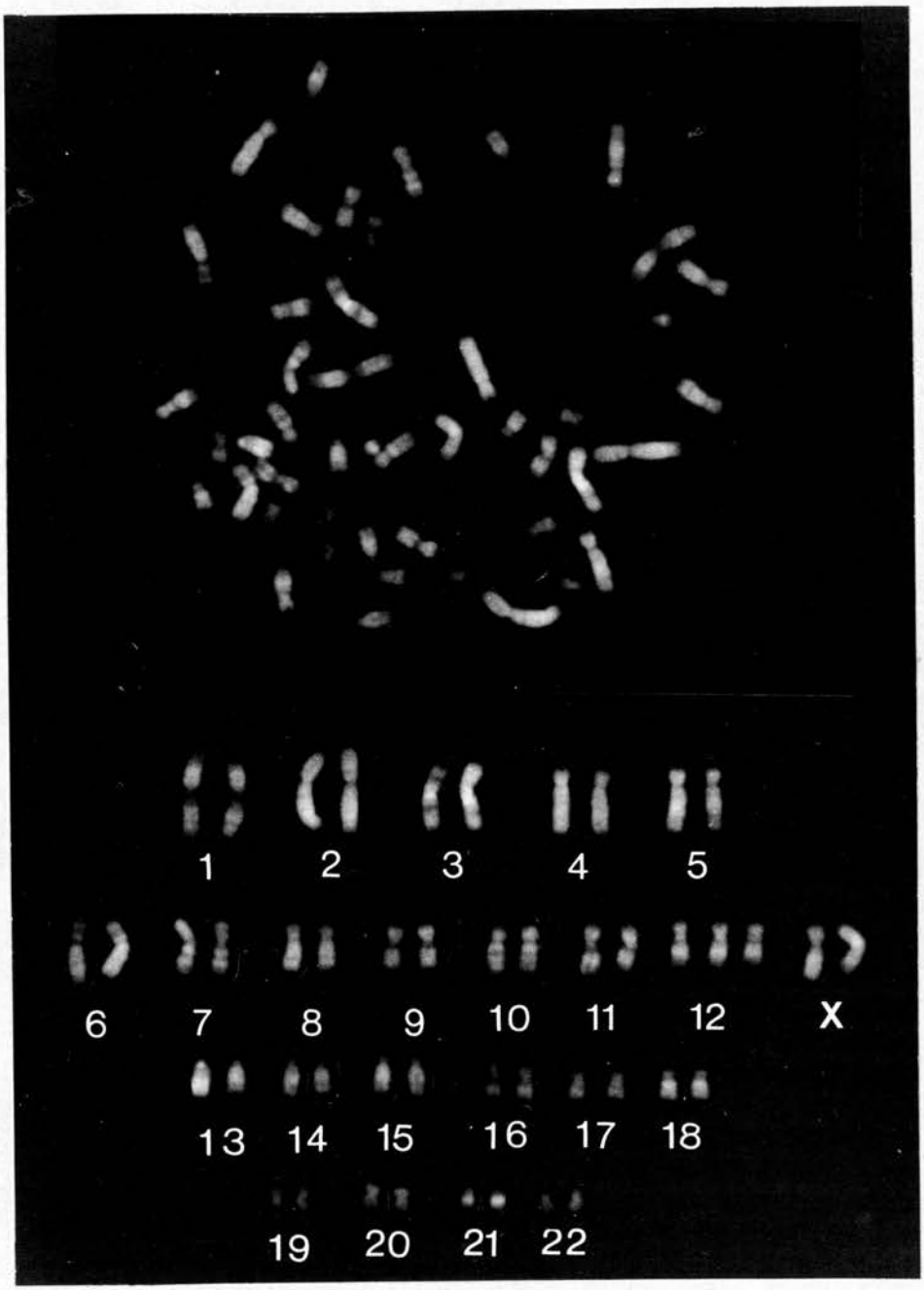
The origin of this line from the peripheral blood of a female patient with chronic myeloid leukaemia, by co-cultivation with lethally irradiated MAR_1 cells has been described in chapter III. Short-term cultures from the patient's leukocytes (without added PHA) revealed a 46,XX karyotype with a Ph^1 chromosome, in every cell (ten examined). Orcein stained preparations from the established line examined after 3 months in culture revealed a normal diploid femal karyotype in each of the 30 cells analysed. There was no evidence of a Ph^1 chromosome.

Quinacrine-stained preparations were made after 27-29 months in vitro. 16 cells were photographed. The modal karyotype (7 cells) comprised 47 chromosomes, the additional one being a D14. Two more cells had 46 chromosomes and one 45. In each there was trisomy 14, other chromosomes having been lost evidently at random. In three of these ten cells, one of the D14 chromosomes appeared to have a small amount of additional weakly fluorescent material on the short arms.

A representative cell is shown in fig.51.

The remaining 6 cells contained from 49 to 60 chromosomes, the last being near-triploid. The extra chromosomes appeared to have been gained at random.

Fig. 52



52. SHA_2 cell representative of the line after 24 months growth. There is trisomy C12.

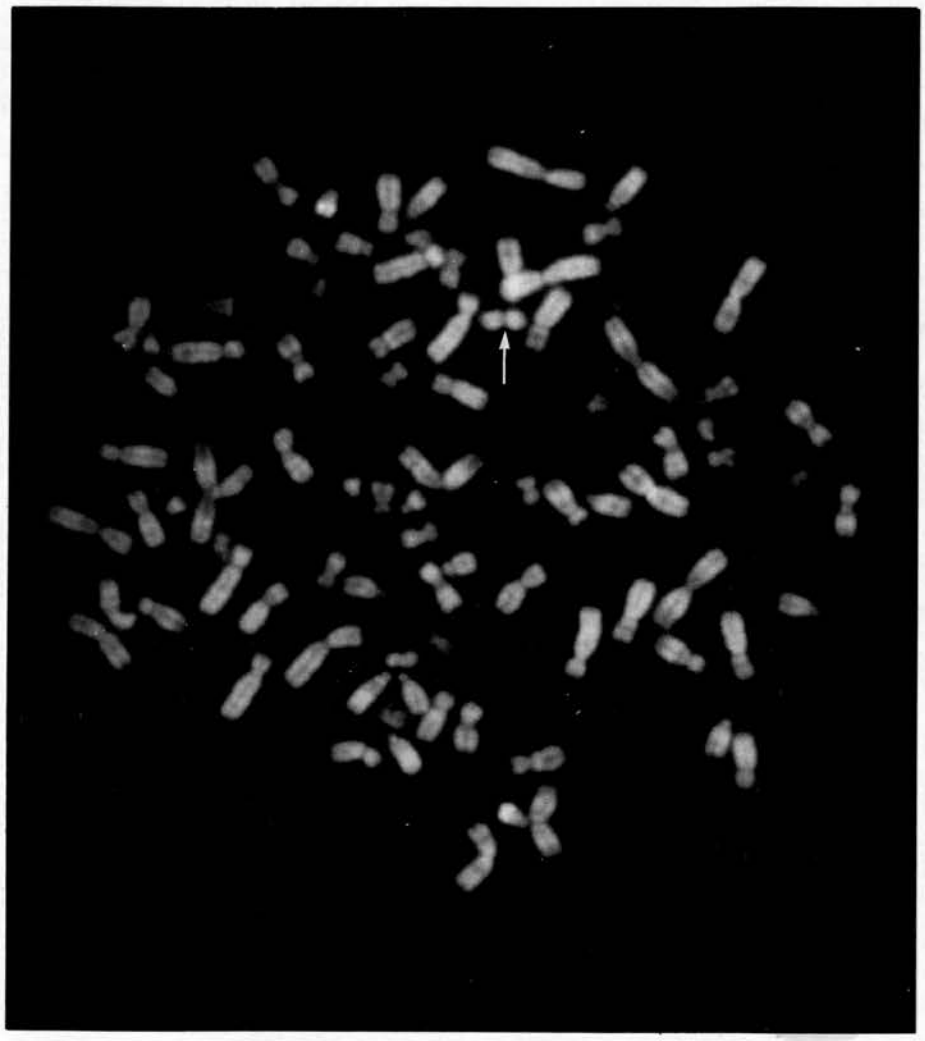
Cell line SHA_2

The origin of this line from the peripheral blood leukocytes of a female patient with chronic myeloid leukaemia, by co-cultivation with irradiated MAR_1 cells has been described in Chapter III. Short term cultures of the donor's fresh blood revealed at 46,XX karyotype with, in each of ten cells examined, a Ph^1 chromosome.

Thirty cells were analysed in orcein stained preparations from the line within three months of its establishment in culture. All had a diploid female karyotype with no evidence of a Ph^1 chromosome.

Quinacrine-stained preparations were made from the line after 24 months growth in vitro. 5 cells were photographed. 4 had 47 chromosomes, the extra one being a C12. The fifth cell had 46 chromosomes, including trisomy 12 and had lost one of the E18 pair. A representative cell is illustrated in fig.52.

Fig. 53



53. WHE$_1$ cell (subtetraploid) representative of the line after 14 months growth. A small metacentric marker chromosome is arrowed.

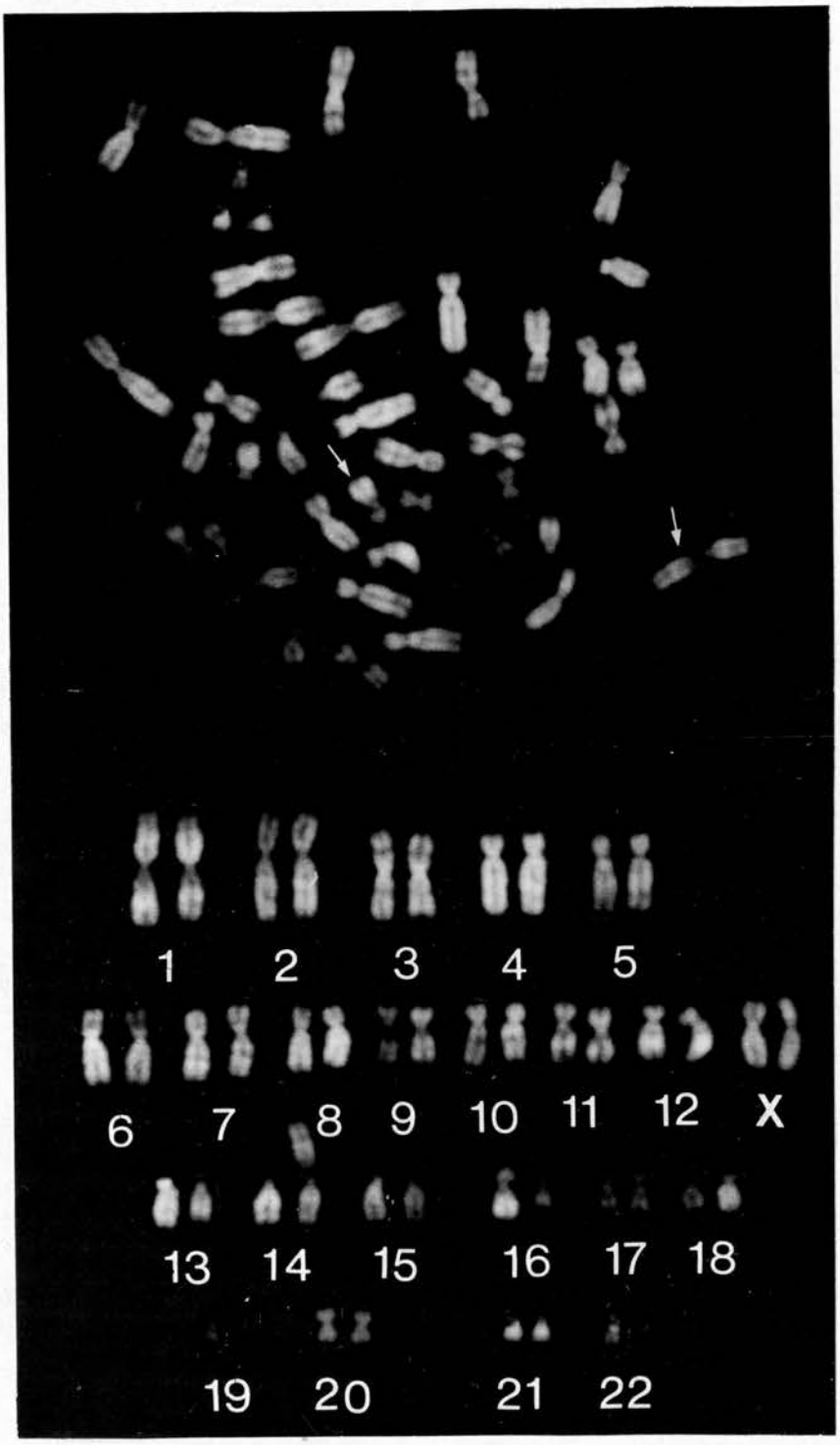
Cell line WHE_1

The origin of this line from cord blood leukocytes of a healthy male infant by co-cultivation with irradiated F137 cells has been described in Chapter III.

Thirty cells were analysed in orcein stained preparations from the line within three months of its establishment. All had a normal male karyotype.

After 14 months growth, quinacrine-stained preparations were made. Over 90% of the dividing cells were noted to be polyploid. 4 cells were photographed. Two had 87 chromosomes and one each 88 and 89. Every cell included the small metacentric marker with darkly staining centromere which is arrowed in figure 53.

Fig. 54



54. ORI$_1$ cell representative of the line after 18-19 months growth. For description of the abnormalities (arrowed) see text opposite).

Cell line ORI_1

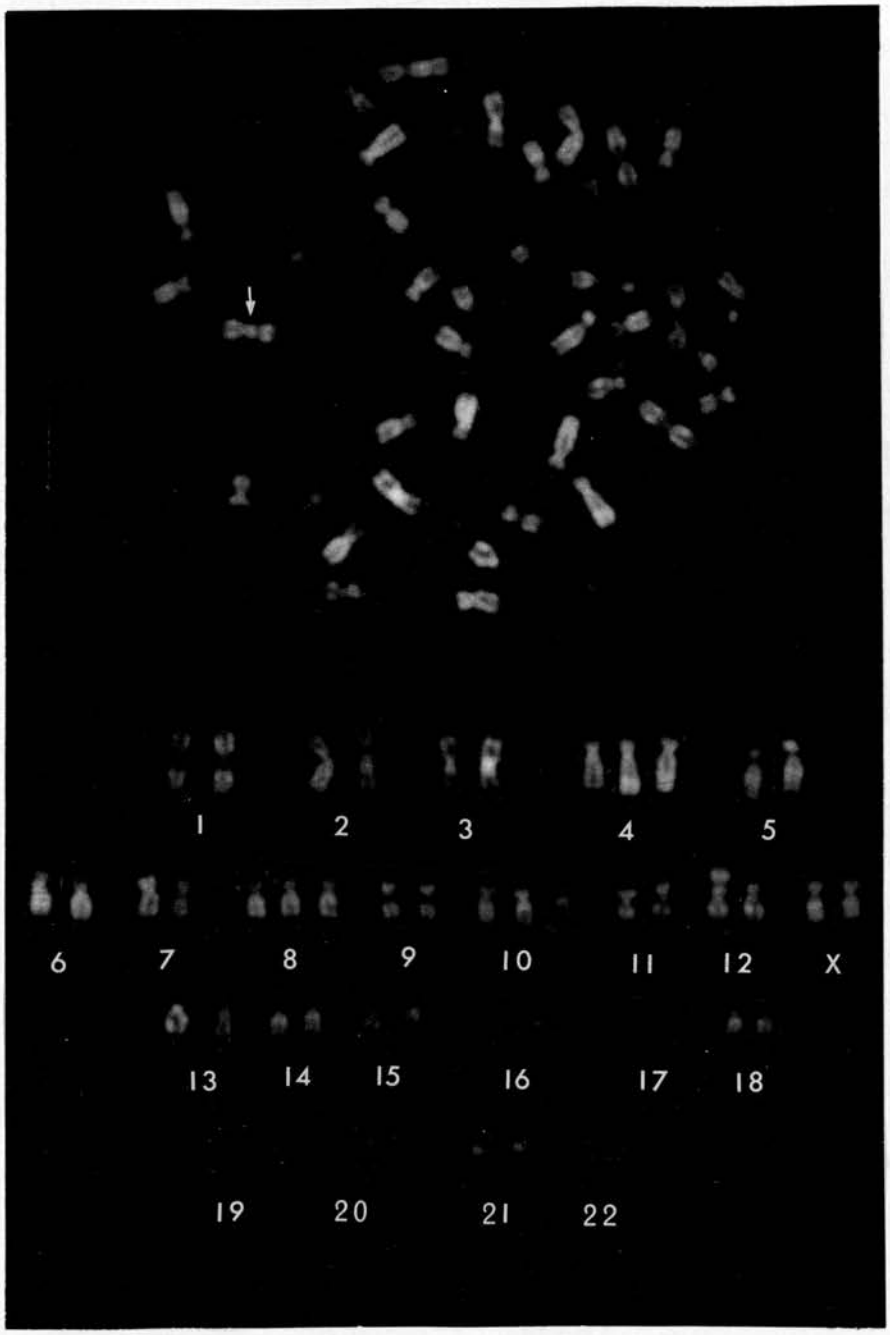
The origin of this cell line from the peripheral blood leukocytes of a healthy adult female by co-cultivation with irradiated F137 cells is described in Chapter III.

Thirty cells were analysed from orcein-stained preparations made within three months of establishment of the line. All had a normal female karyotype. This was confirmed in quinacrine-stained preparations made after eight months growth. Six cells were photographed and all had a normal female karyotype.

After 18-19 months *in vitro* further quinacrine-stained preparations were made and twelve cells were photographed. All had an identical karyotype comprising 46 chromosomes. One of the B16 pair had additional brightly fluorescent material on the long arms and one of the D14 pair had the long arms of an A1 chromosome translocated onto its short arms. Two normal A1 chromosomes were also present so that this line now has a partial A1 trisomy.

A representative cell is shown in fig.54. The abnormalities are arrowed.

Fig. 55



55. CLA_4 cell representative of the line after 18-19 months growth. For description of the abnormalities (arrowed) see text opposite.

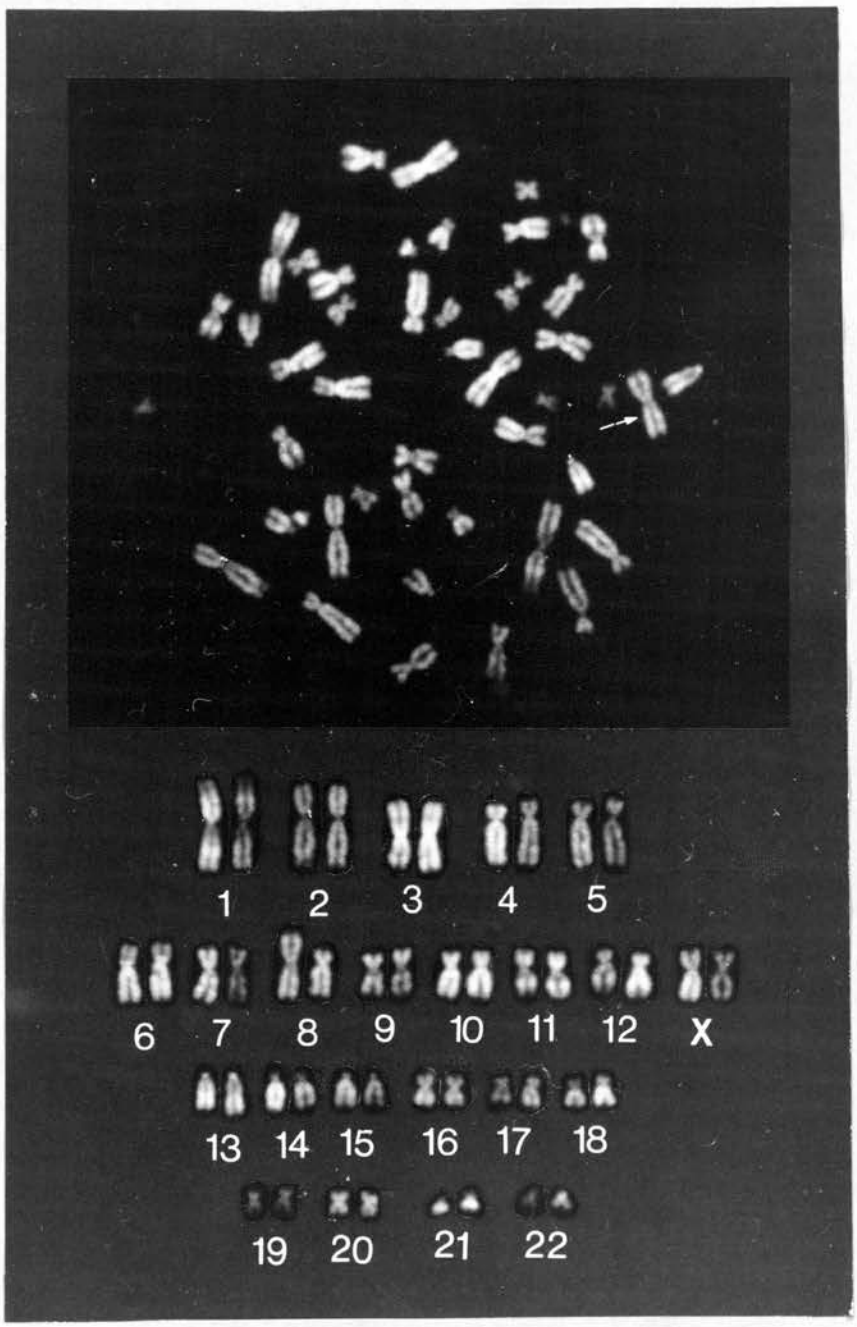
Cell line CLA_4

The origin of this line from the cord blood leukocyte of a healthy female infant co-cultivated with irradiated F137 cells has been described in Chapter III.

Thirty cells were analysed from orcein-stained preparations within three months of establishment of the line. All had a normal female karyotype.

After 22-23 months growth *in vitro*, quinacrine-stained preparations were made. Six cells were photographed. Four contained 49 chromosomes, the extra ones being a B4, a C8 and a C10. In addition there was a considerable amount of extra material on one of the C12 pair. From its fluorescent banding pattern this extra material appeared to be derived from the long arms of a C11. A representative cell is illustrated in fig.55. The abnormalities are arrowed. Of the remaining two cells, one had 46 chromosomes with a normal female karyotype, except for the enlarged C12 marker just described and the other had 47 chromosomes, again including the C12 marker, but with an additional C9 chromosome.

Fig. 56



56. LAM_2 cell representative of the line after 17 months growth. There is additional material on the short arm of one C8 (arrowed).

Cell line LAM_2

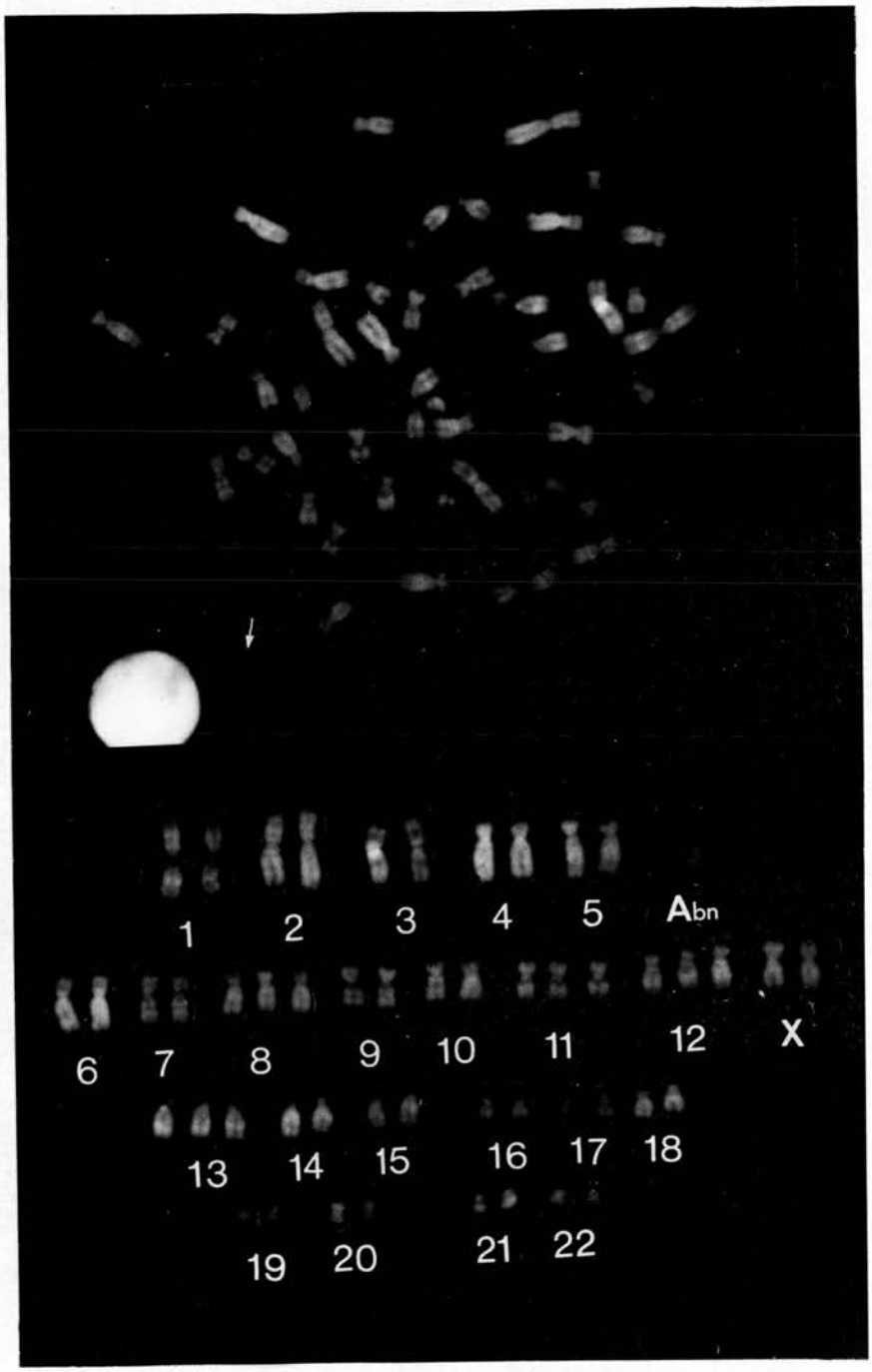
The origin of this line from the cord blood leukocytes of a healthy female infant, co-cultivated with irradiated MAR_1 cells has been described in chapter III.

Thirty metaphase spreads were analysed from orcein-stained preparations made within three months of establishing the line. All had a normal female karyotype.

Quinacrine-stained preparations were made from the line after it had been grown for 17 months. 4 cells were photographed. Two of these had 46 chromosomes, the only abnormality being marked elongation of the short arms of one of the C8 pair. This feature is arrowed in the cell illustrated in fig.56.

Of the other two cells, one had only forty-one chromosomes (including the marker C8). The other had 48 including an extra C9 and two of the C8 markers in addition to a normal C8.

Fig. 57



57. WEB_1 cell from a sample of the line which had been in culture for 22 months. For description of the abnormalities (arrowed) see text opposite.

Cell line WEB_1

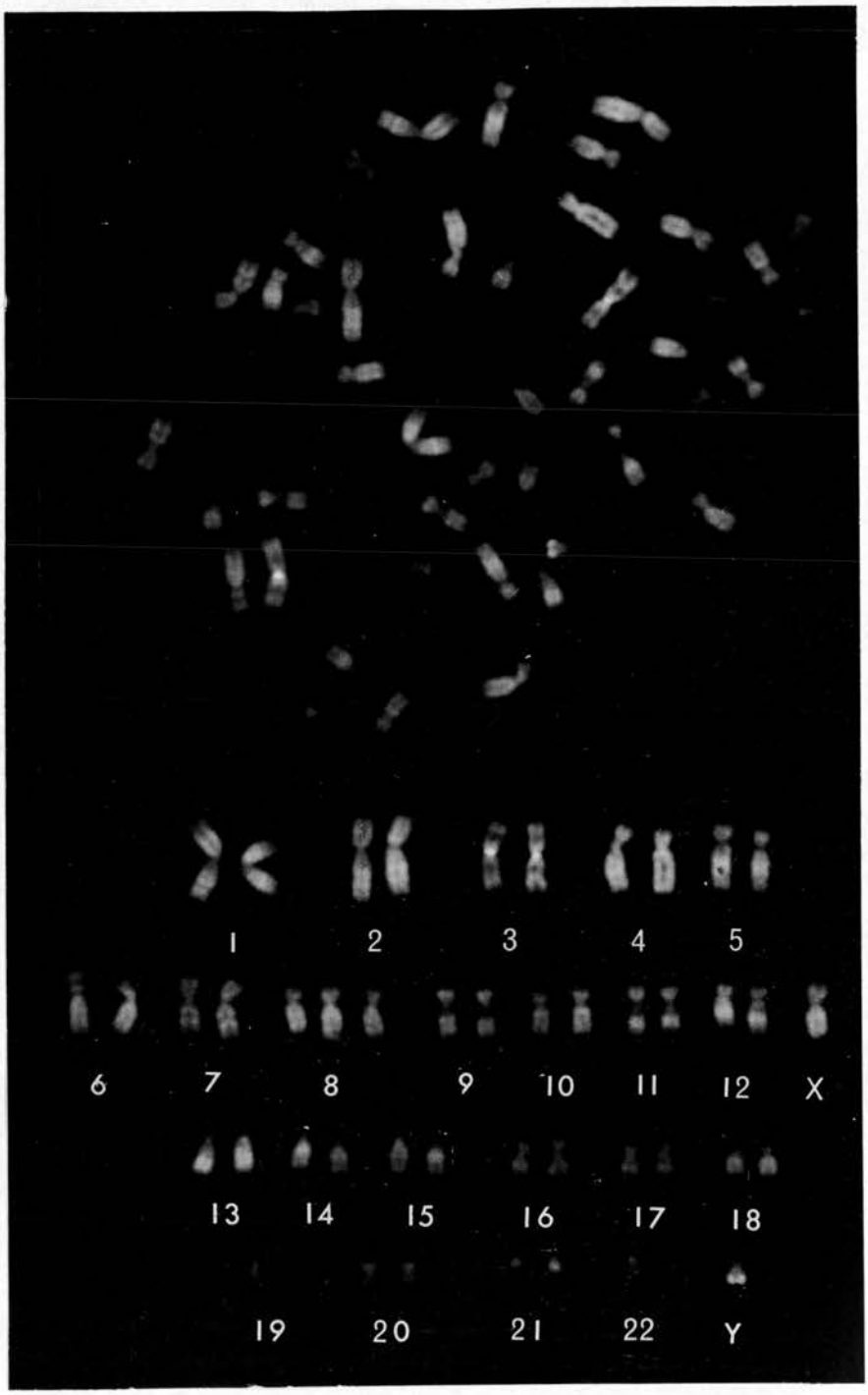
The origin of this line from the cord blood leukocytes of a healthy female infant, co-cultivated with irradiated MAR_1 cells has been described in chapter III.

Thirty metaphase spreads have been analysed from orcein-stained preparations made within three months of establishing the line. All had a normal female karyotype.

Quinacrine-stained preparations were made from the line after 22-23 months growth *in vitro*. Nine cells were photographed. The modal karyotype (4 cells) comprised 48 chromosomes including an additional C8 and D13. Two had 50 chromosomes, the extra ones being a C8, a C11 and a C12 and D13. Two more had 51 chromosomes and differed from those just described only by the addition of a very small weakly fluorescent acrocentric marker which could be distinguished morphologically from a G22. One of these cells is illustrated in fig.57. The abnormal marker is arrowed.

The last cell had 49 chromosomes including trisomy 11, 12 and 13.

Fig. 58



58. ODY_1 cell representative of the line after 12-13 months growth. There is trisomy C8.

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Non-Identity of Apparently Similar Chromosome Aberrations in Human Lymphoblastoid Cell Lines

A FEW chromosome aberrations seem to recur with significant frequency among the many aneuploid human lymphoblastoid lines established from various sources. (For discussion and references see ref. 1.) These include trisomy *C*, a marker chromosome ("m_B") larger than the chromosomes of group 4/5 but with a similar arm ratio and an acrocentric marker ("m_D") similar to an enlarged *D* group chromosome.

Since clones of cells bearing one or more of these abnormalities seem to have a selective advantage for growth *in vitro* it is important to know whether these apparently morphologically identical abnormalities consist of the same genetic material in each case. By using quinacrine fluorescence[2,3] to identify features specific to particular chromosomes in the human complement we can answer this question. Chromosomes in cells from fourteen lymphoblastoid lines, which originated in this laboratory or had been maintained here for more than 1 yr (Table 1), were examined by a technique described before[3].

From each line karyotypes from photographs of four to twelve metaphase spreads, showing good fluorescent banding patterns, have been studied in detail. In most of the aneuploid lines, the amount of chromosomal rearrangement revealed by quinacrine staining is greater than could be anticipated from orcein preparations, and some lines are composed of several distinct clones. In these cases, many more cells require to be examined before the definitive karyotype can be established. From the preliminary studies, however, the following results have been obtained.

Trisomy *C* was detected, on orcein staining, in lines JIYOYE and F137. In each of the seven JIYOYE cells examined with fluorescence there was trisomy of the number 9 chromosome and an additional metacentric, about the size of a number 16 which appeared to be composed of a number 7 chromosome with partial deletion of the long arm (partial trisomy 7) (Fig. 1*a*).

Twelve F137 cells have been photographed. In eight of these there was an extra chromosome morphologically belonging to group *C*. In five cases this was due to trisomy 7 (Fig. 1*b*) but in the other three the extra chromosome seemed to have been derived from a number 3 by partial deletion of one arm (Fig.

Table 1 Cell Lines Examined

Line	Source	Modal chromosome number	Diploid or aneuploid on orcein staining
JIYOYE*	Burkitt's lymphoma	48–50	Aneuploid
RAJI*	Burkitt's lymphoma	46	Aneuploid
F137†‡	Chronic lymphatic leukaemia	47	Aneuploid
F89†‡	Subacute lymphatic leukaemia	46	Aneuploid
GOL_1‡§	Infectious mononucleosis	46	Aneuploid
$G\text{-}S_1$‡§	Chronic lymphatic leukaemia	47	Aneuploid
Bla_1¶	Acute leukaemia (adult)	45	Aneuploid
COA_1‡§	Myelofibrosis	46–48	Aneuploid
FLE_1‡§	Infectious mononucleosis	46	Diploid
DUN_1‡§	Infectious mononucleosis	46	Diploid
SAD_1‡§	Infectious mononucleosis	46	Diploid
HUN_1‡§	Acute leukaemia (child)	46	Diploid
MIT_1¶	Acute leukaemia (adult)	46	Diploid
ORI_1¶	Healthy adult	46	Diploid

* Unpublished results of Pulvertaft.
† Ref. 4.
‡ Ref. 5.
§ Ref. 6.
¶ My unpublished results.

1*c*). In all five cells with trisomy 7, two of the number 7 chromosomes had a prominent subterminal secondary constriction on the long arm. This was not present in the other lines examined. An abnormality of this type has been reported in many cell lines, its frequency apparently depending on the technique used to establish each culture (see refs. 1 and 5 for further refs.).

Trisomy 7 was also present in all five RAJI cells examined. This could not have been predicted from the orcein preparations since another *C* group chromosome (number 8) was missing from every cell (Fig. 1*d*).

Three examples of marker m_B occurred among the eight aneuploid lines. Two were present in every $G\text{-}S_1$ cell and one in each RAJI cell. The fluorescent banding patterns showed that each had been formed in a different way.

One of the pair in $G\text{-}S_1$ appeared to match, in the centromere and proximal part of the long arm, with a number 3 chromosome and the distal part of the long arm was probably derived from a number 7. This line is monosomic for chromosomes 3 and 7. The other marker in $G\text{-}S_1$ corresponded to a number 4 with additional, brightly fluorescent, material on the end of the long arm. In RAJI the marker also seemed to be derived from a number 4 but the extra material at the end of the long arm appeared very dark on quinacrine staining (Fig. 1*e–f*).

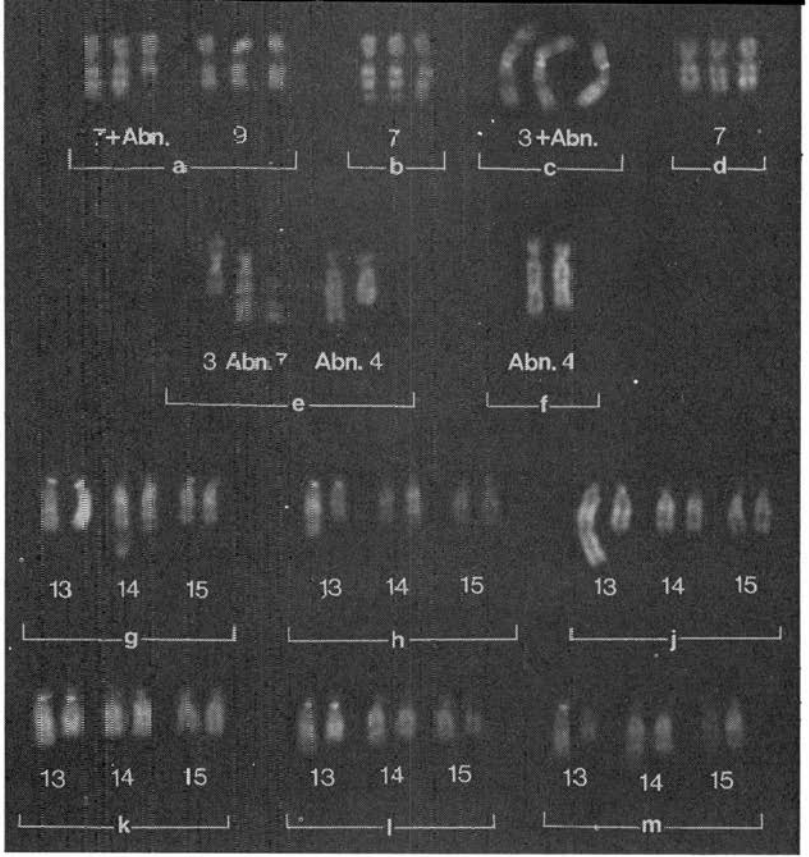

Fig. 1 The chromosomes in each group, *a–m*, are from a single cell. *a*, JIYOYE: trisomy 9 and partial trisomy 7. *b*, F137: trisomy 7. Two of the chromosomes show a prominent subterminal secondary constriction of the long arm. *c*, F137: abnormal chromosome, morphologically belonging to group *C* but, on banding pattern, probably derived from a number 3 as shown. *d*, RAJI: trisomy 7. *e*, G-S_1: two abnormal chromosomes of type "m_B", one probably derived from a number 3 and a number 7, the other from a number 4. *f*, RAJI: an abnormal chromosome of type "m_B" probably derived from a number 4 with additional non-fluorescent material on the long arm. *g*, JIYOYE: the chromosomes of group *D*, showing enlargement of a number 14. *h*, F89: the chromosomes of group *D*, showing enlargement of a number 13. *j*, GOL_1: the chromosomes of group *D*, showing enlargement of a number 13. (Probably duplication of the long arm.) *k*, F137: the chromosomes of group *D*, showing enlargement of a number 13. *l*, F137: the chromosomes of group *D*, showing enlargement of a number 13 and of a number 14. *m*, F137: the chromosomes of group *D*, showing probable 13/14 translocation.

An enlarged *D* chromosome was a feature of every cell of lines JIYOYE, F137, F89 and GOL_1. In JIYOYE marker m_D was derived from a number 14 (Fig. 1*g*), and in F89 and GOL_1 from a number 13. The latter two, however, had quite different banding patterns (Fig. 1*h* and *j*). Ten of the twelve F137 cells had a large number 13 (Fig. 1*k*) but in four cells there was one member of the 14 pair which was almost as large (Fig. 1*l*). In one cell from this line a number 14 was clearly the largest and there was a partial deletion of a number 13, possibly representing a 13/14 translocation (Fig. 1*m*).

In this relatively small sample of aneuploid lines, the apparently similar recurrent aberrations Trisomy *C*, m_B and m_D have each evolved in at least three different ways. It seems likely therefore that aneuploidy arises in lymphoblastoid cells by a wide variety of non-disjunctional, breakage and recombination events, so that the apparent recurrence of distinctive markers in several lines reflects chiefly the limitations of standard staining techniques for the identification of individual chromosomes. These limitations apply not only to obviously aneuploid lines since it is theoretically possible for major rearrangements of genetic material to be concealed in an apparently diploid karyotype. No abnormality has yet been detected, however, on constructing quinacrine fluorescence karyotypes of the six diploid lines listed in Table 1.

In permanent human cell lines, all chromosomes may not be equally susceptible to loss, reduplication or breakage while it is likely that certain aberrations have a more profound effect than others on the viability of the involved cell and its progeny. Thus, for example, an abnormality involving chromosome number 7 was present in four of the eight aneuploid lines in this series. Since up to twelve separate aberrations could be recognized in each cell of any one line, however, there may be more significance in the fact that a normal pair of numbers 1, 10, 15, 17, 18, 20 and 22 was present in every line and no reduplication of these chromosomes could be detected. More definite information on the frequency (and importance to the cell) of aberrations affecting the different chromosomes of the human complement should prove to be one of the most interesting outcomes of further studies along these lines.

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Human Lymphoblastoid Cell Lines. II. Cytogenetic Studies [1]

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SUMMARY—Cytogenetic studies were undertaken on 14 human lymphoblastoid cell lines. Four were aneuploid, all from elderly patients with malignant disease, and eight from young patients with infectious mononucleosis were diploid. It is uncertain whether the age or the diagnosis of the donor is the important factor in determining the karyotype of an established line. The evidence suggests that aneuploidy usually arises *in vitro* from an inoculum of diploid cells. The modal karyotype of a mass culture changes by the emergence of a clone growing more rapidly than the parent line. The "marker C" chromosomes, described by others, was not found in any of 12 lines initiated in the authors' laboratory but was present in 89% of divisions in a line obtained from another center. There is no good evidence for the recurrence of any other specific chromosome aberration in different cell lines.—J Nat Cancer Inst 47: 1203–1214, 1971.

MANY CYTOGENETIC STUDIES on human lymphoblastoid cell lines have now been reported (*1–25*). The findings have raised several points of interest, notably the possible relationship between Epstein-Barr virus (EBV) infection and certain chromosome aberrations (*7*, *8*, *13*, *22*, *24*, *25*), the significance of an abnormal karyotype as a criterion of malignancy in cultured cells (*20*, *21*, *26*), and the theoretical implications of the chromosomal changes commonly observed in cell lines after a period in culture (*12*).

With these points in mind, 14 cell lines were examined, none of which has been the subject of previous cytogenetic studies.

MATERIALS AND METHODS

The cell lines studied are detailed in table 1. All were maintained at 37°C in a humidified atmosphere of 7% CO_2 in air. They were fed twice weekly and subcultured once or twice weekly. The nutrient medium was Ham's F10 (Flow) supplemented with 20% fetal calf serum and 10% tryptose phosphate broth (Difco).

Chromosome preparations were made from growing cells by the method described by Tough *et al.* (*17*). Twelve lines were examined initially within 3 months of becoming established in culture (*27*). Two lines, F89 and F137, were kindly supplied by Dr. E. M. Jensen. These had been grown for approximately 18 months before this study and, in addition, had been stored in liquid nitrogen for 2 years (*28*). At the initial examination, 30

[1] Received June 7, 1971; accepted August 11, 1971.

[2] We are grateful to Dr. E. M. Jensen who kindly supplied cell lines. The photographs were made by Mr. Norman Davidson and Mrs. Heather Houston.

TABLE 1.—Initial cytogenetic findings in 14 cell lines

Cell line	Donor			EBV present?†	Cytogenetic data				
	Age (yr)	Sex	Diagnosis*		Number of cells analyzed	Chromosome count distribution			Karyotype
						<45	45	46	47	
GOL_1	22	F	I. M.	+	30	2	1	27		46, XX
MAR_1	29	M	I. M.	+	30		1	29		46, XY
SAN_1	18	F	I. M.	+	30		1	29		46, XX
DUN_1	19	M	I. M.	+	30	1	1	28		46, XY
DEW_1	18	F	I. M.	+	30		2	28		46, XX
FLE_1	30	M	I. M.	+	30			30		46, XY
BER_1	17	M	I. M.	|	30			30		46, XY
SAD_1	13	M	I. M.	+	30			30		46, XY
G-S_1	86	F	C. L. L.	+	50			50		46, XX, 3-, C-, mar^1+, mar^2+
COA_1	67	F	Myelofibrosis (Atypical)	|	50	1	2	47		46, XX, inv(2p-q+), inv(Cp-q+)
COA_2				|	50	3		47		46, XX
HUN_1	11	M	A. L. L.	+	50		2	45	3	46, XY (3 cells 47, XY, C+)
F89	93	M	Sub L. L.	+	50			36		46, XY, C-, C-, B+, mar^1+, Dq+
								14		46, XY, C-, C-, C-, B+, mar^1+, mar^2+, Dq+
F137	?	M	C. L. L.	+	50		1	10	39	47, XY, C+, Dq+

*I. M. = infectious mononucleosis. C. L. L. = chronic lymphatic leukemia. A. L. L. = acute lymphoblastic leukemia. Sub L. L. = subacute lymphatic leukemia. All lines except F89 and F137 had been established for less than 3 months at the time of this analysis. F89 and F137 had been in culture for approximately 18 months.

†Based on electron microscopy and immunofluorescence. Percentage of virus-positive cells varies with time but never >5% in any line. For details of F89 and F137 *see* (*28*). For all others *see* (*27*).

metaphase spreads from each line were counted and fully analyzed. If 2 or more had the same chromosome abnormality, a further 20 were analyzed.

Further chromosome preparations were made from most lines after different periods in culture. Usually, 10 metaphase spreads were analyzed on each occasion but, where a change in the modal karyotype became apparent, 100 cells were counted and analyzed from each of a series of preparations covering the period of cytogenetic change.

In three cases, at the time of setting up a long-term culture, analyses were done on phytohemagglutinin (PHA)-stimulated 72-hour cultures from the same blood sample, with a modified method of Hungerford (*29*). In one case, chromosome studies were also made on direct preparations from bone marrow with a modified method of Tjio and Whang (*30*). These results are summarized in table 2.

RESULTS

Table 2 records the cytogenetic findings in short-term blood cultures and in direct bone marrow preparations.

Abnormal Karyotypes

Of the 12 lines examined, within 3 months of becoming established in culture, 10 had a normal diploid karyotype with a constitution appropriate to the sex of the donor (table 1). In the remaining 2 lines, distinctive cytogenetic abnormalities were present. These are described below.

COA_1.—All cells contained 46 chromosomes. However, in every case a chromosome #2 and a group C chromosome were absent. These were replaced by 2 abnormal marker chromosomes which appeared to have arisen as a result of pericentric inversions (fig. 1a). Short-term PHA-stimulated cultures of this patient's blood showed a normal female karyotype as did COA_2, the second line derived from the same patient (fig. 1b).

G-S_1.—The modal number in cells from this line was 46. In all 50 cells 1 chromosome from group C and 1 chromosome #3 were missing. There were 2 submetacentric marker chromosomes, 1 slightly larger than a #2, the other

Table 2.—Cytogenetic findings in short-term blood cultures and direct bone marrow preparations

Corresponding cell line (from same donor)	Tissue	Total cells analyzed	Chromosome count distribution				Karyotype
			<45	45	46	>46	
G-S_1	Peripheral blood + PHA*	25		1	24		46, XX
G-S_1	Bone marrow	25	1		24		46, XX
HUN_1	Peripheral blood + PHA	10			9	1	46, XY
COA_1 and COA_2	Peripheral blood + PHA	8			8		46, XX

*PHA = Phytohemagglutinin.

distinguishable from group B by its slightly smaller short arms (fig. 2a). Short-term blood and bone marrow cultures from this patient showed a normal female karyotype.

Because there were no previous reports of cytogenetic studies on cell lines from patients with chronic or subacute lymphatic leukemia, we examined lines F89 and F137 (*28*). The karotypes were abnormal in both.

F89.—This line consisted of 2 distinct cell populations sharing the same chromosome abnormalities and therefore having a common origin. The modal number of chromosomes in both was 46. In every cell, 2 chromosomes were missing from group C, but an additional chromosome in group B and an abnormal medium-sized metacentric were present, whereas 1 member of group D appeared to have extra material on the long arms. The 2 populations were distinguished by the presence in 1 of an abnormal chromosome replacing a #2 (figs. 3a and b).

F137.—There was an abnormal D group chromosome with additional material on the long arms (fig. 4) in each of the 50 cells analyzed; 39 of these cells had 47 chromosomes including an extra one in group C. The cells with fewer than 47 chromosomes probably arose through random loss from the modal line, though they included 7 cells in which the missing chromosome was from group C and these may have represented a separate subline.

One or two C chromosomes had a prominent subterminal secondary constriction of the long arms in 7 of the 50 cells. This corresponds to the "marker C" observed previously (*7*, *8*, *13*, *14*, *19*) though the chromosome pair affected in this line appears to be #7 or 8, whereas in published reports, with one exception (*21*), it was described as one of the smaller members of group C, commonly #10. This anomaly was not seen in any of the other lines examined. The percentage of F137 cells with this and other minor aberrations (fig. 5), has varied with time (table 3).

TABLE 3.—Distribution of marker C and other minor aberrations in F137 cells (based on analysis of 100 cells)

	Period in culture (months)					
	*18	20	21	27	31½	35
Percent cells with 1 marker C	9	32	38	48	76	60
Percent cells with 2 marker Cs	2	38	34	41	1	1
Total percent cells with marker C chromosome	11	70	72	89	77	61
Percent cells with subterminal secondary constriction on a #3	6	2	3	1	0	0
Percent cells with other aberrations†	3	6	20	6	8	11

*Approximate.

†Includes secondary constrictions or despiralization of a D-group chromosome, chromatid, and chromosome gaps affecting chromosomes #1, 2, and group C.

TABLE 4.—Karyotypic evolution in GOL_1-4299

GOL_1-4299	Period in culture (months)			
	7	11½	12½	13½
Percent cells of type b (46, XX, C-, Abn+ Dq+)	0	78	96	100

Karyotypic Evolution

Changes in the modal karyotype with time were studied in lines GOL_1 and G–S_1 (tables 4–5). The proportion of type b cells in F89 (table 6) and the percentage of F137 cells showing a marker C or other minor aberrations (table 3) were also studied for many months.

GOL_1.—After 10 months in culture (plus 1 month's storage in liquid nitrogen) this diploid line had become pseudotetraploid with a modal chromosome number of 91; one of these was an extra metacentric chromosome which appeared to have resulted from a Robertsonian translocation involving 2 group D chromosomes (fig. 6).

A subline, GOL_1-4299, was established from a sample that had been frozen after 4 months' growth. Initially this had a normal karyotype (fig. 7a) but 6 months later most of its cells showed 1 group D chromosome with extra material on the long arms (fig. 7b). One member of group C was missing and was replaced by an abnormal submetacentric chromosome slightly larger than a #16. Cells with these abnormalities (type b) replaced the normal diploid (type a) cells over the period indicated in table 4.

G-S_1.—This line was initially grown in medium supplemented with human serum. After 5 months in culture, a sample was subcultured into medium supplemented with fetal calf serum and maintained as a separate subline G-S_1-2. Three months later the process was repeated for a further subline, G-S_1-3.

Between the 6th and 10th months in culture, the modal karyotype in G-S_1- 2 changed from the previous analysis (type a cells, fig. 2a) to one in which the chromosome number was 48, with additional chromosomes in groups C and G (type b cells, fig. 2b). In subline G-S_1-3, by 16 months 40% of cells were of type b but 47% had a chromosome count of 47. These type c cells differed from type a

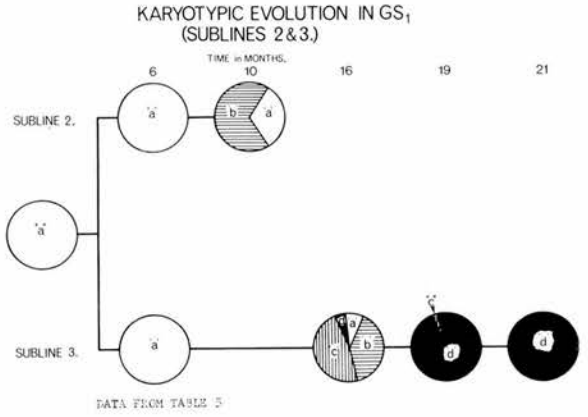
TABLE 5.—Karyotypic evolution in G-S_1 (sublines 2 and 3)

G-S_1-2	Period in culture (months)			
	6	10		
Percent cells, type a ((46, XX, 3-, C-, mar^1+, mar^2+)	100	32		
Percent cells, type b (48, XX, 3-, G+, mar^1+, mar^2+)	0	68		
G-S_1-3	6	16	19	21
Percent cells, type a (46, XX, 3-, C-, mar^1+, mar^2+)	100	8	0	0
Percent cells, type b (48, XX, 3-, G+, mar^1+, mar^2+)	0	40	0	0
Percent cells, type c (47, XX, 3-, mar^1+, mar^1+)	0	47	2	0
Percent cells, type d (47, XX, 3-, C-, D+, mar^1+, mar^1+)	0	5	98	100

TABLE 6.—Karyotypic evolution in F89

F89	Period in culture (months)				
	18*	20	21	26	28
Percent cells, type b (46, XY, C-, C-, C-, B+, mar^1+, mar^2+, Dq+)	29	5	4	6	5

*Approximate. *Note:* Data in tables 4, 5, and 6 are based on analysis of 100 metaphase spreads ignoring nonmodal (including polyploid) cells which never exceeded 10% of the total.



TEXT-FIGURE 1.—Diagrammatic representation of data from table 5 indicating the pattern of karyotypic evolution in 2 sublines of G-S_1 for 21 months.

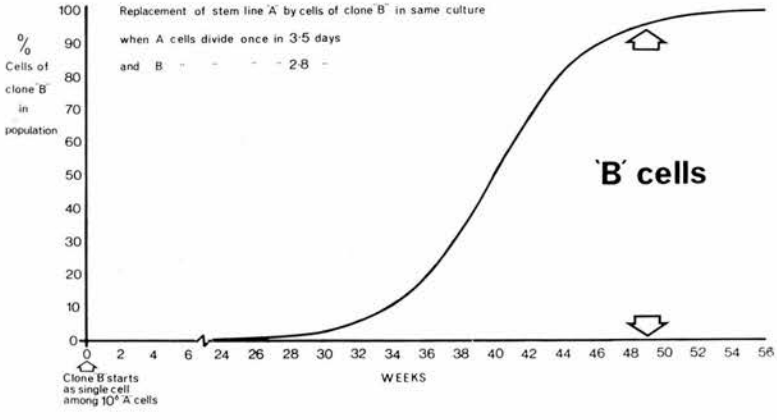
by the addition of a second large submetacentric marker, loss of the smaller marker, and the gain of a chromosome in group C (fig. 2c). At this time, 5% of cells had 47 chromosomes including the same markers as type c but had lost 1 chromosome from group C and gained 1 in group D (type d, fig. 2d). Three months later, type d cells comprised 98% of this subline (table 5 and text-fig. 1).

During the period when the GOL_1 line was changing from a diploid to a polyploid mod, clumps of cells adhered to the glass of the culture flask (fig. 8). G-S_1-3 cells showed similar behavior while type d cells increased in number. In both cases the cells could be detached from the glass by vigorous agitation of the vessel and after 5 or 6 weeks, the lines resumed their normal pattern of gowth as free-floating clumps. This phenomenon was described previously in a Burkitt lymphoma cell line (*31*) and probably the change of behaviour in culture is associated with the emergence of a new karyotype. It is not, however, an invariable accompaniment of cytogenetic change, since it was not observed in the G-S_1-2 subline or in GOL_1-4299.

DISCUSSION

Except for cell lines grown from patients with known constitutional chromosomal aberrations, it was possible on only 2 reported occasions (both cases of Burkitt's lymphoma) to detect identical cytogenetic abnormalitites in cells dividing *in vivo* and in lymphoblastoid lines subsequently grown from the same tissue (*1*, *19*). Our observations on GOL_1 and G-S_1 show that an aneuploid line can evolve *in vitro* from an inoculum of diploid cells and shed some light on the mechanism whereby the modal karyotype of an established line undergoes change. This does not seem to operate by the repetition of an inevitable specific error at mitosis ultimately affecting every cell, but by the gowth of a new clone with a faster division rate than the parent line.

The data from tables 4 and 5 can readily be fitted to a mathematical model (text-fig. 2) which illustrates the general pattern of change in a culture consisting of 2 cell types with unequal division rates. The numerical values are realistic, since, under routine maintenance conditions, average doubling times do not approach the figures attainable under optimum conditions (often less than 48 hours). The shape of the curve is not, however, dependent on the actual values of the mean doubling times.



TEXT-FIGURE 2.—Mathematical model predicting relatively sudden change in modal karyotype of a cell line as result of a rapidly growing and cytogenetically distinct clone arising many months before any change can be detected.

In the G-S_1 line, the first type b cell must have been formed before the 2 sublines were separated, even though this clone was not detected until much later. This is consistent with the mathematical model which predicts an apparent latent period of several months before a new clone can comprise a measurable proportion of the total culture. Thereafter, the parent line is overtaken very rapidly, as is well illustrated by the mergence of type b cells in GOL_1-4299 and by clone d in G-S_1-3.

If a rapidly growing clone arises at an early stage in the life of a culture, when the stemline consists of relatively few dividing cells, the abnormal clone may comprise almost 100% of the culture by the time it is sufficiently well established for cytogenetic studies to be undertaken. This is the probable explanation for the initial findings in G-S_1 and COA_1, and is consistent with our observations (unpublished) that, in general, aneuploid lines grow faster than diploid ones.

The mechanisms whereby a chromosomal aberration may increase the growth rate of cells are not known. Some deductions are possible, however, from the findings in F89 in which b cells (fig. 3b) appear to represent a clone derived from type a. Since b cells comprised 29% of the culture on first examination (table 6) they must have enjoyed an advantage over type a under the culture conditions prevailing in the original laboratory (*28*). The change to different culture conditions appears to have reversed this advantage so that the proportion of b cells has declined. This suggests that the additional chromosome material present in b cells increased their growth rate by improving their adaptation to a particular environment. The fact that the percentage of b cells has now stabilized at about 5% of the total, rather than continuing to decline exponentially, may mean that further adaptation has taken place unassociated with a detectable change in the karyotype.

A C-group chromosome with a prominent subterminal constriction on the long arms (marker C), such as that found in F137, has been the subject of some controversy. It was suggested that the marker C is not a specific entity, but that these constrictions are randomly distributed throughout the chromosomes of lymphoblastoid cells (*25*). This is clearly not true in F137, since at least one marker C was demonstrable in up to 89% of the cells, whereas other chromosome or chromatid gaps affected, at most, 23% (table 3). A constriction in the subterminal region of a #3 chromosome (fig. 5) was noted in 6% of the cells. None of the cells so affected contained more than one marker C. This may indicate that the subterminal regions of a #3 or of a C-group chromosome represent alternative sites of action for the same process. The absence of the marker C from all cell lines initiated in this and at least one other laboratory (*21*) contrasts with its regular occurrence in most reported series (*7*, *8*, *13*, *14*, *19*) and suggests that its appearance is influenced by culture conditions prevailing during the early period of growth *in vitro*. This study confirms that its presence in a cell line is not a direct consequence of productive EBV infection (*24*), but since DNA/DNA hybridization experiments (*32*) indicate that at least part of the EBV genome may be present in every lymphoblastoid cell line, an association between EBV and the marker C chromosome cannot be ruled out.

With this possible exception, there is no evidence for the recurrence of any specific chromosomal aberration among different cell lines. An extra group-C chromosome, frequently observed (*7*, *16*, *20*, *21*), was present in F137; similarly F89, F137, and GOL_1-4299 have an enlarged group-D chromosome, a feature described in at least 6 other lines (*1*, *5*, *10*, *17*, *20*). However, little significance can be attached to such apparent correlations until the chromosomes involved have been accurately identified, for example, by the use of quinacrine fluorescence (*33*, *34*). The fact that lines G-S_1 and F137 have totally dissimilar karyotypes, though derived from patients with the same disorder, argues against any specificity in their chromosomal aberrations.

There is evidence to suggest that aneuploidy is particularly common in cell lines derived from patients with malignant disease (*26*). The findings reported here might appear to support such a conclusion. However, it is apparent (table 1) that the donors of the lines COA_1 and G-S_1 were much older than the other patients. Possibly, therefore, the donor's age rather than his disease may influence the karyotype of an established cell line. Until more lines derived from elderly subjects without malignant disease have been examined cytogenetically, this point must remain in doubt.

Since human lymphoblastoid lines appear to have an almost indefinite lifespan, they promise to be useful experimental tools, particularly in the field of genetics. The availability of clones from a single cell line, each differing from the stemline in a defined region of the karyotype, holds out a real prospect of contributing substantially to the mapping of human chromosomes.

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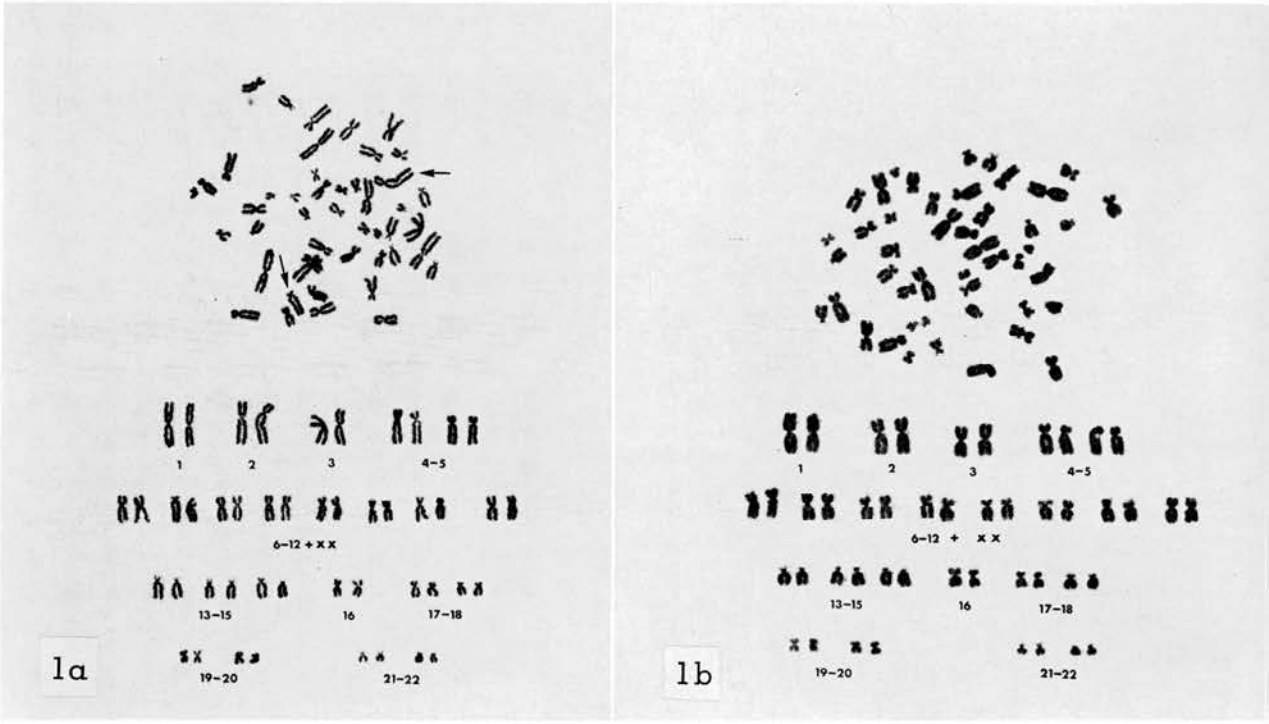
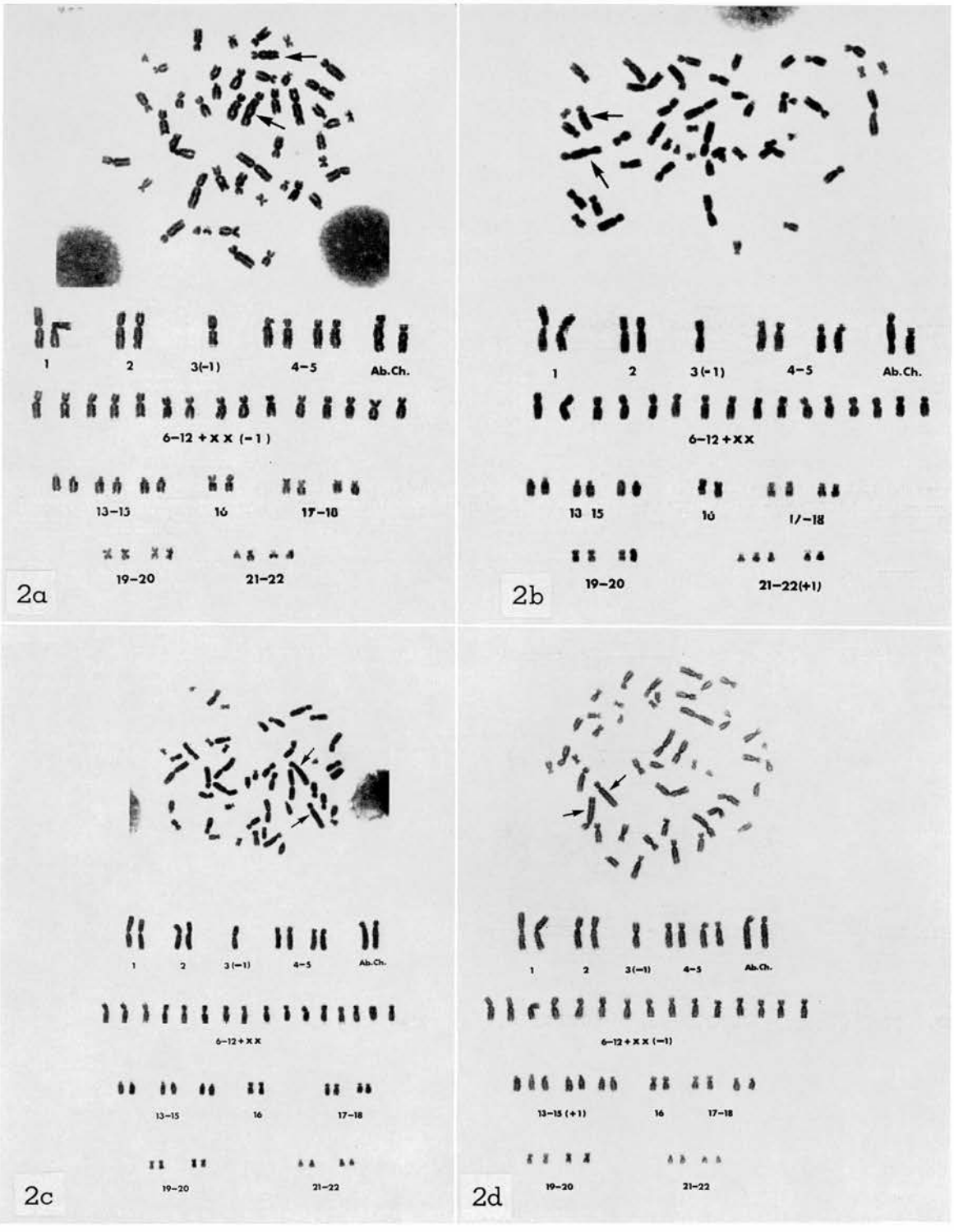
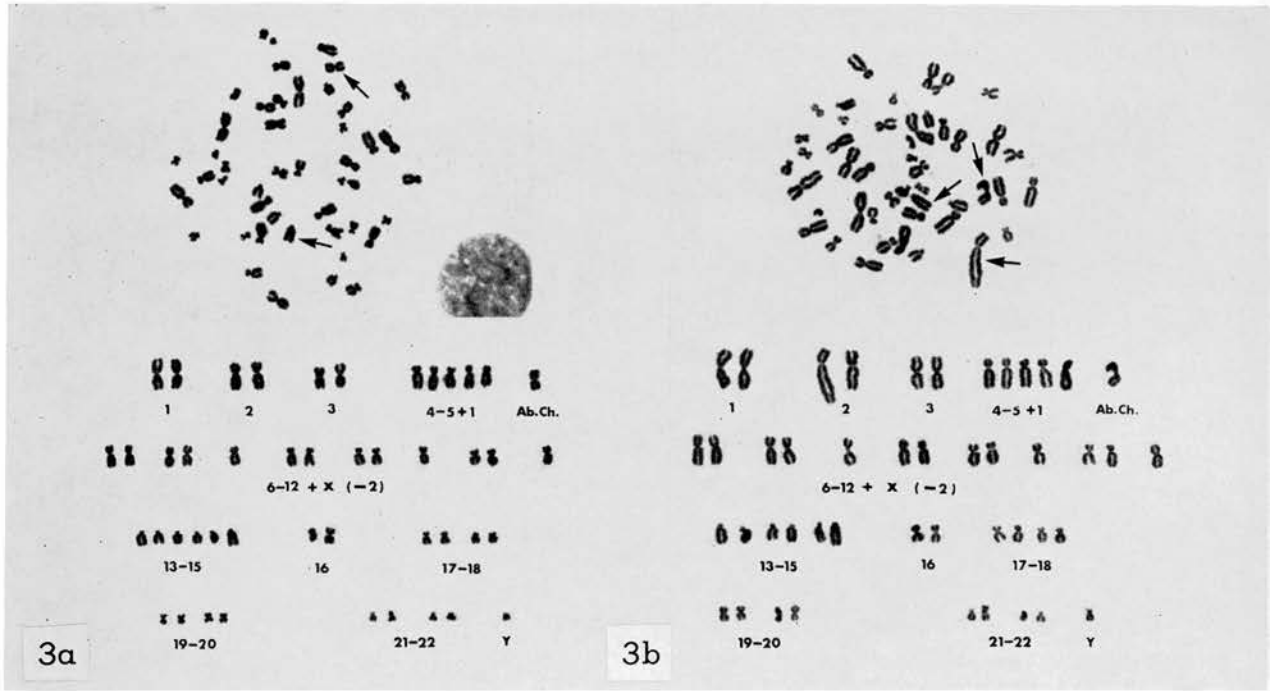
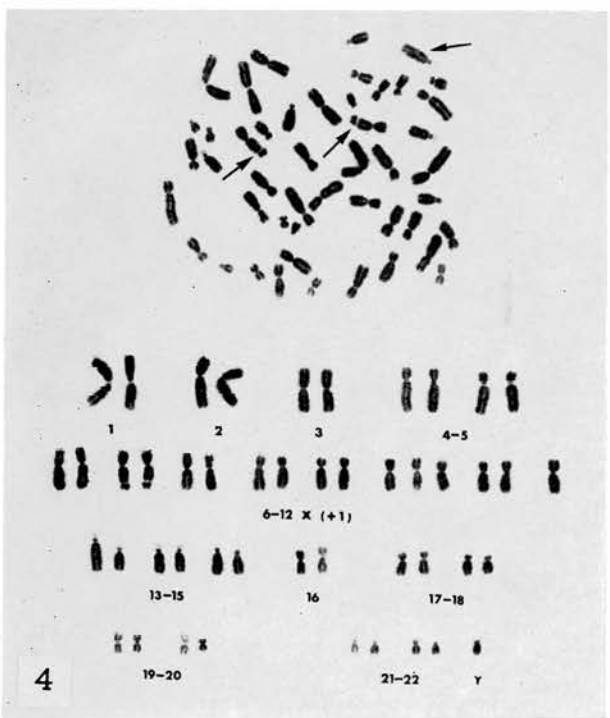


FIGURE 1.—a) Karyotype of COA_1 cell line. *Arrows* indicate the chromosomes involved in pericentric inversions; b) Normal diploid female karyotype of COA_2 cell line.



JRE 2.—a) Karyotype of type a cell from G-S_1 line; b) Karyotype of type b cell from G-S_1 line; c) Karyotype of type c cell from G-S_1 ne; d) Karyotype of type d cell from G-S_1 line; *arrows* indicate abnormal submetacentric chromosomes.





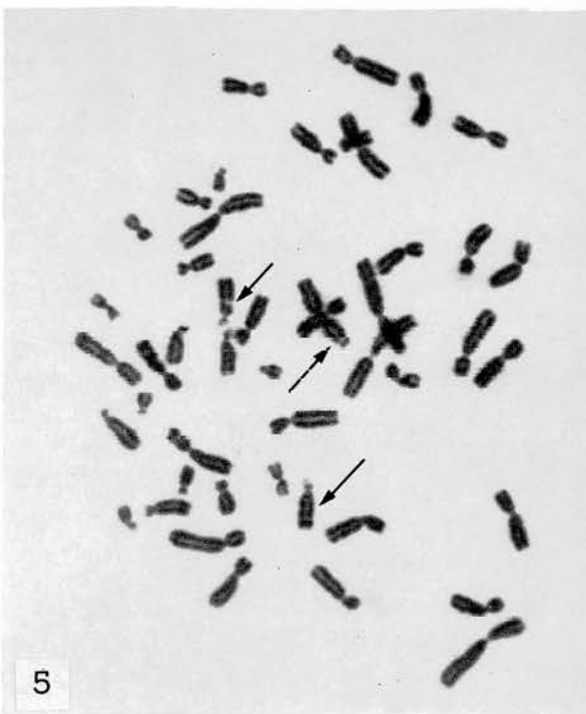


FIGURE 3.—a) Karyotype of type a cell from F89 line; b) Karyotype of type b cell from F89 line. *Arrows* indicate abnormal metacentric c mosome, member of group D with enlarged long arms and (in b) large submetacentric marker replacing a # 2 chromosome.

FIGURE 4.—Karyotype of cell from F137 line. *Arrows* indicate pair of C-group chromosomes with subterminal secondary constriction ar chromosome from group D with enlarged long arms.

FIGURE 5.—Metaphase spread from a cell of F137 line. *Arrows* indicate subterminal secondary constriction in a chromosome # 3 and c matid gaps in 2 chromosomes of group D.

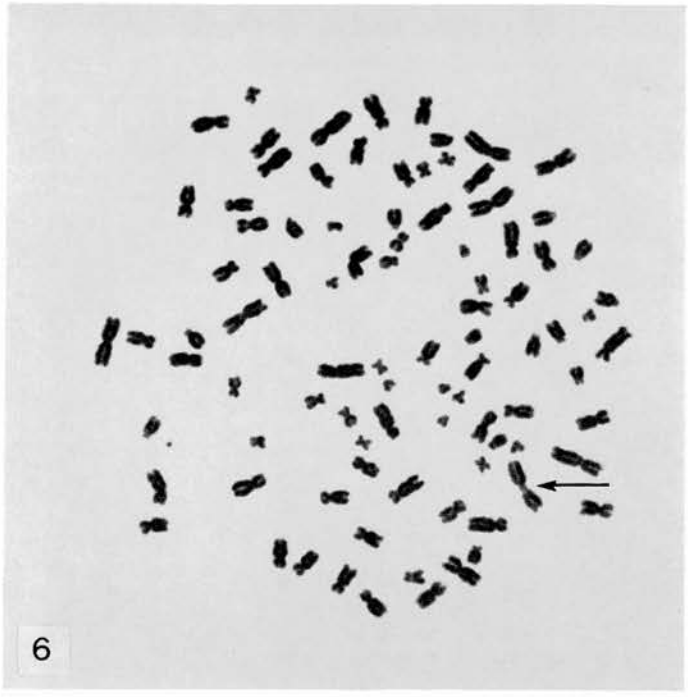
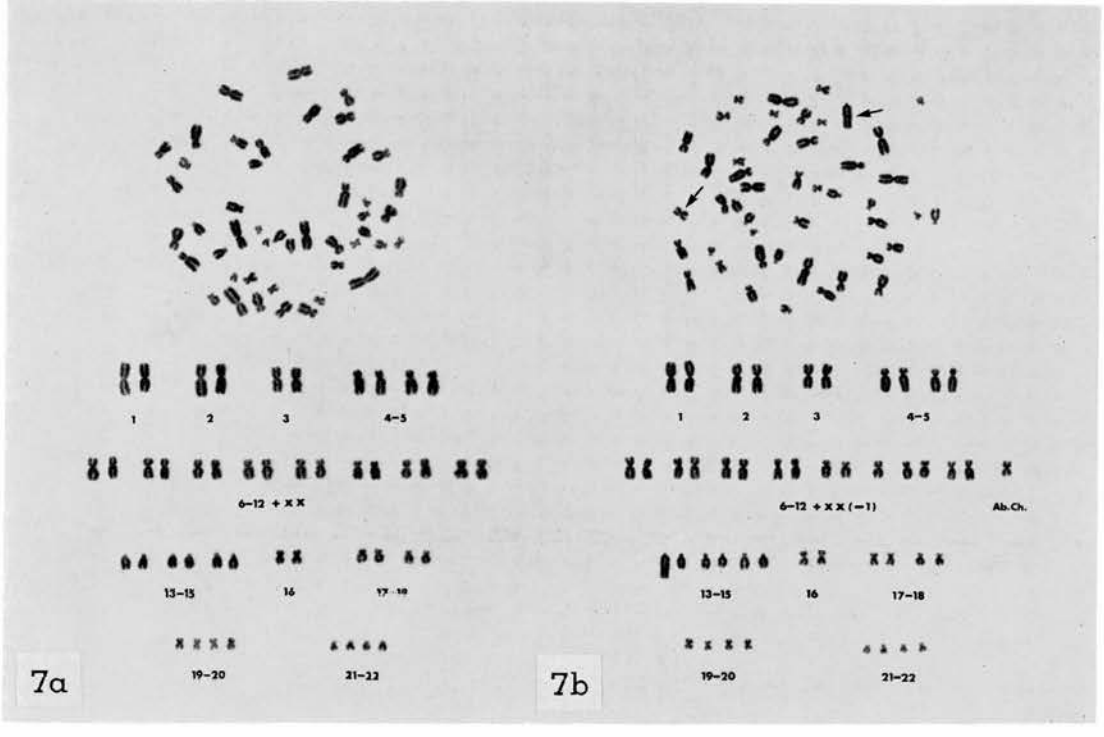


FIGURE 6.—Metaphase spread of subtetraploid (91 chromosomes) cell from line GOL_1. *Arrow* indicates abnormal metacentric chromosome which appears to have been formed from a Robertsonian translocation involving 2 group D chromosomes.



E 7.—a) Normal diploid female karyotype of type a cell in subline GOL_1-4299; b) Karyotype of type b cell from subline GOL_1-4299 *ows* indicate abnormal small metacentric chromosome and one from group D with enlarged long arms.

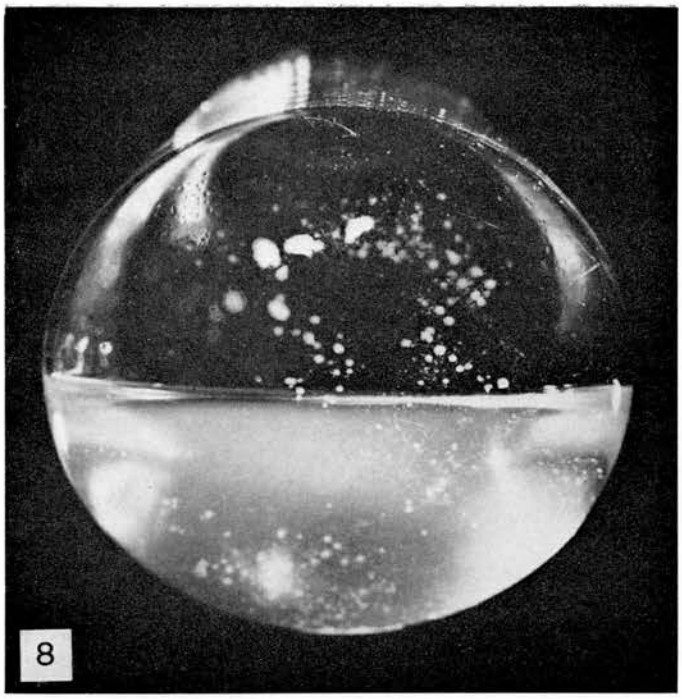


FIGURE 8.—Large clumps of GOL_1 cells adhering to the bottom of tilted culture flask. Phenomenon observed while modal karyotype of this line was changing from diploid to subtetraploid.

Cell line ODY_1

The origin of this line from the peripheral blood cells of an elderly male patient with Waldenströms macroglobulinaemia co-cultivated with irradiated F137 cells has been described in Chapter III.

Thirty metaphase spreads were analysed from orcein-stained preparations made within three months of establishing the line. All had a normal male karyotype.

Quinacrine-stained preparations were made from the line after 12-13 months growth. Eight cells were photographed. the modal karyotype (7 cells) comprised 47 chromosomes, the additional one being a C8. One had 49 chromosomes with two extra C8's and an extra C11.

A typical modal cell is illustrated in fig.58.

Discussion

Almost every line which has been maintained by me for more than one year has now been examined for chromosome abnormalities by quinacrine-staining. With the exception of FLE_1 which was still diploid at 13 months, all those examined have become abnormal by this stage. (by 24 months FLE_1 was predominantly sub-tetraploid). This applies regardless of the age or health of the donor from which the original cells were obtained. Furthermore, when the chromosome analysis of all my cell lines, undertaken at the earliest possible stage in culture is reviewed it is apparent that the aneuploid lines included MON_1 (from a child with non-malignant disease) and three lines from adults with malignant disease ($G\text{-}S_1$, COA_1 and BLA_1). Conversely, one line from a child with malignant disease (HUN_1) and seven from adults with malignant disease (LAW_1, LAW_2, SHA_1, SHA_2, ART_1, MIT_1 and ODY_1) were initially diploid. In the material which I have studied therefore, there appears to be little correlation between the chromosome constitution (diploid or aneuploid) of a newly established line and the age or diagnosis of the donor from which it has been derived.

With the passage of time in culture, most of the lines seem to become abnormal by the acquisition of extra chromosome

material. A striking exception is HUN_1 which has lost the Y chromosome. In many cases it is not possible to identify the exact origin of all the extra material, but there is enough data to establish whether or not particular chromosome aberrations recur in different cell lines with a frequency greater than can be accounted for by chance. In the preceding paper, reprinted from Nature 233, p.555-556, I suggested that the identification of recurrent marker chromosomes in lymphoblastoid cell lines from analysis of orcein-stained preparations was largely spurious. This seems to be confirmed by the additional data presented in this chapter.

Table XIV records those aberrations which were found in more than one cell line on examination of quinacrine-stained material, excluding those which were found in only a single cell from a given line. The list is far from impressive when one considers that it is culled from an examination of nineteen aneuploid lines, that some of the abnormalities were present in only one of several samples of a given line and that in some cases the aberrations, though affecting the same region of the same chromosome, may be distinguished by their fluorescent staining pattern. It remains possible, of course, that a highly specific chromosome aberration is present in many, or even all established human lymphoblastoid lines, but that it affects so minute an area of the karyotype that it has not yet been recognised.

Manolov and Manolova (1971) have reported that an additional narrow fluorescent band was present on the end of the long arms of one of the D14 chromosome pair in fresh biopsy material and in established cell lines from 10 out of 12 Burkitt lymphoma patients from whom they had examined quinacrine-stained preparations. I have found a similar aberration in JIYOYE (from Burkitt's lymphoma) and in F89 and F137 (from lymphatic leukaemia) but not in RAJI, the only other Burkitt lymphoma line which I have examined. In another paper, the same group reported quinacrine fluorescence studies on fresh biopsy material and established cell lines from a total of ten Burkitt lymphoma patients. Most of the karyotypes were regarded as normal and among the few abnormalities which were observed none was found to recur in material from more than one patient. (Manolov, Manolova, Levan and Klein, 1971).

Although evidence for a specific chromosome aberration in established lymphoblastoid cells is therefore meagre, at best, cytogenetic studies do throw important light on the cellular origins of those lines and on their relevance to human disease.

Five of my lines, SHA_1, and 2, LAW_1 and 2, and ART_1 were grown from the peripheral blood of patients with chronic

TABLE XIV

Chromosome aberrations observed in quinacrine stained preparations from more than one cell line

Chromosome principally affected	Nature of Aberration	Lines in which the aberration was present
3	Trisomy	COA_1 and JIYOYE (32-35 months)
3	Partial deletion of one arm (translocated elsewhere)	$G\text{-}S_1$ and BLA_1
4	Additional material on the long arms	$G\text{-}S_1$ (21-32 months) and RAJI
7	Break with loss or translocation of major part of long arms	$G\text{-}S_1$ and JIYOYE (not 'Flow')
8	Trisomy	WEB_1, CLA_4 and ODY_1
9	Additional material on short arms	F89 and BLA_1
9	Trisomy	JIYOYE (not 'Flow') and F137, minor population at 7-13 months.
12	Trisomy	JIYOYE (3-6 months) and SHA_2
13	?Reduplication of long arms	GOL_1, 4299 and RAJI
13	Extra material on long arms	F137 and F89
13	Extra material on short arms	F137 (7-13 months) and JIYOYE (3-6 months)
14	Extra material on long arms	F137, JIYOYE and F89
16	Extra material on long arms	$G\text{-}S_1$ and ORI_1
21	Trisomy	JIYOYE (20-35 months) and F89 (30 months)
Abnormal	Small acrocentric marker extra	$G\text{-}S_1$ (32 months) and WEB_1

myeloid leukaemia. In each case, short term blood cultures from these patients revealed the Ph^1 chromosome, characteristic of the condition (Nowell and Hungerford, 1960) in the great majority of cells. All of the lines, however, were diploid on first examination and in none was a Ph^1 chromosome found at any stage. There is, in fact, only one report of a marker chromosome with the morphology of a Ph^1 being identified in a cell line derived from a patient with chronic myeloid leukaemia. Analysis in that case was carried out on orcein-stained preparations and the "Ph^1" chromosome was found in only about 23% of the cells (Lucas, Whang, Tjio et al., 1966). When the line was reexamined some months later the "Ph^1" chromosome was no longer present (Moore, Kitamura and Toshima, 1968).

In the case of the MIT_1 line, a direct preparation was made from the marrow of the patient (who suffered from acute leukaemia) by Miss M.L. O'Riordan, using a modification of the method of Tjio and Whang (1962). She found that 5 of 10 dividing cells contained a distinctive chromosome aberration (a C/D translocation), the remainder having a normal diploid female karyotype. On initial examination of 30 orcein stained metaphase spreads from this line, no abnormality was detected

and this was confirmed, after 4 months growth, on quinacrine stained preparations (6 cells examined, all 46,XX, no abnormality).

It is suggested, therefore, that in these cases the lymphoblastoid lines have originated, not from malignant cells proliferating *in vivo* but from normal tissue.

In outstanding contrast to these findings are the reports, now accounting for three very thoroughly examined cases, of identical chromosome aberrations being present in direct preparations of Burkitt's lymphoma biopsies and in lymphoblastoid cell lines subsequently derived from the same material. (Stewart, Lovelace and Whang, 1965; Gripenberg, Levan and Clifford, 1969; Manolov, Levan, Nadkarni *et al*, 1970). Nadkarni, Nadkarni, Clifford *et al*. (1969) have also shown in a single case, that an unusual IgM surface specificity was present both on fresh Burkitt lymphoma cells and in a number of lymphoblastoid lines derived from the same tumour. In this situation, therefore, there is good evidence that the proliferation of the lymphoblastoid cells *in vitro* represents a continuation of pre-existing malignant proliferation of the lymphoid cells *in vivo*.

It has been known for many years that the dividing cells of Burkitt's tumours show a relatively high incidence of chromosome aberrations (Jacobs, Tough & Wright, 1963) but more recently, evidence from cytogenetic and isoenzyme studies on serial tumour biopsies has shown that they are typically of monoclonal origin and that they progress by the emergence of successive 'waves' of altered clones derived from the parent clone (Gripenberg, Levan and Clifford, 1969; Fialkow, Klein, Gartler and Clifford, 1970). The same appears to be true of at least some other human and animal tumours (Lajtha, 1967; Fialkow, Martin, Klein et al., 1972). This behaviour seems to be very similar to that seen in cultured human lymphoblastoid cell lines and one may postulate that what happens in vitro when a cell line is established from the peripheral blood of a healthy donor, namely the proliferation of cells derived from normal lymphoblastpid tissue, leading to the emergence of successive waves of aneuploid clones, is precisely the same phenomenon which, when it takes place unchecked in vivo, is recognised as Burkitt's lymphoma.

At present this remains no more than a working hypothesis which does, however, point to two particular areas where the use of human lymphoblastoid cell lines may be of direct relevance to clinical oncology, namely in clarifying the role of EB virus in relation to long-term lymphoproliferation

and secondly in looking for practical means of inhibiting or regulating the cellular proliferation once it has been established.

CHAPTER VII

Antigenic properties of cells from established lines

In view of the association between EB virus and human lymphoblastoid cell lines, there has been widespread interest in the identification of virus-related antigens in such cultures, and of corresponding antibodies in sera from patients with Burkitt's lymphoma and other disorders.

Immunofluorescence

Shortly after the establishment of the cell lines EB-1,2 and 3 (Epstein & Barr, 1964; Epstein, Barr & Achong, 1965; 1965a) the Henle's described an indirect immunofluorescence test which demonstrated a reaction between human serum immunoglobulins and a proportion of the cells of these lines. In principle, acetone-fixed smears of cultured Burkitt lymphoma cells were exposed to serum from selected individuals, washed, then exposed to fluorescein-isothiocyanate-conjugated anti-human IgG. Positive reactions (i.e. distinct fluorescence in a proportion of cells) were invariably found, even at high dilution, when the test sera were derived from Burkitt lymphoma patients. At somewhat lower titres, positive results were also recorded with over 80% of sera from adults, whether these were healthy control subjects or from a diagnostically mixed hospital population. Only about 30% of sera from American children were positive,N(Henle & Henle, 1966). The authors suggested that the fluorescence-positive cells were those containing virus particles, a conclusion which was supported by work from the

same centre which demonstrated that isolated non-enveloped virus particles from the EB_3 cell line were coated and agglutinated by sera which reacted strongly in the indirect immunofluorescence test (Henle, Hummeler & Henle, 1966), and this report was shortly confirmed by a second group (Mayyasi, Schidlovsky, Bulfone & Buscheck, 1967). Zur Hausen and the Henles also showed that when fluorescence-positive cells were individually identified and examined by thin-section electron microscopy, they invariably contained EB virus particles (Zur Hausen, Henle, Hummeler et al. 1967). The test is thus believed to detect an antigen or antigens associated with the capsid of the EB virus particle, commonly referred to as 'VCA' (Viral capsid antigen). If a serum with a very high titre of anti-VCA activity is available in sufficient quantity, the IgG fraction can be conjugated directly with fluorescein-isothiocyanate (FITC) and used as the test reagent in order to convert the procedure to a single stage method for identification of EB virus (Moses, Glade, Kasel et al., 1968; Dunkel & Zeigel, 1970). Epstein and Achong developed a very similar direct immunofluorescence test for EB virus, using a serum raised in Rabbits against isolated EBV particles, adsorbed with human foetal thymus powder and conjugated with FITC. These authors also established the authenticity of their test by demonstrating the presence of virus particles in individually identified fluorescence-positive cells on electron microscopy

(Epstein & Achong, 1967; 1968). Hampar and his group (Hampar, Gerber, Hsu et al., 1970) have used ferritin-conjugated sera from rabbits immunised with concentrated EB virus particles as well as a similar conjugate from a human serum with a high titre of anti-VCA activity, in order to produce direct electron micrographic evidence that such material has a specific affinity for the capsid of naked EB virus particles in a human lymphoblastoid cell line.

The indirect immunofluorescence test as described by the Henles has proved of considerable value in establishing the distinction between EB virus and other herpes-like viruses of animals and man (Henle and Henle, 1966a). It remains the most widely used screening procedure for the identification of EB virus in cultured cell lines giving, in the hands of most authors, results which agree well with those of electron microscopy, (for example, Hinuma, Konn, Yamaguchi et al., 1967; Diehl, Henle, Henle & Kohn, 1968; Zajac & Kohn, 1970; Macek, Seidel, Lewis et al., 1971; Steel & Edmond, 1971). It has also been extensively applied to the screening of various populations for the distribution of antibody directed against EB virus (Levy & Henle, 1966; Goldman, Reisher and Bushar, 1968; Pereira, Blake and McRae, 1969; Diehl, Taylor, Parlin et al., 1969; Svedmyr & Demissie, 1969) and in particular to the identification of diseases (in addition to Burkitt's lymphoma) which are associated with elevated or rising titres of this antibody, notably infectious mononucleosis, (Henle, Henle & Diehl, 1968; Niederman, McCollum, Henle et al., 1968; Pereira, Blake & McRae, 1969; Stevens, Pry and Manaker, 1970;

Joint investigation, 1971), post perfusion syndrome (Henle, Henle, Scriba et al., 1970), sarcoidosis (Hirshaut, Glade, Octavio, et. al., 1970; Wahren, Carlens, Espmark, et al., 1971) and nasopharyngeal carcinoma (Henle, Henle, Hung-Chiu et al., 1970).

Complement fixation

In 1967, Gerber and Birch described a complement-fixing antibody reactive with EB virus-positive P_3^J (JIYOYE) cells but not with virus-free RAJI cells. The antibody was present in high titre in sera from all Burkitt lymphoma patients, but was also widely distributed among healthy American adults (over 90% positive) while 57% of American children over the age of 3 years had detective antibody of this type. Several species of non-human higher primates also showed a high incidence of complement-fixing antibody against EB virus (Gerber & Birch, 1967). Using this complement-fixation test, an association between the post-perfusion syndrome and EB virus infection was strongly suggested (Gerber, Walsh, Rosenblum et al., 1969).

It seemed likely that the antibody activity detected by Gerber and Birch corresponded to the anti-VCA antibody demonstrable by the Henles' indirect immunofluorescence method, although the latter workers had found that many positive reactions in complement-fixation tests between human sera and crude extracts of lymphoblastoid-cell-line cells could not be related to the presence of EB virus in the cell fractions. They suggested that

in these situations complement-fixing reactions were taking place with a variety of antigens (Henle & Henle, 1967). Positive results in complement fixation tests between extracts of disrupted lymphoblastoid cells (permanent lines from Burkitt tumour biopsies) and approximately 50% of African patients' sera were reported by McCormick, Stenback, Trentin et al. (1969). These authors did not report any difference between Burkitt lymphoma patients and controls in the percentage of mean titre of positive reactions, but they did observe that only those cell lines which harboured EB virus particles, demonstrable by electron microscopy, contained the antigen responsible for complement fixation. Gerber and Deal (1970) made a direct comparison of complement-fixing antibody directed against concentrates of EB virus particles ("V" antigen), and against particle-free supernatants from lymphoblastoid cell cultures ("S" antigens). They found that sera with a high titre of anti-VCA antibody (by indirect immunofluorescence) invariably had a comparable level of anti-V complement fixing activity, implying that an identical antibody was being detected by the two methods. Over 50% of "V"-positive sera, however, also displayed anti-"S" activity. No "V"-negative sera had anti-"S" antibody. The correlation between anti-VCA and anti-S titres was not close. These findings indicated that the "S" antigen was EB virus-related, but presumably did not correspond to virus coat protein.

Pope, Horne and Wetters (1969) had made similar observations on the presence in some anti-VCA positive sera of a complement-fixing antibody reactive with a soluble extract of cultured human lymphoblastoid cells. Of major importance was the demonstration by both groups that "S" antigen, though EB-virus associated, was found in material prepared from cultures such as RAJI in which EB virus particles could not be detected. Virtually identical findings were then reported by a third group (Vonka, Benyesh-Melnick and McCombs, 1970) working independently. On further study of the "V" complement-fixing antigen from four human lymphoblastoid cell lines, Walters and Pope (1971) found that it consisted of two components, one of which was resistant to heating at 56°C, the other labile at this temperature. The heat-resistant component, on isolation from sucrose density gradients, corresponded to complete (non-enveloped) EB virus particles. The heat-labile fraction was subviral but not soluble. It was detected only in cultures in which EB virus was replicating but its nature is not precisely known. Hollinshead, Lee and Alford (1971) found complement fixing activity, in sera from eight Burkitt lymphoma patients and from one infectious mononucleosis patient, directed against sonicated extracts of cell interiors from human lymphoblastoid cell lines. They considered that this activity corresponded to a mixture of the anti "V" and anti "S" antibodies described above. In addition, however, they

detected complement fixation reactions between a few of the sera and high molecular weight soluble extracts of the cell membranes from some of the cultured lines. A similar, or identical, antigen was demonstrated on whole cells and isolated membranes from fresh Burkitt tumour biopsies and on peripheral blood leukocytes from a patient with acute leukaemia in relapse. It was not found on normal blood leukocytes and could be separated by disc gel electrophoresis from the histocompatibility isoantigens. Its relationship, if any, to EB virus remains unknown.

It is possible that the membrane-associated complement-fixing antigen described by Hollinshead et al. has some bearing on the complement-dependent cytotoxic activity of human sera directed against cells from human lymphoblastoid lines. Such activity was detected by Herberman and Fahey (1968) in over 90% of sera from African and American Burkitt lymphoma patients. Control groups showed a lower incidence (though still over 50%) of positive reactions and a lower mean titre. Activity appeared to be specific for human lymphoblastoid lines, but was unrelated to the presence or absence of EB virus particles in the cultures. Later work by the same group (Herberman, 1969; Fass and Herberman, 1970; Herberman and Nam, 1971) showed that the cytotoxic activity was directed not only against cultured lymphoblastoid cells but also against freshly isolated cells from Burkitt lymphoma biopsies and against leukaemic marrow myeloblasts. Normal peripheral blood leukocytes did not appear to be damaged by

the sera but evidently carry small amounts of the appropriate antigen since cytotoxic activity could be removed from positive sera by absorption with buffy coat cells from healthy subjects. In acute lymphoblastic leukaemia patients and during the acute phase of infectious mononucleosis, serum levels of the antibody were found to be very low (Herberman & Nam, 1971) suggesting that *in vivo* absorption by the abnormal leukocytes might be occurring.

Immunodiffusion/Precipitation

Old and his colleagues (Old, Boyse, Oettgen *et al.*, 1966; Oettgen, Aoki, Geering *et al.*, 1967; Old, Boyse, Geering *et al.*, 1968) developed a precipitation technique for the detection of anti-EB viral antibody. They used supernatants from a JIYOYE subline which gave a very high yield of virus particles and placed a concentrate of this material in the centre well of an Ouchterlony agar diffusion plate. Test sera were placed in peripheral wells and a positive reaction was denoted by a single or double precipitin line between the test sample and the centre well. Sera from the majority of patients with Burkitt's lymphoma and with nasopharyngeal carcinoma gave positive reactions. Lymphosarcoma and leukaemia patients also had an appreciable incidence of positive reactions (44% and 25% respectively) but of the sera from healthy control subjects or from those with other forms of

malignant disease, only 8-13% were positive. The authors showed that the antibody which they were detecting was a IgG globulin which did not interact with herpes simplex virus; they found a good correlation between a positive reaction in their test and a high titre of anti-VCA antibody by the Henles indirect immunofluorescence method. They were unable to obtain suitable antigen from human lymphoblastoid lines other than JIYOYE but attributed this to the fact that the JIYOYE line gave a much higher yield of virus particles than any other currently available. Thus the precipitation reaction appeared to be a 'high-threshold' test for anti-EB virus activity.

Broadly similar findings were reported by Konn et al. (Konn, Yohn, Hinuma et al., 1969) who studied a series of 475 human patients with a variety of disorders. Precipitating antibody against an extract of the EB virus-positive P_3J (JIYOYE) cell line was characteristically present in those sera which gave a high titre reaction in the Henle's indirect immunofluorescence (anti-VCA) test. However, a consistently higher proportion of sera from patients with malignant disease (of varying types) gave positive results in the immunodiffusion test than those from patients with non-malignant disease even when allowance was made for differences in anti-VCA titre. These authors found up to 3 precipitin lines formed between human sera and JIYOYE cell extracts. Reactions of identity were demonstrated for all three

with lines formed between the same antigen and rabbit antisera from animals immunised with purified EB virus. On electron microscopy only one of the lines corresponded to antibody-coated EB virus particles. The other two were, however, EB virus-related since the reactivity could be removed from positive sera by absorption with EB-virus positive but not with EB virus-negative lymphoblastoid cell lines.

When the above system was modified by reducing the scale to provide a micro-immunodiffusion test, the results continued to demonstrate a positive association between the presence of EB virus in the cell line from which the antigen was derived and the formation of precipitin lines with certain human sera (Stevens, Pry, Blackham and Manaker, 1970). However, the sensitivity of the method appeared to be radically altered since the precipitating antibody could now be detected with a sensitivity comparable to that of the Henles' anti-VCA antibody (Stevens, Pry and Blackham, 1970) and in some cases precipitating antibody was found in sera considered negative by anti-VCA immunofluorescence (Fink, Isibul and Waggoner, 1969).

Reedman, Pope and Moss (1972) have used immunodiffusion to demonstrate an antigen which is EBV-associated but present in extracts of RAJI and other non-virus-producing lines. It is believed to correspond to the soluble "S" antigen detectible by complement fixation. A second precipitation line was

attributed to these authors to an "F" antigen, but they found it impossible to define the origin of this activity or events to establish whether it is related to EB virus.

Membrane antigens

An immunofluorescence reaction differing from that of the Henles has been described and estensively studied by Klein and his colleagues (Klein, Clifford, Klein & Stjernward, 1966; Klein, Clifford, Klein et al., 1967, 1967a). These authors use living preparations of cultured lymphoblastoid cells or fresh biopsy material from Burkitt tumours as the antigen. In its original form, the test consists of exposing such cells to the serum under study, followed by the addition of FITC-conjugated anti-human immunoglobulin. Antibody activity was found in the IgG fraction of sera from patients with Burkitt's lymphoma; it was readily distinguished from iso-antibody (for example anti-HL-A) since there was no reaction with peripheral blood leukocytes or marrow cells.

More recently, the method has been modified as follows. The gamma-globulin fraction from a serum, "Mutua", known to give a strong positive reaction in this test at high dilution, and free from detectible iso-antibodies has been directly conjugated with FITC. A test serum is added to the cell suspension, unbound immunoglobulin removed by washing and

the cells then exposed to FITC-conjugated Mutua globulin. The results are expressed as a "blocking index", calculated by subtracting the percentage of fluorescence-positive cells after incubation with unlabelled test serum followed by the Mutua-conjugate from the percentage of positive cells incubated with Mutua conjugate alone, divided by the latter figure (Goldstein, Klein, Pearson & Clifford, 1969). It is characteristic of this reaction that the fluorescence is confined to the cell membrane and the antigen has been designated "MA" ("membrane antigen"). All cell lines which can be shown, by anti-VCA immunofluorescence, to harbour EB virus particles react positively in tests for MA although the Kleins did not find a close relationship between the percentage of cells containing virus particles and the numbers giving positive results in the membrane fluorescence reaction. Lines, such as Kudi, Ogun and RAJI, which appeared not to release EB virus particles were MA-negative (Klein, Clifford, Klein et al., 1967a; Klein, Pearson, Nadkarni et al., 1968) but could become MA-positive following the admixture of lethally-irradiated cells from an EB virus-positive lymphoblastoid line (Klein, Klein and Clifford, 1967). Thus the membrane antigen appeared to be EB virus-associated though not identical with the viral capsid antigen (VCA). This conclusion was further supported by the observation that infectious mononucleosis was almost invariably associated

with a rising titre of anti-MA antibody, peak levels being attained, however, much later than the peak of anti-VCA activity (Klein, Pearson, Henle et al., 1968). In an elegant electron microscopy study, Dunkel and Zeigel (1970) showed that material from anti-MA positive sera coated the surface of virus-releasing cultured lymphoblastoid cells. Identical material coated the envelopes of extracellular virus particles. It seems likely therefore that the MA is a virus-coded determinant on the plasma membrane of a cell, the membrane itself forming the viral envelope as the particles are shed into a vacuole or to the exterior (Darlington & Moss, 1969). Dunkel and Zeigel found that cells which did not contain visible virus particles were not coated with antibody, an observation which is in conflict with Klein's report that there may be a tenfold higher percentage of MA reactive than VCA-reactive cells in a given culture (Klein, Pearson, Nadkarni et al., 1968). Recently the latter authors have reiterated their original view (Pearson, Dewey, Klein et al., 1970; Klein, Gergely and Goldstein, 1971) suggesting that the discrepancy may be accounted for by the fact that the expression of membrane antigens is highly dependent on precise culture conditions (Yata and Klein, 1969; Yata, Klein, Hewetson and Gergley, 1970).

At an early stage, Klein and his colleagues found that different sera with comparable levels of anti-MA activity did not show reactions of complete identity in cross-blocking experiments (Pearson, Klein, Henle et al., 1969; Svedmyr, Demissie, Klein and Clifford, 1970) suggesting that there might be more than one component to the membrane antigen. This possibility was

investigated further by conjugating two non-identical anti-MA sera with fluorochromes of different colours (Tetramethylrhodamine-isothiocyanate and fluorescein isothiocyanate) and examining the distribution of the labelled compounds on the surface of cultured human lymphoblastoid cells (Klein, Gergely and Goldstein, 1971). By this technique the authors concluded that the two types of sera 'Kipkoech' type and 'Mutua' type react with the same macromolecular complex antigen, 'Kipkoech' sera (which completely block 'Mutua' sera but are not reciprocally blocked by them) containing antibody directed against a component of the antigen which is larger than, and includes, the portion that interacts with 'Mutua' antibody. Cross-blocking experiments with human sera from a variety of sources suggests strongly that anti-VCA activity is also the result of a complex of related antibodies (Svedmyr, Demissie, Klein & Clifford, 1970).

'Early' Antigens

Finally the Henles and their colleagues (Henle, Henle, Zajac et al., 1970) detected a further EB virus-related antigen in cell lines (RAJI and 64-10) which appeared virus-free until infected in vitro with an EB virus concentrate from the P_3 HRI-K clone of JIYOYE. Neither line supported the replication of EB virus particles for more than a few cycles as judged by immuno-fluorescence with a direct anti-VCA conjugate, but indirect immunofluorescence with sera from patients with infectious mononucleosis, Burkitt's lymphoma or nasopharyngeal carcinoma

yielded a high percentage of positive results even when known anti-VCA-positive sera from healthy subjects failed to react. The fluorescence was intracytoplasmic and the reaction appeared to be a consequence of transient or abortive EB virus infection of the cell lines since it did not occur if the virus was exposed to heat (56°C), ultraviolet light or to a serum containing anti-EB virus antibodies, before inoculation of the RAJI or 64-10 cultures. The reaction developed within 8 hours of inoculation of the cell line (some 4 hours earlier than the appearance of VCA-positive cells) and the antigen responsible has been designated "Early antigen" ("EA"), the assumption being made that it is comparable to the early non-virion but virus-coded antigens detected in the cytoplasm of cultured cells following infection with herpes simplex virus. (Geder, Vaczi, Gonczol et al., 1967; Geder, Vaczi, Heney et al., 1967). A study on almost identical lines to that described above has been reported by Himuma, Sairenji and Ohta-Hatano (1971). These authors also detected an early antigen (which they designated 'N' antigen) in a lymphoblastoid line (NC37) infected in vitro with EB virus.

Relationships between antigens

With so many antigens now identified in, or on the surface of, cultured human lymphoblastoid cells, questions inevitably arise as to their relationship to each other and to the human diseases associated with EB virus infection. Collaborative studies between laboratories with particular experience and

expertise in the techniques associated with different antigen/antibody systems have gone a considerable way towards resolving the first of these issues. Klein, Pearson, Henle et al. (1969) found that when sera from Burkitt lymphoma patients, Nasopharyngeal carcinoma patients, infectious mononucleosis patients and various control subjects were compared for anti-VCA and anti-MA activity, there was concordance in 80% of cases (similar titres for both antibodies) but striking discordance in the remaining 20%. It was possible to absorb out anti-MA activity from "Mutua" serum by exposure overnight to 7.5×10^7 cultured Burkitt lymphoma cells per ml of serum; this procedure did not affect the level of anti-VCA activity in the same serum (Pearson, Klein, Henle et al., 1969). Furthermore, in the same report, the authors noted that in sera from a number of unaffected relatives of Burkitt lymphoma patients they detected significant levels of anti-MA antibody in the complete absence of anti-VCA activity. In at least one such case, the anti-MA antibody appeared, on cross-blocking tests, to be directed against a more restricted part of the membrane-associated antigen-complex than the antibody present in Mutua serum. Subsequently, at least four surveys of patients with malignant disease have been reported in which anti-MA and anti-VCA activities were measured in the same sera. Among African and Hong-Kong Chinese patients with Nasopharyngeal carcinoma, over 90% had high titres of at least one antibody and

in over 60% high levels of anti-VCA coexisted with corresponding titres of anti-MA, (Mutua) and of anti-MA (Kipkoech). Among controls, including patients with tumours of the post-nasal space other than carcinomas, fewer than 20% had high titres in all three tests (De Schryver, Friberg, Klein et al., 1969). Gunven, Klein, Henle et al. (1970) confirmed the near universal occurrence of high titres of anti-EBV (MA and VCA) antibodies in African Burkitt lymphoma patients as compared with healthy relatives and unrelated control subjects. They found a concordance rate of 75% for the two tests and noted that among discordant sera, the samples with high anti-MA but low anti-VCA activity were not (as suggested earlier) confined to relatives of Burkitt lymphoma patients. Among sixty Swedish patients with Hodgkin's disease, fifty-nine with chronic lymphatic leukaemia and twenty-three with lymphocytic lymphomas, Johanssonand his colleagues (Johansson, Klein, Henle and Henle, 1970, 1971) found two groups of patients with very high geometric mean titres of anti-MA antibody, namely those with Hodgkin's sarcoma (poorly differentiated histologically with lymphocyte depletion and carrying a bad prognosis) and those with poorly differentiated highly malignant forms of lymphocytic lymphoma. The anti-MA values in sera from these individuals were comparable with those previously found in samples from Burkitt's lymphoma

and Naso-pharyngeal carcinoma sufferers. Patients with chronic lymphatic leukaemia, particularly those presenting initially with lymph node enlargement, had mean anti-MA activities somewhat higher than control sera but the difference did not reach statistical significance. (Levine, Merril, Bethlenfalvay et al., 1971, have also recorded a high geometric mean titre of anti-VCA activity in sera from chronic lymphatic leukaemia patients but found no association between antibody titre and white cell count). Over the series as a whole, concordance rates for anti-VCA and anti-MA titres in each serum were over 70%. The authors speculate that in the more malignant forms of Hodgkin's disease and lymphocytic lymphoma, EB virus may play a carcinogenic or co-carcinogenic role and they support the view reached on the basis of surveys quoted in Chapter I that there is no general association between proliferation of lymphoid tissue and a high or rising anti-EB virus antibody titre.

A comparison of anti-VCA, anti-MA and precipitating antibody levels was undertaken by Klein, Geering, Old, Henle et al. (1970) in a total of 151 sera from 131 donors who included African patients with Burkitt's lymphoma, nasopharyngeal carcinoma and other tumours of the head and neck, from patients recovered from infectious mononucleosis and from healthy control subjects. Concordance rates between any two of the three antibodies were over 70% and all three tests gave concordant results in 67% of cases.

However, there were sufficient exceptions to establish that the three antigens responsible were distinct from each other. In one Burkitt lymphoma patient, the titres of antibody to the three antigens varied in quite different ways as the disease passed through clinical phases of regression and relapse. This is discussed more fully below.

VCA, MA and EA (early antigen) production were followed in lymphoblastoid cell lines experimentally infected with EB virus by Pearson, Henle and Henle (1971). Three apparently virus-free clines, RAJI, 64-10 and SKL-1 each reacted differently to the introduction of concentrated virus from the HRI-K clone of JIYOYE. The patterns of response again clearly distinguished the three antigens from one another while confirming that all were EB virus-related. Both EA and MA were detectible in (or on) many more cells than those actually containing virus particles (as judged by anti-VCA fluorescence). Gergely, Klein and Ernberg (1971) found that the DNA antagonists Iodo-deoxyuridine (IUDR) and cytosine arabinoside (Ara-C) blocked the synthesis of new virus particles in two EB virus-carrying lymphoblastoid lines from Burkitt's tumours, thus VCA-positive cells virtually disappeared from the cultures. Up to 40% of cells, however, became EA positive. When the DNA inhibition in Ara-C treated cultures was reversed by the addition of deoxycytidine, there was a prompt return of cellular DNA synthesis with the appearance of many VCA-positive cells. This indicated that the production of viral capsid

antigen, but not of early antigen, is dependant upon cellular DNA synthesis. Similar studies on experimentally injected RAJI cells (Gergely, Klein and Ernberg, 1971a) showed that MA production was also independent of cellular DNA synthesis though neither EA nor MA appeared when protein synthesis was blocked by puromycin. Throughout this series of experiments, infection of the RAJI cells appeared to be totally abortive since no VCA-positive cells were found and, in the absence of metabolic inhibitors, EA and MA-positive cells disappeared within a few days.

Two-colour immunofluorescence tests have also enabled a clear distinction to be made between the EB virus associated early, membrane and viral capsid antigens and have confirmed that there is no cross-reactivity between HL-A and EB virus-associated cell surface receptors (Klein, Gergely and Goldstein, 1971; Gergely, Klein and Ernberg, 1971). In these studies, all VCA-positive cells also carried EA and MA. Among VCA-negative cells, there was a small percentage which were EA-positive, some being MA-positive also and others MA-negative.

The conclusions which can be drawn from all the foregoing evidence have been summarised in table XIVa . One point which requires further elaboration is the nature of the MA activity detected on the surface of fresh Burkitt lymphoma biopsy cells since EB virus is not detectible in such preparations (by electron microscopy or by anti-VCA immunofluorescence)

TABLE XIVa

Antigens associated with cultured human lymphoblastoid cells.

Antigen Designation	Possible Equivalents	Cellular Localisation	Relation to EB virus
Early Antigen (EA)	'N' antigen (Hinuma et al.)	Nuclear and cytoplasmic	Coded directly by EB virus DNA but can be produced in a cell which does not support replication of EBV
Viral Capsid Antigen (VCA)	Heat-resistant component of 'V' complement-fixing antigen	Nuclear and cytoplasmic, mainly in disintegrating cells	Probably structural components of non-enveloped virus particles
Membrane antigen (MA)		Cell surface, also virus envelope	Dependent on presence of replicating virus in cell line, though not necessarily in every MA-positive cell. Anomalous position of MA on fresh Burkitt's lymphoma tissue (see text).
Soluble antigens(s)		Supernatants of human lymphoblastoid cell line cultures.	Only detectable with sera which also have positive anti-VCA titres, but soluble antigen is obtained from supernatants of apparently virus-free cultures such as RAJI.

TABLE XIVa cont'd

Antigens associated with cultured human lymphoblastoid cells

Antigen Designation	Possible Equivalents	Cellular Localisation	Relation to EB virus
'Heat labile component of 'V' complement-fixing antigen		Insoluble sub-viral fraction of cell sonicates	Only found in cell lines in which EB virus is replicating. Possibly non-structural components of virus.
Complement-fixing surface antigen detected by cytotoxic reaction (Herberman et al)	Complement-fixing antigen in cell-membrane extracts (Hollinshead et al.)	Cell surface	Probably none. Similar (? identical) antigen present on leukaemic lymphoblasts and, in smaller quantities on normal peripheral blood leukocytes.

and since, when applied to cultured lymphoblastoid cells, positive MA reactions are obtained only in those lines in which EB virus is found to replicate. Klein himself has recently discussed this paradox (Klein, 1970) and suggests either that a slightly different antibody in anti-MA-positive sera is responsible for the reaction with fresh biopsy cells or that the 'suppression' of EB virus particles in tumour tissue in vivo operates through a different mechanism from that responsible in apparently virus-free cultured lines, with the result that membrane antigen can be elaborated in the former situation but not in the latter.

Some idea of the possible functions of the different EB-virus-related antibodies detected in human sera can be gained from studies on the neutralisation of EB virus infectivity. Two groups (Pope, Horne and Scott, 1969; Miller, Lisco, Kohn and Stitt, 1971) have shown that initiation of new cell lines by exposure of peripheral blood leukocytes or marrow cells to EB virus in vitro is prevented by prior incubation of the virus-containing material with sera which have a high titre of anti-VCA antibody. Neither group, however, tested their material for the presence of anti-MA or anti-EA activity and in view of the high concordance rates reported above, it is unlikely that the sera contained anti-VCA antibody alone. Walters and Pope (1971) separated material from EB virus-positive lymphoblastoid cells (disrupted by

freeze-thawing) on sucrose density gradients and identified a fraction with maximum capacity to induce long-term proliferation in foetal marrow leukocytes. They considered that this fraction consisted principally of naked EB virus with very little admixture of enveloped particles. Hence they deduced that naked virus particles are infective and that neutralisation of infectivity is a function of anti-VCA antibody. This view is in conflict with evidence from Pearson, Dewey, Klein et al. (1970) who showed that when RAJI cells were the target for in vitro infection with EB virus, there was a close correlation between the anti-MA titre of a given serum and the capacity of that serum to neutralise the infection. Only a poor correlation was observed between anti-VCA levels and neutralising potential. Later, the same group (Pearson, Henle and Henle, 1971; Gergely, Klein & Ernberg, 1971a) demonstrated that when anti-MA activity was removed from a serum by differential absorption, leaving anti-EA and anti-VCA activity intact, the neutralising capacity of the serum was lost. This suggests very strongly that infectivity for human tissues is associated mainly with the complete enveloped form of the EB virus.

Of more direct concern to the clinician is the relevance of EBV-associated antibodies to the outcome of those human diseases suspected of being carried by EB virus. It is assumed that cases of spontaneous regression of Burkitt's lymphoma are evidence of an immunological defence mechanism against the

tumour and considerable efforts have been made in a number of centres to identify serum factors with anti-tumour activity. Klein, Clifford, Klein and Stjernsward (1966) found a good correlation between the serum level of anti-MA antibody and the duration of response to chemotherapy in a series of Burkitt lymphoma patients. Klein, Clifford, Henle et al. (1969) followed the changes in titre of anti-MA (by blocking index) anti-VCA and of precipitating antibodies in the serum of one Burkitt lymphoma patient during a prolonged (4 year) remission followed by massive tumour recurrence. Anti-VCA levels showed only minor fluctuations throughout the course of the disease; anti-MA activity was high during remission but virtually disappeared six months before recurrence was clinically evident. As the tumour mass enlarged, anti-MA antibody reappeared and remained at a high level until death. Precipitating antibodies could not be detected during remission, but appeared shortly after tumour recurrence, reaching a high level before death. When antibody to early antigen in sera from Burkitt's lymphoma patients was examined in relation to the clinical stage of the disease, it was found that persistance of anti-EA activity during regression was associated with a high risk of subsequent recurrence; occasionally, however, the antibody was undetectable in moribund patients with very large tumour masses (Henle, Henle, Klein et al. 1971). In infectious mononucleosis, anti-EA antibody

appears during the course of the illness but declines to undetectible levels within a few months of recovery (Henle, Henle and Niederman, 1971). The interpretation of these data is speculative at present, however, it seems possible that circulating anti-MA may have an inhibitory effect on the growth of Burkitt tumour cells and that relapse in the patient described by Klein et al. was a consequence of the decline in anti-MA activity during the preceding months. Subsequent growth of an antigenic tumour mass may then have provoked a secondary (but ineffective) rise in anti-MA titre and the simultaneous appearance of precipitating antibodies. Persistance of anti-EA activity, in the absence of clinically evident disease may indicate that EB virus remains in the tissues in a state which can still provoke lymphoproliferation. The disappearance of the antibody in moribund patients can then be attributed to absorption by mssive quantities of the corresponding antigen. The finding that local X-irradiation of Burkitt's tumour deposits and of nasopharyngeal carcinoma is regularly followed by a rise in anti-MA antibody in the patient's serum (Einhorn, Klein and Clifford, 1970) has been related to the increased exposure of membrane antigen in cultured lymphoblastoid cells following irradiation in vitro (Yata and Klein, 1969). In most Burkitt lymphoma and nasopharyngeal carcinoma patients there

is a rise, albeit small, in the titre of anti-VCA in the serum during the first monthafter local radiotherapy to the tumour. In the Burkitt's lymphoma group anti-EA levels also rise sharply but this is not true of the nasopharyngeal carcinoma patients in whom levels of anti-EA fall within 2-3 months of the procedure (Einhorn, Henle, Henle et al., 1972). It has not yet been possible to relate these changes to the response to treatment and the reason for the difference is at present quite obscure. Equally difficult is the question as to why antibody to membrane antigen should seem to be protective in some situations but ineffective in others, even when an antibody rise can be demonstrated and the tumour cells can be shown to be coated with IgG (Klein, Clifford, Henle et al., 1969). In animal systems it has been claimed that recurrence of a tumour after a period of remission is due to the emergence of an immunoresistant cell type (Fenyo, Klein, Klein and Swiech, 1968) in which case, antibody coating of the tumour cell may have no influence on their growth or may even enhance it by protecting the tumour from cell-mediated immunological attack (Hellstrom and Hellstrom, 1969).

More recently Henle, Henle and Klein (1971) have identified two distinct patterns of distribution of EBV associated early antigen in abortively infected human lymphoblastoid cell lines, possibly representing two separate components of an early antigen

complex. Antibodies eliciting the two patterns of immunofluorescence were titrated separately in sera from patients with infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma. The relative proportion of the two antibody types appeared to be characteristic for each of the conditions although there was an appreciable overlap among the three groups of patients. If this finding can be confirmed it may prove to have an important bearing on the distinction between oncogenic and self-limiting EB virus-induced diseases. At present, however, no meaningful interpretation can be made of the results.

Sera from Burkitt tumour patients and from control donors have been assessed for their effect on the growth of cultured lymphoblastoid cells derived from Burkitt's lymphoma (Osunkoya, 1967). Growth-promoting capacity was poorest in samples from Burkitt's patients in remission, optimum in those from healthy subjects or from patients with disorders other than Burkitt's lymphoma, and intermediate in those from Burkitt's patients with active disease. There was, however, no correlation between relative inhibition of <u>in vitro</u> growth and the level of anti-MA activity in the sera under test.

Efforts have been made to exploit the putative anti-tumour activity of sera from Burkitt's lymphoma patients in sustained remission by infusing their plasma into newly diagnosed patients.

Early reports of such trials were encouraging (Ngu, 1967; Clifford, Singh, Stjernsward & Klein, 1967) but a later controlled trial has failed to confirm that any benefit ensues from the procedure (Fass, Herberman, Zeigler and Morrow, 1970).

To date it has proved simpler to investigate the possible role of humoral antibody in the defence against EB-virus-induced tumours, but evidence from studies of both animal and human malignant disease suggests that cell-mediated immune mechanisms may be even more important (for reviews see Hellstrom and Hellstrom, 1969; Alexander and Hall, 1970; Mathe, 1971). In this context it is of interest that X-irradiated cells from a number of human lymphoblastoid cell lines provoke vigorous DNA synthesis in freshly isolated peripheral blood lymphocytes from an unrelated individual when the two cell populations are mixed in vitro (Hardy & Ling, 1969; Hardy, Ling and Knight, 1969; Hardy, Knight and Ling, 1970). The activation requires direct cell-cell contact and is similar to the one-way mixed lymphocyte reaction (Bach and Voynow, 1966) but is of approximately tenfold greater intensity and the effect is maximal when the proportion of lymphoblastoid cells to fresh blood lymphocytes in the mixture is approximately 1:5, whereas the optimum ratio for mixtures of small lymphocytes is 1:1 (Han, Moore and Sokal, 1971).

In collaboration with Dr. D.A. Hardy (Department of Experimental Pathology, University of Birmingham) I have applied similar methods to the search for cell-mediated immunological reactions between cells from established lymphoblastoid lines and fresh blood lympho-

cytes from the same donor (i.e. in an autochthanous system).

Materials and methods

All procedures were carried out under sterile conditions.

Activating cells. These were derived from the human lymphoblastoid cell lines maintained in this laboratory as described in chapters II and III. For convenience, the abbreviation "LCL" cells (lymphoblastoid cell line cells) is used hereafter. For use in mixed culture experiments, cells from each line were spun down from a growing culture at 150 x g for 10 minutes and resuspended in fresh growth medium (Ham's F_{10} + 10% tryptose phosphate broth + 20% foetal calf serum) to give a final viable count of 1-2 x 10^6/ml. Viable counts were made in a Neubauer's haemocytometer after dilution of a small aliquot of cell suspension 1:1 with 0.45% Nigrosin in buffered salt solution (Kaltenbach, Kaltenbach & Lyons, 1958).

Irradiation of the cells in batches of 3-9 x 10^6 was carried out as described in Chapter III, to a total dose of 3000 rads. When cells from a number of lines were being used in a single experiment, they were irradiated simultaneously, in separate sterile containers. Following irradiation, the cells were washed twice with the medium used for "responding cells" (see below) and resuspended in the same medium at a final concentration which allowed for convenient dispensing into the culture tubes (usually 2 x 10^5/ml).

Responding cells. Peripheral blood (30-50 ml) was obtained from patients who had recovered from infectious mononucleosis, or

from healthy volunteers, by venepuncture into a plastic syringe and immediately transferred to polycarbonate tubes (15-25 ml each). The blood was stirred for 10 minutes with 'orange' sticks to remove fibrin clot. To the defibrinated blood was added one-third of its own-volume of a 3% gelatin solution prepared as detailed below (Coulson and Chalmers, 1964). The blood/gelatin mixture was allowed to stand at 37°C in the polycarbonate tubes until the red cells had sedimented to occupy 50% of the total volume (approx. 30 minutes). The serum-gelatin layer was removed by gentle aspiration into fresh polycarbonate tubes. The leukocytes were separated from this suspension by centrifugation (150 x g for 20 minutes), washed twice in growth medium (Eagles MEM, Glasgow modification, supplemented with 15% pooled serum gelating prepared from the supernatants of defibrinated blood samples of healthy donors with Penicillin 100 μ/ml and Streptomycin 100 μg/ml) and resuspended in the same medium to give a final concentration of 2×10^6 viable cells/ml. Counts were made in a Neubauer haemocytometer using Nigrosin as described above, or 2% trypan blue (Ling, 1968) as indicators of viability. The morphology of the leukocytes was examined under the same conditions. All preparations consisted of over 90% lymphocytes and contained fewer than 5% dead cells. The number of red cells was determined by dilution of a small aliquot of the final suspension 1:200 with formol citrate (Sodium citrate 3.0 G Formaldehyde, 1.0 ml, Distilled water to 100 ml) and counting in a Neubauer

haemocytometer. The proportion of red cells to lymphocytes in different preparations ranged from 2:1 to 4:1.

Mixed culture procedure

Cultures were set up under sterile conditions in 3" x ½" round-bottomed glass test tubes with loose-fitting alloy (Oxoid) caps. The numbers of fresh blood lymphocytes and of irradiated lympho-blastoid cell line cells ('LCL' cells) in each mixed culture varied slightly between experiments (see individual experiments for details) but typically comprised 10^6 fresh lymphocytes and 10^5 irradiated LCL cells which was achieved by adding 0.5 ml of each of the cell suspensions to a culture tube. All cultures were set up in triplicate and within each experiment two types of controls were included, one being of identical composition to the mixed cultures with respect to erythrocytes and irradiated LCL cells, but containing no blood lymphocytes, the other consisting of fresh blood lymphocytes alone. Erythrocyte suspensions for the former type were prepared by aspirating cells from the sedimented layer remaining after removal of the serum-gelatin phase from the defibrinated blood samples. The red cells were washed twice with the growth medium described under "responding cells" and resuspended in that medium in a concentration identical to that of the erythrocytes in the lymphocyte suspension prepared from the same donor, the cell count being determined as described above.

The interval between irradiation of LCL cells and the setting-up of mixed cell cultures was usually less than 6 hours and never greater than 18 hours. Within a single experiment, this interval was the same for all irradiated cell lines used.

Mixed cultures and controls were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 3 to 8 days. Twenty-four hours before harvesting, 0.5 μCi of (^{3}H)-methyl labelled thymidine (specific activity 150 mCi per m.mole, Radiochemical Centre, Amersham) was added to each culture tube.

Measurement of lymphocyte activation .

The amount of tritiated thymidine incorporated into the DNA of the cultured cells was taken as a measure of lymphocyte activation (Ling, 1968) and was determined by the following procedure which is based on that described by Ling and Holt (1967).

Cultures ready for harvesting were spun down at 800 x g for 5 minutes in an 'MSE Major' centrifuge, identical centrifugation being applied between each of the succeeding steps. The supernatant was discarded and the cells resuspended in 1 ml of normal saline. The cells were re-sedimented, the saline discarded and the pellet shaken up in 1 ml of 10% trichloroacetic acid. The procedure was repeated with fresh 10% trichloroacetic acid and then with 1 ml of absolute methanol, following which three drops (0.1 ml) of N_1 sodium hydroxide were added to the cell pellet which was

dispersed by shaking and the tubes transferred to a water bath at 60°C for 1 hour to allow the cellular material to be digested. 1 ml of absolute methanol was then added and the solution transferred to a scintillation vial with 10 ml of scintillation fluid (composition detailed below).

The tritium content in each vial was measured on a Packard 'Tri-carb' liquid scintillation counter, each sample being counted for ten minutes, followed by one minute of external standard counting. The efficiency of counting for each sample was determined by comparing the external standard value with the standard quenching curve obtained on the same machine at the beginning of the experimental run. Results are expressed as disintegrations per minute (dpm) calculated as

$$\frac{\text{recorded counts in 10 minutes}}{10} \times \frac{100}{\text{counting efficiency (\%)}}$$

The results reported are means for triplicate cultures. Individual values rarely varied from the mean by more than 10%. On a few occasions one culture was lost through a laboratory accident or a single value differed grossly from the other two. In these cases the mean for the remaining values has been taken.

Composition of Solutions

Gelatin

This was prepared by dissolving fine grain pigskin gelatin (British Glue and Gelatin Research Association) 3G in 100 ml

of freshly boiled physiological saline. The solution was sterilised by Seitz filtration while still hot and allowed to cool to 37°C before use. Gelatin solution for sedimentation of red cells and polymorphs from defibrinated blood was always used within 24 hours of preparation.

Scintillation fluid

15 G 2,5 diphenyloxayole (Koch-Light Laboratories Ltd.)

0.3 G 1,4-bis-(2-(5-(phenyl-oxayolyl))benzene (Koch-Light Labs.Ltd.)

dissolved together for 24 hours at room temperature in 2.5 L. Xylene (Analar grade).

2 volumes of the above solution mixed thoroughly with one volume of Triton-X-100 (Lennig Chemical Co., London W.C.1).

Experiments and Results

The first experiments, involving two patients who had recovered from infectious mononucleosis and two control subjects, are described in the accompanying paper, reprinted from Lancet 1, 1322-1323 (1970). It is clear from these preliminary results that stimulation does occur in an autochthonous mixture of fresh blood lymphocytes and irradiated LCL cells.

Preliminary Communication

EVIDENCE OF ALTERED ANTIGENICITY IN CULTURED LYMPHOID CELLS FROM PATIENTS WITH INFECTIOUS MONONUCLEOSIS

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Summary X-irradiated lymphoid cells of established cell lines derived from two patients with infectious mononucleosis were found to stimulate fresh lymphocytes from the same individuals several months after recovery from the illness. This is believed to reflect a degree of antigenic disparity between the cultured lymphoblasts and autologous fresh lymphocytes.

INTRODUCTION

LYMPHOBLASTOID cell lines have been established in a number of centres from the peripheral blood of patients in the acute or convalescent phase of infectious mononucleosis.[1–4] Many of these lines can be shown to carry the herpes-like virus of Epstein and Barr (E.B. virus). Seroepidemiological evidence suggests that this virus may be the causative agent of infectious mononucleosis,[5,6] but the relationship between cells growing in culture, E.B. virus, and the circulating atypical mononuclear cells characteristic of the illness remains speculative.

Similar cell lines have been established from patients with Burkitt's lymphoma, leukæmia, and other conditions as well as from healthy individuals.[7,8] Cells from such lines excite a potent blastogenic reaction when added to freshly isolated lymphocytes from healthy individuals, similar to that observed in a one-way mixed-lymphocyte reaction, but of greater intensity.[9,10]

We here report on the findings when lymphoid cells of lines initiated from the blood of infectious mononucleosis patients are cultured with their own fresh lymphocytes after recovery from the illness.

MATERIALS AND METHODS

The lymphoblastoid cell lines GOL_1 and MAR_1 were used.[4] GOL_1 comes from a 22-year-old female (A) and MAR_1 from a 29-year-old male (B). Both were convalescent after acute infectious mononucleosis when blood was taken for long-term culture, and both subsequently recovered uneventfully. At the time of this study they were symptom-free. Both patients had anti-E.B.-virus titres of 1/80 in the blood specimens from which the long-term cultures were grown,[4] and although E.B. virus could not be demonstrated in GOL_1 cells at the time of the experiments reported here, the line became virus positive (by immunofluorescence and electron microscopy) one month later, after an apparently spontaneous alteration in cultural behaviour and a change in karyotype from diploid to polyploid.[11] Virus was present in 1–2% of MAR_1 cells. Both lines were routinely maintained in Ham's F10 medium (Flow Laboratories) supplemented with 10% tryptose phosphate broth (Difco), 20% fetal calf serum (Flow Laboratories), and antibiotics. GOL_1 cells had been in culture for 6 months, frozen in liquid nitrogen for 1 month, then returned to culture for 5 months before use in these experiments. The MAR_1 line had been in culture for $10^1/_2$ months without interruption. DEW_1, a cell line from a third glandular fever patient, was used as an additional control. This line had been in culture for $4^1/_2$ months. E.B.-virus particles could be demonstrated in less than 1% of cells.[4]

Fresh blood (50 ml.) was obtained from the first two (A and B) and from two healthy male controls (C and D). The blood was defibrinated immediately and a lymphocyte-rich suspension prepared by gelatin sedimentation. 18 hours before setting up mixed lymphocyte cultures, cells from each of the established lines were irradiated (total dose 3000 rad, 250 kV X ray source, dose-rate 33 rad per minute Thoraeus I filter).

From the sedimented blood a cell suspension containing 2×10^6 lymphocytes and $4–8\times10^6$ erythrocytes per ml. of medium (Eagle's minimum essential medium supplemented with 15% pooled normal human serum) was prepared and 1 ml. volumes were distributed into $3\times{}^1/_2$ in. sterile, capped, glass tubes. To some of the tubes, 10^6 irradiated cells in the same medium from the established lines were added as shown in the table. Control cultures were of identical composition with respect to erythrocytes and irradiated cells, but contained no blood lymphocytes. The degree of stimulation of the blood lymphocytes was assessed by measurement of the mean uptake of tritiated thymidine in triplicate cultures.

THYMIDINE INCORPORATION IN MIXED CELL CULTURES

Culture	Radioactivity incorporated (d.p.m.)	
	Mean	S.D.
A control	5773	737
A+X GOL_1 (autologous)	33,607	1861
A+X MAR_1	110,390	5436
A+X DEW_1	122,860	4764
B control	2124	329
B+X GOL_1	84,669	5220
B+X MAR_1 (autologous)	12,333	3005
B+X DEW_1	82,120	4216
C control	1313	183
C+X GOL_1	98,349	*
C+X MAR_1	72,164	1694
C+X DEW_1	76,653	2712
D control	1450	*
D+X GOL_1	39,264	7637
D+X MAR_1	87,664	6501
D+X DEW_1	97,738	155
C R.B.C.+X GOL_1	341	..
C R.B.C. +X MAR_1	209	..
C R.B.C. +X DEW_1	410	..

Cultures were harvested on day 5. 0·5 μCi of [^{3}H]-methyl labelled thymidine (150 mCi per mmole) was added to each, 24 hours before harvesting.*

X = X irradiated. R.B.C. = red blood-cell controls.

D. = standard deviation of mean (triplicate values). * = duplicates only

RESULTS

The control cultures indicate that thymidine incorporation into irradiated cells was negligible over the period of the study. In all cases where fresh lymphocytes were mixed with irradiated cells from cultured lines, the lymphocytes were significantly activated. Thymidine uptake in the two autologous mixtures (A+GOL_1X and B+MAR_1X) was about six times greater than the unstimulated level, compared with the nineteen to seventy-five fold increase observed in mixtures of unrelated cells.

DISCUSSION

We have found that lymphoblastoid cell line cells from infectious mononucleosis patients are potent activators of fresh lymphocytes from unrelated donors. This is in line with experimental findings on cell lines from other sources.[9] No previous studies have been reported, however, in which cell lines have been tested against fresh autologous lymphocytes. Our experiments show that stimulation does occur in such mixtures.

It is highly likely that the stimulation of small lymphocytes by irradiated lymphoid cell line cells has an immunological basis.[10] One would, therefore, expect the level of stimulation in this system to reflect the degree of antigenic disparity between the cell types in each combination. Our observation of a much greater degree of activation in mixtures of unrelated cells than in autologous mixtures is in accord with this expectation. Even in autologous mixtures, a sixfold enhancement of thymidine incorporation was observed. It is logical to assume that this lymphocyte stimulation too was produced by surface antigens on the cultured lymphoblasts and suggests the existence of an appreciable antigenic disparity between cells originating from the same donor. There are several ways in which this antigenic disparity may have arisen:

(1.) The in-vitro environment of the cultured cells may have caused some alteration in surface determinants—e.g., by adsorption of antigenic groups from the medium.

(2) The in-vitro environment has induced a mutation or favoured the emergence of a line from an antigenically distinct mutant cell in the initial blood-sample. If this explanation is correct, the demonstration that fresh lymphocytes can be stimulated by autologous cells could be interpreted as providing experimental support for the concept of an immunological surveillance mechanism.[12]

(3.) In view of the reported association between infectious mononucleosis and E.B. virus, the cells growing in culture may carry a virus-related surface antigen. Recognition of such an antigen by immunocompetent cells might then be related to the process of recovery from the lymphoproliferative disorder, infectious mononucleosis.

We are not able to choose between these alternatives at present. Further experiments are however in progress to determine the kinetics of stimulation by autologous cells, the nature of the stimulatory antigen and the cytotoxic capacity of the lymphocytes after stimulation.

We thank Dr. N. R. Ling for his advice and encouragement.

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As part of a programme designed to identify the antigens responsible for this reaction, tissue typing of the cell lines has been undertaken by Dr. Heather Dick of the Department of Bacteriology, Glasgow University, and by Mrs. P. Mackintosh, Birmingham Regional Blood Transfusion Centre. Both have used a microcytotoxicity test (Kissmeyer-Nielsen & Kjerbye, 1967). In many cases, fresh blood lymphocytes from the donors of the cell lines and from control subjects participating in the mixed-lymphocyte culture experiments have been typed by these workers and by Dr. A.R. Sanderson (McIndoe Memorial Unit, East Grinstead) using the same technique. The interpretation of the results for HL-A typing of cultured lymphoblastoid cells presents many difficulties but in general the independent findings of Dr. Dick and Mrs. Mackintosh are in good agreement (personal communication). There is a clear relationship between the antigens identifiable on fresh lymphocytes and on LCL cells from the same donor, but the cultured cells tend to react with many more antisera than do the fresh blood cells (Dick and Steel, 1971; Dick, Steel and Crichton, 1972; and unpublished results of Dr. Dick and Mrs. Mackintosh). Family studies have not yet been carried out to determine whether 'new' antigens appearing on cultured cells have been inherited from the parents but not expressed on circulating lymphocytes (as has been shown in the case of short-term cultures by Noer, Van der Hagen and

Berg, 1971) or whether the recognised degree of cross-reactivity between certain HL-A antigens (Amos, 1969) is more apparent in the case of cultured cells. This latter hypothesis is in keeping with the observations that in cytotoxicity testing, cultured lymphoblastoid cells are extremely sensitive, requiring particular care in the choice of complement source (McDonald, Jacobbi and Williams, 1970). Furthermore, preliminary electron microscopic studies have shown that the HL-A determinants are densely distributed all over the surface of LCL cells, whereas they are confined to isolated patches on the surface of small lymphocytes (Kourilsky, Silvestri, Levy et al., 1971). Most reports of HL-A typing of cultured lymphoblastoid cells have recognised that apparently 'extra' antigens appear on them, while reproducibility of the results and closeness of fit with the typing of the donor's fresh lymphocytes seem to be inversely related to the number of antisera in the test panel and to the sensitivity of the technique applied (Rogentine and Gerber, 1969; Bernoco, Glade, Broder et al., 1969; McDonald, Jacobbi and Williams, 1970; Moore and Woods, 1971; Ferrone, Pellegrino and Reisfeld, 1971; Juji, Kano and Milgrom, 1971). An additional factor contributing to discrepancies between the apparent HL-A phenotype of fresh lymphocytes and cultured lymphoblastoid cells from the same donor is the presence in a small number of tissue-typing antisera of cytotoxic activity reactive with cultured cells of every line tested so far

(Dick, Steel and Crichton, 1972; Mackintosh, personal communication). This cytoxic factor may be related to that described by Herberman and his associates (see introduction to this chapter), though it appears to be less widely distributed in human sera. Furthermore, Dick has shown that it is not removed by absorption with normal human lymphocytes though it can be absorbed with lymphoblastoid cell line cells, leaving the anti-HL-A specificity of the serum intact or only slightly reduced in titre (Dick, Steel and Crichton, 1972 and unpublished observations). The same sera have also shown anomalous cytotoxicity (i.e. unrelated to HL-A type) against the peripheral blood leukocytes from some patients with acute leukaemia and from at least one moribund patient with cerebrovascular disease (Dick and Steel, 1971 and unpublished observations). Very similar findings have been recorded by Bernoco, Glade, Broder et al. (1969). These last observations make it unlikely that the anomalous cytotoxic activity is directed against a specifically EB virus-related cell surface antigen. Also against such an interpretation is the finding that none of three sera showing anomalous cytotoxicity against LCL cells have high levels of EB-virus-associated anti-VCA antibody (Dick, Steel and Crichton, 1972). The antigen may therefore be a determinant common to cells of lymphoid origin, not normally expressed on the surface of small lymphocytes, but appearing when the cells undergo blastogenic change, or when they are damaged in some way.

In seeking to identify the antigens responsible for activation of fresh blood lymphocytes by antochthonous LCL cells we have considered the following as major possibilities; first, macromolecules or haptenic groups adsorbed from the culture medium, notably from the foetal calf serum which has been shown, by immunisation studies, to affect the antigenic properties of cultured HeLa cells (Hamburger, Pious and Mills, 1963) and of lymphoblastoid cell line cells (Eng and Landon, 1970); secondly, changes in the HL-A phenotype of cultured LCL cells either induced by EB virus or arising spontaneously in vitro; thirdly, the acquisition of surface neo-antigens, again either EB virus-induced or as a consequence of adaptation to the cultured state, and fourthly, alterations in cell-surface antigens produced, as artefacts, by the irradiation procedure.

These possibilities were examined in a further series of experiments.

Table XV gives details of twelve healthy adult (aged 18-32 years) donors of fresh blood lymphocytes used in these experiments. Eight had suffered from infectious mononucleosis, six to twenty-four months earlier. From all of them, cultured lymphoblastoid cell lines had been established. A further line, ORI_1, had been grown by co-cultivation from the peripheral blood of one of the four donors (M.O.) who had not had infectious mononucleosis. The fifteen cell lines from which irradiated cells were used in the series of experiments are also listed in table XV.

Table XVI records the results of experiments in which fresh lymphocytes from each of nine donors were mixed with irradiated LCL cells from an autochthonous and an allogeneic line. The first two sets of figures are taken from the experiments reported in the paper reproduced earlier in this chapter. In four cases (indicated by an asterisk in the table), penicillin and streptomycin were excluded from the culture medium during the 6-7 days of the experiments.

Four conclusions may be drawn from these results. First that activation of fresh blood lymphocytes by autochthonous LCL cells is a consistent finding, not confined to lymphocytes from patients who are known to have suffered from infectious mononucleosis. Secondly, that the phenomenon is not dependent on the presence of antibiotics (as possible antigens) in the growth medium, thirdly that the level of activation is not consistantly higher in an allogeneic than an autochthonous

TABLE XV: Donors of fresh lymphocytes and lymphoblastoid cell lines used in mixed experiments

DONOR			CORRESPONDING CELL LINE		
Initials	Sex	HL-A type (fresh lymphocytes) #	Name		Most consistent HL-A specificities ##
E.D.	♀	2, 7, ?3	DEW_1		2,7,3, ?FJH
D.D.	♂	8,11, ?5 ✓	DUN_1	✓	1,8,?9
I.G.	♀	N.D.	GOL_1		1,10,8
K.F.	♂	1, 7, 8	FLE_1		1, 7,8
E.B.	♀	1,2,8,W18	KAT_1		1,2,8,W18, ?Maki
V.B.	♀	2,7,10	LIZ_1		2,7,10
A.M.	♂	1,3,7,8	MAR_1		1,3,7
M.O.	♀	1,2,8,12	ORI_1		1,2,8,12, ?3,?7,?13
K.Y.	♀	1,10,?5,?9	YUD_1		1,10,?8,?W5
D.M.	♂	1,10,12,LND	None		-
D.V.	♂	1,8,9	None		-
M.S.	♂	1,2,8,12	None		-
Not Available	♀	N.D.	CLA_4		?2,W28,12,W19
"	♂	3,7	HUN_1		1,3,7,8
"	♂	N.D.	SAD_1		2,3,7,4c*,Walland
"	♀	N.D.	$G\text{-}S_1$		7,13
"	♀	N.D.	LAM_2		3, 8
"	♂	N.D.	F137		1,3,LA-W,7

ND = Not done

\## Most results confirmed independently by Dr.A.R. Sanderson, Dr.H.M.Dick and Mrs. P. Mackintosh, but in a few cases, lymphocytes have been typed on only one occasion.

\# # This column records only those specificities which have been consistently detected on repeated typing by Dr.Dick and Mrs. Mackintosh. It excludes reactions detected with sera showing anomalous (i.e. unrelated to HL-A) cytotoxicity.

✓ HL-A 11 detected on fresh lymphocytes of DD and HL-A 1 detected on corresponding LCL cells DUN_1 may in fact be represented more accurately as a rare variant of HL-A 1 which cross-reacts with HL-A 11 (Dr. H.M. Dick, personal communication).

TABLE XVI

Stimulation of fresh lymphocytes by autochthonous or allogeneic LCL cells.

Fresh lymphocyte Donor	Period of culture (days)	Radioactivity incorporated into fresh lymphocytes (dpm)			Allogeneic Line used	## Number of identified Histo-incompatibilities in Allogeneic mixture
		# Control (No.LCL cells)	Autochthonous mixture	Allogeneic mixture		
I.G.	5	5773	33,607	122,860	DEW_1	N.D.
A.M.	5	2124	12,333	82,120	DEW_1	1
* D.D.	6	5084	80,214	59,295	DEW_1	3
* K.Y.	7	1511	47,727	38,281	GOL_1	1
* K.F.	7	2916	16,562	22,841	YUD_1	1
* E.D.	7	6134	23,526	42,887	FLE_1	2
M.O.	6	6710	40,564	35,612	DUN_1	0
V.B.	5	4059	88,438	68,139	GOL_1	1
E.B.	6	7065	24,378	49,352	CLA_4	1

* No antibiotics in the medium during these experiments.

In additional control cultures containing X-irradiated LCL cells and erythrocytes but no fresh blood lymphocytes, counts were invariably less than 500 dpm.

This column records the number of those HL-A antigens consistently found on the LCL cells, but absent from the fresh blood lymphocytes in a given allogeneic mixture.

All results are expressed as the means of triplicate cultures. Each tube contained 10^6 viable fresh lymphocytes. Mixed cultures, in addition, contained 10^5 X-irradiated LCL cells, except in the case of experiments with M.O. fresh cells in which each tube contained 2×10^5 irradiated LCL cells.

mixture, and fourthly that there is no obvious relationship between the level of activation achieved in a given allogeneic mixture and the number of identifiable histo-incompatibilities involved.

The second of these conclusions cannot be regarded as completely established by our experiments since it can be argued that penicillin and/or streptomycin molecules may have become firmly bound to the surface of cultured cells, during their prolonged period of culture, so that the absence of antibiotics from the growth medium used during the course of a six or seven day experiment does not exclude the possibility that these drugs, acting as haptens, are responsible for the activation of antochthonous fresh lymphocytes.

Nevertheless, it should be noted that none of our lymphocyte donors gave a history of allefgy to penicillin or to streptomycin, while *in vitro* studies on the activation by penicillin and other antibiotics of peripheral blood lymphocytes from patients known to display delayed-type hypersensitivity to these drugs have never shown levels of activation comparable to those found in our experiments and in many instances have yielded inconclusive results (Hirschhorn, Bach, Kolodny *et al.*, 1963; Ripps, Fellner and Hirschhorn, 1965; Vischer, 1966; Ripps and Fellner, 1966).

The possible effect of irradiation on cell surface antigens was investigated by subjecting freshly isolated peripheral blood lymphocytes from four donors to the irradiation procedure described above, the total dose being 3000 rads. Mixed leukocyte cultures were then set up using 10^5 of these cells plus 10^6 autochthonous or allogeneic fresh lymphocytes (non-irradiated). Tritiated thymidine was added 24 hours before harvesting and the uptake measured exactly as described above. The results (Table XVII) indicate that while the counts in non-activated lymphocyte cultures from two patients who had had infectious mononucleosis (DD, and ED) were rather high, an increase in the counts (implying activation of the lymphocytes) was seen only in allogeneic and not in autochthonous mixtures. Hence the irradiation procedure per se cannot account for the apparent antigenic differences between irradiated LCL cells and antochthonous fresh lymphocytes.

To investigate further the relative intensity of activation in autochthonous and allogeneic mixtures and its relationship, if any, to the level of recognisable histo-incompatibility between the two cell populations, Dr. Hardy and I undertook a further series of experiments in which the kinetics of the activation process was examined by measuring the uptake of tritiated thymidine (in triplicate cultures) at three or more different time intervals

TABLE XVII

Lack of stimulation of fresh blood lymphocytes by X-irradiated autochthonous small lymphocytes

Irradiated lymphocytes	^{3}H-Thymidine Uptake in Autochthonous mix (dpm)		^{3}H-Thymidine uptake in Allogeneic mix (dpm)		Donor of allogeneic fresh lymphocytes.
	Control	Mix	Control	Mix	
X-DD	5,084	7,962	2,605	12,385	M.S.
X-KY	1,511	817	1,785	9,500	DH
X-KF	2,916	2,261	1,117	4,213	MS
X-ED	6,134	3,243	1,522	3,506	DH

All results are means of triplicate cultures harvested on day 7 after setting-up.

Mixed cultures contained 10^6 fresh lymphocytes plus 10^5 X-irradiated fresh lymphocytes. Control tubes contained fresh lymphocytes only.

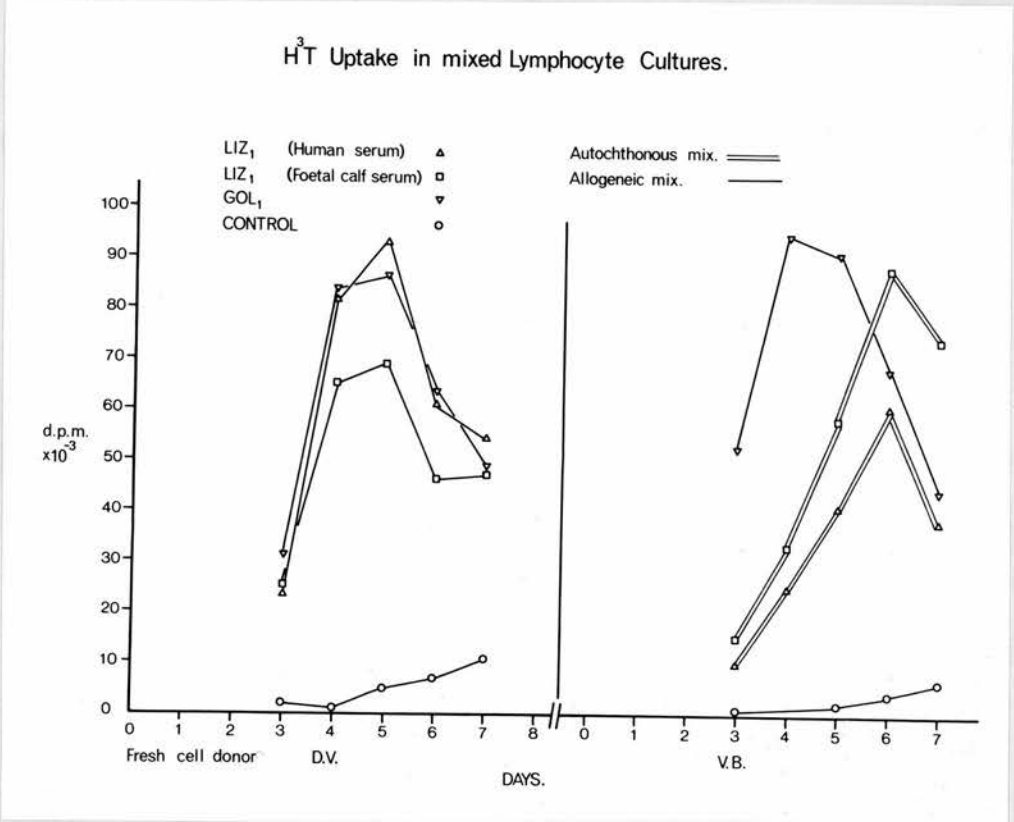
after setting up the mixed cultures. In the course of this series we also studied the effect of eliminating foetal calf serum antigens by growing the LCL cells in medium supplemented instead with 10% unpooled human AB Rh + ve serum for at least six weeks (12-20 cell cycles) before use in a mixed culture experiment. In two cases we compared the response to different numbers of irradiated LCL cells from the same line and on one occasion we used, as the responding cells, peripheral blood lymphocytes which had been frozen down in liquid nitrogen for three weeks before the experiment. Details of the freezing procedure are as follows.

Using sterile techniques throughout, from 60 ml of defibrinated peripheral blood, the lymphocytes were separated by gelatin sedimentation as described above, giving a yield of 9×10^7 viable lymphocytes. These were resuspended in a total volyme of 5 ml of freezing medium consisting of Eagles MEM with 35% autochthonous serum-gelatin and 10% glycerol. The suspension was dispensed into 5 x 1 ml ampoules which were sealed. The ampoules were cooled at $1^{\circ}C$ per minute using a programmed temperature controller (Canalco) until the contents were about to freeze solid (minus $17^{\circ}C$). The cooling rate was then increased to $3^{\circ}C$ per minute to avoid the damage to cells caused by transient rewarming just at the point of freezing as adjacent cells freeze solid and release the latent heat of crystallisation (see temperature plot

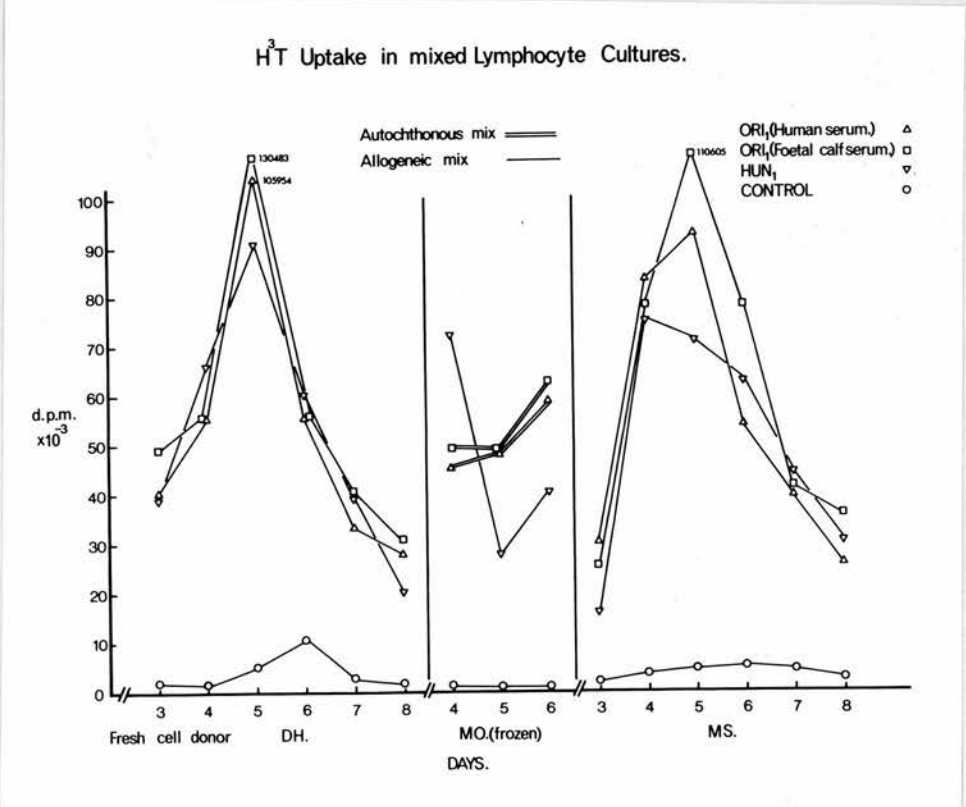
of Naginton and Greaves, 1962). When the temperature had reached -70°C the ampoules were stored in liquid nitrogen. Immediately before use in the mixed culture experiment, the ampoules were thawed rapidly by agitation in a water bath at 37°C. The cells were then washed once in Eagles MEM and resuspended at 2×10^6 viable cells/ml in Eagles MEM with 15% pooled human serum-gelatin. The viability of the recovered cells on this occasion (determined by Nigrosin exclusion as described earlier) was 65%.

The results of the experiments are illustrated graphically in figs.59 to 65. Each point represents the mean of triplicate cultures. Control curves are values for cultures of 10^6 fresh lymphocytes without added LCL cells. The alternative control cultures (X-irradiated LCL cells with donor erythrocytes but no fresh lymphocytes) never yielded more than 500 d.p.m.

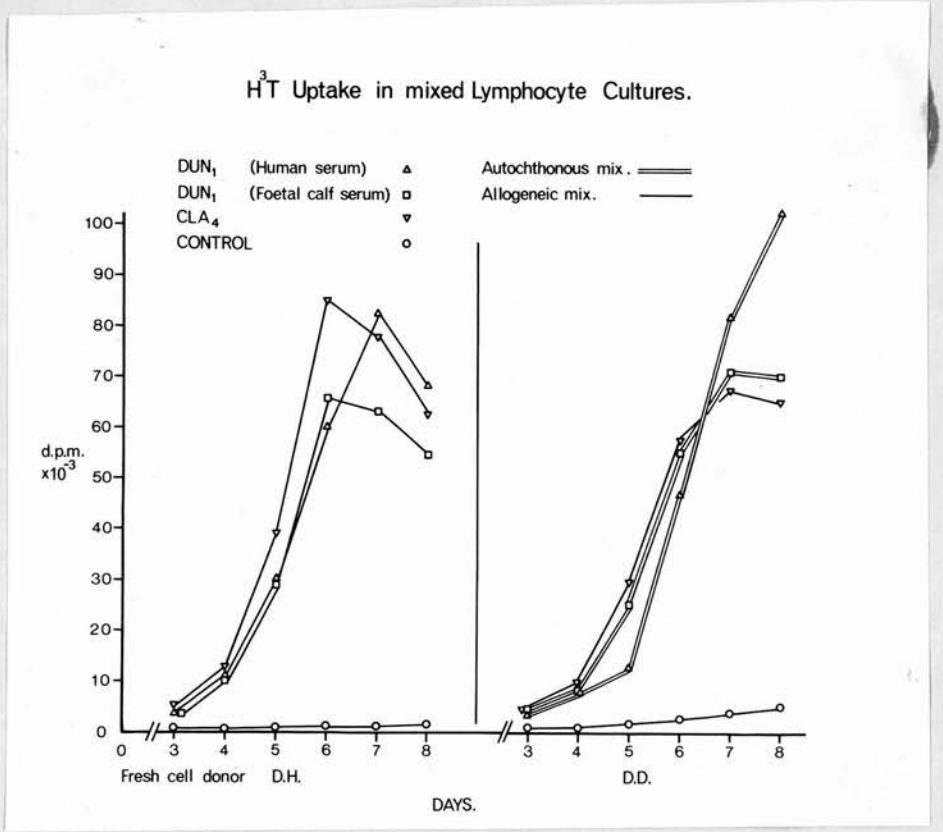
The peak level of activation in allogeneic mixtures was reached on day 4 in some cases (figs. 59 and 60) but on other occasions, as late as day 7 (figs.61 and 62). Within each experiment, however, the kinetics of activation appeared to be very similar for fresh lymphocytes from the two (or three) donors. Our impression is that the response rate is affected by the precise culture conditions, the most variable factor in practice being the concentration of CO_2 in the incubator and hence the pH of the culture medium. It is important therefore to compare results only within single experiments, since this


H^3T Uptake in mixed Lymphocyte Cultures.
LIZ_1 (Human serum)
LIZ_1 (Foetal calf serum)
GOL_1
CONTROL
Autochthonous mix.
Allogeneic mix.
d.p.m. $x10^{-3}$
Fresh cell donor
D.V.
V.B.
DAYS.

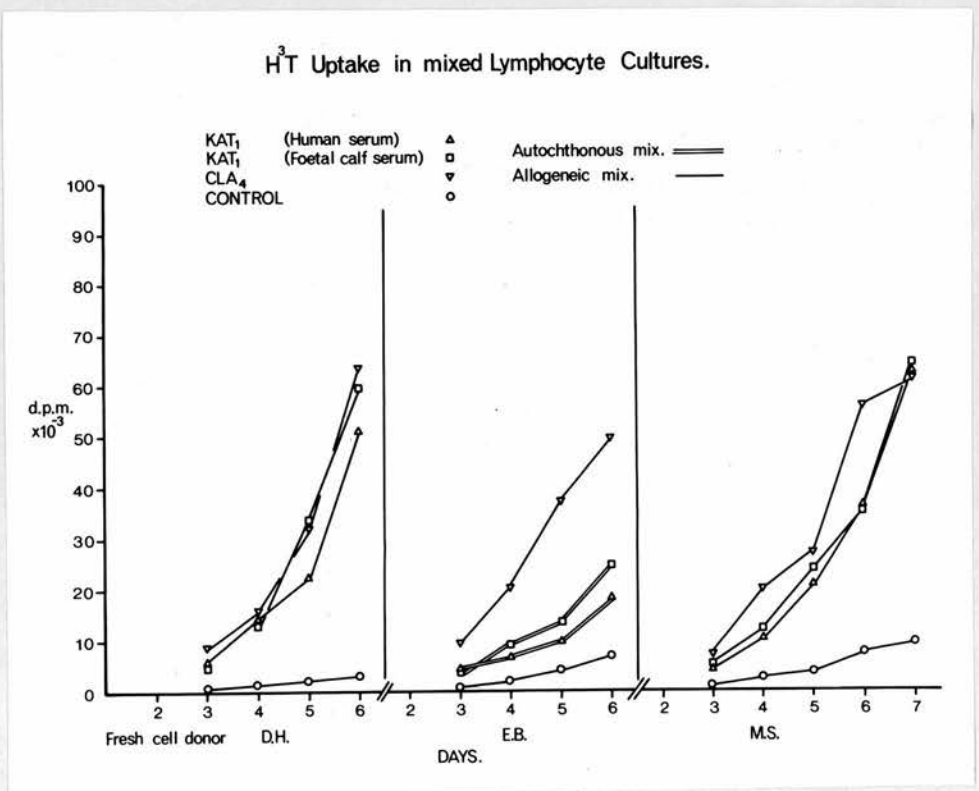
59.


H^3T Uptake in mixed Lymphocyte Cultures.
Autochthonous mix
Allogeneic mix
ORI_1(Human serum.)
ORI_1(Foetal calf serum.)
HUN_1
CONTROL
130483
105954
110605
d.p.m. $x10^{-3}$
Fresh cell donor
DH.
MO.(frozen)
MS.
DAYS.

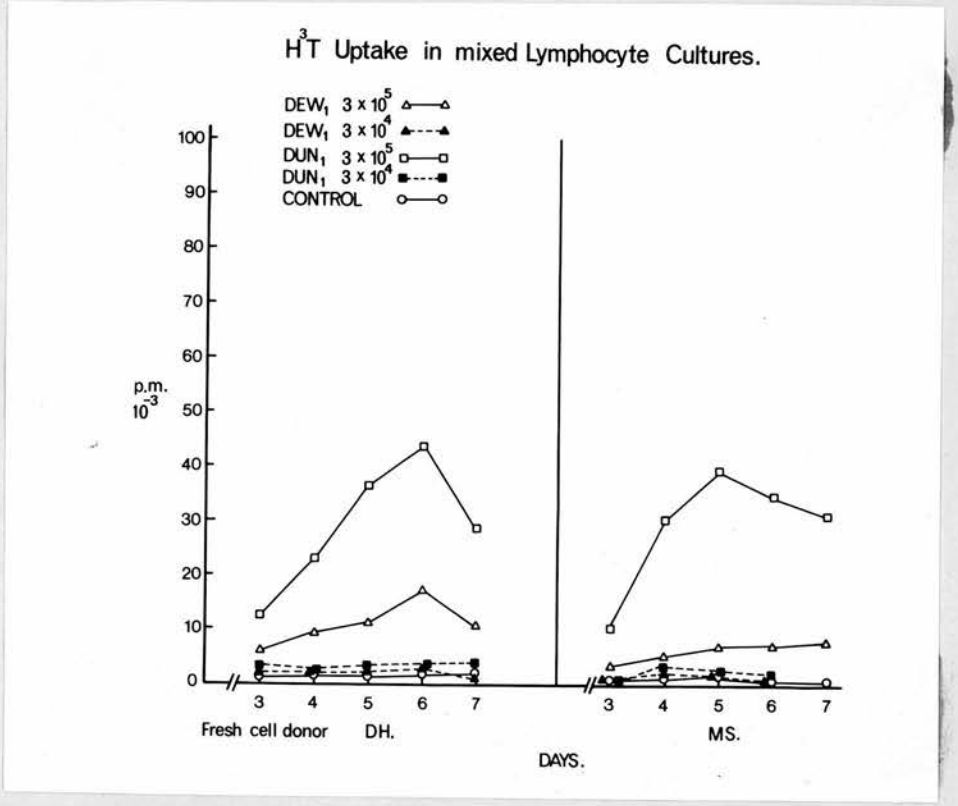
60.


H^3T Uptake in mixed Lymphocyte Cultures.
DUN_1 (Human serum)
DUN_1 (Foetal calf serum)
CLA_4
CONTROL
Autochthonous mix.
Allogeneic mix.
d.p.m. x10^{-3}
Fresh cell donor
D.H.
D.D.
DAYS.

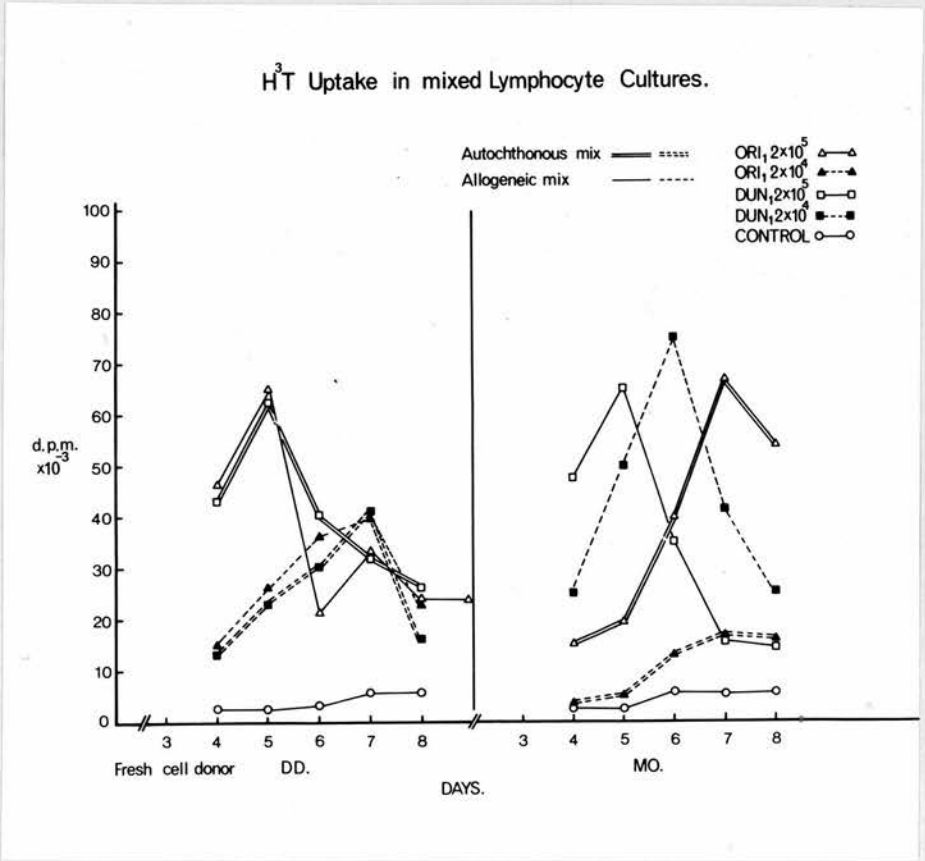
61.


H^3T Uptake in mixed Lymphocyte Cultures.
KAT_1 (Human serum)
KAT_1 (Foetal calf serum)
CLA_4
CONTROL
Autochthonous mix.
Allogeneic mix.
d.p.m. x10^{-3}
Fresh cell donor
D.H.
E.B.
M.S.
DAYS.

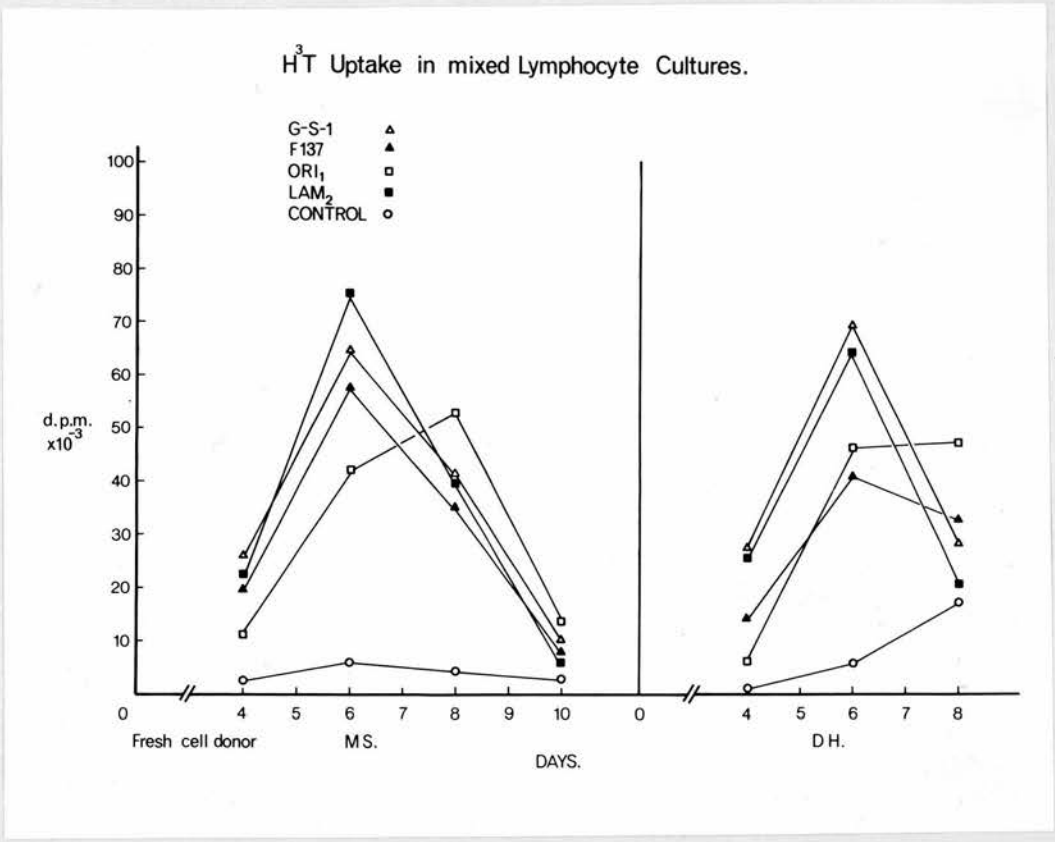
62.


H3T Uptake in mixed Lymphocyte Cultures.
DEW1 3 x 10^5
DEW1 3 x 10^4
DUN1 3 x 10^5
DUN1 3 x 10^4
CONTROL
p.m. 10^-3
100
90
80
70
60
50
40
30
20
10
0
3 4 5 6 7
3 4 5 6 7
Fresh cell donor DH.
MS.
DAYS.

63.


H3T Uptake in mixed Lymphocyte Cultures.
Autochthonous mix
Allogeneic mix
ORI1 2x10^5
ORI1 2x10^4
DUN1 2x10^5
DUN1 2x10^4
CONTROL
d.p.m. x10^-3
100
90
80
70
60
50
40
30
20
10
0
3 4 5 6 7 8
3 4 5 6 7 8
Fresh cell donor DD.
MO.
DAYS.

64.


H3T Uptake in mixed Lymphocyte Cultures.
G-S-1
F137
ORI1
LAM2
CONTROL
d.p.m. x10-3
100
90
80
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10
0
4
5
6
7
8
Fresh cell donor
M S.
D H.
DAYS.

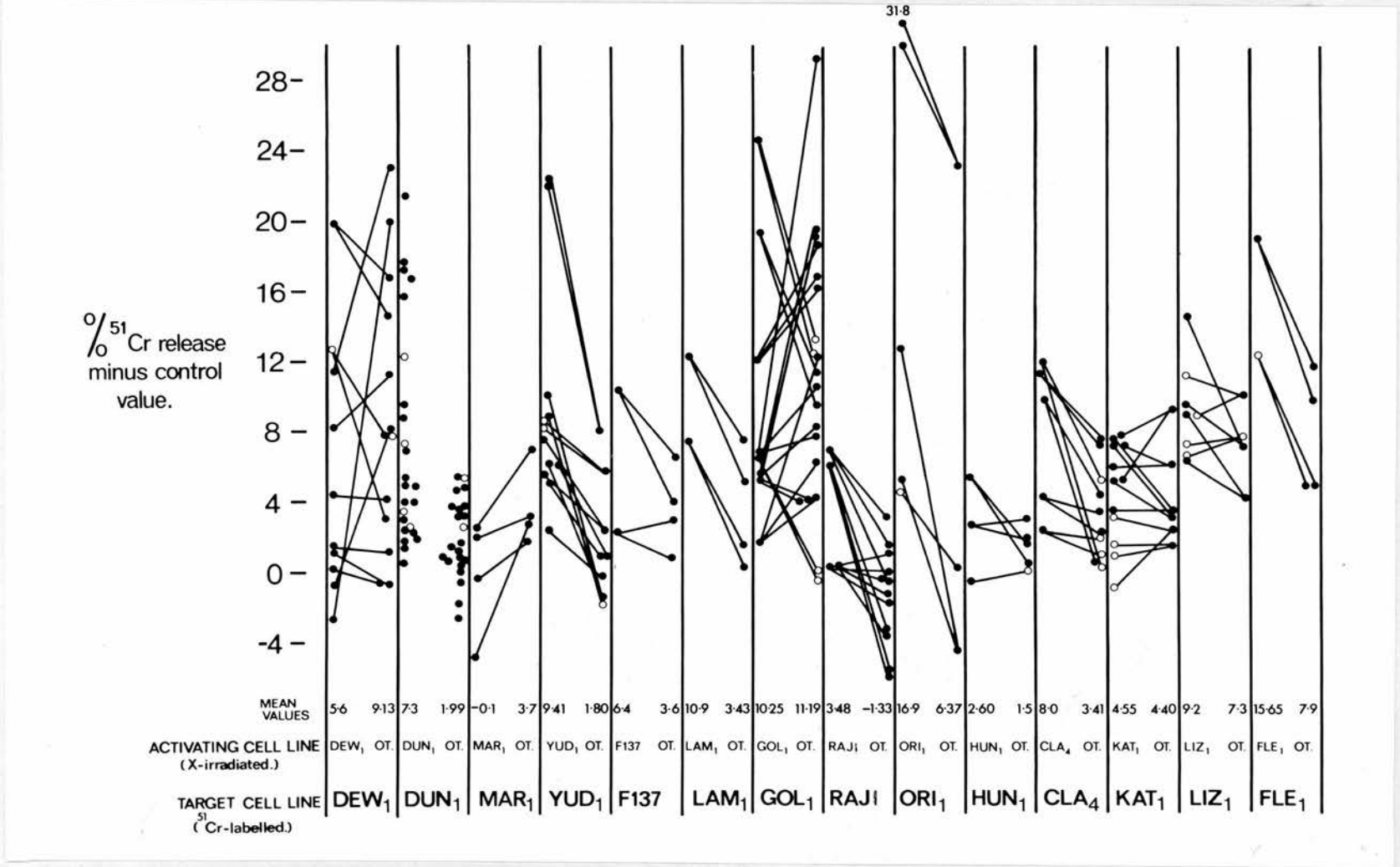


Fig. 66

66. See text for description "OT" = Other cell lines.

ensures that culture conditions were identical for all the tubes.

It is clear that in most cases activation in an autochthonous system proceeds at a slower rate than in the corresponding allogeneic mixture (figs. 59, 60, 62 and 64) although the peak level achieved may be similar in the two situations. The autochthonous mixture DD + X-DUN, proved an exception; on both occasions activation was comparable in rate and in peak level to that observed in the corresponding allogeneic cultures, DD + X-CLA_4 and DD + X-ORI_1 (figs 61 and 64). The impression gained from measurement of tritiated thymidine uptake at a single point after setting up mixed cultures would obviously depend on the exact time interval chosen. For example, the experiment in which M.O. fresh lymphocytes were stimulated with 2×10^5 irradiated LCL cells from the autochthonous line ORI_1 and the allogeneic line DUN_1 (fig.64) would appear to give totally different results depending on whether the reaction was measured on day 5,6 or 7.

Culture of LCL cells for six weeks in medium supplemented with human serum in order to eliminate foetal calf antigens may have resulted in a slight reduction in the antigenicity of these cells when tested in an autochthonous, though not in an allogeneic situation (figs. 59 and 62) but it can be concluded that foetal calf antigens are not the major factors responsible for activation in either type of mixture.

In one experiment (fig.64) reduction of the number of irradiated cells in the mixed cultures from 2×10^5 to 2×10^4 delayed the activation process, but had an inconsistent effect on the peak level. On another occasion (fig.63), 3×10^6 X-irradiated cells from two lines failed to provoke any measurable activation at all. In that case, the lymphoblastoid lines had been growing much more slowly than usual in the weeks before the experiment and contained a relatively high proportion (approx. 40%) of dead cells. The reasons for fluctuations in the growth rate of the lines are not known, though they are possibly related to the variable properties of different batches of foetal calf serum. It has already been shown by Hardy, Knight and Ling (1970) that the activation of fresh blood lymphocytes by irradiated LCL cells is, at least in part, dependent on the level of metabolic activity in the latter and this presumably accounts for the different results obtained in these two experiments with small numbers of irradiated cells.

The single experiment using blood lymphocytes after 3 weeks storage in liquid nitrogen (fig.60) demonstrated that such cells remain capable of responding to both allogeneic and authochthonous LCL cells, peak activation being achieved earlier in the former situation. However, the number of lymphocytes available limited the experiment to three days of observation which is insufficient to determine whether the response differs in any way from that of fresh lymphocytes not subjected to freezing.

The value of demonstrating that stored lymphocytes can still respond to antochthonous irradiated LCL cells lies in the fact that this will permit studies to be made on lymphocytes from cord blood which, from the epidemiological studies discussed in Chapter I, can confidently be regarded as free from previous exposure to EB virus. If such lymphocytes are activated *in vitro* by an autochthonous lymphoblastoid line (grown by co-cultivation) this will imply that the phenomenon is not part of an anamnestic response to a previously-encountered viral antigen but is comparable to the allogeneic mixed lymphocyte reaction in that cells are recognised as 'foreign' without an earlier 'priming' exposure (Ling, 1968).

When the results in figs.59 to 65 are analysed in terms of the HL-A phenotypes of the cell populations involved, (table XW) there is no evidence of any relationship between the rate or peak level of activation achieved and the number of identifiable histo-incompatibilities in a given allogeneic mixture. For example, M.S. and ORI, both carry HL-A types 1,2,8,12 while DH is typed 1,10,12,LND, yet ORI cells provoked virtually identical reactions in fresh blood lymphocytes from MS and DH (fig.60).

Following the publication of our preliminary results in the paper reproduced above, similar studies were reported by five other groups (Green and Sell, 1970; Junge, Hockstra & Dienhardt, 1970; Flier, Glade, Broder & Hirschhorn, 1970; Knight, Moore

Clarkson, 1971; Han, Moore and Sokal, 1971). Flier and his colleagues found that in some autochthonous mixtures no activation took place but all the other groups, in confirmation of our findings, observed marked stimulation in both allogeneic and autochthonous mixtures. There was wide variation in the relative levels of activation observed in the two situations but since all the studies were carried out at a single point in time after setting up mixed cultures, this variation can readily be explained as indicated above. Green and Sell (1970) and Han, Moore and Sokal (1971) also found that activation in an autochthonous situation cannot be attributed solely to antigens derived from foetal calf serum.

There is disagreement as to whether direct cell-cell contact is essential for activation of fresh lymphocytes by irradiated LCL cells. Hardy, Knight and Ling (1970) found that supernatants from LCL cultures failed to stimulate allogeneic lymphocytes and that separation of the two cell populations by a millipore membrane effectively blocked the reaction. Green and Sell (1970), Flier, Glade, Broder and Hirschhorn (1970) and Han, Moore and Sokal (1971) also reported negative results from attempts to activate fresh lymphocytes with cell-free filtrates from growing cultures. However, Junge (1970) did detect blastogenic activity in cell-free supernatants of three lymphoblastoid lines, using a micro-culture method which gives rather low scintillation counts. The

discrepancy may be a reflection of different techniques employed by different workers, but at present it remains a very important area of disagreement.

Cytotoxic reactions of fresh lymphocytes after activation by autochthonous or allogeneic LCL cells

Hardy, Knight and Ling (1970) and Hardy, Ling and Aviet (1970), showed that lymphocytes activated by allogeneic irradiated LCL cells acquired cytotoxic potential against these cells. There did not appear to be any specificity in the reaction in the sense that activated lymphocytes would destroy cells both of the line used in the activation phase and of other lines. Although lymphocytes activated by phytohaemagglutinin (PHA) are cytotoxic to Chang liver cells in culture (Holm and Perlmann, 1969), there was no evidence of a similar non-specific activity against LCL cells. In this system, cytotoxic activity appeared to be induced only by activation with irradiated LCL cells (Hardy, Ling and Aviet, 1970). It seemed that both the activation and the cytotoxic phase of the reaction might correspond to an immunological defence mechanism operative in vivo against deviant cells. Dr. Hardy and I have, therefore, studied the cytotoxic capacity of fresh lymphocytes stimulated by autochthonous LCL cells.

Materials and Methods

Peripheral blood lymphocytes were obtained and activated in vitro by X-irradiated (3000 rads) autochthonous or allogeneic LCL cells as previously described.

For use as target cells, lymphoblastoid cultures growing under the conditions described in Chapter II at a concentration of 10^6 viable cells/ml were incubated for 24 hours with $Na_2\ ^{51}CrO_4$ (1 μCi/ml)(Radiochemical Centre, Amersham). The cells were then washed twice with fresh medium (Eagles MEM, Glasgow modification, with 15% pooled human serum-gelatin and antibiotics) and resuspended in the same medium, usually at a concentration of 2×10^6/ml (for variations see individual experiments). To study cytotoxicity, 0.5 ml of supernatant was carefully removed (without disturbing the cells) from a culture of lymphocytes + X-irradiated LCL cells (without tritiated thymidine) which had been maintained for 6 days. The fluid was replaced by 0.5 ml of ^{51}Cr-labelled LCL cell suspension. After 6 hours (occasionally longer, see individual experiments) the tubes were centrifuged (500-800 xg for 5 minutes) and the supernatants carefully transferred to counting vials. The deposits were dissolved in 0.1 ml of N_1 NaOH at 60°C for 1 hour then washed into counting vials with 1 ml of physiological saline. γ activity in supernatants and deposits was estimated in a Nuclear Enterprises gamma counter, each vial being counted for 300 seconds. The percentage ^{51}Cr released for each sample was calculated as

$$100 \times \frac{\text{counts in supernatant-background count}}{\text{counts in supernatant + counts in deposit-(2 x background)}}$$

All cultures were set up in triplicate and the results are expressed as means of the values obtained.

Control cultures included, a) unstimulated lymphocytes plus ^{51}Cr-labelled LCL cells, b) donor red cells incubated for 6 days with X-irradiated LCL cells, plus ^{51}Cr-labelled LCL cells, and c) ^{51}Cr-labelled LCL cells subjected to three cycles of freezing and thawing to determine the maximal release of radioactivity. In a few experiments an additional control was included, namely fresh lymphocytes activated for 6 days with X-irradiated small lymphocytes (autochthonous or allogeneic), plus ^{51}Cr-labelled LCL cells.

In all cases, parallel triplicate cultures were labelled with tritiated thymidine and counted, as described earlier, to determine the level of activation achieved in the fresh lymphocytes on the same day that cytotoxicity was being measured or (in 2 cases) on the preceding day.

Our preliminary results are recorded in the attached paper, reprinted from Experientia 27: 1336-1338, 1971.

Futher experiments were then carried out to investigate a) the relationship between the level of activation (H^3T uptake) achieved and the degree of cytotoxic potency subsequently acquired by the activated lymphocytes and b) the specificity of the cytotoxic response in terms of efficiency of killing of target cells from different lines.

The first four experiments used fresh blood lymphocytes from healthy control subjects and did not include any autochthonous mixtures in the stimulation phase. The results are presented in

Separatum EXPERIENTIA *27*, 1336 (1971)
Birkhäuser Verlag, Basel (Schweiz)

Cytotoxic Potential of Lymphocytes Stimulated with Autochthonous Lymphoid Cell Line Cells

Human lymphocytes are activated by contact with histocompatibility antigens on the lymphocytes of another donor. The reaction, which occurs with lymphocytes of all normal individuals and with lymphocytes from cord bloods, requires no prior immunisation of the cell donors with the antigens concerned (for refs. see [1]). An immunological basis for the reaction is indicated, however, by the demonstration, in rats, that lymphocytes from tolerant animals are specifically unresponsive to cells of the tolerance inducing strain [2,3]. Human lymphocytes are also activated by cells from human lymphoblastoid cell lines (LCLs) established from the blood cells of normal individuals and patients with various disorders. The reaction is essentially similar to a 'one-way' mixed lymphocyte reaction but of greater intensity [4]. Lymphocytes activated by culture with X-irradiated cells from a particular cell line are cytotoxic to these cells and the cells of other lines [5].

New surface antigens on the LCL cells as well as histocompatibility antigens must be capable of activating lymphocytes since cells from autochthonous LCLs are stimulatory [6–9], although if the conditions are carefully controlled, to a lesser degree than cells from a histoin-

with ^{51}Cr by incubation for 24 h with $Na_2{}^{51}CrO_4$ (1 μCi/10^6 cells/ml).

Mitotic activation of fresh blood lymphocytes (10^6/ml in 20% pooled human serum gelatin/Eagles MEM) incubated with X-irradiated LCL cells (added to give a final concentration of 10^5 cells/ml soon after X-irradiation) was assessed by the incorporation of thymidine-[methyl-^3H] 0.5 μCi; 150 mCi/mmole) into the trichloroacetic acid-insoluble fraction over the interval day 6–7. Details of the methods are to be found in refs. 4 and 12. Suitable controls lacking X-irradiated LCL cells or fresh lymphocytes were treated identically.

The cytotoxic capacity of the activated blood lymphocytes was assessed by the chromium release technique. Control cultures contained donor red cells and either blood lymphocytes of the donor or the X-irradiated LCL cells used as stimulant but not both[5]. In each experiment the response of blood lymphocytes from an unrelated donor as well as from the original LCL donor were compared. As a further control (to cover any possible change induced by X-irradiation) X-irradiated blood lymphocytes were cultured with autochthonous or allogeneic blood lymphocytes.

Stimulation (enhanced ^3H-thymidine incorporation) induced by autochthonous LCL cells occurred with the lymphocytes from all six patients studied. The activated lymphocytes were cytotoxic to ^{51}Cr-labelled autochthonous and allogeneic cells. The results of two typical experiments are shown in Tables I and II. The stimulation and cytotoxicity of the lymphocytes in the autochthonous situation was not a result of changes induced by the X-irradiation since X-irradiated blood lymphocytes did not stimulate autochthonous lymphocytes nor was it due to foetal calf serum antigens in the culture fluid since human serum was used as the serum supplement in all experiments. Moreover, GREEN and SELL[9] have reported that foetal calf serum antigens were not important in their autochthonous stimulations.

It may be concluded that neo-antigens on the LCL cells initiate a lymphocyte stimulation, in vitro, resulting in the death of the LCL cells. The fact that allogenic as well as autochthonous LCL cells can be killed by lymphocytes stimulated with autochthonous LCL cells suggests that either the killing phase is of limited specificity or that there are cross reacting antigens shared by the various LCL cells. These points are currently under investigation.

If it is accepted that 'deviant' lymphocytes, similar to LCL cells, may arise in vivo as a result of viral transformation or other cause, the in vitro phenomena described must closely mimic an immunological surveillance mechanism operative in the host.

Résumé. Les petits lymphocytes du sang, après avoir été mis en contact pendant plusieurs jours avec les cellules lymphoïdes d'une lignée autochthone, peuvent tuer les cellules de cette lignée et d'autres lignées. Ces investigations peuvent apporter de nouvelles données sur l'opération de contrôle immunologique.

D. A. HARDY and C. M. STEEL[13]

Department of Experimental Pathology, University of Birmingham, Birmingham B15 2TJ (England); and University Department of Medicine, and M.R.C. Clinical and Cytogenetics Unit, and Western General Hospital, Edinburgh (Scotland),
3 June 1971.

[12] N. R. LING and P. J. L. HOLT, J. Cell. Sci. *2*, 57 (1967).

[13] We are grateful to Dr. N. R. LING for his help and guidance. D. A. HARDY gratefully acknowledges financial support from the Medical Research Council and the John Squire Fund.

tables XVIII to XXI. Six later experiments in the same series included autochthonous activation mixtures in the activation phase and examined the acquisition of cytotoxic potential against both allogeneic and autochthonous ^{51}Cr-labelled LCL cells. These results are presented in tables XXII to XXVIII.

Analysis of results of cytotoxicity experiments

Before discussing the above results in detail, it is necessary to comment upon the limitations of the ^{51}Cr-release technique as a means of measuring cytotoxic interactions between cell populations *in vitro*.

It must be appreciated that the percentage of ^{51}Cr released into the supernatant is not directly proportional to the number of cultured cells which have been killed, since repeated freezing and thawing, which leaves no viable cells as judged by Nigrosin exclusion, results in a maximum release of less than 90% of the total ^{51}Cr, the remainder presumably being bound to the sedimented cell debris. In two cases, the ^{51}Cr release on freeze-thawing was less than 50% (Tables XX and XXII). The reasons for this are not clear, but they may be related to variations in the rate of cooling of cultures which in turn may affect the degree of disruption of individual cells.

A second reason for a discrepancy between the extent of cell killing and the proportion of ^{51}Cr released into the supernatant is that the activated lymphocytes which are responsible for

TABLE XVIII

Stimulation of fresh blood lymphocytes of donors DH and MS by X-irradiated cells from lines DEW_1 and DUN_1 with subsequent testing of cytotoxic potential of activated lymphocytes against ^{51}Cr-labelled DEW_1 and DUN_1 cells.

Activation mixture (^3H-Thymidine uptakes recorded in figure 63)		% ^{51}Cr release from : -			
		^{51}Cr DEW_1 (5×10^5/culture)		^{51}Cr DUN_1 (5×10^5/culture)	
Fresh Cells	X-irradiated LCL cells	Mean	Standard Error	Mean	Standard Error
DH lymphocytes (10^6)	None	20.470	0.492	29.726	1.609
"	+ X-DEW_1 (3×10^5)	28.658	1.032	31.084	0.909
"	+ X-DEW_1 (3×10^4)	21.943	1.276	29.783	0.293
"	+ X-DUN_1 (3×10^5)	31.739	2.924	36.697	1.736
"	+ X-DUN_1 (3×10^4)	43.467	0.857	45.514	2.574
MS lymphocytes (10^6)	None	27.470	1.106	28.754	0.649
"	+ X-DEW_1 (3×10^5)	26.704	2.813	26.254	0.336
"	+ X-DEW_1 (3×10^4)	25.090	1.781	26.878	0.957
"	+ X-DUN_1 (3×10^5)	35.540	1.708	31.212	0.767
"	+ X-DUN_1 (3×10^4)	47.543	3.186	50.206	2.140

DH red cells only/..

TABLE XVIII - cont'd

DH red cells only	none	20.327	1.009	25.385	0.336
"	+ X-DEW$_1$ (3×10^5)	17.982	0.403	25.731	0.288
"	+ X-DEW$_1$ (3×10^4)	19.519	0.889	25.463	0.588
"	+ X-DUN$_1$ (3×10^5)	19.462	1.932	23.761	0.150
"	+ X-DUN$_1$ (3×10^6)	19.797	0.140	25.055	0.419
^{51}Cr-labelled cells subjected to 3 cycles of freezing and thawing		89.567	0.171	84.228	0.108

Cytotoxicity tested on day 6 after setting-up activation mixtures.
Incubation of ^{51}Cr-labelled cells with activated lymphocytes (or controls) 6 hours.

TABLE XIX

Stimulation of fresh blood lymphocytes from donors DH and MS by X-irradiated cells from lines MAR_1 and YUD_1 with subsequent testing of cytotoxic potential of activated lymphocytes against ^{51}CR-labelled MAR_1 and YUD_1 cells.

Activation mixture		Activation level on day 5 (CPM)	% ^{51}Cr release from:			
			^{51}Cr MAR_1 (10^5 cells/culture)		^{51}Cr YUD_1 ($4x10^5$ cells/culture)	
Fresh Cells	X-irradiated LCL cells*		Mean	Standard Error	Mean	Standard Error
DH lymphocytes (10^6)	none	1903	38.240	1.562	12.472	1.339
"	+ $X\text{-}MAR_1$ a.	17406	37.996	1.510	10.949	0.016
"	+ $X\text{-}MAR_1$ b.	50099	40.394	1.817	12.277	0.577
"	+ $X\text{-}YUD_1$ a.	64912	40.136	2.671	18.614	1.404
"	+ $X\text{-}YUD_1$ b.	72932	41.495	2.336	14.904	0.635
MS lymphocytes (10^6)	none	1466	39.410	2.120	11.581	0.378
"	+ $X\text{-}MAR_1$ a.	5397	34.749	0.796	10.923	0.361
"	+ $X\text{-}MAR_1$ b.	27590	42.099	2.570	12.549	1.237
"	+ $X\text{-}YUD_1$ a.	48433	42.101	0.678	16.760	1.037
"	+ $X\text{-}YUD_1$ b.	53293	46.497	1.414	17.648	0.960

TABLE XLX - cont'd

DH red cells only	none	500	39.948	0.228	13.947	0.488
"	+ X-MAR$_1$ a.	"	39.349	1.841	14.447	0.166
"	+ X-MAR$_1$ b.	"	42.436	4.838	14.213	0.216
"	+ X-YUD$_1$ a.	")	not done		13.765	duplicate only
"	+ X-YUD$_1$ b.	")			14.250	0.841
^{51}Cr-labelled cells subjected to 3 cycles of freezing and thawing			83.299	4.920	86.160	Not done

* 'a' = LCL cells irradiated 48 hours before setting-up activation mixtures.

'b' = LCL cells irradiated 3 hours before setting-up activation mixtures

5 x 10^4 irradiated LCL cells used in all activation mixtures.

Cytotoxicity tested on day 6 after setting-up activation mixtures.
Incubation of ^{51}Cr-labelled cells with activated lymphocytes (or controls) 6 hours.

/ ^{51}Cr-MAR$_1$ cells were badly clumped, only small numbers could be dispersed into an adequate suspension for counting.

TABLE XX:

Stimulation of fresh blood lymphocytes from donors DH and MS by X-irradiated cells from lines DEW_1 and DUN_1, with subsequent testing of cytotoxic potential of activated lymphocytes against ^{51}Cr-labelled DEW_1 and DUN_1 cells.

Activation mixture		Activation level on day 7 (dpm)	% ^{51}Cr release from:-			
			^{51}Cr-DUN_1 (10^6/culture)		^{51}Cr-DEW_1 (10^6/culture)	
Fresh cells	X-irradiated LCL cells*		Mean	Std.Error	Mean	Std.Error
DH lymphocytes (10^6)	none	699	19.706	0.761	27.218	1.390
"	+ X-DEW_1 a	2661	23.486	3.900	28.331	0.838
"	+ X-DEW_1 b	20142	25.299	1.075	31.522	0.915
"	+ X-DUN_1 a	1742	21.524	0.768	26.643	1.197
"	+ X-DUN_1 b	9350	22.767	0.981	31.277	1.511
MS lymphocytes (10^6)	none	430	19.009	1.152	28.027	0.502
"	+ X-DEW_1 a	2430	19.985	0.351	28.344	1.729
"	+ X-DEW_1 b	10454	22.291	0.341	29.508	0.876
"	+ X-DUN_1 a	1661	19.540	0.361	27.593	0.350
"	+ X-DUN_1 b	10585	23.088	1.477	29.227	1.992

MS red cells only/

TABLE XX - cont'd

MS red cells only	none	500	Not done		24.777	1.052
"	+ X-DEW$_1$ a	"	18.950	0.987	25.440	0.494
"	+ X-DEW$_1$ b	"	17.369	1.010	24.521	0.497
"	+ X-DUN$_1$ a	"	17.313	0.273	25.239	0.740
"	+ X-DUN$_1$ b	"	17.076	0.683	25.317	0.814
^{51}Cr-labelled cells subjected to 3 cycles of freezing and thawing			38.093	1.571	83.277	3.182

* 'a' = LCL cells X-irradiated 48 hours before setting up activation mixtures.
'b' = LCL cells X-irradiated 3 hours before setting up activation mixtures.

5×10^4 X-irradiated cells in each activation mixture.

Cytotoxicity measured on day 8 after setting-up activation mixtures.
Incubation of ^{51}Cr-labelled cells with activated lymphocytes (or controls) 6 hours.

TABLE XXI:

Stimulation of fresh lymphocytes from donors DH and MS by X-irradiated cells from lines $G\text{-}S_1$, F137, ORI and LAM_1 with subsequent testing of cytotoxic potential of activated lymphocytes against ^{51}Cr-labelled $G\text{-}S_1$, F137 and LAM_1 cells.

Activation mixture (^{3}H-thymidine uptake recorded in fig. 65)		% ^{51}Cr release from:-					
		$^{51}Cr\text{-}G\text{-}S_1$ (10^6/culture)		^{51}Cr-F137 (10^6/culture)		$^{51}Cr\text{-}LAM_1$ (10^6/culture)	
Fresh Cells	X-irradiated LCL cells	Mean	Std.Error	Mean	Std.Error	Mean	Std.Error
DH lymphocyte (10^6)	+ none	15.903	0.469	14.751	0.115	18.787	0.583
"	+ $X\text{-}G\text{-}S_1$ (10^5)	37.767	2.600	22.015	0.104	24.420	0.426
"	+ X-F137 (10^5)	19.242	0.270	25.176	0.345	23.960	1.152
"	+ $X\text{-}ORI_1$ (10^5)	27.472	0.804	21.261	0.712	26.383	0.322
"	+ $X\text{-}LAM_1$ (10^5)	21.228	0.186	18.726	0.586	31.069	0.384.
MS lymphocytes (10^6)	+ none	17.017	0.141	15.703	0.094	Lost	
"	+ $X\text{-}G\text{-}S_1$ (10^5)	28.826	2.050	20.806	1.468	25.888	0.293
"	+ X-F137 (10^5)	16.742	0.501	18.006	0.348	19.725	0.861
"	+ $X\text{-}ORI_1$ (10^5)	19.175	0.758	18.764	1.018	18.318	1.383
"	+ $X\text{-}LAM_1$ (10^5)	18.018	0.484	16.631	0.327	25.428	0.951

DH red cells only/

TABLE XXI - cont'd

DH red cells only	none	17.312	1.083	15.473	0.781	17.992	0.837
"	+ X-G-S_1 (10^5)	24.673*	0.771	19.518*	1.422	30.579*	1.767
"	+ X-F137 (10^5)	18.403	0.231	16.837	0.837	22.059	1.260
"	+ X-ORI_1 (10^5)	18.403	0.324	16.707	0.748	23.821	0.290
"	+ X-LAM_1 (10^5)	19.468	0.718	15.585	0.156	26.443	1.100
^{51}Cr-labelled cells subjected to 3 cycles of freezing and thawing		82.995	0.646	81.441	0.382	60.054	2.319

Cytotoxicity tested on day 7 after setting up activation mixtures.

Incubation of ^{51}Cr-labelled cells with activated lymphocytes (or controls) 6 hours.

*No explanation can be found for these high values in controls containing red cells + X-G-S_1 but because of them, results involving fresh lymphocytes activated by X-G-S_1 are excluded from subsequent analyses.

TABLE XXII

Stimulation of fresh blood lymphocytes from donors DD and DV by X-irradiated cells from lines GOL_1, DUN_1 and RAJI, with subsequent testing of cytotoxic potential of activated lymphocytes against ^{51}Cr labelled GOL_1, DUN_1 and RAJI cells.

Activation mixture		Activation level on day 6 (dpm)	% ^{51}Cr release from:					
			^{51}Cr-GOL_1		^{51}Cr-DUN_1		^{51}Cr-RAJI	
Fresh Cells	X-irradiated LCL cells		(5×10^5/culture)		(5×10^5/culture)		(5×10^5/culture)	
			Mean	Std.Error	Mean	Std.Error	Mean	Std.Error
DD lymphocytes (10^6)	none	1468	16.634	0.794	14.423	0.390	31.297	1.311
"	+ $\underline{DUN_1}$ F a (10^5)	25291	28 962	1.230	24.033	0.755	32.368	0.944
"	+ $\underline{DUN_1}$ H a (10^5)	12660	22.804	0.569	18.388	1.116	29.540	0.903
"	+ GOL_1 a (10^5)	3252	18.498	0.446	14.816	0.525	31.218	0.766
"	+ RAJI a (10^5)	8339	20.975	0.716	16.086	0.747	31.664	2.886
"	+ $\underline{DUN_1}$ f b (10^5)	69881	45.931	3.066	31.766	1.328	34.540	Duplicates
"	+ $\underline{DUN_1}$ H b (10^5)	lost	24.530	3.127	19.366	1.640	32.726	0.487
"	+ GOL_1 b (10^5)	6901	23.527	1.073	15.192	0.599	30.838	0.521
"	+ RAJI b (10^5)	21474	27.381	0.409	18.289	1.144	38.239	1.891
DV lymphocytes (10^6)/								

TABLE XXII - cont'd

DV lymphocytes (10^6)	none	1860	12.205	0.439	14.578	0.353	31.975	0.361
"	+ DUN_1 F a (10^5)	36373	31.811	1.177	31.251	3.115	30.817	0.349
"	+ DUN_1 H a (10^5)	31007	31.464	4.605	28.127	0.693	31.502	0.940
"	+ GOL_1 a (10^5)	10365	17.957	0.175	15.420	0.624	28.390	Duplicates
"	+ RAJI a (10^5)	17435	20.535	0.815	16.058	0.409	32.308	0.389
"	+ DUN_1 F b (10^5)	11027	28.421	1.303	19.809	0.583	26.330	0.360
"	+ DUN_1 H b (10^5)	11947	31.184	1.363	23.423	0.905	26.105	1.346
"	+ GOL_1 b (10^5)	12782	24.405	0.427	18.454	1.346	28.513	0.515
"	+ RAJI b (10^5)	28787	29.064	1.759	19.283	0.825	38.083	1.802
DD red cells only	none	500	15.531	0.931	14.868	0.296	33.574	0.445
"	+ DUN_1 F a (10^5)	"	14.856	1.234	15.311	0.203	33.416	0.742
"	+ DUN_1 H a (10^5)	"	15.371	0.795	14.064	0.077	33.671	1.028
"	+ GOL_1 a (10^5)	"	16.449	0.405	14.823	0.231	34.360	0.843
"	+ RAJI a (10^5)	"	15.148	0.645	14.454	0.183	32.424	2.114
"	+ DUN_1 F b (10^5)	"	14.955	0.150	14.354	0.833	33.003	0.712
"	+ DUN_1 H b (10^5)	"	16.622	0.399	15.867	0.829	34.241	0.832.
"	+ GOL_1 b (10^5)	"	16.056	0.127	14.906	0.575	34.423	1.042
"	+ RAJI b (10^5)	"	16.719	1.366	14.604	0.418	32.749	0.502

TABLE XXII - cont'd

	Mean	Std.Error	Mean	Std.Error	Mean	Std.Error
^{51}Cr-labelled cells subjected to 3 cycles of freezing and thawing	63.180	2.135	74.720	0.962	48.691	1.986

* ('a' = cells irradiated 48 hours before setting up activation mixtures) autochthonous activation mixtures
'b' = cells irradiated 3 hours before setting up activation mixtures) underlined

'F' = LCL cells grown in foetal calf serum 'H' = LCL cells grown in 10% ABRh+ve human serum for 6 weeks before experiment.

Cytotoxic tested on day 6 after setting up activation mixtures. 6 hour incubation.

TABLE XXIII

Stimulation of fresh blood lymphocytes of donors MO and DD by X-irradiated cells from lines ORI_1 and DUN_1 with subsequent testing of cytotoxic potential of activated lymphocytes against ^{51}Cr-labelled DUN_1 cells.

Activation mixture ⁄ (Activation levels recorded in Fig. 64)		% ^{51}Cr-released from ^{51}Cr-DUN_1 cells (5×10^5)	
Fresh Cells	X-irradiated LCL cells	Mean	Std.Error
MO fresh lymphocytes (10^6)	none	8.848	0.276
"	+ X-ORI_1 (2×10^3)	14.359	1.346
"	+ X-ORI_1 (2×10^4)	11.457	1.540
"	+ X-DUN_1 (2×10^5)	10.714	0.174
"	+ X-DUN_1 (2×10^4)	26.367	1.191
"			
DD fresh lymphocytes (10^6)	none	9.572	0.198
"	+ X-ORI_1 (2×10^5)	14.400	Duplicates
"	+ X-ORI_1 (2×10^4)	12.837	0.754
"	+ X-DUN_1 (2×10^5)	21.892	1.230
"	+ X-DUN_1 (2×10^4)	14.660	0.338
MO red cells only	none	8.820	0.268
"	+ X-ORI_1 (2×10^5)	8.474	0.085
"	+ X-ORI_1 (2×10^4)	8.807	0.179
"	+ X-DUN_1 (2×10^5)	8.425	0.190
"	+ X-DUN_1 (2×10^4)	8.565	0.242
^{51}Cr-labelled cells subjected to 3 cycles of freezing and thawing		83.105	0.484

Cytotoxicity tested on day 7 after setting-up activation mixtures.
Incubation of ^{51}Cr-labelled cells with activated lymphocytes (or controls) 6 hours.

⁄ Autochthonous mixtures underlined.

TABLE XXIV

Stimulation of fresh lymphocytes from donors DH, MS and MO with X-irradiated cells from lines OR_1 and HUN_1 with subsequent testing of cytotoxic potential of activated lymphocytes against ^{51}Cr-labelled ORI_1 and HUN_1 cells.

Activation mixture (Levels of activation recorded in fig. 60)		% ^{51}Cr release from:-			
Fresh Cells	X-irradiated LCL Cells*	^{51}Cr-ORI (4×10^5/culture)		^{51}Cr HUN_1 (8×10^5/culture)	
		Mean	Standard Error	Mean	Standard Error
DH lymphocytes (10^6)	none	27.759	0.923	10.530	0.263
"	+ X-ORI_1 H (10^5)	59.544	1.500	12.582	0.339
"	+ X-ORI_1 F (10^5)	57.762	3.067	13.631	0.251.
"	+ X-HUN_1 (10^5)	50.915	1.230	13.345	0.697
MS lymphocytes (10^6)	none	36.931	1.517	10.967	0.589
"	+ X-ORI_1 H (10^5)	42.230	3.838	11.494	0.306
"	+ X-ORI_1 F (10^5)	49.726	2.884	12.846	0.399
"	+ X-HUN_1 (10^5)	32.557	1.009	16.471	1.364
**M.O. lymphocytes (10^6)	none	20.592	0.822	9. 222	0.067
"	+ X-ORI_1 H (10^5)	25.354	2.570	9.311	0.077
"	+ X-ORI_1 F (10^5)	Not done		Not done	
"	+ X-HUN_1 (10^5)	20.832	1.048	8.850	0.244

DH red cells only/

TABLE XXIV - cont'd

DH red cells only	none	21.586	0.094	10.228	0.062
"	+ X-ORI$_1$ H (10^5)	20.427	0.537	10.049	0.175
"	+ X-ORI$_1$ F (10^5)	22.526	0.751	10.402	0.144
"	+ X-HUN$_1$ (10^5)	22.905	0.288	10.040	0.071
^{51}Cr-labelled cells subjected to 3 cycles of freezing and thawing		88.065	0.334	88.923	1.726

/ Autochthonous mixtures underlined
* 'H' = LCL cells grown in 10% ABRh+ve human serum, replacing calf serum, for 6 weeks before this experiment.
'F' = LCL cells grown with 20% foetal claf serum as medium supplement.
** MO Lymphocytes had been stored for 3 weeks in liquid nitrogen as described in text.
Cytotoxicity measured on day 5 after setting up activation mixtures.
Incubation of ^{51}Cr-labelled cells with activated lymphocytes (or controls) 6 hours.

TABLE XXV

Stimulation of fresh blood lymphocytes from donors DD and DH with X-irradiated cells from lines DUN_1 and CLA_4 with subsequent testing of cytotoxic potential of activated lymphocytes against ^{51}Cr-labelled DUN_1 and CLA_4 cells

Activation mixture (Level of activation recorded in Fig.61)		% ^{51}Cr released from			
Fresh cells	X-irradiated LCL cells*	^{51}Cr-DUN_1 (10^6/culture)		^{51}Cr-CLA_4 (10^6/culture)	
		Mean	Standard Error	Mean	Standard Error
DH lymphocutes (10^6)	none	15.618	0.495	14.396	0.198
"	+X-DUN_1 H (10^5)	17.377	0.579	16.615	0.243
"	+X-DUN_1 F (10^5)	17.813	0.483	18.090	0.566
"	+X-CLA_4	17.320	0.106	18.657	1.243
DD lymphocytes (10^6)	none	15.008	0.411	12.086	0.453
"	+X-DUN_1 H (10^5)	17.387	0.282	14.143	0.286
"	+X-DUN_1 F (10^5)	18.531	0.535	13.139	0.309
"	+X-CLA_4 (10^5)	14.520	0.151	14.509	0.528

DD red cells only/

TABLE XXV - cont'd.

DD red cells only	none	15.591	0.377	13.143	0.084
"	+ X-DUN$_1$ H (10^5)	15.653	0.069	13.670	0.528
"	+ X-DUN$_1$ F (10^5)	15.532	0.549	13.596	0.187
"	+ X-CLA$_4$ (10^5)	15.948	0.165	13.290	0.239
Nil	Nil	15.869	0.568	12.857	0.431
^{51}Cr-labelled cells subjected to 3 cycles of freezing and thawing		71.554	1.649	77.688	0.959

Cytotoxicity tested on day 6 after setting-up activation mixtures.
Incubation of ^{51}Cr-labelled cells with activated lymphocytes (or controls) 8 hours.

* Autochthonous mixtures underlined.
'H' = LCL cells grown in medium supplemented with 10% ABRh+ve human serum for 6 weeks before experiment.
'F' = LCL cells grown continuously in medium supplemented with 20% foetal calf serum

TABLE XXVI

Stimulation of fresh lymphocytes from donors EB, MS and DH with X-irradiated cells from lines KAT_1 and CLA_4 with subsequent testing of cytotoxic potential of activated lymphocytes against ^{51}Cr-labelled KAT_1^1 and CLA_4^4 cells.

Activation mixture (Levels of activation recorded in fig.62)		% ^{51}Cr-release from:					
Fresh cells	X-irradiated LCL cells	^{51}Cr-KAT_1H (10^6/culture)		^{51}Cr-KAT_1 F (5×10^6/culture)		^{51}Cr- CLA_4 (10^6/culture)	
		Mean	Std.Error	Mean	Std.Error	Mean	Std.Error
EB fresh lymphocytes (10^6)	none	43.816	0.487	35.943	0.743	36.927	0.479
"	+ X-KAT_1 H (10^5)	44.901	0.622	35.040	1.359	37.240	0.512
"	+ X-KAT_1 F (10^5)	45.395	0.259	39.160	0.968	43.220	0.883
"	+ X-CLA_4 (10^5)	45.624	0.536	38.502	0.502	48.987	0.364
MS fresh lymphocytes (10^6)	none	45.441	0.700	39.114	0.908	40.825	1.148
"	+ X-KAT_1 H (10^5)	50.531	0.243	46.633	0.837	41.550	1.087
"	+ X-KAT_1 F (10^5)	52.554	2.108	42.697	0.522	45.274	2.174
"	+ X-CLA_4 (10^5)	47.588	0.537	42.557	0.645	50.098	1.831
DH fresh lymphocytes (10^6)/							

TABLE XXVI - cont'd

DH fresh lymphocytes (10^6)	none	45.260	0.407	37.206	0.846	37.401	1.122
"	+ X-KAT$_1$ H(10^5)	51.230	0.209	45.069	1.965	44.802	1.239
"	+ X-KAT$_1$ F(10^5)	52.447	2.098	42.506	1.472	44.907	0.652
"	+ X-CLA$_4$ (10^5)	51.389	0.342	46.565	0.678	48.908	1.529
EB red cells only	none	45.973	0.617	36.793	0.751	35.026	0.677
"	+ X-KAT$_1$ H (10^5)	45.339	0.290	35.509	0.506	34.891	0.652
"	+ X-KAT$_1$ F (10^5)	47.750	0.416	36.262	0.850	34.683	0.934
"	+ X-CLA$_4$	44.253	0.550	35.777	0.684	34.670	0.554
^{51}Cr-labelled cells subjected to 3 cycles of freezing and thawing		82.130	0.705	73.902	1.001	86.753	0.801

/ Autochthonous mixtures underlined.
'H' = LCL cells grown in medium supplemented with 10% ABRh+ve human serum for 6 weeks before experiment
'F' = LCL cells grown continuously in medium supplemented with 20% foetal calf serum.

Cytotoxicity tested on day 5 after setting up activation mixtures.
Incubation of ^{51}Cr-labelled cells with activated lymphocytes (or controls) 17 hours.

Table XXVII

Stimulation of fresh lymphocytes from donors DV and VB by X-irradiated cells from lines LIZ_1 and GOL_1 with subsequent testing of cytotoxic potential of activated lymphocytes against ^{51}Cr-labelled LIZ_1 and GOL_1 cells.

Activation mixture / (levels of Activation recorded in Fig.59)		% ^{51}Cr released from:					
Fresh cells	X-irradiated LCL cells	^{51}Cr-LIZ_1 H (10^6/culture)		^{51}Cr-LIZ_1 F (10^6/culture)		^{51}Cr-GOL_1 (10^6/culture)	
		Mean	Std.Error	Mean	Std.Error	Mean	Std.Error
DV lymphoblasts (10^6)	none	31.103	0.518	30.638	0.265	31.383	0.807
"	+ X-LIZ_1 H (10^5)	40.065	0.775	45.219	0.481	41.040	1.252
"	+ X-LIZ_1 F (10^5)	37.447	0.561	40.218	1.706	42.850	0.506
"	+ GOL_1 (10^5)	35.291	1.090	38.271	0.480	50.803	3.111
VB lymphocytes (10^6)	none	28.236	0.624	31.859	0.595	31.264	1.339
"	+ X-LIZ_1 H (10^5)	39.467	1.046	38.406	1.340	43.624	1.123
"	+ X-LIZ_1 F (10^5)	37.216	2.203	39.259	0.984	44.672	1.389
"	+ X-GOL_1 (10^5)	38.483	2.757	39.673	0.372	55.833	3.263

VB red cells only/

TABLE XXVII - cont'd

VB red cells only	none	29.167	0.213	32.749	0.615	29.382	0.917
"	+ X-LIZ$_1$ H (10^5)	27.993	0.803	31.629	0.712	29.221	0.524
"	+ X-LIZ$_1$ F (10^5)	27.675	0.172	32.721	0.350	28.862	0.692
"	+ X-GOL$_1$ (10^5)	27.540	0.644	31.971	0.871	28.961	0.670
^{51}Cr-labelled cells subjected to 3 cycles of freezing and thawing		78.742	0.464	81.659	0.980	79.036	0.380

/ Autochthonous mixtured underlined.

'H' = LCL cells grown in medium supplemented with 10% ABRh+ve human serum for 6 weeks before this experiment.

'F' = LCL cells grown continuously in medium supplemented with 20% foetal calf serum.

Cytotoxicity tested on day 5 after setting-up activation mixtures. Incubation of ^{51}Cr-labelled cells with activated lymphocytes (or controls) 9 hours.

TABLE XXVIII

Stimulation of fresh lymphocytes from donors KF and MS by X-irradiated cells from lines FLE_1 and YUD_1 (sublines A and B) with subsequent testing of cytotoxic potential of activated lymphocytes against ^{51}Cr-labelled FLE_1, YUD_1 and SAD_1 cells.

Activation mixture: Fresh cells	X-irradiated LCL cells	Activation level on day 7 (DPM)	% ^{51}Cr release from: * ^{51}Cr-FLE_1 (10^6/culture)	^{51}Cr-YUD_1 A (6×10^5/culture)	^{51}Cr-SAD_1 (10^6/culture)
			Mean	Mean	Mean
KF lymphocytes (10^6)	none	2916	20.5	22.9	28.4
"	+ X-KF (small lymphocytes)	4651	20.5	22.0	27.5
"	+ X-FLE_1	10562	32.9	21.2	30.1
"	+ X-YUD_1 A	Lost	25.3	32.0	31.9
"	+ X-YUD_1 B	22841	25.3	32.8	29.8
MS lymphocytes (10^6) +	none	1117	24.8	21.5	29.3
"	+ X-KF (small lymphocytes)	6604	24.0	21.1	26.8
"	+ X-FLE_1	13857	43.8	29.6	29.1
"	+ X-YUD_1 A	10074	36.4	43.2	30.3
"	+ X-YUD_1 B	3683	34.6	43.9	31.3

KF red cells only/

TABLE XXVIII - cont'd

KF red cells only	none	2816	18.6	Lost	27.7
"	+ X-KF (small lymphocytes)	2391	18.4	18.1	27.0
"	+ X-FLE_1	2555	18.1	20.6	25.9
"	+ X-YUD_1 A	499	17.1	19.3	25.0
"	+ X-YUD_1 E	367	18.7	19.8	26.0

/- 10^5 irradiated cells added in each case, 'X-KF (small lymphocytes)' = 10^5 fresh blood lymphocutes from this donor X-irradiated as described in text to total dose of 3000 rads. YUD_1 A and YUD_1 E are sublines of YUD_1 maintained separately but under identical conditions for 4 months before this experiment.

* Figures in this table have been calculated by hand and standard errors not yet available.

Cytotoxicity tested on day 7 after setting up activation mixtures.

Incubation of ^{51}Cr-labelled cells with activated lymphocytes (or controls) 6 hours.

lysis of the target cells are themselves capable of taking up the label as it is released. Direct evidence that this does in fact occur can be drawn for example from the experiments recorded in Tables XIX and XXII where some fresh lymphocytes stimulated with X-irradiated MAR_1-a, DUN_1-b or GOL_1 cells proved ineffective in the cytotoxic phase. ^{51}Cr release from labelled YUD_1 or RAJI cells exposed to those particular activation mixtures actually appeared to be less than that measured in labelled cultures exposed to control non-activated lymphocytes. It therefore seems clear that a fraction of the released ^{51}Cr finds its way into the activated lymphocytes. How large this fraction may be cannot be estimated at present, but it may produce an appreciable distortion of the results, particularly when the level of ^{51}Cr release is low. Increasing the incubation period in the cytotoxic phase from 6 to 17 hours (Table XXVI) increased the mean percentage of ^{51}Cr release both in control cultures and in those exposed to activated lymphocytes, but did not seem to enhance specific cell-mediated killing.

There was wide variation in the levels of ^{51}Cr release in control cultures exposed to cells which are not believed to be cytotoxic (i.e. non-activated lymphocytes or red cells). This is presumably a reflection of the variable susceptibility of LCL cells at any given time to damage in the course of pipetting and other manipulations involved in these experiments as well asto the toxic action of ^{51}Cr itself. Such susceptibility is

not a constant characteristic of each lymphoblastoid cell line since, for example the ^{51}Cr release from control cultures of DUN_1 cells varied from less than 10% (table XXIII) on one occasion to almost 20% (table XX) on another. It may be related to the unexplained fluctuations in growth rate and in percentage of viable cells within each line which has been discussed earlier in this Chapter.

In spite of these variable factors and inherent inaccuracies in the release technique, there is no reason to doubt that, within a controlled experiment, increasing amounts of ^{51}Cr released into the supernatant reflect increasing numbers of labelled cells killed. The method has the advantage of allowing easy and objective measurement as well as a high degree of reproducibility, the standard errors being so small that differences of 2-3% in ^{51}Cr release between sets of triplicate cultures are meaningful.

Cytotoxic potential in relation to level of activation achieved

Because of the numbers of cells required (both fresh lymphocytes and LCL cells) we have not yet been able to study the cytotoxic potential of a given lymphocyte population at different stages after initiating the activation phase. This imposes severe limitations upon an analysis of cytotoxic potential in relation to the level of activation achieved. However, Hardy, Ling, Wallin and Aviet (1970) found that cytotoxic activity in fresh lymphocytes activated by exposure to X-irradiated EB_2 cells was acquired

with approximately the same time course as the increase in DNA synthesis, both being maximal by the 6th day. The rate of ^{3}H-thymidine uptake then declined rapidly but the cytotoxic potential persisted for some days longer at close to its peak level. If this holds good for lymphocytes stimulated by LCL cells of non-Burkitt lymphoma origin, then all of our experiments have been carried out close to the optimum period for demonstrating cytotoxic activity.

Special conditions may apply when the activation mixture has been autochthonous or when the labelled target cells are from the same line as that used in the activation phase. The influence of level of activation on subsequent cytotoxic efficiency must, therefore, be assessed first in experiments (or parts of experiments) where neither of these situations obtains.

Tables XVIII, XIX, XX and XXII allow an examination of the effect of varying the level of activation in the fresh lymphocytes, all other factors being constant. This is achieved by using as the stimulant, either different numbers of irradiated cells from a single line or the same number of cells but with a long (48 hour) or short ($\leqslant$3 hour) post-irradiation delay. As expected,

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larger numbers of irradiated cells provoke more vigorous activation (fig.63), while the figures for ^{3}H-thymidine uptake in tables XIX, XX and XXII indicate that the longer post-irradiation delay was usually associated with a decline in antigenic potency, although since the data refer to a single point in time only, this conclusion must be regarded as tentative.

Of the eight combinations in tables XIX and XX where lymphocytes from the same donor, activated to different levels by irradiated cells from a single line, have been tested for cytotoxic potency against ^{51}Cr-labelled cells from a second line, the efficiency of killing was, in every case higher in the more intensely-activated lymphocytes. Table XVIII, however presents a different picture. X-irradiated DEW_1 cells, (3×10^4) appeared neither to activate nor to confer cytotoxic potency upon lymphocytes from donors DH and MS. X-irradiated DEW_1 (3×10^5) produced an appreciable level of activation in cells from both donors (fig.63) but these cells appeared not to be toxic to ^{51}Cr-labelled DUN_1 cells. X-irradiated DUN_1 (3×10^4) was a very feeble activating influence compared with 3×10^5 of the same cells (fig.63) yet the cytotoxic potential against ^{51}Cr-labelled DEW_1 cells was much greater in lymphocytes from both donors exposed to the smaller of the two activating doses.

In table XXII the data for activated lymphocytes from subject DD allow only two sets of activation mixtures to be compared since the authochtonous mixture DD+ x -DUN_1 is excluded. In one (DD+ x -RAJI, killing ^{51}Cr-GOL_1) an increased level of activation was associated with an increased cytotoxic potential, while in the other, DD lymphocytes activated with X-GOL_1 cells showed no cytotoxic capacity against labelled RAJI. It is of interest that in this pair of activation mixtures, the level of activation was very low. In the same table, eight sets of activation mixtures involving fresh lymphocytes from donor DV can be assessed. Four show a correlation between level of activation and cytotoxic potency and one (DV lymphocytes activated by X-DUN_1H) show equal killing effect against ^{51}Cr-labelled GOL_1 though there is a difference of 250% in ^{3}H-thymidine uptake at the time of cytotoxicity testing. Note that the lymphocytes of this donor, in contrast to those of DH, showed a higher level of activation (on day 6) in response to X-DUN, 'a' (48 hours post-irradiation) than to X-DUN_1 'b' (3 hours post-irradiation). It is possible that activation had in fact been more vigorous in the latter mixture with an early peak level and that by day 6 the curves had crossed (cf. figs 59 and 64) but it is of interest that where any difference in the cytotoxic potency could be detected, it was correlated with the level of activation recorded at the actual time of cytotoxicity testing. Of the three remaining sets of activation mixtures using this donor's lymphocytes two show no

measurable killing effect at the higher activation level and a 'negative' ^{51}Cr release at the lower level (DV lymphocytes activated by X-DUN_1 H and F killing ^{51}Cr-labelled RAJI) or a 'negative' ^{51}Cr-release at both activation levels (DV lymphocytes activated by X-GOL_1, killing RAJI). The interpretation of 'negative' (lower than control values) ^{51}Cr-release has already been discussed. It is noteworthy that in those cases in which it was found, the level of activation was invariably low.

From all the above data, the following conclusions may be drawn regarding the phenomenon of non-specific killing of ^{51}Cr-labelled LCL cells by activated lymphocytes ('Non-specific' in this context implies the ^{51}Cr-labelled target cells and the X-irradiated activating LCL cells were from two unrelated lines).

1. Cytotoxicity is dependent upon some degree of activation being attained since non-activated lymphocytes never, (and very weakly activated lymphocytes rarely) produce an elevation of ^{51}Cr release from labelled cultures above the control values attained when the target cells are exposed to donor erythrocytes. Table XXV indicates that donor erythrocytes, in turn, do not alter the ^{51}Cr release from the values obtained when the labelled 'target' culture is incubated alone without the addition of any other cell population.

2. There is a general association between the level of activation attained in the donor's fresh lymphocytes at the time of cytotoxicity

testing and the degree of cytotoxicity displayed by the activated cells against an unrelated line. The correlation, however, is not close and there are a few marked and unexplained exceptions. Kinetic studies on the phenomenon of non-specific cytotoxicity are required before the relationship to activation levels can be clarified further.

Specific Cytotoxicity in relation to level of activation achieved

'Specific cytotoxicity' in the context of the ensuing discussion refers to the release of ^{51}Cr from a labelled culture by activated lymphocytes when the X-irradiated activating cells and the ^{51}Cr-labelled target cells belong to the same lymphoblastoid cell line.

Tables XVIII, XIX, XX and XXII have again been analysed to determine the relationship between activation level (^{3}H-thymidine uptake) and subsequent specific cytotoxic potency. Autochthonous activation mixtures are excluded and will be treated separately. A total of eighteen pairs of activation mixtures can be compared. Of these, fourteen show a positive correlation between level of activation and degree of specific cytotoxicity, one shows no specific cytotoxic potential at all (MS lymphocytes + $X\text{-}DEW_1$, table XVIII), and three show a negative correlation for the two parameters. These exceptions are as follows. In table XVIII, lymphocytes from both donors, DH and MS, though very weakly stimulated by 3×10^4 X-irradiated DUN_1 cells (fig.63) nevertheless kill ^{51}Cr-labelled DUN_1 more effectively than do

lymphocytes more vigorously stimulated by 3 x 10^5 X-irradiated cells of the same line; while in table XIX, DH lymphocytes stimulated by YUDI 'b' (3 hours post-irradiation) are more effective than those stimulated by YUD_1 'a' (48 hours post-irradiation) when tested for cytotoxicity against ^{51}Cr-labelled YUD_1.

Taken as a whole therefore the data suggest that the potency of specific cytotoxicity, like non-specific cytotoxicity, is to some extent related to the level of activation achieved at the time of testing although there are some notable deviations from this general rule.

Relationship of activation level to cytotoxic potency (specific and non-specific) when the activation mixture is autochthonous

From tables XXII and XXIII five pairs of autochthonous activation mixtures (DD + X-DUN_1 and MO + X-ORI_1) can be identified, in which the level of activation was varied by altering the numbers of irradiated cells in the activation mixture. Cytotoxicity was then measured against ^{51}Cr-labelled cells of the same LCL line (two cases) or of an unrelated line (three cases). Of the five pairs, there was a positive correlation between level of activation and cytotoxic potency in four and a negative correlation in one. The exception was found in table XXIII (DD + X-DUN_1 2 x 10^5 or 2 x 10^4, killing ^{51}Cr-labelled DUN_1) since DD fresh lymphocytes on day 7

show a higher level of activation in response to 2 x 10^4 X-DUN_1 cells than to 2 x 10^5 of the same cells (fig.64). In this case, the cytotoxic potency against ^{51}Cr-labelled DUN_1 cells appears to be better correlated with the peak level of activation (occurring on day 5 in response to the higher number of irradiated cells) than to the actual level prevailing on the day of the test. These limited observations, however, suggest that the general relationship between activation level and cytotoxic potency against LCL cells is the same whether the fresh lymphocytes are activated by material from an autochthonous or an allogeneic line.

Evidence for a distinction between specific and non-specific toxicity of activated lymphocytes against LCL cells.

In order to compare the overall level of ^{51}Cr release from labelled cells exposed to fresh lymphocytes activated by irradiated material from an unrelated line ('non-specific' cytotoxicity) with the level attained when the activating and target LCL cells are from the same line ('specific' cytotoxicity) one must take account of two variable factors, namely the level of activation achieved in the fresh lymphocutes and the vulnerability (or susceptibility to killing) of different batches of ^{51}Cr-labelled cells. These two variables are probably not independent; for example in table XXII, X-GOL_1, proved to be a very weak activator of allogeneic lymphocytes and

at the same time ^{51}Cr-GOL_1 cells were highly vulnerable to killing by fresh lymphocytes activated by unrelated lines. The same appears to be true of DEW_1 in the experiment recorded in table I. It may be postulated that weak antigenicity and high susceptibility to cell-mediated killing are both reflections of a particular metabolic state of the cell line.

Because the great majority of the experiments recorded in tables XVIII to XXVIII and in the paper reprinted from Experientia 27: 1336-1338, (1971) have a cross-over design (i.e. two or more activating lines and two or more target lines, all or nearly all the possible combinations being tested) it is possible to analyse all the data in the form illustrated in fig.66. For each ^{51}Cr-labelled target cell line the values for excess ^{51}Cr release over the control levels are represented as sets of points in two columns. The control values chosen are those for ^{51}Cr release from target cells exposed to non-activated fresh lymphocytes, except in the case of LAM_1 cells in table XXI where this figure is not available and the 'red cell control' value has been substituted. The left hand column records those values obtained when the activating cell line was the same as the target line and the right hand column contains the values obtained when X-irradiated activating material and ^{51}Cr-labelled target cells were from unrelated lines.

Open circles represent values obtained when the activating mixture was autochthonous, so that an open circle in a left hand column indicates that not only activation but also the subsequent killing phase reflect interactions between the fresh lymphocytes and established LCL cells of the same donor.

Lines have been drawn connecting the points obtained in the course of a single experiment using activated lymphocytes from a single donor. (These have been ommitted from figure 66 in the case of DUN_1 target cells since 30 lines are required and these cannot readily be distinguished in the space available). When such a line slopes downwards from left to right, it indicates that 'specific' killing has been more effective than 'non-specific' and when the slope is reversed the opposite applies. If differences in killing potency were entirely attributable to differences in activation level and in target line vulnerability, one would expect equal numbers of lines to slope in each direction. One may regard this as the null hypothesis which predicts that of the 142 lines-connecting comparable points, seventy-one will slope in each direction, standard deviation being 5.97. The observed distribution is 104 sloping down from left to right and 38 sloping in the opposite direction. This difference from the predicted figure is highly significant ($p<0.01$) indicating that there is a component of the cytotoxic reaction which depends upon specific recognition of the cell line used in the activation phase.

A more rigorous test of the data presented in fig.66, which takes account of the actual difference between the mean value of ^{51}Cr release on 'specific' killing and the corresponding value on 'non-specific' killing for each target line, can be applied as follows.

For each of the 14 target cell lines let m_s = mean % ^{51}Cr release (excess over control value) on 'specific' killing and m_n = corresponding value for 'non-specific' killing.

Then $d_i = m_s - m_n$ for each line

$$\bar{d} = \frac{\Sigma d_i}{14} \quad \text{and} \quad S^2 = \sum_{c=1}^{14} (d_i - \bar{d})^2$$

$$s^2 = \frac{S^2}{13}$$

The function $\dfrac{\bar{d}}{\sqrt{\frac{s^2}{14}}}$ which has a value of 2.082 is then subjected to an upper-tailed t test with 13 degrees of freedom and it is found that $p < 0.05$ implying that in the series of fourteen cell lines, taken as a group, the mean values of ^{51}Cr release on specific killing are significantly higher than the corresponding values on non-specific killing.

The same conclusion may be drawn from an analysis of those experiments in which there was cross-over activation by, and killing of, two cell lines and an additional 'control' target line unrelated to the other two (tables XXI and XXII). In table XXI, DH and MS lymphocytes are activated by X-F137, X-ORI_1 and X-LAM_1 and subsequently tested for cytotoxic activity against ^{51}Cr-labelled G-S_1, F137 and LAM_1 (Activation mixtures including X-G-S_1 ignored because of high 'red cell control' values). The order of effectiveness of these activated cells against the different target lines is shown in table XXIX, together with a corresponding analysis of the data from table XXII. Overall there is a clear tendency for an activation mixture involving irradiated material from a given line to move higher in the cytotoxic scale when the target cells are from the same line compared with its rank order when tested against an unrelated line.

Discussion

The observation that there is an element of specificity in the cytotoxic phase greatly strengthens the case for regarding this *in vitro* system as having an immunological basis and increases its usefulness as a model for possible *in vivo* defence mechanisms against deviant cells.

It is consistent with this view that, in the cytotoxic phase, the nature of the serum (human or foetal calf) supplementing the medium in which the activating or the target

TABLE XXIX

Order of effectiveness of activated cells against different ^{51}Cr-labelled target lines.

'A' data from table d

'B' data from table e

	^{51}Cr-labelled target line	$G\text{-}S_1$	F137	LAM_1
A	D.H. lymphocytes +	X-ORI_1	X-F137	X-LAM_1
		X-LAM_1	X-ORI_1	X-ORI_1
		X-F137	X-LAM_1	X-F137
	M.S. lymphocytes +	X-ORI_1	X-F137	X-LAM_1
		X-LAM_1	X-ORI_1	X-ORI_1
		X-F137	X-LAM_1	X-F137

Activation mixtures in descending order of cytotoxic potency

TABLE XXIX - cont'd

^{51}C-labelled target line	GOL_1	DUN_1	RAJI
DD lymphocytes +	X-DUN_1 F b	X-DUN_1 F b	X-RAJI b
	X-DUN_1 F a	X-DUN_1 F a	X-DUN_1 F b
	X-RAJI b	X-DUN_1 H b	X-DUN_1 H b
	X-DUN_1 H b	X-DUN_1 H a	X-DUN_1 F a
	X-GOL_1 b	X-RAJI b	X-RAJI a
	X-DUN_1 H b	X-RAJI a	X-GOL_1 a
	X-RAJI a	X-GOL_1 b	X-GOL_1 b
	X-GOL_1 a	X-GOL_1 a	X-DUN_1 H a
B			
DV lymphocytes +	X-DUN_1 F a	X-DUN_1 F a	X-RAJI b
	X-DUN_1 H a	X-DUN_1 H a	X-RAJI a
	X-DUN_1 H b	X-DUN_1 H b	X-DUN_1 H a
	X RAJI b	X-DUN_1 F b	X-DUN_1 F a
	X-DUN_1 F b	X-RAJI b	X-GOL_1 b
	X-GOL_1 b	X-GOL_1 b	X-GOL_1 a
	X-RAJI a	X-RAJI a	X-DUN_1 H b
	X-GOL_1 a	X-GOL_1 a	X-DUN_1 F b

Activation mixtures in descending order of cytotoxic potency

cells were grown proved quite irrelevant to the outcome (i.e. there is no evidence to suggest that recognition of foetal calf antigens contributes to the cytotoxic reaction in the experiments recorded in tables XXII, XXIV, XXV, XXVI or XXVII). Identification of the antigen, or antigens responsible for the recognition of cultured cells as 'foreign' even by autochtonous fresh lymphocytes remains speculative but an attractive hypothesis is that they are altered HL-A determinants, possibly modified through interaction with new (? virus coded) antigens on the cell surface or by a change in the physical conformation of the cell surface which accompanies the reversion to a primitive "blastoid" form. This takes account of the difficulties encountered in HL-A typing of the cell lines. Whether the antigenic change in established lymphoblastoid cell lines is directly virus-coded or an indirect consequence of the change in behaviour and morphology, one might expect some cross-reactivity between the cells of different lines in the specificity of the new determinants and, as mentioned earlier in this chapter, cross-reactive antigens on all (or nearly all) established lines have been described (Bernoco, Glade, Broder, et al., 1969; Dick & Steel, 1971; Dick, Steel & Crichton, 1972; Mackintosh et al., in preparation). If these common or cross-reactive antigens are important in the

immunological reactions against LCL cells, they provide a rationale for the phenomenon of 'non-specific' cytotoxicity as defined earlier. The higher level of 'specific' cytotoxicity is less readily understood, particularly when the activating mixture is autochthonous, but one may postulate either that the new antigens interact with the existing surface determinants in such a way as to form the equivalent of an entirely altered antigenic complex or alternatively that there is a two-phase process in cell-mediated defence, first recognition of a given cell as deviant (which may be on the basis of a minor aberration in the surface antigens) and secondly the mounting of a cytotoxic attack specified by all the surface determinants of the deviant cell, regardless of how large a proportion of these may bem identical with the surface antigens of the activated cells. Whatever the precise mechanism it is obvious that any immunological defence mechanism operative in vivo against deviant cells must, to be effective, incorporate the same principle of potent and specific response to minimal antigenic discrepancy between the normal and aberrant tissue.

If the lymphoproliferative action of EB virus *in vitro* has any bearing on the development of Burkitt's lymphoma and Nasopharyngeal carcinoma *in vivo* then it may be important to determine whether patients with these disorders are characterised by any measurable defect of cell-mediated immunological defence against their own abnormal proliferating cells. So far these is only scanty information on this point. It has been shown (Fass, Herberman and Zeigler, 1970; Bluming, Fass and Zeigler, 1971) that Burkitt's lymphoma patients often display a delayed hypersensitivity reaction to an intradermal inoculation of autochthonous tumour extract and that the quality and duration of chemotherapeutically induced remission is correlated with the intensity of the reaction measured at the start of treatment. Stjernsward, Clifford and Svedmyr (1970) have found that fresh blood lymphocytes from such patients are rarely activated *in vitro* by exposure to cells extracted directly from tumour biopsies but in unpublished studies (carried out in Professor Klein's laboratory in Stockholm) the same group has found consistently high levels of activation of blood lymphocytes exposed to autochthonous LCL cells, the lymphoblastoid lines being established from tumour biopsy material The possibility has been raised that fresh biopsy cells do not activate autochthonous blood lymphocytes because the former are coated with antibody which masks the antigenic sites. (Klein, personal communication). It seems

therefore, that the interaction of humoral and cell-mediated immune responses to proliferating lymphoma tissue will need to be studied in some detail before the significance of immunlogical defences (and their impairment) is likely to become clear in relation to the development of EB virus-associated malignant disease.

Finally, the question arises as to whether the cells of established lymphoblastoid lines do themselves have cytotoxic potential. This applies particularly in the case of those cultures initiated by co-cultivation with irradiated material from an unrelated line, since the fresh blood lymphocytes in such a mixture must, at least during the first week, have possessed the capacity to destroy cells of established lines and specifically those of the line used in co-cultivation.

Since we had available two lines (CLA_2 and CLA_4) grown from aliquots of the same placental blood sample by co-cultivation with irradiated cells from different lines (CLA_2 with irradiated MAR_1 and CLA_4 with irradiated F137, see chapter III), Dr. Hardy and I were able to carry out a simple cross-over experiment in which ^{51}Cr-labelled cells from MAR_1 and F137 were exposed to each of the CLA lines. The situation was then reversed, ^{51}Cr-labelled CLA_2 and CLA_4 cells being tested for lysis when mixed with F137

or MAR_1. controls consisted of each of the ^{51}Cr-labelled lines incubated alone. The results (table XXX) show that the established lines retained neither specific nor non-specific cytotoxic potential directed against other (unrelated) lines. This implies either that the cells from which established lines are derived are not the same ones responsible for the cytotoxicity demonstrable in short term mixed cultures of fresh lymphocytes with irradiated LCL cells, or else that the activated fresh lymphocytes undergo some further change, associated with the loss of cytotoxic potential during the process of their establishment in long term culture.

TABLE XXX

Lack of cytotoxic potential in established LCL cells.

Composition of Culture 5×10^5 of each cell type	% ^{51}Cr release Mean	Std.Error
CLA_2 + ^{51}Cr-F137	16.625	0.279
CLA_4 + ^{51}Cr-F137	15.133	0.925
Medium + ^{51}Cr-F137	17.151	1.207 *
^{51}Cr-F137 Freeze-thawed 3 times	86.30	Not Done
CLA_2 + ^{51}Cr-MAR_1	17.074	0.503
CLA_4 + ^{51}Cr-MAR_1	19.029	0.637
Medium + ^{51}Cr-MAR_1	21.593	1.796 *
^{51}Cr-MAR_1 Freeze-thawed 3 times	83.54	Not Done
F137 + ^{51}Cr-CLA_2	15.709	0.252
MAR_1 + ^{51}Cr-CLA_2	18.747	0.673
Medium + ^{51}Cr-CLA_2	18.781	0.883 *
^{51}Cr-CLA_2 Freeze-thawed 3 times	84.31	Not Done
F137 + ^{51}Cr-CLA_4	14.594	0.029
MAR_1 + ^{51}Cr-CLA_4	16.204	0.253
Medium + ^{51}Cr-CLA_4	18.741	0.125
^{51}Cr-CLA_4 Freeze-thawed 3 times	85.84	Not Done

Mixtures incubated for 6 hours

*Duplicates only

CHAPTER VIII

An assessment of the potential value of human lymphoblastoid cell lines in biomedical research

Interest in human lymphoblastoid cell lines as research tools has by now extended into widely dispersed fields of medicine and biology and it is not difficult to predict that this process will continue in the foreseeable future. In discussing those areas in which their application is likely to prove most profitable, the first consideration must be an assessment, as far as this is possible, of the potential hazards associated with this type of culture. At present, three categories of risk can be recognised; transmission of EB virus from cultures to non-immune individuals, accidental inoculation of viable cells into human subjects and contamination of the cultures with organisms (particularly viruses) pathogenic to man, leading to further spread of these agents, possibly with enhanced virulence.

As regards transmission of EB virus to non-immune individuals, there appears to be a very substantial risk that this will occur at some time. Laboratory technicians being, in the main, young adults, an appreciable percentage will be EB virus antibody-negative. The mode of transmission of EB virus has yet to be clearly defined but the observation that the agent can be recovered from throat washings during the acute stage of infectious mononucleosis (Chang & Golden, 1971; Pereira, 1972) suggests that droplet-spread is a likely mechanism. In the routine handling of cultures it is difficult to avoid occasional accidents leading to dispersal of small amounts of material in aerosol form, producing, in the case of EB virus-

infected cultures, the equivalent of a droplet spray from an infectious mononucleosis sufferer. Moore (1970) has reported that in over five years experience of handling some 600 lymphoblastoid cell lines, often in enormous quantities, no case of infectious mononucleosis has occurred among his laboratory staff, and that several of his technicians, handling such cells regularly, have remained EB virus antibody-negative (by indirect immunofluorescence for anti-VCA activity) for periods in excess of 3 years. Thus it may be the case that, given reasonable standards of care and technical skill, the actual risk to non-immune laboratory personnel, of developing infectious mononucleosis through transmission of EB virus from a cultured lymphoblastoid cell line is comparable to that of acquiring the disease through contact with a human patient or carrier of the agent. If proper precautions should be neglected, however, and the presence of a potentially pathogenic virus disregarded, one must assume that outbreaks of infectious mononucleosis among susceptible personnel would follow rapidly. These cultures should therefore not be handled in an open laboratory or in a microflow cabinet blowing air outwards. Discarded cells and supernatant should be tipped directly into 'Chloros' or similar solution and any spillages wiped up promptly with an excess of the same disinfectant.

Infectious mononucleosis is clearly one possible outcome of accidents involving inoculation of living lymphoblastoid cells into a human subject (Grace, Blakeslee & Ralph, 1969; Moore & Gerner,1970)

however, of greater concern is the possibility that the cells may continue to grow in vivo. The experiments of Dr. Hardy and myself described in the preceding chapter suggest that an immunological attack would be mounted against the lymphoblastoid cells even if inoculated into the original donor, but it may be unsafe to assume that this could not be outstripped by the proliferative capacity of the cultured cells. A number of lines have been shown to grow vigorously in hosts of another species, albeit after suitable immunosuppressive preparations (Adams, Foley, Uzman et al., 1967; Southern, Burchenal, Clarkson et al., 1969; 1969a; Deal Gerber and Chisari, 1971) and there are recorded instances of growth of tumours in human subjects following accidental or deliberate inoculation of tumour tissue from another individual (see review by Gross, 1971). It must be emphasized that in respect of this potential hazard, no distinction has been established between cell lines from donors with or without malignant disease, indeed the absence of any such distinction is suggested by the hetero-transplantation experiments referred to above.

As auto-inoculation of viable cells ought to be a rare accident and inoculation of one person by another even rarer, it is impossible to make a realistic assessment of the real risk of tumour-induction by lymphoblastoid cell cultures. The observation

that successful hetero-transplantation depends on the presence of a rather large number of viable cells in the inoculum (Southern, Burchenal, Clarkson et al., 1969; Deal, Gerber and Chisari, 1971) may imply that there is a margin of safety in practice. Efforts should be directed towards minimising the risk of this type of accident, for example by ensuring that all needles and Pasteur pipettes are washed-through with 'Chloros' immediately after they have been used in the handling of lymphoblastoid cultures, to avoid the danger of viable cells adhering to sharp implements.

Apart from EB virus, it is known that other agents can multiply in cultures of human lymphoblastoid lines. These have included Herpes simplex virus (Bedoya, Rabson and Grimley, 1968), Coxsackie A, Vesicular Stomatitis Virus, Vaccinia, ECHO 11, Adenovirus 5, Poliovirus 1, 2 and 3, Rhinovirus 2, Reovirus 1 and Mumps virus (Wallace, 1969), and an unidentified virus found by Achong, Mansell, Epstein and Clifford (1971) in a line grown from a nasopharyngeal carcinoma biopsy. Mycoplasma has been recovered from a number of lines (Traul, personal communication, 1972) and indeed a few cultures in this laboratory were found to carry Mycoplasma Arginini during the latter part of 1970 (Steward & Young, 1971). The infected samples were discarded and repeated checks on all linessince then have yielded negative results.

The full range of organisms which could grow in this type of culture is unknown, but it is not unlikely that it will include a number pathogenic to man. Within a laboratory, therefore, the cells might constitute a source of infection hazardous to the personnel, a risk which is increased by the longevity of the individual cell lines.

As in the case of all human tissue cultures, there is at least a theoretical risk that organisms replicating in the cells in vitro may become adapted to human tissue in such a way as to increase their virulence for the human host (Fenner and Cairns, 1959). Once again, the fact that a single cell line may be propagated for many years should be regarded as adding to this danger by increasing the time available for mutation or other forms of adaptation to take place in any contaminant organism.

Regular examination of cell lines by electron microscopy should provide a substantial measure of protection against the carriage of unrecognised contaminant pathogens but as in the case of EB virus, recognition of the potential risks by those involved in handling the cultures is likely to prove the most effective means of ensuring safety.

Given that these hazards are not shared (or at least not in the same degree) by primary cultures of human fibroblasts or epithelial cells, the mere convenience of using a cell line which grows in suspension (i.e. does not require trypsinisation before

each subculture) and which is relatively easily maintained *in vitro* is not sufficient reason for replacing traditional monolayer cultures by lymphoblastoid cells for purposes already adequately served by the former, for example the routine quality control of culture media or assays of interferon. On the other hand, the unique properties of human lymphoblastoid cell lines render them particularly valuable in both clinical and theoretical areas of oncology and genetics.

Oncology

The relationship of lymphoblastoid cell lines to Burkitt's lymphoma has suggested very strongly that EB virus is potentially oncogenic in man, the development of tumours being prevented in the normal situation by immunological defences in the host. This argument has been developed in Chapters I and VII, but the case is as yet far from proven. The observation that EB virus will induce long-term proliferation of lymphoid cells *in vitro* and that immunological reactivity against autochthonous LCL cells can be demonstrated also *in vitro*, suggests that this system may prove to be a valid model for the study of factors determining individual variation in the susceptibility of lymphocytes to the proliferative action of the virus and for the efficacy of cell-mediated immunological defences against such proliferating cells.

As discussed in Chapter III, there is already evidence that lymphocytes from foetal tissue or from cord blood are more readily induced to grow as long term lines following EB virus infection *in vitro* than are those from children or adults. Having arrived at a co-cultivation system giving a consistently high success rate in terms of the initiation of new lines from cord blood lymphocytes, the next stage should be to compare the success rates under identical conditions using peripheral blood lymphocytes from healthy individuals of different ages. The results should also be analysed according to the donors' previous histories of infectious mononucleosis and the levels of antibody to EB virus in their sera. Thereafter the study should be extended to include lymphocytes from patients with a relatively high risk of developing malignant disease (e.g. Down's syndrome and Fanconi's anaemia) and from patients living in areas where Burkitt's lymphoma is common, noting in this case what evidence there may be for chronic malarial infestation in each donor. Such a study, carried out on a sufficiently large scale, should establish whether any variations in susceptibility to EB virus-induced lymphoproliferation *in vitro* can be correlated with environmental or genetic factors suspected of contributing to the risk, *in vivo*, of malignant disease in general and of Burkitt's lymphoma in particular. If a coherent pattern should emerge from this type of survey, the logical

extension would be a detailed investigation of the mechanisms whereby susceptibility to the proliferative action of EB virus *in vitro* can be modified. This would probably follow the lines by which a similar problem is being approached in relation to the transformation of human fibroblast cultures by SV40 (Aaronson, 1970).

On the same basis, it is clearly important to extend studies on *in vitro* immunological reactions between peripheral blood lymphocytes and autochthonous cultured lymphoblastoid cells. As discussed in Chapter VII, there is already some limited evidence for the capacity of fresh biopsy cells from Burkitt's tumours to provoke a delayed-hypersensitivity reaction in the autochthonous host and to stimulate his peripheral blood lymphocytes *in vitro*. It may be of considerable importance in coming to an understanding of the factors involved in the genesis and prognosis of Burkitt's lymphoma if the *in vitro* reaction can be quantified and measured repeatedly throughout the course of the disease in individual patients, as has been done for some of the EB virus.associated humoral antibodies (Chapter VII). This would require a supply of tumour cells from each patient, carefully preserved in deep freeze. If, at the same time, a lymphoblastoid cell line could be established from the original tumour biopsy, a comparison could be made between the cell-mediated immune responses to the original tumour cells and to

lymphoblastoid line derived from them. Cytogenetic evidence (Chapter VI) suggests that in such circumstances the lymphoblastoid line is derived from the malignant cells of the tumour, but it is not known whether the antigens responsible for stimulation of autochthonous lymphocytes are equally expressed on the cultured cells and on those of the fresh biopsy. Questions to be asked and, one hopes, answered in an investigation on these lines should include the following. Is the in vitro lymphocyte activation and/or cytotoxic response to autochthonous LCL cells modified by serum factors from patients with Burkitt's lymphoma, nasopharyngeal carcinoma or other malignant diseases? Is the response, in the case of Burkitt's lymphoma patients, related to cutaneous hypersensitivity reactions to autochthonous tumour extracts, to levels of circulating antibodies against EB virus or to immunoglobulin coating of the tumour cells in vivo? Is the response of lymphocytes from patients with 'big spleen' disease or, other evidence of chronic malarial infestation, different quantitatively or qualitatively from that of healthy subjects when both are challenged with autochthonous lymphoblastoid cell line cells?

From this there may emerge a clear indication of whether Burkitt's lymphoma is associated with an impaired cell-mediated defence mechanism against EB virus-induced lymphoproliferation and, if so, whether this impairment a) is mediated by a

circulating humoral factor and b) is a characteristic of patients with chronic malarial infestation but without Burkitt's lymphoma.

In relation to less specific, more fundamental problems of oncogenesis, the fact that a DNA virus will induce abnormal and apparently unlimited proliferation of human cells *in vitro* is obviously of the greatest interest. Considerable efforts are being expended in a number of centres in order to determine the number of EB viral DNA-equivalents are integrated into each host cell genome and how precisely the integration takes place (Zur Hausen, Schulte-Holthausen, Klein *et al.*, 1970; Nonoyama & Pagano, 1971). The vital issue,of course, is to identify the essential cellular functions which are usurped by the viral DNA and in consequence of which continuous proliferation of the cell ensues. Since the DNA of EB virus is (by the standards of polyoma or SV40) very large (Crawford and Black, 1964; Weinberg and Becker, 1969), it would seem to be an unpromising candidate for a pointer to the molecular basis of malignancy (for discussion see Winocour, 1971). Nevertheless, studies of the surface antigens of lymphoblastoid cells in culture (Chapter VII) can be interpreted not only in terms of immunological defences against deviant cells, but also as manifestations of altered cell function which may provide clues to the nature of the virus interaction.

In addition, the size of EB viral DNA may prove to be an advantage in attempting to localise the site of its integration

into the host cell genome, for example in examining individual metaphase chromosomes by in situ hybridization with C^{14} labelled virus-specific homologous RNA (Pardue & Gall, 1969; Jones, 1970).

Finally the fact that lymphoblastoid cell lines are not completely stable *in vitro* but undergo chromosomal (and presumably other) changes over periods of months and years (see chapter VI) warrants further study in the light of the knowledge that the cells of malignant tumours also undergo evolution (including karyotypic evolution) *in vivo*. The two processes may be very similar. I have suggested (chapter VI) that lymphoblastoid lines change by the emergence of successive clones of cells each more completely adapted than its precursors, to the conditions in which it is growing. Fialkow and his colleagues have provided evidence (from analysis of G-6PD types) that at least some human tumours, including Burkitt's lymphoma, are of clonal origin (Fialkow, Klein, Gartler & Clifford, 1970; Fialkow, Martin, Klein et al., 1972). Thus the development of resistance to successive chemotherapeutic agents, and the increasingly malignant behaviour characteristic of the progression of Burkitt's lymphoma, leukaemia and other tumours may be a function of the same 'instability' of the proliferating cells, permitting the emergence of sublines derived from the parent clone but selected for the capacity to grow rapidly in spite of the host's intrinsic resistance and in the presence of whatever extrinsic therapeutic attack may be mounted.

It is not known whether these changes with time in tumour cells and in cultured lymphoblastoid cells can be attributed to successive modifications in a single common cellular function, but an analysis of the characteristics of established lines at different ages *in vitro* may throw light on this area. For example there have been claims that malignancy in man is associated with a specific chromosome aberration (see Muldal, Elejalde & Harvey, 1971) and, as discussed in chapter VI several allegedly specific changes in the karyotype of lymphoblastoid cells in culture have been reported, many appearing only after the lines have been grown for long periods. The availability of staining methods which permit accurate identification of every chromosome has already disposed of some of these claims (see chapter VI) but they also offer the opportunity of very detailed analysis of the karyotype which may lead to the positive identification of changes that are correlated with an increased growth rate in the affected cells. Although this type of investigation is still at an early stage, it would appear that human lymphoblastoid lines are ideally suited to the purpose.

Similarly, there is interest in the possibility that malignancy may be associated with alterations in the surface histocompatibility antigens of the tumour cells (e.g. Seigler, Kremer, Metzgar et al., 1971; Pegrum, Balfour, Evans and Middleton, 1971). Analysis of changes in the pattern of HL-A determinants on

lymphoblastoid cells with time in culture may therefore prove to be a worthwhile project, although it must be recognised that such a study poses major difficulties both of technique and of interpretation.

Finally, lymphoblastoid cell cultures offer scope to the biochemist who may be interested in the metabolic pathways of cells which can proliferate indefinitely in vitro or in the physico-chemical characteristics of their enzymes and other constituents. To date there has been only one published study on the metabolism of these cells. Rice and McCurdy (1971) report that the respiratory quotient (RQ) is higher and the oxygen consumption lower in cell lines originating from patients with malignant disease than in those established from healthy subjects or from patients with non-malignant disease. The two classes of cell line were also distinguished by the response in terms of RQ and oxygen consumption to leukognenol, an incompletely characterised extract obtained from Penicillium gilmanii or from mammalian liver cells. It is paradoxical that two of the lines of "malignant" origin in this study were from patients with chronic myeloid leukaemia. The chromosome constitution of these cultured cells was not reported but it has been found in the great majority of cases (see Chapter VI) that the Ph' chromosome is not present in lymphoblastoid lines grown from Ph'-positive chronic myeloid leukaemia patients and

hence it is believed that the lines are derived from lymphocytes which are not 'malignant' in any sense. Rice and McCurdy found that the RQ and oxygen consumption of these two lines corresponded to the pattern observed in lines from acute-leukaemia, multiple myeloma and Burkitts lymphoma and differed from that of the 'non-malignant' lines. It will be of interest to see if their observations are confirmed by other groups.

Iso-enzyme patterns for all the lines described in Chapters II and III have been examined at the Galton Laboratories, University College London (Prof. H. Harris). Some twenty-six loci have been examined in each line and in the case of fifteen lines a direct comparison has been made with the fresh blood cells of the original donor. Preliminary results indicate that the iso-enzymes are very stable even in aneuploid lines, but in a few cases, changes in the pattern of one or more enzymes have been observed with time. These changes have included alteration in electrophoretic mobility, deletion and re-activation of an enzyme lost at an earlier stage in culture (Prof. H. Harris, Dr. S. Povey and Dr. B. Watson, personal communication). There is no indication that any of the changes observed so far affect the growth rate of the cell line, but as the range of iso-enzymes which can be studied grows wider, it is possible that an aberration common to all established lines may be recognised.

Genetics

Whereas both short term cultures of peripheral blood and *in vitro* studies of skin fibroblasts have an important role to play in the diagnosis of genetically determined disorders of man and in the investigation of the precise biochemical lesions (Hsia, 1970; Krooth and Sell, 1971; Mellman, 1971), long-term lymphoblastoid lines may offer unique opportunities for the study of genetically determined defects of immunoglobulin production (Schimke and Kirkpatrick, 1970). In more general terms they may also be utilised for experiments on the repair or circumvention of molecular defects, for example through the introduction of extraneous nucleic acid by transducing bacteriophage (Merril, Geier and Petricciani, 1971) or by cell-fusion (Schwartz, Cook & Harris, 1971). In such a situation the relative stability of lymphoblastoid lines through months and years in culture will provide a much more exacting test of the attempted therapy than is possible using fibroblast cultures.

With this type of project in mind, a number of lines have now been established from patients with genetically determined disorders (Blume, Glade, Gralnick et al., 1969; Choi,& Bloom, 1970). As described in chapter II we have grown a single line from a patient with cystic fibrosis and arrangements have been made to collaborate with geneticists in several British centres in order to create a bank of cell lines from patients in their care.

The longevity of the cell lines can also be exploited in the analysis of the normal genetic processes of human tissue. For example, if sufficient genetic markers can be characterised (iso-enzymes, immunoglobulins and possible HL-A phenotypes) the mutation rates for the loci may be measured by establishing large numbers of clones (ideally from single cell isolates) of a given line and looking at each locus in all of the clones for evidence of variation.

If, at the same time, the karyotype of each clone is determined, using a quinacrine fluorescence technique (see Chapter VI) the data obtained should contribute to the process of gene assignment and chromosome mapping. Equally, the study should provide information on gene-linkage in man. A long-term genetic experiment of this type is already under way (Prof. H.J. Evans, Dr. M.E. Arthur). It is anticipated that a vast amount of data will require to be collected and analysed before meaningful results are obtained and a system of computer storage and handling of the information has therefore been devised.

An alternative approach to the same problem is the use of cell fusion (Harris 1970), creating heterokaryons between human and other mammalian cells from which chromosomes are gradually shed. Human lymphoblastoid cells can be used in such a system (Caspersson, Zech, Harris et al., 1971) though they do not appear to offer any advantage over fibroblasts and indeed pose greater technical difficulties.

The epigenetics of lymphoblastoid cells must also attract considerable attention, particularly in relation to their specialised function in the synthesis of immunoglobulins. There is already a substantial body of information, mainly from animal studies, on the cellular and molecular mechanisms involved in this process (Talmage, Radovich & Hemmingsen, 1970; Herzenberg, McDevitt & Herzenberg, 1968). Attention has been directed to the question of whether a single cell can synthesize more than one class of heavy chain either simultaneously or sequentially (Takahahi, Tanikagi, Yagi et al., 1968; Bloom, Choi and Lamb, 1971). If so, it will be of great importance to identify the factors which control the selection of one or the other biochemical pathway. One current view is that specific antibody production is induced by a chemical factor, possibly messenger RNA, passed to an immunoglobulin-producing cell by a macrophage which has been exposed to the appropriate antigen (Cohn, 1968). One may speculate that this theory will lend itself to investigation <u>in vitro</u> using cultured macrophages and lymphoblastoid cells.

A second possible specialised function of lymphoblastoid cells which is of particular interest to immunologists is the production in the cultures of humoral factors which influence the behaviour of human lymphocytes and macrophages (Granger, Moore, White et al., 1970). The precise role of these factors in immunological reactions is unknown and initially the principal use of lymphoblastoid lines in this connection may be as a source of large quantities of purified material for chemical and biological assay.

Other possible applications

Agents such as low-dosage radiation, carcinogenic chemicals and even drugs which are suspected of causing damage to living tissue as a result of very prolonged exposure may produce measurable effects in lymphoblastoid lines over comparable periods. Exploitation of the cell lines as a monitoring system for such purposes is still in the realms of speculation, but there are obvious advantages in using human tissue and at present lymphoblastoid cells seem to be the only material with the necessary property of longevity *in vitro*.

Finally, the fact that many lines produce a single species of immunoglobulin may permit them to be used as a source of material for the analysis of the biological properties of the different classes and for the preparation of monospecific antisera. This is a particularly attractive proposition in the case of rare immunoglobulins such as IgE (Nilsson, 1971).

The above list is not exhaustive and is likely to become rapidly out of date. No mention is made of the use of living cells as therapeutic agents in man although such a purpose has been envisaged, notably by Moore (Moore & Minowada, 1969). In the light of current knowledge, the ethics of this procedure must be open to question and no convincing benefit has accrued from the few occasions on which it has been attempted (Moore & Gerner, 1970).

CHAPTER IX

General conclusions

It is evident that human lymphoblastoid cell lines offer scope for a vast amount of research work in a wide variety of fields. Some technical problems remain to be overcome, chiefly the rather low overall success-rate in establishing new lines from individual blood samples. It is highly desirable, particularly in relation to human genetics, to be able to initiate a long-term culture from the majority of specimens, if not all, since most genetically-determined disorders, which may be amenable to study *in vitro*, are rare and tend to be detected in children, from whom repeated blood samples cannot be readily obtained. The second technical point is the yield of EB virus particles from established lines. As indicated in Chapter IV, my experience, in common with that of several other workers, has been that the amount of EB virus released by the cells of a given line tends to diminish with time. Since the interaction of the viral DNA with the host cell genome seems likely to be a topic worthy of strenuous investigation, a reliable source of EB virus in reasonable quantity would be a distinct asset. There is considerable interest at present, in many centres, in means of increasing the virus yield from any given line. This was discussed in Chapter IV and it is probable that one or more of the techniques recently described will answer this particular need.

In this context, very little further evidence seems to be required before it can be established beyond doubt whether EB virus DNA is present in every human lymphoblastoid cell line (and, by inference, responsible for the long term proliferation). My own findings (Chapters III and IV) suggest that inability to detect virus particles in the cells of a particular line cannot be taken to imply that all, or even a part of the viral genome is absent from that line.

The counterpart *in vivo* of the lymphoblastoid cell which proliferates *in vitro* has yet to be positively identified. Morphologically the cultured cell resembles the incompletely differentiated members of the primitive lymphoid series, but although there is good reason, from cytogenetic studies, to regard many of the lines as the cloned progeny of a single cell, they have a much wider range of attributes than any single member of the lymphoid series *in vivo*. Collectively they exhibit phagocytosis, secrete immunoglobulins and liberate humoral substances which modify the behaviour of immuno-competent cells. Whether any single cell from a lymphoblastoid line can perform all of these functions, or whether a proportion of the cells undergo a measure of differentiation along various specialised paths is a matter which requires further study.

Both morphologically and cytogenetically I have found no consistent differences between the lines established from patients with non-malignant disease (including healthy subjects) and from those with a variety of malignant disorders of the reticulo-endothelial system. Virtually all of the lines, regardless of origin have become aneuploid after one year of growth *in vitro*. There was no evidence, among nineteen aneuploid lines examined, for the recurrence of specific chromosome aberrations in more than one line. The observed aneuploidy evidently comes about by the emergence of successive clones of cells each better adapted than its parent line (or clone) to the conditions of growth *in vitro*. Similar patterns of growth have been recorded in human tumours *in vivo*, notably in Burkitt's lymphoma. In Burkitt's lymphoma, furthermore, identical chromosome aberrations have been found in direct preparations from fresh biopsy material and in lymphoblastoid lines subsequently derived from the same tissue. It therefore seems possible that the growth of Burkitt's tumour *in vivo* and of human lymphoblastoid cell lines *in vitro* are two manifestations of the same process.

Since lymphoblastoid cell lines can be established from the peripheral blood of a substantial proportion of healthy infants while Burkitt's lymphoma is a rare condition even in the climatically defined regions of "high" incidence, it is clear that some factor must normally be operating to limit the potential for lymphoproliferation *in vivo*.

The studies reported in Chapter VIII are at least consistent with the view that the limiting factor is an immunologically-based surveillance mechanism. Dr. Hardy and I have found that not only is there invariably a mixed-lymphocyte type of reaction between fresh blood lymphocytes and irradiated lymphoblastoid cells from an antochtonous line, but that this is followed by the development, in the fresh cells, of cytotoxic potential directed against many lymphoblastoid lines and most specifically against the particular line used in the activation phase.

The nature of the antigen or antigens responsible for this phenomenon is one immediate object of further study, but equally important is the question as to why this mechanism should fail (if indeed it has failed) in Burkitt's lymphoma patients.

Thus the position has now been reached where lymphoblastoid cell lines can form an integral part of a comprehensive *in vitro* model system for the study of human oncogenesis, tumour evolution and tumour control.

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