# AN INVESTIGATION INTO DNA-SYNTHESIZING CELLS IN THE BLOOD USING AUTORADIOGRAPHICAL TECHNIQUES

A dissertation presented for the Degree of Master of Science of the University of St. Andrews.

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To Fiona, Sara and Oliver

## DECLARATION

I declare that the following research has been carried out and the thesis composed by myself and that it has not been submitted previously in part or complete for any other degree.

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CONTENTS

## ABSTRACT

DNA synthesizing cells have been demonstrated in the blood following the incubation of blood samples with $^{3}$H-TdR. Autoradiographic techniques using stripping film and emulsion are described and compared.

In addition to a control group, blood samples have been obtained from newborn infants, patients recovering from operations and patients suffering from the following disorders:- infectious mononucleosis, Hodgkin's disease, non-Hodgkin's lymphoma, chronic lymphocytic leukaemia, acute leukaemia, myeloma and a miscellaneous group of conditions which include anaemia, pancytopenia, polycythaemia, malignancy and infection.

In all of the conditions studied the number and type of labelled cells was recorded. The most striking results were observed in infectious mononucleosis and acute leukaemia. The significance and possible functions of the DNA synthesizing cells is discussed and the efficacy and value of autoradiography as a useful tool is considered.

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# INTRODUCTION

Malpighi described the capillary circulation in the lungs of a frog and recognised red cells. Later in 1665 he made similar observations of red blood cells in the hedgehog. Swammerdam in 1663 while demonstrating his great skill in dissection observed the red blood cells of a frog. Leeuwenhoek, on the other hand, with the aid of his own constructed microscope first described red cells in 1673 and discussed their deformability in 1675. Towards the end of the 17th and during the early 18th century, Leeuwenhoek's observations widened and his many publications were much quoted by other scientists.

Further advances were made after the intoduction of dyes by Ehrlich. He showed that a relationship existed between the cells and a variety of aniline dyes divided into three groups of acid, basic and neutral. This led to eosinophils, basophils and neutrophils being differentiated and lent greater enthusiasm to the microscopical study of haematology. Indeed, although many of the discoveries of that era are now of historical value, blood cells and their precursors have assumed ever increasing importance in the course of many years so that the microscopical study and recognition of cells is equally significant today. Information reported on the changes observed and the presence of abnormal cells is of obvious importance in the diagnosis and management of any disorder.

In the laboratory in which this present study was carried out with a daily workload of approximately 250 blood counts, less than 30% require blood film examinations. This is based upon a protocol which excludes examination of films on samples with haematological values within normal parameters.

Less than 10% of the films examined exhibit the typical characteristics of blood dyscrasias. The most common findings of those that show abnormalities are iron deficiency, normocytic, normochromic and macrocytic anaemias, infectious mononucleosis, other viral illness and haemolytic anaemia.

It is apparent then that the studies of Ehrlich and later those of Pappenheim, another outstanding figure in Haematology and blood cell morphology in particular are still appropriate today. Pappenheim was also a great supporter of the theory that the origin of all blood cells was a single primitive common cell. This hypothesis was also shared by his friend, Ferrata. In his work of 1912, Ferrata described the "haemocytoblast" and proposed it as the common precursor of all blood cells, Baserga and Zavagli (1981). This cell was therefore classified as a pluripotent stem cell. Although many suggestions have been made as to its possible identity the exact nature and morphological classification of this cell remains unknown.

In more recent years, certain anaemias have been recognised as hereditarily-determined deficiencies of enzymes such as glucose - 6 - phosphate dehydrogenase (G-6PD) and Pyruvate Kinase, within the red blood cell. New techniques of enzyme detection and assay are available and are of obvious value considering that G-6PD is probably the most common hereditary enzymatic defect of clinical significance. It is considered by McCulloch and Till (1977) and generally agreed that the disorders chronic myeloid leukaemia, (CML), acute myeloblastic leukaemia (AML) and polycythaemia rubra-vera (PV) are clonal in origin and are the product of a single cell. This was determined by the recognition of genetically determined

markers such as the presence of a single iso-enzyme of G-6PD or by the presence of any chromosomal abnormalities such as the Philadelphia chromosome in the haemopoietic cells of any of these disorders.

Recent advances have seen the emergence of techniques for the identification and study of leucocyte enzymes. Familiar enzymes of the series such as peroxidase and alkaline phosphatase have been known for some years and similar characteristic enzymes in lymphocytes have lately been observed. The most important of these is terminal deoxynucleotidyl transferase (TdT), levels of which have been increased in most cases of acute lymphoblastic leukaemia (ALL) and in many cases of malignant lymphoma, Catovsky (1980). Important differences of enzyme content and iso-enzyme pattern exist between normal and leukaemic cells and between cells from different types of leukaemia. It is hoped that these cell markers will be an aid to more accurate diagnosis and perhaps better treatment of leukaemia and lymphomas, Lancet (1977). The earlier demonstration of T and B cell markers has also been shown to be of considerable value in the classification and prognosis of lymphoproliferative disorders especially in ALL, Catovsky (1977).

At the same time, although the results of cytochemical staining techniques, such as PAS and Sudan Black, have scientific interest, their significance in the identification of blood cells and in the diagnosis of disease still remains obscure. This is especially evident in the cases where inconclusive and inconsistent results are obtained.

Historically then the study of haemopoiesis has been the subject of exhaustive research for many decades. The origin,

development and function of red cells, white cells and platelets is widely documented and normal haematological values for the constituents of human blood are well established, Dacie and Lewis (1975). Haemoglobin (Hb) is one of the most important and probably the most common determination in laboratories. It is subject to many variations including the influences of age, sex, physical exercise and diurnal variations. The establishment of a reliable standard for cyanmethaemoglobin led to the wide routine use of this method of estimation. The present measurement of Hb as used by Coulter Electronics in their instruments is derived from this method but with the advantage of a more rapid conversion time. The accuracy of measurements of packed cell volume (PCV) and red blood counts (RBC) established by microhaematocrit and manual techniques respectively have always been questioned. The RBC count in particular, fraught with inaccuracies due to cell distribution within the counting chamber and counting errors had been abandoned as unreliable. The accuracy therefore of data such as mean cell volume (MCV) and mean cell haemoglobin (MCH) derived from the RBC count had also been questioned. In recent years, the inadequacies of previous techniques have been overcome. This has been brought about largely by the advent of electronic cell counters. The facilities of red cell counting, sizing and computation of PCV combined with greater precision and accuracy have contributed to much of the efficiency and information of haematological investigation. The most recent and advanced fully automated counting system, the Coulter Model S Plus, generates several other parameters additional to those mentioned above, namely a white cell

count, a platelet count and values for platelet size and distribution. Increasing workloads together with an ever-increasing variety of tests have also developed a greater demand for quality control to ensure validity of results. Frequent quality control checks using reference materials followed by statistical analysis of observed values are now commonplace in most laboratories. Furthermore, the use of computer facilities interphased with the system enables continuous monitoring to be carried out throughout every workload. It is evident then that the last two decades have provided the most technological advances in haematological analysis. It may be argued however, whether all of the data produced is clinically of any value or does the sheer amount tend to obscure and confuse rather than inform? Useful and comprehensible information should always be the priority as against the mere production of irrelevant data.

The most fundamentally important part of any haematology investigation is inspection of the blood film. The diagnosis of a blood disorder may often be made from the film by simply observing the presence or absence of abnormalities affecting the red cells, leucocytes and platelets. However, the descriptive details of any morphological changes may be unhelpful and confusing unless definite conclusions can be reached and useful information reported. The examination and classification of the cells in the blood and bone marrow is based simply on morphological criteria. There is no apparent insight into the growth characteristics and proliferation of the cell population. The erythroid series can be recognised as a continuous sequence of maturing cells from the large basophilic proerythroblast progressing through

polychromatic and orthochromatic erythroblasts with the gradual expulsion of the nucleus and increasing development of haemoglobin to the fully mature red cell. It is generally recognised that while the information obtained by visual examination can be significant, it is limited in nature. It is more valuable to study living cells by in-vitro and in-vivo techniques. Over the years attention has been directed from the mere observation of fixed preparations of cells to a closer appreciation of their function and proliferation by means of cell culture techniques, animal experimentation and the use of radioactive isotopes.

The present day in-vitro cell culture techniques have become increasingly more complex and sophisticated. Colony formation in-vitro of haemopoietic precursor cells has provided a unique opportunity to study the kinetics and growth patterns of these cells. The culture system in-vitro normally has three main requirements; responsive precursor cells from bone marrow or other haemopoietic tissue, a haemopoietic hormone to serve as stimulus and a semi-solid matrix that would support growth and help promote the formation of a colony. In 1967, Senn et al, compared the colony forming ability of normal human bone marrow with that of leukaemic marrow. It was shown that the latter failed to produce colonies in contrast to that of normal marrow. However, Robinson et al (1971) subsequently showed that colony growth could be obtained from the peripheral blood of patients with leukaemia in relapse and suggested that differences in techniques and particularly differences in the medium and the stimulus used were the main reasons for success in comparison to previous studies. Colony stimulating factor (CSF) in the form of glycoprotein is necessary in-vitro to stimulate cell proliferation

and produce colonies of a particular cell class in semi-solid cultures. The number of colonies formed depends on the number of progenitor cells and the concentration of the stimulus present in the culture. Peripheral blood levels of neutrophil polymorphs and monocytes do not necessarily reflect the rates of production of these cells in-vivo. This is probably due in part to the irregular release of cells from the bone marrow. Information can now be obtained from cell culture techniques to assess the growth pattern and differentiation capacity of the committed precursor. This information will obviously have great bearing on the investigation of various haematological disorders and can be used to confirm and expand on the observations made from conventional morphological studies. Cell culture techniques can prove to be of value in several situations such as in the investigation of neutropenia or myeloproliferative disorders. The proliferation and differentiation capacity of any precursor cells can be studied in-vitro to assess the nature of any abnormality in cell production. Since marrow transplantation is of increasing importance in the treatment of aplastic anaemia and leukaemia, assessment of the numbers and viability of progenitor cells by in-vitro techniques is extremely useful, Metcalf (1979). Despite the technical problems inherent in cell culture and assuming adequate normal control cultures are included, the technique has an important role to play.

Apart from cell culture techniques, the use of animals, mice in particular, has been an invaluable tool in haematological research for many years. The mouse as a result of meticulous in-breeding is available in a variety of specific

genotypes offering unique opportunities for the study of several types of inherited anaemias, Harrison (1979). The spleen colony assay for in-vivo colony forming cells, Till and McCulloch (1961), was carried out on the mouse using bone marrow cells and despite several other attempts to produce similar spleen colonies in other animals, this has been the only success. The cells that form macroscopic colonies on the spleens of irradiated mice are known as CFU-S (colony forming unit - spleen) and are believed to be the earliest precursors detectable as single cells. These cells differentiate to produce granulocytes, erythrocytes and platelets. The next closest descendents are colony forming units in semi-solid agar cultures (CFU-C), as described previously, which produce granulocytes and the CFU-E that respond to erythropoietin in-vitro and give rise to erythroid cells. Since the identity of the CFU-S is still in doubt means were sought to examine the proliferative activity of the cell in bone marrow by assessing the proportion of cells engaged in DNA synthesis (S-phase) at any one time. In the experiment by Becker et al (1965), the mouse was instrumental in a procedure known as the "thymidine-suicide" technique. In essence, the fraction of CFU-S in DNA synthesis was measured by studying the effect of a high concentration of high specific activity tritiated thymidine on their ability to form spleen colonies in mice. The cells in DNA synthesis incorporate the isotope, are killed by irradiation and are unable to produce spleen colonies. The difference in the number of colonies formed on the spleens of mice injected with the sample exposed to the isotope and those injected with a control sample is a measure of the CFU-S in S-phase

of the cell-cycle at the time of exposure.

Several reports have demonstrated that the "thymidine-suicide" of CFU-S is less than 10% in a normal steady state production and so the majority of CFU-S in bone marrow are out of cell-cycle in Go phase or in a prolonged G1 phase. Further behaviour patterns of the CFU-S population have been examined under different situations of stress due to radiation or drugs. The subsequent observation of the response has thrown light on the nature and activity of the haemopoietic stem cell (CFU-S) and its role in haemopoiesis, Schofield (1979). It would appear that on demand for proliferation during recovery time from partial depletion or damage, the CFU-S is stimulated to undergo more rapid production in an effort to restore the steady state. This recovery can be indicated by an elevated thymidine-suicide measurement. At present, the method of regulation of stem cell population is still unknown although recent work by Wright and Lord (1978) suggests that control of proliferation is dependent on the precise balance of stimulator and inhibitor factors.

It is evident that radioisotopes play an important role in the investigations of cellular proliferation described above. Radioisotopes have been utilised in different forms and in various techniques in research and routine analytical procedures for many years. The range of application had widened with newer techniques and an ever-increasing range of radioactive labelled compounds is now available. Radio-isotopes are particularly suited to the study of the blood and haemopoietic organs and many diagnostic isotope procedures have become routine tests in clinical haematology. The most common investigations include estimations of blood volume and

red cell survival using Chromium-51, studies of iron metabolism and erythropoiesis by means of Iron-59 and investigations of megaloblastic anaemia with Cobalt-58. These techniques are all used to study particular situations and the isotopes employed are detected and measured by pulse counting instruments or scintillation counters. These measurements, while accurately providing the total radioactivity in a source, do not indicate the variation or distribution throughout the sample. Much more critical information is provided by autoradiography especially at a cellular level by allowing the identification of the type and the number of cells labelled.

In essence, autoradiography is a method whereby the location and pattern of distribution of radiation, in the form of labelled substances can be observed in tissue at a cellular level. The technique of autoradiography for the light microscope involves the application of a thin layer of photographic emulsion in close contact with a sample containing a radioactive isotope, such as blood, bone marrow or histological section on a microscope slide. The emulsion consists of a suspension of silver bromide crystals supported in a matrix of gelatin. Similar to the well known photographic process, these crystals become activated when exposed to the charged $\beta$-particles from the radioactive material in the specimen. When the emulsion is subsequently treated with a developing agent, these activated crystals become converted into metallic silver grains. The crystals which are not activated remain unchanged and are dissolved out of the emulsion by the process of fixation leaving behind a pattern of developed silver grains in the form of

a latent image. This final image reflects the pattern of the distribution of the radioactive label throughout the specimen which can be readily identified microscopically. Autoradiography differs from the pulse counting technique in that the image formed as a result of the passage of the charged particles through the crystals, becomes permanent after development. This achieves an accurate record of the exact location and distribution of radioactivity within the sample, the performance of which cannot be matched by pulse counters. The formation of grains in response to the reaction allows for the discrete examination of any particular area of the specimen including observations of the structure of individual cells such as nuclear or cytoplasmic labelling. The main contribution of autoradiography is that it allows the location and rate of any cellular activity to be observed without undue disruption of the surrounding tissue structure.

Autoradiography is not restricted to microscopic specimens. Large and often irregular objects such as complete skulls, brains or whole bones may require scanning to find the distribution within them of radioactive material. The combination of autoradiography with the electron microscope, while greatly widening the potential of the study of sub-cellular structures has also increased the complexity of the techniques of autoradiography used and strained the limitations of electron microscope resolution, Rogers (1979). The analysis of the finished autoradiographs is also more difficult at this level of magnification than at light microscope level. Despite the difficulties and the time-consuming nature of the process, it remains a most valuable method for studying the function and distribution of labelled

molecules at such high resolution.

Tritium ($^{3}H$), Carbon-14 ($^{14}C$) and Iodine-125 ($^{125}I$) are three of the isotopes used in autoradiography with $^{3}H$ probably one of the most commonly used in such methods. It produces a very sharp image with good resolution and the B-particles emitted have low kinetic energies which has the advantage that the grains will be localised nearer to the source of radiation and directly over the nucleus in one plane. $^{14}C$ produces B-particles with high energy and as a result travels further in the section and emulsion than $^{3}H$. The grains therefore tend to be produced more remotely from the source and so the resolving power is less. $^{125}I$ on the other hand has energy similar to $^{3}H$ and the resolving power is almost as good, Flitney (1977). The half-life of $^{3}H$ of 12.3 years approximately, is sufficiently long to enable most experimentation to be carried out with virtually no loss in activity.

Initially, much of the improvement in the resolution of tissue autoradiographs was due to Pelc (1947) in his experiments using Iodine-131. In 1957, tritium labelled thymidine ($^{3}H$-TdR) became available and was recognised as a valuable specific label for DNA synthesis since it is incorporated into the DNA of proliferating cells in-vivo or during in-vitro incubation. The resulting labelled cells can be detected using autoradiography. Those cells which are in DNA synthesis are the only ones which label and can be assumed to be destined for mitosis. Mature cells, incapable of proliferation, do not incorporate this label. The continuous sequence of events of mitosis divided into prophase, metaphase, anaphase and telophase for convenience, has been observed and described microscopically.

It represents the formation of two new cells with an equal distribution of chromosomes. On the other hand, knowledge of the cell cycle leading up to and including mitosis, was largely unknown because there was no visual evidence of morphological change. With the development of autoradiography and the later advent of a specific radioactive label for DNA, the stages of interphase were recognised and studied. The nuclear cycle is divided in relation to the time of DNA synthesis. Prior to the DNA synthesis stage (S Phase) is a post-mitotic period referred to as G1 which is followed by a post-DNA synthesis,pre-mitotic phase known as G2. Mitosis (M) follows G2. One complete passage through the stages of interphase and the phases of mitosis is termed a cell cycle, the duration of which is termed the generation time. Within the cell cycle, G2 cells have double the DNA content of cells in G1 phase, in preparation of mitotic division. Cells in the S-phase have DNA contents which vary between the two values depending on the stage of DNA synthesis. In 1963 a Go phase was suggested by Lajtha. This was a true resting stage in which cells apparently do not participate in the cell cycle and so they neither synthesize DNA nor undergo mitotic division but are capable of returning to activity after stimulation. Since cells in G1 phase and Go are identical with respect to DNA content, it is not possible to differentiate between a population of Go cells and cells in a prolonged G1 phase.

The study of cell kinetics using autoradiography and $^{3}$H-TdR has provided a clearer understanding of the proliferative potential and behaviour of differing cell populations. Following in-vivo administration of $^{3}$H-TdR various measurements

can be made. During this labelling period any cells that are synthesizing DNA are labelled together with any cells that pass from G1 into DNA synthesis during this time. Samples obtained after 60 minutes "flash-labelling" show a labelling index (LI) which is equal to the ratio of the number of cells in DNA synthesis to the total number of cells in the population while the label was available. The LI can also be determined after incubation in-vitro with $^{3}$H-TdR for the same time. Following administration of $^{3}$H-TdR, cells labelled in the S-phase move through G2 into mitosis from which two daughter cells, each labelled with approximately half the activity of the parent, emerge. If further division arises, labelled cells then move through G1, the next S phase, G2 and subsequent mitosis. Non-dividing daughter cells remain labelled during maturation. The duration of these phases of the cell cycle can be determined by various techniques. One of the commonest methods used is that which utilises the incidence of labelled mitotic figures. In essence, the method involves labelling cells in the S phase of a population and observing that block of labelled cells as it passes through mitosis. The first labelled mitotic figures will appear after cells which were in late S at the time of labelling pass through the G2 phase and enter mitosis. Thus the period of time between introduction of $^{3}$H-TdR and the appearance of labelled mitoses will be equal to the duration of G2. After the appearance of the first labelled mitotic figures the proportion increases until all the mitotic figures are labelled. Cells which were in G1 while the label was available then begin to enter mitosis and the proportion of labelled mitosis decreases. Eventually the labelled cells will divide again and so the labelled mitotic

figures will reappear. From this information a series of labelled mitoses curves can be drawn fitted to the percentage of labelled mitotic figures at various time intervals after the administration of tritiated thymidine, Wimber (1963), Thomas (1974). The duration of the cell cycle and its subdivisions can be deduced by measurements of the intervals on the mitosis curve. The double labelling method using $^{14}C$ and $^{3}H$-TdR has also proved useful in the study of the various phases of the cell cycle of asynchronous cell population, Wimber (1963).

There has never been total agreement on the actual durations of the cell cycle or of its phases. This is a result of the difficulties involved in measuring any system where the cells have a continuous passage through different but related compartments, as occurs in the case of maturing erythroid precursors during the period of observation, rather than existing in separate units. This movement produces a variation in the labelling patterns during this period. The incidence of mitotic figures in any cell population can be used as a measure of the rate of cell production. The mitotic index % can be determined by the ratio of the number of mitotic figures to the total number of nuclei examined in the population. The mitotic index will be increased if the proportion of dividing cells is increased or if the duration of mitosis is increased relative to the total duration of the cell cycle. The incidence of labelled cells (labelling index) is similarly affected by the same criteria, Thomas (1974). In any given population of cells, the duration of DNA synthesis is normally much longer than the duration of mitosis which may be measured in minutes. As a result, the

labelling index (LI) is usually much higher than the mitotic index. The frequency and location of dividing cells therefore can be investigated more readily by studying labelled cells rather than mitotic figures. Furthermore, non-dividing labelled daughter cells can be traced readily during subsequent maturation and migration by autoradiographs prepared at various intervals after administration of the label. This sequence was demonstrated by Bond et al (1959) who followed the course of in-vivo $^{3}$H-TdR labelled S-phase erythroid cells and peripheral neutrophils. He observed the continuous progression of labelled cells from the basophilic to the polychromatic and finally into the non-proliferative orthochromatic normoblast compartment. A similar study was also carried out by Stryckmans et al (1966) to estimate the DNA synthesis time of erythropoietic and granulocytic cells in humans.

It is evident then, that the advent of DNA labels and $^{3}$H-TdR in particular, combined with autoradiography are valuable tools in the study of cell proliferation. The technique has not only provided useful additional information concerning the kinetics of haemopoiesis but has supplemented the established techniques of cell culture and animal experimentation. Culture methods alone are inappropriate to measure the in-vivo lifespan of any of the elements of the blood. Investigations involving infusion of $^{3}$H-TdR in human subjects followed by autoradiography are used to measure the production rates in proliferating cell compartments. These studies are difficult and time consuming and ethical considerations must be taken into account. Nevertheless, they do provide information that cannot be obtained by any other

method, although similar in-vivo studies have been exploited in animal models, particularly mice, to great advantage. On the other hand, in-vitro culture methods enables the study of the effect of stimulators such as erythropoietin, on the rate of proliferation, by measurement of colony size. Colony formation is related to the concentration of erythropoietin in culture, Testa (1979).

It is apparent then that by combination of all of these experimental resources, continuous progress has been made in the overall understanding of cell kinetics and in the mechanisms involved in the regulation of haemopoietic proliferation. Autoradiographical procedures have been applied to numerous varied experiments and are not restricted to any particular area of pathology. Wickramasinghe (1975) utilised $^{3}$H-TdR autoradiography to describe the kinetic disturbances in megaloblastic erythropoiesis and the subsequent response to therapy. Similar investigations were carried out by Rearden and Masouredis (1977) using $^{125}$I-anti D to assess the uniformity of the D antigen content of the individual red cells.

The present study was prompted by the observation that small numbers of cells capable of DNA synthesis are present normally in the peripheral blood, Rubini et al (1961) and also by similar evidence that haemopoietic stem cells (CFU-S) are normal constituents of circulating blood, Barnes et al (1967). The knowledge that DNA synthesizing cells can be demonstrated in-vitro after a brief exposure (approximately one hour) of the cells to $^{3}$H-Thymidine also influenced the initiation of such a study.

With these findings in mind, it was decided to use autoradiography with $^{3}$H-TdR to investigate the presence and

significance of DNA synthesizing cells in the blood in different states and disorders.

In an investigation by Rubini et al (1965) various physical and chemical factors which affect $^{3}$H-TdR cell labelling were evaluated using autoradiography. It was found that adequate labelling was achieved during one hour's incubation at 37$^{o}$C followed by exposure for 7 to 14 days. These conditions formed the basis of this present study. Generally, increased numbers of labelled cells were detected in those circumstances where there was an increased stress on the bone marrow. This group included conditions of viral and bacterial infection and malignancies such as Hodgkin's disease, acute leukaemia and myeloma. Cord blood samples were also in this category. Morphologically the labelled cells were classified as monocytes, large lymphocytes and atypical lymphocytes, blast cells, myelocytes, lymphoid cells, early erythroblasts and plasma cells. As indicated above and from the work of Rubini et al (1961), Killmann (1968) and Harris (1973) the DNA synthesizing cells appear to be present in the circulation in increased numbers and in several cell types due to different haemopoietic stress situations. An immunological function is probably served in most cases where these cells occur. It is possible however, though by no means certain, that a proportion of the DNA synthesizing cells serve as haemopoietic stem cells. The most obvious circumstance for their presence would appear to be in cord blood samples under conditions demanding a greater degree of haemopoietic activity than normal. Thus during foetal development the activity of the bone marrow is reflected by increased numbers of blood-borne stem cells, Prindull and Hespe (1978).

In general then, it would seem possible though not conclusive from the available evidence that most of the labelled cells present are associated with an immune response and a small proportion could have a stem cell role. The significance of these circulating stem cells is not fully understood but it is probable that they have been recruited from one area and transported in the blood to some other location in the body. This situation could arise under circumstances of haemopoietic stress where the DNA synthesizing cells are migrating to an inactive or depleted area of the bone marrow with the purpose of increasing haemopoiesis, Harris (1973).

The study of cell kinetics in acute leukaemia is one area in particular where autoradiography has made an important impact. It is now recognised that cell division is not more rapid in the leukaemic population than in normal cell populations. It was noted also in this study, in agreement with other investigations, that labelling took place consistently and virtually exclusively in large blast cells with an absence of labelling in small blasts. These latter cells are confined to the Go phase of the cell cycle but can resume proliferation under certain circumstances. The application of cell kinetics to the design of chemotherapy programmes is still vitally important in the treatment of leukaemia. This has never been more apparent than in recent years due to the development of cycle-specific and cycle non-specific drugs. Only the latter group of drugs have the potential to eradicate the Go or out of cycle cells. As a result of the strategic combinations of these drugs and equally strategic timings of administrations, the maximum effectiveness can be achieved, Mauer (1975). Although the cell kinetics used to monitor the developments

and administration of these anti-leukaemic drugs are extremely useful, the autoradiographical process itself is tedious and time consuming and cannot be justified as a routine procedure. Nevertheless, autoradiography utilising the liquid emulsion technique which has many advantages as compared to stripping film, will continue to be a valuable instrument in the study of cellular proliferation. Its most important function is that it allows the critical visual examination of proliferating tissue at a cellular level when compared to scintillation and pulse counting techniques.

The recent introduction of DNA flow cytometry has provided another means of obtaining kinetic information. This extremely precise and rapid method measures the DNA content of large numbers of cells by utilising fluorescent dyes. The percentages of G1, S and G2+M phase cells can therefore be determined. Since this method does not allow for cell identification, the results obtained for mixed cell populations must be treated with caution. The advantage of autoradiographical procedures over all other techniques is the demonstration and identification of different cell populations.

In the present investigation, therefore, autoradiography was adopted to demonstrate and identify cells in DNA synthesis circulating in the peripheral blood. The significance of the presence of these cells, while not fully appreciated, has raised speculation as to their origin and function in view of the assumption that DNA synthesizing cells may actually divide as they circulate in the blood. These observations and findings are discussed in this study.

# MATERIALS

## 1. GLASSWARE

All glassware was initially immersed overnight in chromic acid cleaning solution, rinsed well in tap water and soaked for 2 hours in distilled water with two changes of the distilled water. The glassware was then dried in a hot air oven at 160°C. Microscope slides thus washed, were immersed in methanol until required.

At the end of each experiment, all glassware which had contained Ilford Emulsion was cleaned by soaking for several hours in normal sodium hydroxide solution, rinsed in tap water, then soaked overnight in Decon 90 solution.

After thorough rinsing in running tap water, the glassware was soaked in distilled water and dried as detailed above.

## 2. CHEMICALS

All chemicals, except where stated, were of British Drug Houses 'ANALAR' grade.

## 3. HEPARIN

Heparin B.P. (Mucous, preservative free). The heparin used was obtained from EVANS MEDICAL LTD., 5000 units in each 5 ml. vial.

The contents of the vial were dissolved in 20 ml. sterile isotonic saline. 20 mls. of freshly drawn blood was mixed with 0.2 ml. of the heparin solution which ensured a final concentration of 20 units of Heparin per 1.0 ml. of blood.

## 4. ISOTOPE

The product used was (Methyl-$^{3}$H) THYMIDINE ($^{3}$H-TdR) Code TRK 418, 250μCi, specific activity 40-50 Ci/m. mol. This was obtained from the RADIOCHEMICAL CENTRE, AMERSHAM.

5. ILFORD G5 and K2 EMULSION

Both Ilford G5 and K2 emulsions were used in the liquid emulsion technique and were obtained from ILFORD LTD., MOBBERLY, CHESHIRE. They were stored at 4°C in the refrigerator as recommended by the manufacturer.

For use, the emulsions were diluted with equal parts of distilled water containing glycerol to a final concentration of 1%. The inclusion of glycerol helped to reduce stress background during subsequent drying of the emulsion. WALLER, (1961). As cited by Rogers (1979).

6. AR 10 STRIPPING FILM

The stripping plate was obtained from KODAK LTD. and was stored and handled according to manufacturer's instructions. It was stored in the refrigerator at 4°C where its shelf-life was approximately 6 months.

The stripping film is packed in boxes of 12 glass plates; each box containing 3 packets of 4 plates wrapped in black paper and surrounded by a polythene bag.

The film itself consists of a double layer compressing a thin layer of nuclear emulsion carried on a base of inert gelatin and mounted on a glass support. The gelatin layer is in contact with the glass.

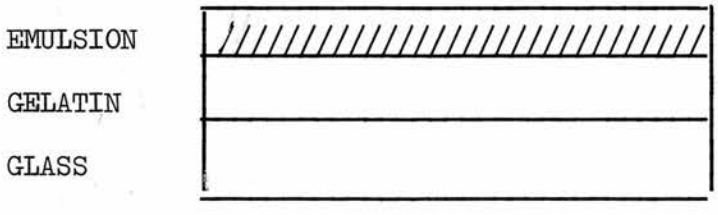



Fig. 1. Cross section of AR 10 STRIPPING FILM.

7. EQUIPMENT FOR AUTORADIOGRAPHY

(i) A Safelight fitted with a 15W bulb and a KODAK safelight filter, WRATTEN series No. 1 (Red).

(ii) A thermostatically controlled water bath working within the range 43 - 45°C.

(iii) Dipping vessel - a plastic container was used to hold the emulsion (approximately 25 ml. capacity).

(iv) 25 ml. and 50 ml. graduated measuring cylinders and a 50 ml. beaker.

(v) A glass universal container containing 11.76 ml. distilled water and 0.24 ml glycerol.

(vi) A clean sharp scalpel blade and a pair of forceps.

(vii) A pair of plastic forceps and a glass stirring rod.

(viii) Hot plate at 40°C.

(ix) Light-proof slide boxes obtained from CLAY-ADAMS containing SILICA GEL.

(x) Thermometer.

8. DEVELOPMENT REQUIREMENTS

D 19 Developer and 'Unifix' Powder (Acid-Fix) were both obtained from KODAK LTD. and prepared according to manufacturer's instructions:-

1) The contents of the smaller pack of KODAK developer powder was dissolved in a quantity of water at 38°C, equal to approximately two-thirds of the final volume which was 5.0 litres.

2) When this was completely dissolved, the larger pack was stirred in until all the powder had dissolved. The final volume was made up with cold water.

3) The solution was well stirred and distributed into tightly stoppered one litre bottles. A full bottle

8. DEVELOPMENT REQUIREMENTS

3) of freshly made unused developer had a shelf-life of 1 - 6 months at a temperature between 18 - 24°C. The KODAK 'Unifix' acid fixer powder is also dissolved in water at a temperature not exceeding 27°C and the final volume was made up as with the Developer. The distribution and storage of the acid fixer is also similar to the developer.

# METHODS

1. COLLECTION OF BLOOD

Samples of venous blood were taken into:-

a) Sequestrene (EDTA-Sodium Salt) 4.0 ml. disposable container.

b) Heparin, 2.0 ml. was added to heparin solution in a 5.0 ml. disposable plastic container (STERILIN) to give a final concentration of 20 units of heparin per ml.

The samples were mixed gently to ensure that anti-coagulation was effective. Samples of blood from umbilical cord obtained from newborn infants were similarly treated.

As soon as possible after collection, using the sequestrene treated sample, total leucocyte counts were performed using a Coulter Counter Model S Plus. A differential leucocyte count was also carried out on a blood film stained by May-Grunwald Giemsa.

2. DILUTION OF TRITIATED THYMIDINE ($^{3}$H-TdR)

a) A 1:100 dilution of $^{3}$H-TdR was made for use. A 10 µl automatic pipette with a sterile disposable tip was used to remove aseptically, 10µ Ci from the stock bottle which was then diluted in 1.0 ml. of isotonic saline to give a concentration of 10µ Ci/ml.

b) 0.2 ml. of this dilution was added to 2.0 ml. of heparinised venous or cord blood to give a final concentration of 1µ Ci/ml. of blood.

3. INCUBATION OF BLOOD

The heparinised blood was incubated with $^{3}$H-TdR with occasional gentle mixing at 37°C for 1 hour. After incubation, blood films were spread on clean slides, air-dried and fixed

in methanol for 5 - 10 minutes. These blood films were processed using either the liquid emulsion or stripping film techniques to be described.

## 4. AUTORADIOGRAPHY

The experiments were based on the methods described by Rogers (1979).

### 4.1 PREPARATION OF AUTORADIOGRAPHS BY LIQUID EMULSION TECHNIQUE

Before working under the safety lighting in the dark room it was ensured that:-

a) The water bath was within the temperature range 43 - 45°C.

b) The slides to be coated were warmed on the 40°C hotplate. This ensured that the emulsion coating the slides drained off more readily than from a cold slide thus helping to achieve a thinner uniform layer.

c) The 50 ml. and 25 ml. graduated measuring cylinders were placed in the water bath along with the glass 'universal' containing 11.76 ml. distilled water and 0.24 ml. glycerol.

d) The plastic container which was used as the dipping vessel was allowed to stand in the water bath and was steadied by standing inside an immersed 50 ml. beaker.

#### (i) DILUTION OF EMULSION

This process was carried out under the safelight.

1) Using plastic forceps, Ilford emulsion strips were transferred from the stock bottle to the 50 ml. graduated measuring cylinder until the 20 ml. mark was reached.

4.1

(i) DILUTION OF EMULSION

2) The measuring cylinder was returned to the water bath for a further 15 minutes to allow the emulsion to dissolve. This was hastened by occasional gentle stirring of the emulsion with a glass rod.

3) The liquified emulsion was then slowly poured from the 50 ml. measuring cylinder into the 25 ml. measuring cylinder until it reached the 12 ml. mark. The contents of the 'universal' container were slowly added to the emulsion in this 25 ml. measuring cylinder to make a 1:1 dilution of the emulsion. This also provided a final concentration of 1% glycerol.

4) The diluted emulsion was now carefully poured into the plastic dipping vessel which was then returned to the water bath to stand vertical in the immersed 50 ml. beaker.

5) The diluted emulsion was gently stirred, using a glass rod and was then allowed to stand for a further few minutes to allow any bubbles to escape.

(ii) SLIDE COATING

It is essential that the emulsion is free of air bubbles and has a uniform thickness. Both of these conditions can be achieved by gently stirring the emulsion and allowing it to settle for a further few minutes. The emulsion is then tested by dipping a clean warmed blank slide, withdrawing and examining it for faults under the safety light.

4.1

(ii) SLIDE COATING

1) The warmed test slides were dipped individually in the emulsion for about 1 second.

2) The vertically held slide was slowly withdrawn from the emulsion and excess emulsion was allowed to drain on to a moist tissue.

3) The back of each slide was wiped before being clamped into the drying box and left overnight in the dark to dry.

4) The following day, under the safelight, the slides were placed in light-proof slide boxes in the presence of drying agent silica gel. The boxes were then put in black polythene bags, bound with tape and placed in refrigerator at $4^{\circ}C$ to expose for 5 days.

(iii) CONTROL PROCEDURES

Appropriate controls were included in every experiment to detect the possible presence of false positives or negatives. False positives can be produced in the emulsion by chemography (the chemical interaction of specimen and emulsion) by heat, by light or by pressure. False negatives can also be caused by chemography and by fading of the latent image during exposure.

In order to detect false positives, a control is included with each batch of tests by exposing an identical blood film which had not been incubated with radioactive isotope.

The best control against false negatives is to take one coated test slide from each batch which is then exposed

4.1

(iii) to light, prior to exposure at 4°C.

Both of these control slides were included with the test slides in the light-proof boxes so that exposure and development proceeded under identical conditions.

(iv) DEVELOPMENT AND FIXATION PROCEDURE

These stages were carried out at a temperature between 18 - 20°C under safelight conditions in the dark-room. Dark-room requirements include stainless steel slide racks and three glass baths containing:-

a) Filtered D 19 Developer

b) Rinse of distilled water

c) Filtered acid fixer

All processing solutions were at a temperature between 18 - 20°C.

After exposure at 4°C, the experimental slides were removed from their light-proof slide boxes and transferred to the stainless steel racks and left for 20 minutes to reach room temperature.

1. The rack, with slides, was placed in the first bath of developer for 3 minutes if liquid emulsion was used and 5 minutes if stripping film was used.
2. The slides were next rinsed in the second bath of distilled water for 30 - 60 seconds.
3. The developed preparations were now placed in the acid fixer bath for 5 minutes (10 minutes if stripping film was used).
4. The fixed preparations were finally washed in gently running tap water for 20 minutes and stained by May-Grunwald Giemsa stain and then dried.

4.1

(iv) The developing and fixing times described above for liquid emulsion and stripping film, were confirmed by trial and error, to be the optimum times for use. The development times of 3 minutes and 5 minutes for the liquid emulsion and stripping film respectively, were found to be effective, in that they provided the shortest times and produced satisfactory results with the least amount of background level.

As a general rule, the time of fixation was taken to be approximately twice that of the development time. The difference in time between both methods were due to the extra layers of formvar and gelatin on the stripping film, which necessitated longer development and fixation in that method.

4.2 PREPARATION OF AUTORADIOGRAPHS BY STRIPPING FILM TECHNIQUE

(i) Slide Coating with Formvar and Gelatin

All experimental and control slides in the process were pre-stained by May-Grunwald Giemsa.

In order to avoid the possibility of chemographic effects of the stain, or the fading of the stain during exposure, the pre-stained preparations were first coated with a thin, impermeable membrane using a 4% solution of polyvinyl formal (FORMVAR) in 1:2 Dichlororethane. This procedure was based on the method described by Giannelli, (1974).

1. Using a pasteur-pipette, a drop of 4% Formvar was allowed to fall onto the clean surface of distilled water, cooled to 10 - 15°C in a dish at least 6 inches deep. On contact with the water, the drop spread and

the solvent evaporated leaving a membrane on the surface. This membrane was then picked up on the pre-stained slide by dipping the slide under the membrane in the dish and carefully removing it from the water so that the membrane completely covered the slide and coated the preparation. The slides were then clamped into the drying box and allowed to dry for 20 minutes under a stream of cool air from a hair drier to hasten the process.

2. After drying, the pores of the membrane were sealed by exposing the slides to the saturated vapours of an 0.5% solution of Formvar for 1 - 2 minutes.
3. As the stripping film tended to adhere less firmly to the Formvar coated slides, thus causing a greater degree of loss of the emulsion during subsequent developing and fixing, it was necessary to coat the slides with gelatin. This was achieved by dipping the Formvar coated slides in the following freshly prepared and filtered solution of gelatin at room temperature:-

| | |
|---|---|
| Gelatin | 0.5 gm. |
| Chrome Alum | 0.25gm. |
| Distilled Water | 100 ml. |

The coated slides which now provided a good adhesive surface for the emulsion, were finally dried in the drying box as before and were ready for the stripping film application.

(ii) DARK-ROOM REQUIREMENTS

Kodak AR 10 Stripping Film was used. The box of film was removed from the refrigerator at least one hour

before required to allow temperature equilibration. The emulsion must be handled in the dark-room under a safelight. Equipment required in the dark-room included:-

a) A bath at least 3 inches deep containing a fresh Potassium Bromide/Sucrose solution:-

| | |
|---|---|
| Potassium Bromide | 10 mgms. |
| Sucrose | 200 gms. |
| Distilled Water | 1 litre |

at a temperature of 20°C

b) A clean sharp scalpel and a pair of forceps were also required.

According to O'Callaghan, Stevens and Wood (1969) leaching of bromide ions from the stripping film can occur during flotation, leading to an increase in background, that may result during long exposures. By floating the film on a solution of potassium bromide and sucrose, they have shown that the background can be improved significantly should this solution be incorporated in the technique, whenever the exposure time is 2 weeks or more.

(iii) DESCRIPTION OF TECHNIQUE

1) Under the safelight in the dark-room, a plate was taken from the packet and placed on the bench with the emulsion layer uppermost.

2) Using a clean sharp scalpel, a margin of about $\frac{1}{4}$ inch was cut around the edge of the plate. The remaining emulsion was further divided into eight rectangular pieces each large enough to coat a microscope slide.

3) Using forceps, a rectangular piece of the emulsion was grasped at its corner and gently and steadily

stripped from the glass support.

4) As the film parted company with the glass, it was placed simultaneously, emulsion surface downwards, on to the surface of the Potassium Bromide/Sucrose solution in distilled water.

5) The film was allowed to float on the surface for 2 - 3 minutes during which time the emulsion absorbed water and spread to reach its maximum expansion. It was then picked up on the test and control slides.

6) These slides were partially immersed in the water at an angle, then carefully lifted out of the water with the film covering the preparation and folded around the back of the slide.

7) The slide was drained of excess water, clamped vertically in the drying box and dried for 30 minutes in a gentle stream of cool air.

8) As the film dried, it made very close contact with the preparation and the slides were finally transferred to light-proof boxes containing silica gel, sealed in black polythene bags and stored in a refigerator at $4^{\circ}C$ for the exposure period of 2 weeks. In order that the work-flow be improved, two baths were utilised and the films were floated out and picked up sequentially. Control slides were included as in the previous technique.

(iv) DEVELOPMENT AND FIXING

After exposure, these stages were carried out in the same dark-room conditions using the same developer and fixer as for Ilford liquid emulsion method described previously. This process was carried out as follows:-

The rack of the slides was:-

1. Developed in D 19 developer for 5 minutes at 20°C
2. Rinsed in bath of distilled water at 20°C for 30 - 60 seconds.
3. Fixed in acid-fixer for 10 minutes at 20°C.
4. Washed in gently running tap water for 20 minutes.

The autoradiographs were allowed to dry and harden for 24 hours before staining by May-Grunwald Giemsa.

## 4.3 DRYING AND EXPOSURE OF EMULSION

Liquid emulsion layers, being much thinner than those of stripping film, dried much more rapidly and this often led to higher background levels caused by stresses in the emulsion. This was avoided by drying the slides slowly at room temperature, without the aid of a hair drier, as described in the stripping film method. In order to find the optimum exposure times for both the liquid emulsion and stripping film, several preparations were set up for different intervals of exposure by each method. The exposure times used were 2 days, 3 days, 5 days, 1 week and 2 weeks.

The films were then developed and processed as described then examined microscopically for the presence and intensity of grains in the labelled cells. The optimum exposure times at 4°C for liquid emulsion and stripping film were found to be 5 days and 14 days respectively. These exposures were established for practical use but in order to allow a degree of flexibility in process they were not adhered to rigidly.

In most cases however, it was found that longer exposures increased the grain count per cell and although

this did not significantly affect the labelling index, occasionally the cellular detail was obscured leaving the cell unclassified.

5. EXAMINATION OF AUTORADIOGRAPHS

The stained autoradiographs prepared by both methods were examined microscopically and differential counts were performed on 500 nucleated cells per slide and scored for the presence of nuclear labelling (Grains). The number and type of each labelled cell was recorded. All cells with 5 or more grains over their nuclei were considered labelled and background levels were acceptable at less than 3 grains over a similar area.

As mentioned previously, control slides were included in every experiment to exclude the presence of false positives or negatives.

# COMPARISON OF AUTORADIOGRAPHICAL TECHNIQUES

A comparison of liquid emulsion and stripping film techniques was carried out using Ilford emulsions and Kodak AR 10 stripping film. The criteria used in the selection of the method of choice were simplicity, effectiveness and efficiency of technique. Although it was established that the information achieved from the two methods was virtually the same (see below), it became evident that the liquid emulsion technique more readily fulfilled the criteria.

The major disadvantages of the stripping film method were the unaccountable intermittent appearance of high background in some experiments which rendered the results unacceptable. The most likely explanation for high background in this method was that the technique itself involved more handling of the film. The process of stripping the film from the glass support sometimes caused visible flashes of static electricity resulting in a very high background. The coating of the preparations with formvar followed by gelatin added to the complexity of the technique and also required that the exposure period be extended for 2 or more weeks because of the additional thickness of the 2 layers. In addition, since the film is required to be floated on the surface of water for 2 - 3 minutes, this not only slowed down the process of coating the slides but occasionally led to contamination of the film from dust on the water surface.

Comparison of the numbers of labelled cells per 500 cells counted using emulsion and stripping film and different exposure times is shown in table I.

Table I

| PATIENT | DIAGNOSIS | EMULSION 5 DAYS EXPOSURE | AR 10 STRIPPING FILM 14 DAYS EXPOSURE |
|---|---|---|---|
| B. M. | CORD | 5 | 7 |
| G. McC. | CHRONIC LYMPHO-CYTIC LEUKAEMIA | 0 | 0 |
| D. M. | HODGKIN'S DISEASE | 2 | 0 |
| H. S. | HODGKIN'S DISEASE | 1 | 1 |
| C. H. | ACUTE MYELOID LEUKAEMIA | 7 | 5 |
| K. L. | CONTROL | 1 | 0 |
| C. P. | CONTROL | 1 | 1 |
| | | MEAN = 2.4 | MEAN = 2.0 |

Duplicate films were treated with liquid emulsion or stripping film and exposed at various lengths of time in order to determine the effect of film type and varied exposure. With either film and for exposures of 1 or 2 weeks the numbers of labelled cells and the means were not markedly different.

The liquid emulsion technique was found to be very quick and simple to use, with the emulsion layer adhering to the specimen and slide more firmly thus providing a thinner layer. This enabled the preparation to be stained through the emulsion, without the presence of formvar and gelatin, after exposure and development had taken place. Batches of 40 - 50 slides can be dipped in liquid emulsion in approximately 1 hour. Whereas virtually double that time is required for an equivalent number processed using stripping film. The problem of high background which occurred with the stripping film was comparatively rare with the dipping technique and the background generally was reduced.

Liquid emulsions are produced in series according to grain size and sensitivity and the two used, G5 and K2, compared favourably with each other, the only disadvantage was that the G5 emulsion tended to produce larger and more dense grains over the nucleus which tended occasionally to obscure the cellular detail. For this reason only, it was decided to use the K2 emulsion for most of the autoradiographical experiments since it produced a much finer grain size with the same length of exposure.

Therefore, for the reasons mentioned, the liquid emulsion and in particular, K2 emulsion, was preferred to stripping film. However, in any laboratory where autoradiography is not a routine technique but may be required only occasionally, then the stripping film may adequately serve the purpose as it has a shelf-life of 6 months and each plate provides sufficient film for a small number of slides.

# RESULTS

## CONTROL SAMPLES

The control samples were taken from healthy adult volunteer laboratory and Radiography Department personnel. The controls served to establish a labelling index range for a healthy adult population. 11 male and 9 female volunteers were used and the ages of those selected ranged from 19 - 53 years. All haematological parameters from each control specimen estimated by means of the Coulter Model S Plus were found to be within the normal ranges. The white cell counts ranged from 4.9 to 13.6 x $10^9$/L with essentially normal differential counts.

After incubation of the blood samples with $^3$H-TdR the films from this control group were made and autoradiographically processed. At various intervals throughout this entire study, a control slide was included in each experiment and processed in conjunction with the test slides.

500 nucleated cells were counted from each film and the number of labelled cells was determined. (Table II). In addition, a number of these control specimens were incubated at 37°C with $^3$H-TdR for several hours and films made at hourly intervals up to 5 hours and these were also processed and examined as described. (Figs. 2 and 3).

## RESULTS

Of the 20 control specimens examined only 5, 3 female and 2 male controls, had labelled cells recorded in their individual 500 cell count. A labelled smear (or damaged) cell (Fig. 4) was seen in one preparation only and with its inclusion the overall labelling index range was 0 - 0.4%. The remaining slides with 0.2% labelling indices, displayed labelled cells

which were mononuclear resembling monocytes or large lymphocytes (Fig. 5). The intensity of grains in one cell prevented identification. No labelled cells of the myeloid or erythroid series were found nor were any mitotic figures seen. The results produced after different exposures of 5 days for liquid emulsion and 14 days for stripping film were virtually the same with labelling index never greater than 0.2%. From figures 2 and 3, increasing the incubation time with $^{3}$H-TdR over 5 hours did not increase the number of labelled cells in any of the preparations examined.

## CONTROLS

TABLE II

| CONTROL | AGE | SEX | WHITE CELL COUNT x $10^{-9}$/L | LABELLED CELLS PER 500 CELLS COUNTED |
|---|---|---|---|---|
| G. P. | 19 | M | 6.3 | 0 |
| W.T.L. | 36 | M | 13.6 | 0 |
| C. P. | 21 | M | 7.3 | 0 |
| B.M.W. | 46 | M | 11.9 | 0 |
| G. F. | 23 | M | 7.5 | 1 |
| W.T.L. | 36 | M | 7.2 | 1 |
| M. W. | 24 | F | 9.0 | 0 |
| D. S. | 24 | F | 7.6 | 2 (including 1 smear cell) |
| E. B. | 36 | F | 7.1 | 1 |
| M. C. | 41 | F | 5.6 | 0 |
| A. M. | 53 | M | 7.5 | 0 |
| A. F. | 22 | F | 7.3 | 0 |
| D. S. | 40 | M | 5.5 | 0 |
| K. L. | 26 | F | 5.6 | 1 |
| S. O. | 47 | F | 5.0 | 0 |
| J. W. | 23 | F | 10.4 | 0 |
| A. D. | 43 | M | 4.9 | 1 |
| T. H. | 36 | M | 6.2 | 0 |
| G. P. | 20 | M | 7.2 | 0 |
| L. S. | 19 | F | 6.9 | 0 |

Fig. 2

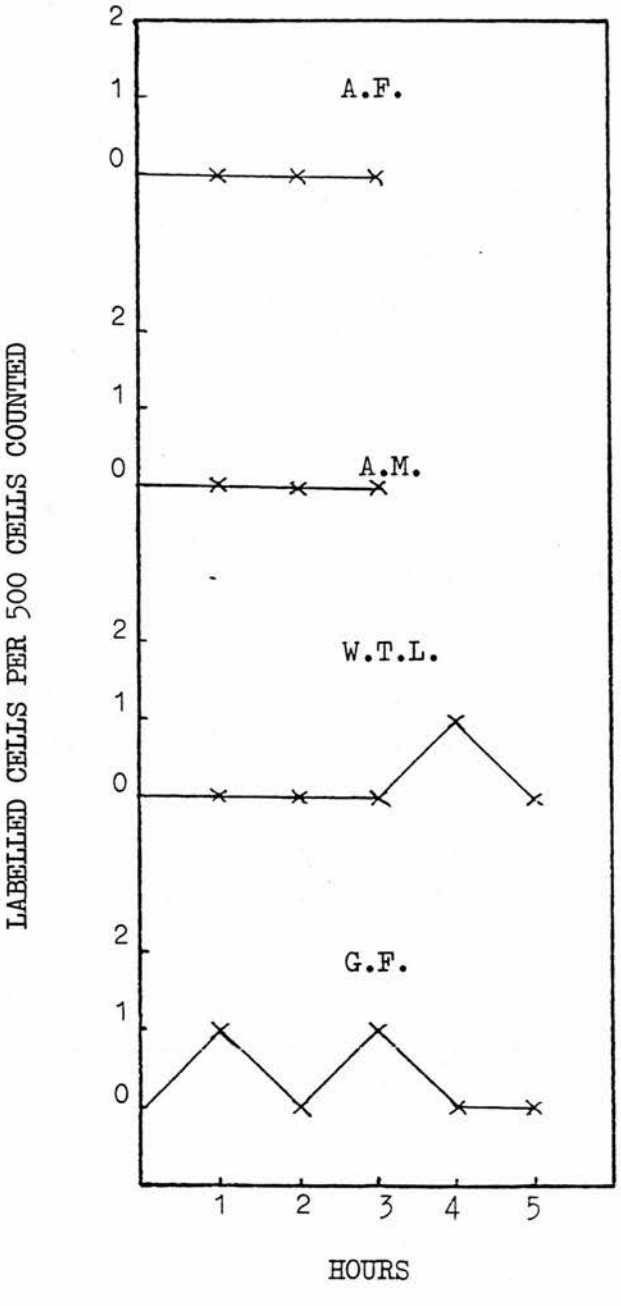
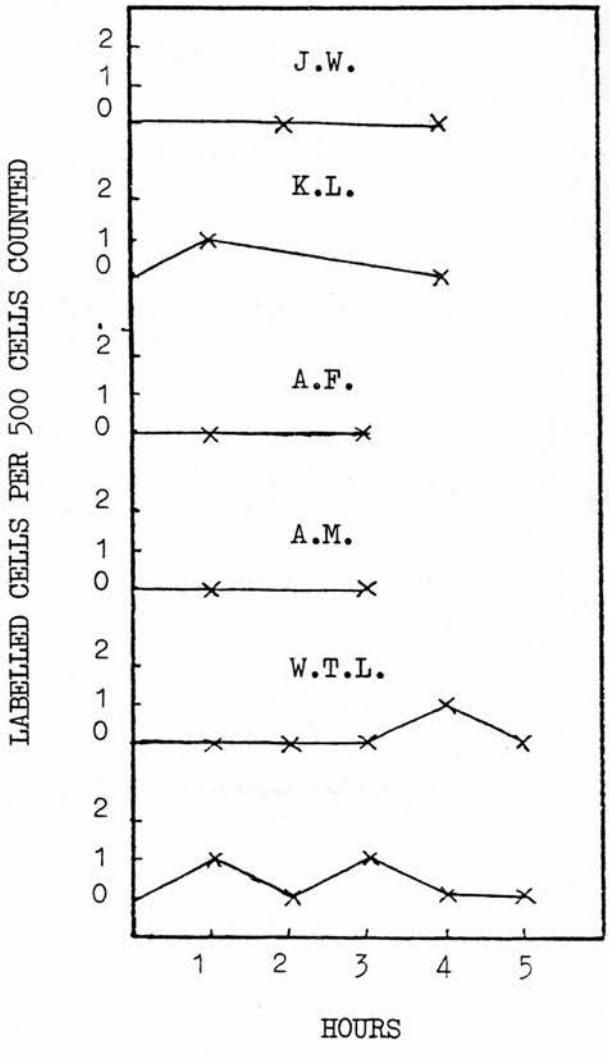



Fig. 3




Figs. 2 and 3 show the results of the sampling of 10 specimens during continuous incubation with $^{3}$H-TdR up to 5 hours.

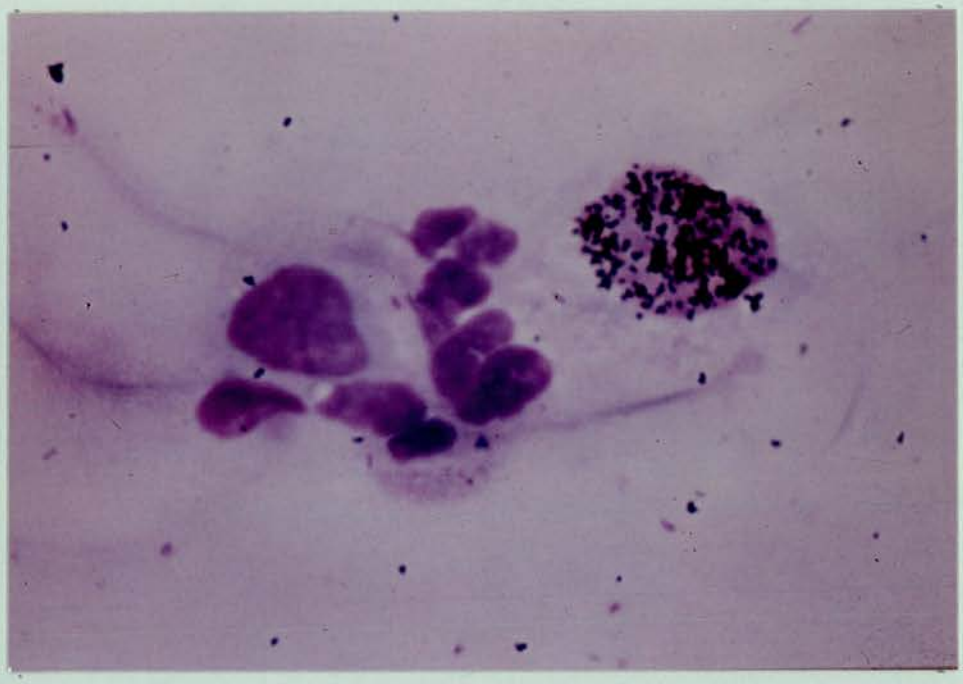


FIG. 4

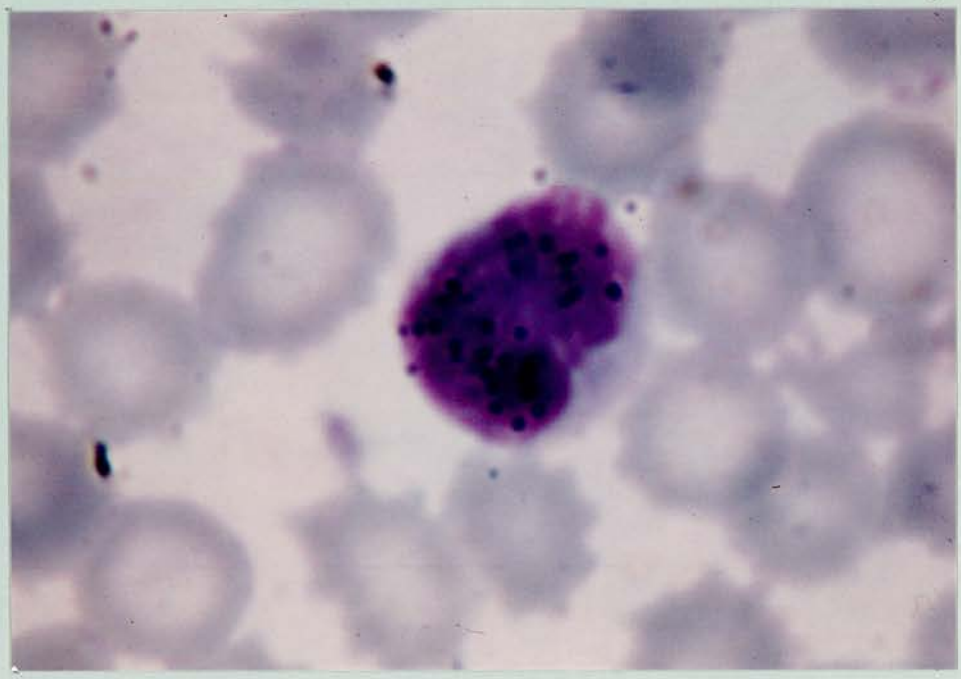


FIG. 5

## CONTROL SAMPLES

## DISCUSSION

The findings in this section suggest that a small number of DNA synthesizing cells can be found in the peripheral blood of a normal healthy population. In terms of the total white cell count the absolute number is probably larger, although it was also shown that no labelled cells were seen in 75% of the control samples examined. The results compare favourably with Bond et al (1958) and Rubini et al (1961). Both of these studies led to speculation that the cells which synthesize DNA in the circulation are part of the haemopoietic stem cell population. It was made apparent also that increased numbers of DNA synthesizing cells are present with various blood disorders. It is possible that these labelled cells do not necessarily have the same stimulus or function as those described in this particular study since the samples here are derived from a normal healthy population. It may be assumed therefore, that these circulating proliferative cells are present as part of a continual transient population which may be increased in number under more demanding situations of haemopoietic stress or may be triggered into a particular response under certain conditions or stimuli such as post irradiation, anaemia or, in the case of infection, an immunologically responsive role would be adopted. Thus, under such circumstances, the particular crisis involved may give rise, through a definite pathway, to DNA synthesizing cells of different appearances and function, Killmann (1968); Harris (1973).

## AUTORADIOGRAPHIC STUDY OF CORD BLOODS

It has been shown by numerous researchers (Winter et al, 1965; Knudtzon, 1974; Prindull et al, 1974, 1975 and 1978) using autoradiography and scintillation counting and also culture techniques, that there is a high proportion of cells in DNA synthesis in the blood of premature and full-term infants. An autoradiographical study was undertaken to demonstrate and describe circulating cells in spontaneous DNA synthesis in cord blood.

Umbilical cord blood specimens were obtained from 33 normal full-term infants, (15 by normal delivery and 18 by induction) and 3 premature infants (normal deliveries following 30 - 35 weeks gestational periods). Autoradiographical experiments were set up using Ilford Emulsion with an exposure time of 5 - 7 days. The films were then developed in Kodak D 19, fixed in Kodak Acid Fixer and stained with May Grunwald/ Giemsa. 500 nucleated cells were counted and differentiated from each film and the number and type of labelled cells was determined.

## RESULTS

The main types and numbers of labelled cells obtained in normal delivery and induction cord bloods are shown in tables III and IV respectively. The mean value of each cell type in both these groups is also recorded.

In 33 normal, full-term cord bloods, the mean total labelled cells estimated in a 500 nucleated cell count between the 2 main groups was $6.3 \pm 2.2$. Although this figure includes several cell types, the mean overall labelling index for the 33 samples was 1.26%. This is increased when compared to the normal control group. The labelled erythroblasts were differentiated, where the intensity of grains permitted, into basophilic and polychromatic cells according to their comparative size and morphological appearance. (Figs. 6 and 7). In each of the full-term groups approximately equal numbers of labelled basophilic and polychromatic erythroblasts were observed. There were 24 labelled basophilic and 22 polychromatic labelled erythroblasts counted in the normal delivery group and 28 basophilic and 23 polychromatic erythroblasts in the induction delivery group. The mean total erythroblasts of both groups was $2.9 \pm 2.0$.

Apart from smear (or damaged) cells, most films also contained myelocytes, some of which were labelled. (Fig. 8). In addition, 7 labelled cells were counted which defied classification due to the heavy labelling which obscured cellular detail. (Fig. 9). The remaining lymphoid cells have been classified as transitional or lymphoid cells by various workers, Yoffey (1974) and Prindull et al (1975). These transitional cells are similar in appearance to

lymphocytes but are generally larger and characterised by their high nuclear cytoplasmic ratio and paler staining nucleus (leptochromatic), (Fig. 10). The lymphoid cells have monocytoid features with occasional slightly basophilic cytoplasm. (Fig.11). Both of these cells have been classified as lymphoid in this particular study. In general, there appeared to be no marked difference in the labelling between both groups of full-term normal delivery and full-term induction delivery cord bloods. The mean total labelled cells in each group per 500 cell count was 6.2 and 6.4 respectively.

Cord bloods were also obtained from premature deliveries at 30 - 35 weeks gestation. (Table V). 3 specimens only were examined and the results compared favourably with the other groups. However, the small sample examined did not provide sufficient evidence as to the expected increase of labelled cells in cord blood of premature infants when compared to the full-term specimens. From the 3 specimens in the premature delivery cord bloods only 6 basophilic and 2 polychromatic labelled erythroblasts were recorded.

## CORD BLOOD LABELLED CELLS IN 500 CELL COUNT

TABLE III

| FULL-TERM NORMAL DELIVERY | TOTAL NUCLEATED CELL COUNT x $10^9$/L | LABELLED ERYTHROBLASTS | LABELLED MYELOCYTES | LABELLED LYMPHOID CELLS | LABELLED SMEAR CELLS | LABELLED UNCLASSIFIED CELLS | TOTAL LABELLED IN 500 CELL COUNT |
|---|---|---|---|---|---|---|---|
| CORD BLOOD 1 | 12.2 | 3 | 2 | 1 | 1 | 0 | 7 |
| CORD BLOOD 2 | 11.8 | 6 | 0 | 1 | 2 | 0 | 9 |
| CORD BLOOD 3 | 15.6 | 3 | 0 | 1 | 3 | 0 | 7 |
| CORD BLOOD 4 | 13.3 | 2 | 2 | 1 | 2 | 0 | 7 |
| CORD BLOOD 5 | 15.8 | 2 | 0 | 1 | 1 | 2 | 6 |
| CORD BLOOD 6 | 13.7 | 5 | 1 | 0 | 1 | 0 | 7 |
| CORD BLOOD 7 | 18.4 | 3 | 0 | 2 | 0 | 0 | 5 |
| CORD BLOOD 8 | 22.7 | 2 | 2 | 1 | 1 | 0 | 6 |
| CORD BLOOD 9 | 13.8 | 3 | 1 | 1 | 0 | 0 | 5 |
| CORD BLOOD 10 | 9.4 | 2 | 0 | 0 | 1 | 2 | 5 |

## CORD BLOOD LABELLED CELLS IN 500 CELL COUNT

TABLE III contd.

| FULL-TERM NORMAL DELIVERY | TOTAL NUCLEATED CELL COUNT x $10^{-9}$/L | LABELLED ERYTHROBLASTS | LABELLED MYELOCYTES | LABELLED LYMPHOID CELLS | LABELLED SMEAR CELLS | LABELLED UNCLASSIFIED CELLS | TOTAL LABELLED IN 500 CELL COUNT |
|---|---|---|---|---|---|---|---|
| CORD BLOOD 11 | 8.7 | 3 | 2 | 1 | 0 | 0 | 6 |
| CORD BLOOD 12 | 11.0 | 3 | 0 | 1 | 1 | 0 | 5 |
| CORD BLOOD 13 | 16.9 | 3 | 1 | 1 | 1 | 0 | 6 |
| CORD BLOOD 14 | 18.1 | 3 | 0 | 2 | 1 | 0 | 6 |
| CORD BLOOD 15 | 10.8 | 3 | 1 | 0 | 2 | 0 | 6 |
| MEAN VALUE OF CELL TYPE | | M = 3.06 | M = 0.66 | M = 0.93 | M = 1.13 | M = 0.26 | M = 6.2 |

## CORD BLOOD LABELLED CELLS IN 500 CELL COUNT

TABLE IV

| FULL-TERM INDUCTION | TOTAL NUCLEATED CELL COUNT x $10^{-9}$/L | LABELLED ERYTHROBLASTS | LABELLED MYELOCYTES | LABELLED LYMPHOID CELLS | LABELLED SMEAR CELLS | LABELLED UNCLASSIFIED CELLS | TOTAL LABELLED IN 500 CELL COUNT |
|---|---|---|---|---|---|---|---|
| CORD BLOOD 1 | 15.6 | 3 | 1 | 0 | 2 | 2 | 9 |
| CORD BLOOD 2 | 12.0 | 4 | 0 | 1 | 1 | 1 | 7 |
| CORD BLOOD 3 | 10.8 | 3 | 0 | 1 | 2 | 0 | 6 |
| CORD BLOOD 4 | 9.7 | 2 | 0 | 2 | 2 | 0 | 6 |
| CORD BLOOD 5 | 13.0 | 2 | 1 | 1 | 3 | 0 | 7 |
| CORD BLOOD 6 | 14.4 | 2 | 0 | 2 | 2 | 0 | 6 |
| CORD BLOOD 7 | 18.7 | 2 | 1 | 2 | 3 | 0 | 8 |
| CORD BLOOD 8 | 16.4 | 2 | 0 | 1 | 3 | 0 | 6 |
| CORD BLOOD 9 | 11.3 | 4 | 0 | 1 | 2 | 0 | 7 |
| CORD BLOOD 10 | 11.4 | 3 | 0 | 2 | 2 | 0 | 7 |

## CORD BLOOD LABELLED CELLS IN 500 CELL COUNT

TABLE IV contd.

| FULL-TERM INDUCTION | TOTAL NUCLEATED CELL COUNT x $10^{-9}$/L | LABELLED ERYTHROBLASTS | LABELLED MYELOCYTES | LABELLED LYMPHOID CELLS | LABELLED SMEAR CELLS | LABELLED UNCLASSIFIED CELLS | TOTAL LABELLED IN 500 CELL COUNT |
|---|---|---|---|---|---|---|---|
| CORD BLOOD 11 | 7.6 | 2 | 2 | 0 | 2 | 0 | 6 |
| CORD BLOOS 12 | 16.7 | 3 | 0 | 1 | 3 | 0 | 7 |
| CORD BLOOD 13 | 12.2 | 4 | 0 | 3 | 1 | 0 | 8 |
| CORD BLOOD 14 | 10.8 | 5 | 1 | 2 | 0 | 0 | 8 |
| CORD BLOOD 15 | 13.2 | 3 | 0 | 0 | 2 | 0 | 5 |
| CORD BLOOD 16 | 8.6 | 2 | 0 | 1 | 1 | 0 | 4 |
| CORD BLOOD 17 | 16.2 | 2 | 0 | 1 | 2 | 0 | 5 |
| CORD BLOOD 18 | 7.9 | 2 | 0 | 0 | 2 | 0 | 4 |
| MEAN VALUE OF CELL TYPE | | M = 2.77 | M = 0.33 | M = 1.16 | M = 1.9 | M = 0.16 | M = 6.4 |

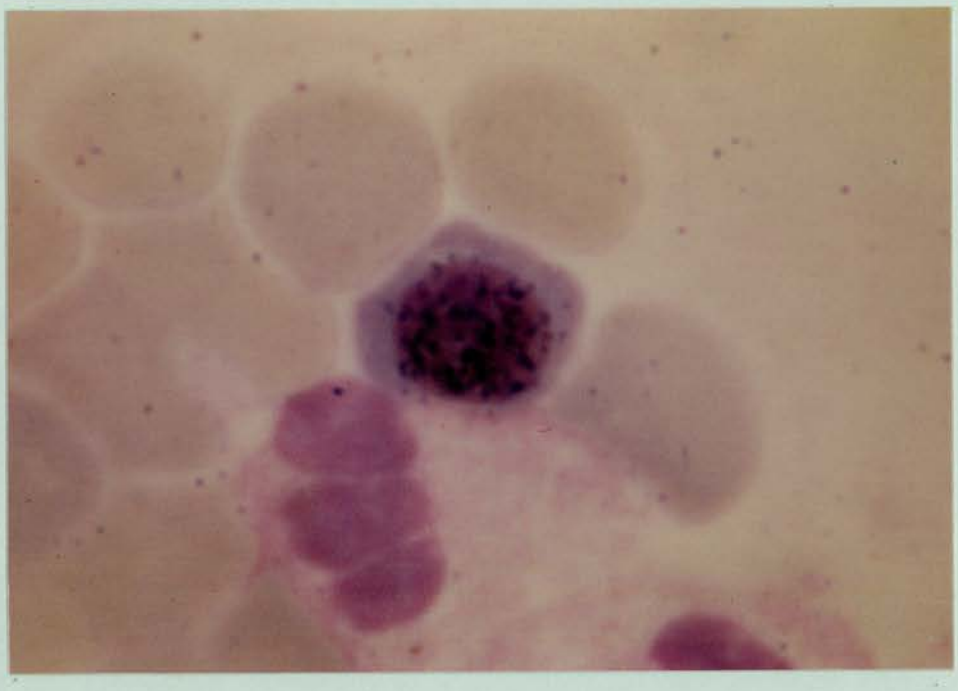


FIG. 6

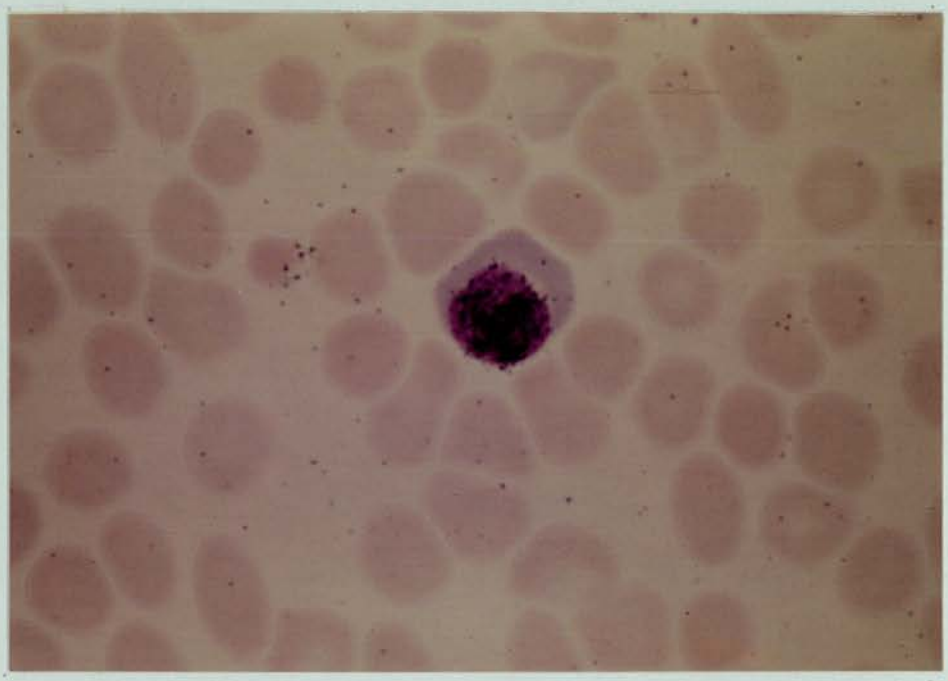


FIG. 7

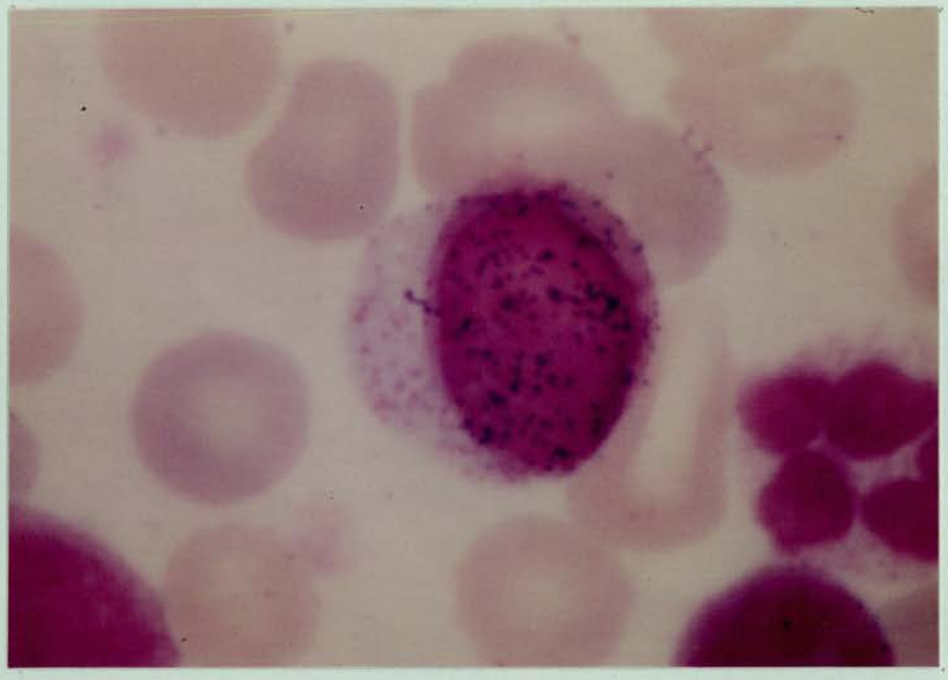


FIG. 8

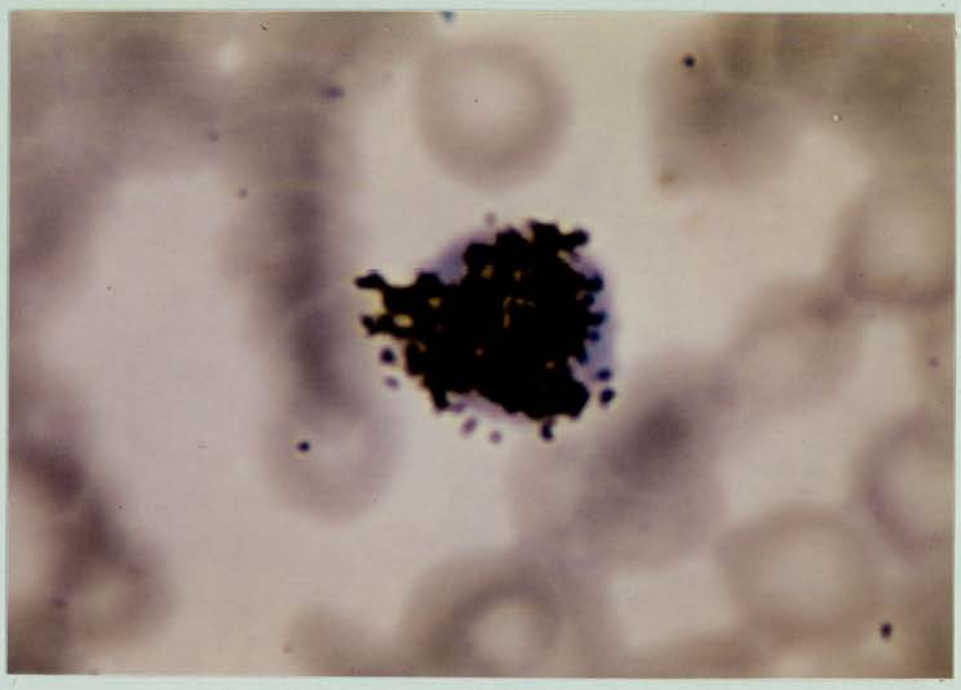


FIG. 9

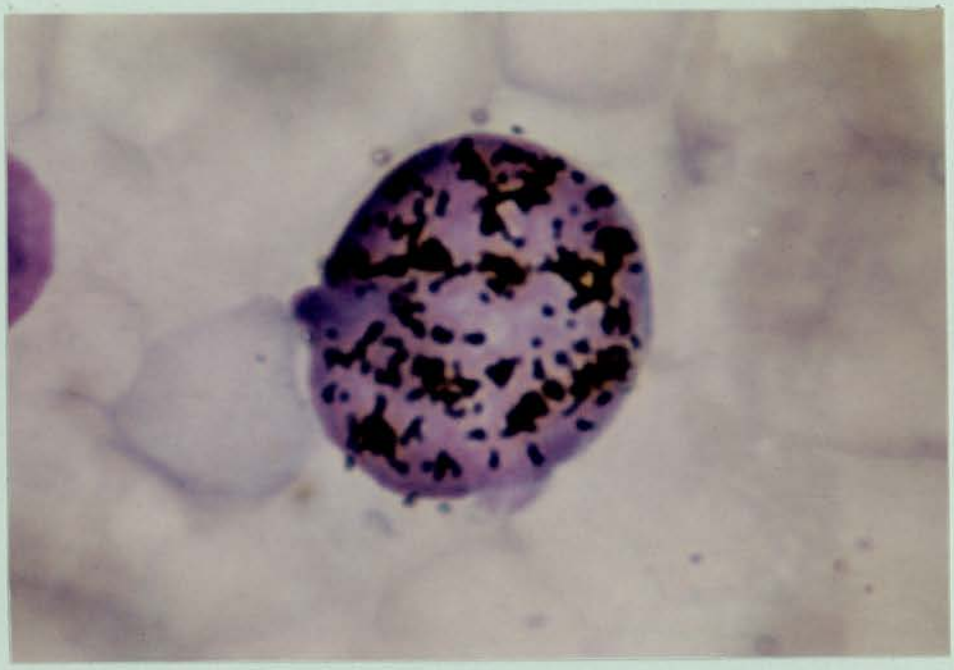


FIG. 10

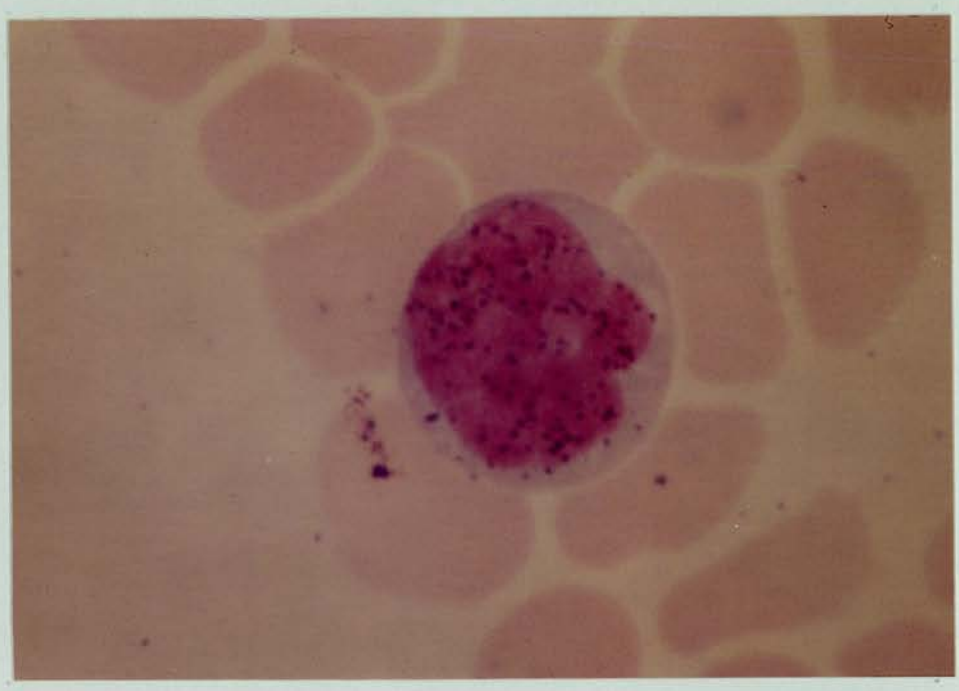


FIG. 11

## CORD BLOOD LABELLED CELLS IN 500 CELL COUNT

TABLE V

| PREMATURE DELIVERY | TOTAL NUCLEATED CELL COUNT x $10^{-9}$/L | LABELLED ERYTHROBLASTS | LABELLED MYELOCYTES | LABELLED LYMPHOID CELLS | LABELLED SMEAR CELLS | LABELLED UNCLASSIFIED CELLS | TOTAL LABELLED IN 500 CELL COUNT |
|---|---|---|---|---|---|---|---|
| CORD BLOOD 1 | 13.2 | 2 | 0 | 1 | 3 | 0 | 6 |
| CORD BLOOD 2 | 14.0 | 4 | 0 | 0 | 1 | 0 | 5 |
| CORD BLOOD 3 | 8.3 | 2 | 2 | 1 | 0 | 0 | 5 |
| MEAN VALUE OF CELL TYPE | | M = 2.66 | M = 0.66 | M = 0.66 | M = 1.33 | 0 | M = 5.33 |

## CORD BLOOD

## DISCUSSION

It has been shown by Winter et al (1965), Prindull et al (1975) and Prindull et al (1977) and (1978) that cells in spontaneous DNA synthesis are significantly greater in number in cord blood than in adult blood. The cells incorporating $^{3}$H-TdR include lymphoid or transitional cells, occasional myelocytes and erythroblasts. The in-vivo study of Bond et al (1959) recorded that basophilic and polychromatic erythroblasts were labelled within minutes of injection of $^{3}$H-TdR. Since orthochromatic erythroblasts are non-dividing cells, they do not label until division of a precursor cell. Bond et al (1959) established that this occurred at about 3 hours after injection and used this information, together with the appropriate DNA synthesis time to deduce the generation time of the polychromatic erythroblast. No labelled orthochromatic erythroblasts were seen in this present study since a 1 hour flash-labelling in-vitro technique was used. Apart from increased numbers of erythroid and myeloid precursors in DNA synthesis, in cord blood, in recent years it is the lymphoid or transitional cells which have attracted most interest, Yoffey (1974), Prindull et al (1975). The transitional cells form a range of sizes from the pachychromatic small lymphocyte to the large basophilic blast cell. The cytoplasm can vary in differing degrees of basophilia from pale to intensely basophilic. The cells observed in this study were classified simply as lymphoid because of the wide variation in morphology.

The number of lymphoid cells incorporating $^{3}$H-TdR reported in this present study do not compare with those of

Prindull et al (1975) and (1977) but different techniques utilising buffy coat concentrations of cells were used in his studies together with scintillation counting. Nevertheless, increased numbers of labelled lymphoid cells were observed, as compared to the normal control group.

Winter et al (1965) noted that in foetal life, virtually all of the circulating lymphocytes are of the transitional type with a high nuclear/cytoplasmic ratio and a leptochromatic nucleus. A higher labelling incidence of these cells had been expected from the premature infants but the small number of cord bloods obtained reduced the chance of significant analysis in this group.

In his study of 1974, Prindull discussed the possibility that the increased numbers of cells in DNA synthesis may be involved in immune reactions or may have a haemopoietic role. The observation was made that while the foetus was indeed capable of an immunological response, the more likely explanation was that since such a sustained lymphocyte production was apparent in all premature and full-term newborn infants, as well as in mid-foetal life, Winter et al (1965), then such cells may represent haemopoietic stem cells. Further evidence for this is provided from Knudtzon (1974) and Prindull et al (1978) where in-vitro colony-forming cells (CFUc) from cord blood, demonstrated by culture techniques, greatly exceeded those from adult blood. A similar conclusion as to their identity was reached when it was established that the most likely candidate for the CFUc was the transitional lymphocyte previously described. It is probable that the circulating CFUc in cord blood and foetal blood indicated an exchange or migration through the blood, between marrow, spleen or liver. Thus these cells in

the blood may reflect the level of activity or expansion of haemopoiesis in premature or full-term infants and which eventually reduce in numbers to normal adult levels as maturation proceeds.

## INFECTIOUS MONONUCLEOSIS (I.M.)

For the purpose of this study, 17 blood samples were obtained from 16 patients with infectious mononucleosis. Diagnosis was based on the following criteria:-

a) Clinical evidence commonly lymphadenopathy, fever, malaise, sore throat,

b) Characteristic appearance of peripheral blood film, lymphocytosis with the presence of atypical lymphocytes,

c) A positive serological test for I. M. using MONOSPOT (Ortho Diagnostics).

The specimens were obtained from 10 male and 6 female patients with an age range 14 - 33 years.

When the haematological values had been determined, the autoradiographic process was carried out as previously detailed. A 500 cell differential count was carried out on each film and the number of labelled cells established. The percentages of lymphocytes and atypical lymphocytes were estimated and the total numbers of each were calculated. The percentage of labelled atypical lymphocytes was determined from a count of 300 of such cells and this provided the labelling index, (L. I.%). The absolute numbers were also estimated. (Table VI.) No mitotic figures were seen in any preparation.

In order to estimate any change in the degree of labelling the following experiment was carried out.

A sample from case no. 15 was divided into 6 aliquots. The normal volume of $^{3}$H-TdR was added to each aliquot sequentially, every hour from zero time. Following incubation with $^{3}$H-TdR, films were made, processed and examined as previously

## INFECTIOUS MONONUCLEOSIS (I. M. )

TABLE VI

| CASE No. | AGE | SEX | WHITE BLOOD CELL COUNT x $10^{-9}$/L | LABELLED CELLS PER 500 CELLS COUNTED | LYMPHOCYTE COUNT[f] | | ATYPICAL LYMPHOCYTES | | LABELLED ATYPICAL LYMPHOCYTES | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | x $10^{-9}$/L | % | x $10^{-9}$/L | % | L.I. % | x $10^{-9}$/L |
| 1 | 24 | M | 10.9 | 10 | 4.4 | 41 | 2.1 | 20 | 8.0 | .168 |
| 2 | 18 | M | 5.8 | 7 | 1.5 | 26 | 3.1 | 54 | 9.0 | .279 |
| 3 | 18 | M | 17.8 | 3 | 5.3 | 30 | 8.9 | 50 | 7.0 | .623 |
| 4 | 20 | F | 5.9 | 5 | 3.1 | 53 | 0.7 | 12 | 3.0 | .021 |
| 5 | 19 | F | 13.3 | 7 | 4.1 | 31 | 5.8 | 44 | 10.0 | .580 |
| 6 | 19 | M | 9.6 | 14 | 3.3 | 35 | 1.5 | 16 | 8.0 | .120 |
| 7 | 21 | F | 7.8 | 3 | 4.3 | 55 | 0.4 | 5 | 1.0 | .004 |
| 8 | 25 | M | 13.0 | 18 | 5.2 | 40 | 6.5 | 50 | 10.0 | .650 |
| 9 | 14 | F | 6.5 | 8 | 3.4 | 53 | 1.3 | 21 | 6.0 | .078 |

TABLE VI contd.

| CASE No. | AGE | SEX | WHITE BLOOD CELL COUNT x $10^{-9}$/L | LABELLED CELLS PER 500 CELLS COUNTED | LYMPHOCYTE COUNT x $10^{-9}$/L | % | ATYPICAL LYMPHOCYTES X $10^{-9}$/L | % | LABELLED ATYPICAL LYMPHOCYTES L.I. % | x $10^{-9}$/L |
|---|---|---|---|---|---|---|---|---|---|---|
| 10 | 18 | M | 24.3 | 30 | 4.8 | 20 | 9.7 | 40 | 15.0 | 1.455 |
| 11 | 16 | F | 16.5 | 34 | 2.4 | 15 | 8.2 | 50 | 14.0 | 1.148 |
| 12 | 19 | M | 8.2 | 5 | 3.7 | 45 | 1.7 | 21 | 7.0 | .119 |
| 13 | 20 | F | 25.4 | 33 | 10.4 | 41 | 10.1 | 40 | 14.0 | 1.114 |
| 14 | 21 | M | 14.6 | 11 | 5.4 | 37 | 5.8 | 40 | 12.0 | .696 |
| 15 | 19 | M | 9.9 | 4 | 4.5 | 46 | 2.4 | 25 | 7.0 | .163 |
| 16 | 33 | M | 20.6 | 24 | 6.3 | 31 | 9.2 | 45 | 14.0 | 1.288 |
| 17 | 33 | M | 7.5 | 0 | 4.5 | 60 | 0.3 | 4 | 0 | 0 |
| MEAN VALUES | | | M = 12.8 | M = 12.7 | M = 4.5 | M = 38.7 | M = 4.5 | M = 31.5 | M = 8.0 | M = .518 |

detailed.

From figure 12 it can be seen that the LI fell over this period from 7% to 2%. It was also apparent that the intensity of the label became less with fewer grains present in the cells. (Figs. 13 and 14).

While the various criteria for the diagnosis of I.M. described above were fulfilled in all cases, the specimens were not always obtained when the patients were in the same stage of the illness. This is best illustrated by the second specimen obtained from the same patient, (case nos. 16 and 17) 4 weeks after the initial sample and in the convalescent phase. It can be seen that although the total white cell count has returned to normal limits, the lymphocytosis persists and the number of atypical lymphocytes and LI is greatly reduced. Similar examples of this can be seen in samples from case nos. 4 and 7.

In the acute phase of the illness there is a correlation between the total labelled cells and the total atypical lymphocyte count ($r = 0.95$); the total labelled cells and the total white cell count ($r = 0.94$) as shown in figure 15. There was also a correlation ($r = 0.93$) between the total white cell count and the total atypical lymphocytes as shown in figure 16. In the same figure the correlation between the total white cell count and the number of labelled cells per 500 cell count ($r = 0.78$) was not statistically significant.

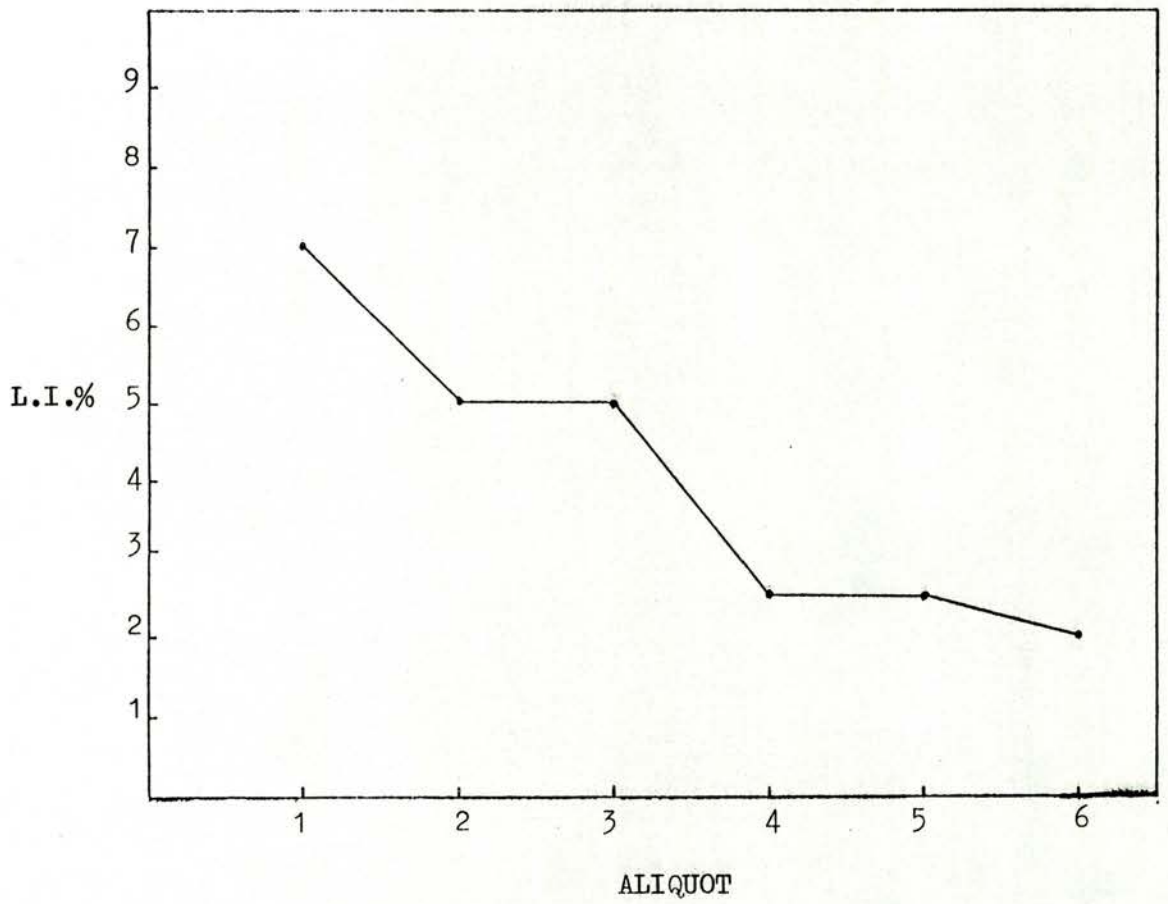



Fig. 12. The labelling index of each aliquot is shown following successive and separate incubations of 1 hour each up to 6 hours.

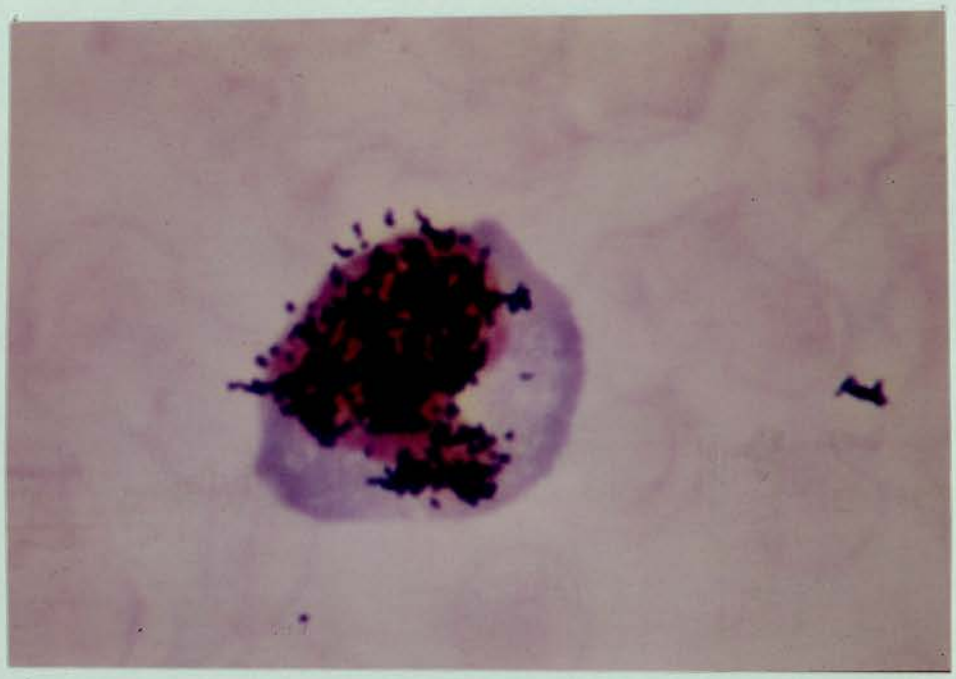


FIG. 13

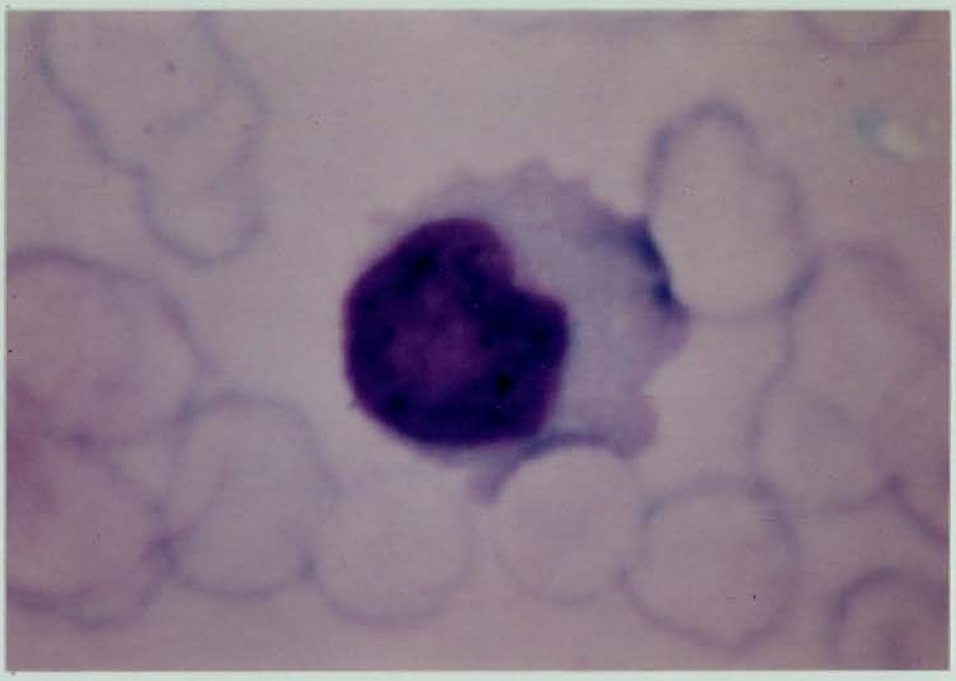


FIG. 14

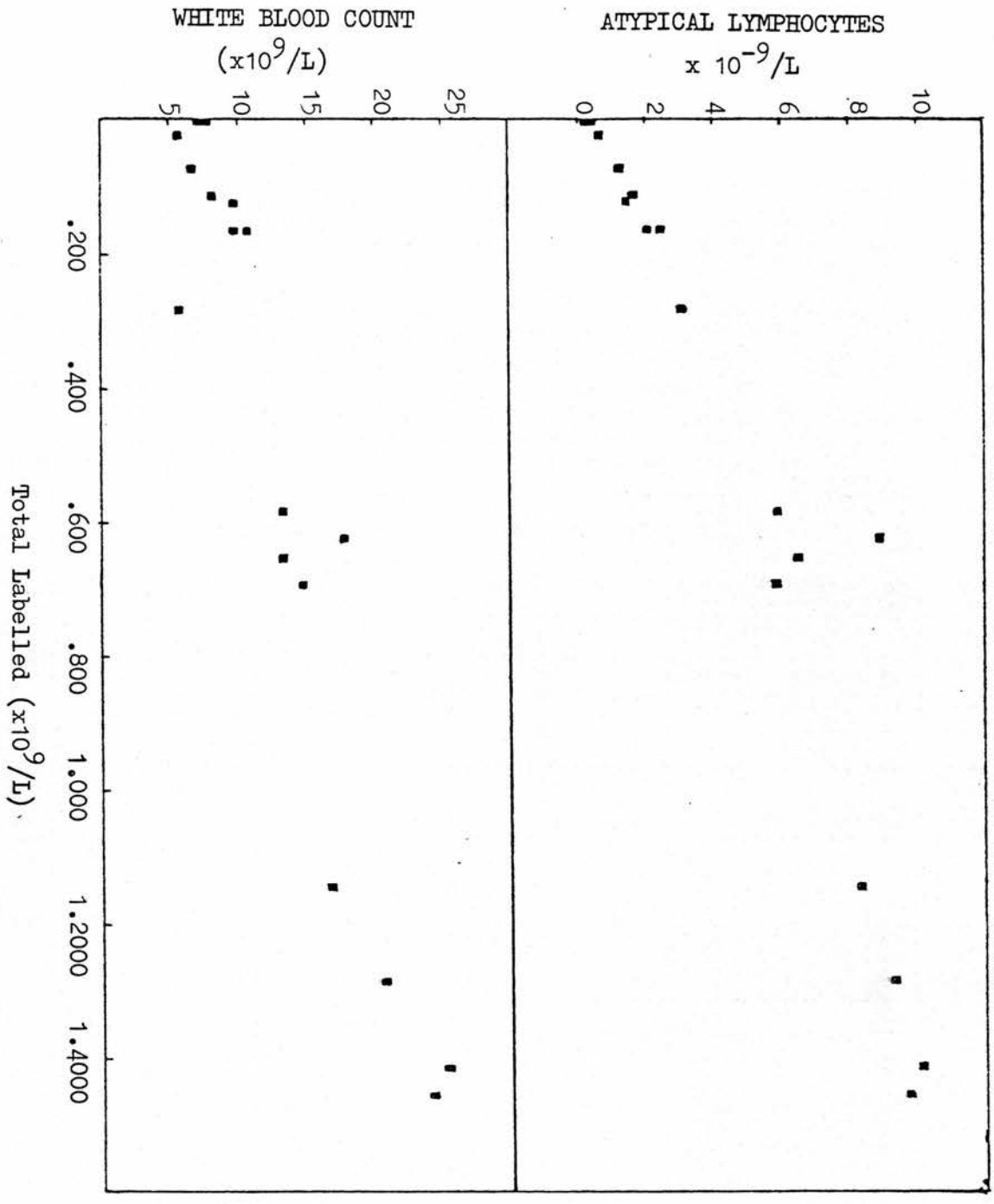



Fig. 15. Scattergram depicting the correlation of the total labelled cells and the total atypical lymphocytes ($r = 0.95$) and the total white cell count ($r = 0.94$)

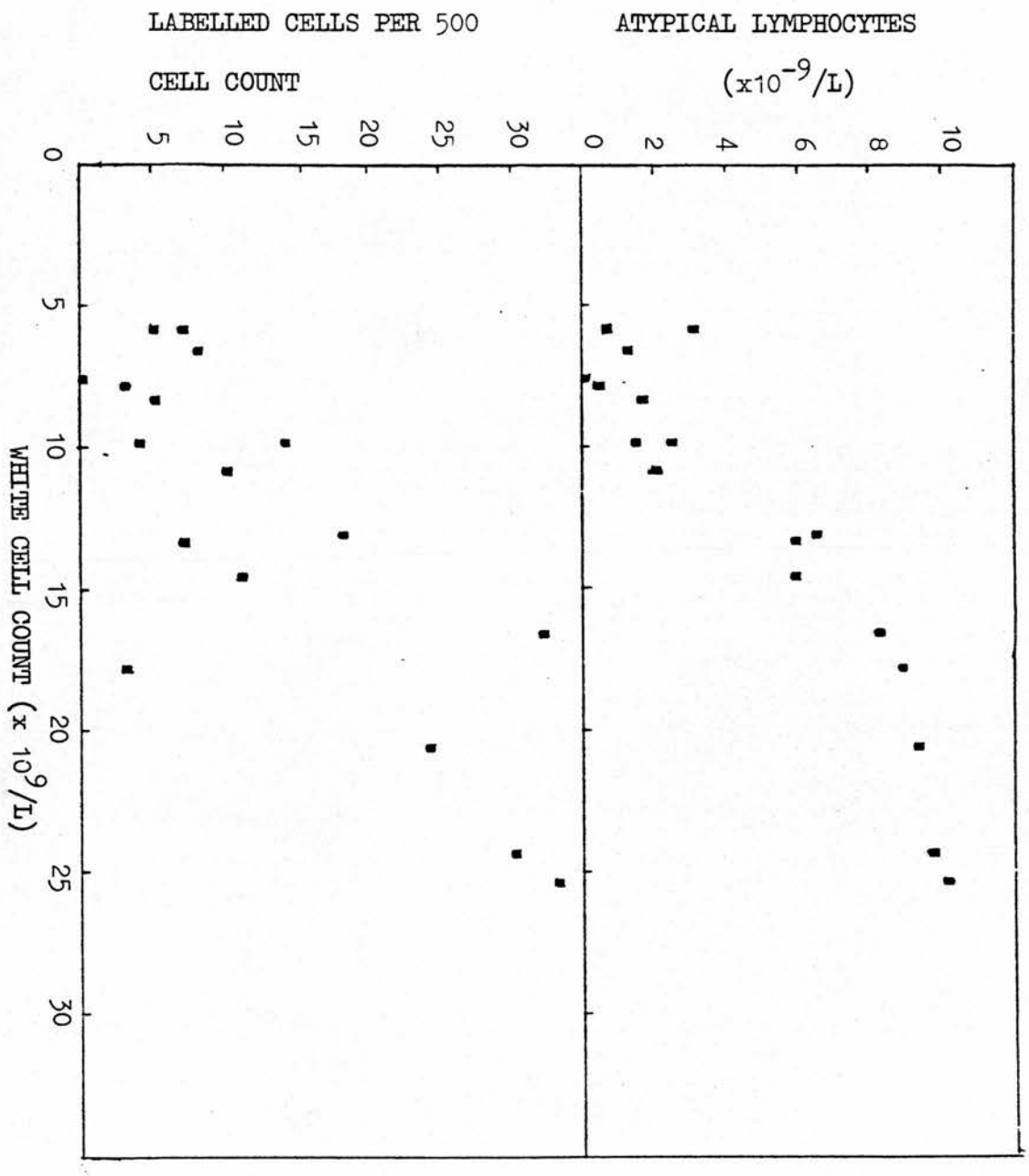



Fig. 16. Scattergram depicting correlation of the total white cell count with the total atypical lymphocytes (r=0.93) and the labelled cell count (r=0.78)

## INFECTIOUS MONONUCLEOSIS

### DISCUSSION

In the I. M. study, the results clearly show that a high proportion of cells synthesise DNA in the peripheral blood. This confirms earlier observations of Gavosto et al (1959); Hale and Cooper (1963); Epstein and Brecher (1965).

Schmid et al (1965) classified the atypical lymphocytes of I. M. into three types characterised by their morphology and the percentage of each in DNA synthesis. In this present study no attempt was made to categorise the atypical lymphocytes using the same criteria. The main reasons for this are that I. M. undergoes rapid changes from day to day and the total number of atypical lymphocytes together with the quantitative distribution of the three cell types, change continuously as the disease progresses. Furthermore, morphological classifications are of limited value in autoradiographical experiments because the cellular detail is often distorted and may even be obscured by the intensity of the labelling.

The experiment illustrated in figure 12 shows how the L. I. decreases over a period of time and the optimum results were achieved when the sample was processed within one hour. This important information must be kept in mind when specimens are not received immediately after withdrawal.

Only one patient - case no. 17 - was investigated after the acute phase of the illness. The results of this however, confirm the findings of most workers that the uptake of $^{3}$H-TdR is at its height during the acute phase and then falls to normal levels during the convalescent stage. From the similar results of case nos. 4 and 7 it can be assumed that they were

also in the stages of convalescence. This was statistically proven in that the higher the total atypical lymphocytes present then the higher was the total of labelled cells and similarly the higher the total white cell count then the higher was the total labelled cells. (Fig. 15). From figure 16 it can be seen that the higher the total white cell count then the higher was the total atypical lymphocytes present. However, the correlation between the total white cell count and the number of labelled cells per 500 cell count was suggestive but not conclusive.

The number of cells found in DNA synthesis in the peripheral blood of patients with I. M. (mean L. I. 8% range 1.0% - 15.0%) may be assumed to undergo mitosis but no mitotic figures were observed in any preparation. This finding is also in agreement with Gavosto et al (1959) and Schumacher et al (1967).

Several possible explanations have been presented for this discrepancy by Epstein and Brecher (1965). The most likely of which was that the cells in DNA synthesis divide only extravascularly. They did not rule out the possibility that the DNA synthesis observed may be viral rather than cellular DNA synthesis. A further explanation could be that the DNA synthesis time (S) takes many hours, whereas the mitotic duration can be measured in minutes and so a disparity will exist between the labelling index and the mitotic index. In addition, the possibility of a prolongation of the pre-mitotic resting phase ($G_2$) can produce the same effect.

Although the exact relationship between DNA synthesis and mitosis in atypical lymphocytosis is uncertain, Malacarne and Dallapiccola (1970) have demonstrated a marked increase in the number of mitoses in the peripheral blood of patients

with I. M. by direct chromosome examination, as compared to a group of healthy donors. This information provides further evidence of the mitotic ability and proliferative capacity of the lymphocytes in I. M.

The Epstein-Barr virus (EBV) is believed to be the cause of infectious mononucleosis, Henle et al (1979), Thomas et al (1980).

B lymphocytes become infected with EBV and this produces a host reaction of increased numbers of activated T lymphocytes directed against the EBV-infected cells. The proliferation of atypical lymphocytes coincides with the raised labelling index leading to the eventual control of the EBV transformed B lymphocyte population. It would appear that the atypical lymphocyte population is a combination of both activated T lymphocytes and EBV transformed B lymphocytes. The lymphocytosis, atypical lymphocytes and increase in DNA synthesis in the acute stage of the disorder, represents a primary reaction consisting of an initial and short-lived increase in B lymphocytes which are infected with EBV. This induces cellular DNA synthesis, Gerber and Hoyer (1971). T lymphocytes then undergo a larger and more sustained proliferative response which also gives rise to the appearances of atypical lymphocytes in the peripheral blood.

Since I. M. is recognised as a self-limiting lymphoproliferative disorder, in recent years interest has switched from the cause to the exact mechanism responsible for the control of the disease. In a recent study by Schooley et al (1981) a theory is presented in which suppressor T lymphocytes, which are developed <u>in-vitro</u> and are capable of inhibiting EBV-B lymphocytes outgrowth, may represent <u>in-vivo</u> an important

control mechanism of EBV-induced B lymphocyte proliferation in I. M. The observation is made that the capability for this inhibition resides within the non-atypical T lymphocyte population. Furthermore, the continuation of this facility years after the primary infection, is proof that it is not a feature of the atypical lymphocyte. These assertions are consistent with results in the present study. Case no. 16/17 in convalescence, has a persistent lymphocytosis of 60% but only 4% atypical lymphocytes. Similar results are evident with case nos. 4 and 7.

EBV is also closely associated with the malignant disorders of Burkitt's lymphoma and nasopharyngeal carcinoma, Henle et al (1979). Because I. M. is a self-limiting disorder, the development of a vaccine against EBV would appear at first to be unnecessary but it could prove valuable against these two malignancies and provide a greater insight into the other factors involved which makes these disorders progressive.

## HODGKIN'S DISEASE (H.D.)

### RESULTS

Specimens were obtained from 8 male and 5 female patients with Hodgkin's Disease. The age of these patients ranged from 17 - 77 years. 25 samples in total were taken and of these 4, 6, 2, 3 and 2 specimens were obtained from case no. 1, 2, 5, 6 and 8 respectively at varying dates. (Table VII). Haematological values were estimated from each of these specimens and the blood films, prepared after incubation with tritiated thymidine, were processed by auto-radiography as previously described. After exposure and subsequent development, the films were examined microscopically and the number of labelled cells recorded in each 500 cell count.

It has been established by numerous workers Crowther et al, 1967, Halie et al, 1974 that abnormal cells occur in the peripheral blood of patients with Hodgkin's Disease with an increase in the number of cells synthesising DNA. This study was undertaken to demonstrate these cells and to describe them where possible.

While it was not always possible to obtain specimens from untreated Hodgkin's patients, specimens were collected at various times during treatment and although the results did not reveal any obvious significance, the presence of similar labelled cells became apparent. The labelled cells recorded ranged from 0 - 7 per 500 cell count in 25 specimens. This produced a labelling index range of 0 - 1.4% and a mean value of 1.2 labelled cells per 500 cells counted. Generally, the cells which labelled in the preparations were of the lymphoid type approximately 15 - 20$\mu$ in diameter, as measured

with a microscope micrometer eyepiece, with basophilic cytoplasm (Fig. 17). Labelled smear cells, whose true identity can only be assumed, were found in a few preparations. Unclassified labelled cells were also noted and these were so described because the cellular detail was virtually obscured due to heavy labelling. These cells were smaller by comparison, approximately 10 -15$\mu$ in diameter.

Case no. 8 had a sharp increase in the number of cells synthesizing DNA in the peripheral blood and both specimens from this case were obtained after X-ray and Chemotherapy treatment. Among the 7 labelled cells counted from case no. 8 were 1 unclassified cell and 1 smear cell. No mitotic figures were seen in any of the preparations examined.

From the scattergram in Fig. 19 no correlation between white cell count and the number of labelled cells could be observed.

## HODGKIN'S DISEASE (H.D.)

Table VII

| CASE No. | AGE | SEX | DATE OF SPECIMEN | WHITE BLOOD CELL COUNT x $10^{-9}$/L | LABELLED CELLS PER 500 CELLS COUNTED | COMMENT |
|---|---|---|---|---|---|---|
| 1 | 46 | F | 17. 7.79 | 3.3 | 0 | Had X-ray treatment and Chemotherapy |
| 1 | 46 | F | 9.10.79 | 3.7 | 2 Lymphoid<br>2 Unclassified | Not on treatment. ? Recurrence |
| 1 | 46 | F | 4. 3.80 | 3.4 | 1 Lymphoid | On Antibiotics - Chest Infection |
| 1 | 46 | F | 20. 5.80 | 2.9 | 0 | |
| 2 | 54 | F | 14. 8.79 | 12.9 | 0 | Diagnosed 1968. X-ray treatment.<br>Progression of disease. Chemotherapy started. |
| 2 | 54 | F | 9.10.79 | 4.8 | 1 Lymphoid<br>2 Smear Cells | For Chemotherapy |
| 2 | 54 | F | 4.12.79 | 6.1 | 2 Lymphoid | Viral Infection. On Chemotherapy |
| 2 | 54 | F | 15. 1.80 | 5.5 | 0 | On Chemotherapy |
| 2 | 54 | F | 25. 6.80 | 5.8 | 0 | Pre-op assessment |
| 2 | 54 | F | 21.10.80 | 7.9 | 0 | |
| 3 | 53 | M | 11. 9.79 | 5.7 | 0 | Diagnosed in 1972. No recurrence |
| 4 | 23 | F | 4. 9.79 | 14.8 | 2 Lymphoid | Lymphocyte Depletion - Widespread Nodes |

## HODGKIN'S DISEASE (H.D.)

TABLE VII contd.

| CASE No. | AGE | SEX | DATE OF SPECIMEN | WHITE BLOOD CELL COUNT x $10^{-9}$/L | LABELLED CELLS PER 500 CELLS COUNTED | COMMENT |
|---|---|---|---|---|---|---|
| 5 | 29 | M | 13.11.79 | 4.5 | 0 | In remission since 1978 |
| 5 | 29 | M | 24. 4.80 | 4.4 | 2 Lymphoid<br>1 Smear Cell | Nodes. ? Recurrence |
| 6 | 30 | F | 12.12.79 | 16.8 | 0 | Nodular sclerosing type diagnosed |
| 6 | 30 | F | 15. 7.80 | 7.6 | 0 | Radiotherapy treatment |
| 6 | 30 | F | 21.10.80 | 14.3 | 0 | |
| 7 | 20 | M | 18.12.79 | 7.9 | 1 Unclassified<br>1 Smear Cell | Stage IIa H. D. In remission |
| 8 | 35 | F | 8. 1.80 | 10.3 | 7 Lymphoid | Stage II H. D. Completed Chemotherapy |
| 8 | 35 | F | 18. 3.80 | 5.5 | 2 Lymphoid<br>1 Smear Cell | Completed Radiotherapy |
| 9 | 20 | M | 8. 1.80 | 7.2 | 2 Lymphoid | In remission. Nodular sclerosing. Diagnosed in 1977. |
| 10 | 55 | M | 18. 3.80 | 5.9 | 2 Lymphoid | |
| 11 | 17 | M | 2. 5.80 | 1.0 | 0 | |
| 12 | 46 | M | 8. 7.80 | 7.9 | 1 Unclassified | |
| 13 | 77 | M | 26. 8.80 | 5.7 | 0 | Has X-ray treatment |

## NON-HODGKIN'S LYMPHOMA (N.H.L.)

### RESULTS

Specimens were obtained from 5 male and 6 female patients with Non-Hodgkin's Lymphoma whose ages ranged from 26 - 78 years. All patients were receiving treatment or had had treatment at various times. The specimens were processed and examined as described. The mean white cell count was $5.5 \times 10^9/L$. (Table VIII). The labelled cells recorded were lymphoid cells approximately 10 - 15μ in diameter with basophilic cytoplasm (Fig. 18) and the labelling index range was 0 - 0.4%. No mitotic figures were seen.

From Fig. 20 no correlation between the two variables is present although the limited number of samples available leaves the results inconclusive.

NON-HODGKIN'S LYMPHOMA (N.H.L.)

Table VIII

| CASE No. | SEX | AGE | WHITE BLOOD CELL COUNT x $10^{-9}$/L | LABELLED CELLS PER 500 CELLS COUNTED | COMMENT |
|---|---|---|---|---|---|
| 1 | F | 70 | 4.7 | 0 | Malignant Lymphoma. On Chemotherapy. |
| 2 | F | 39 | 1.5 | 0 | Nodular Lymphoma. On Chlorambucil. |
| 3 | F | 49 | 3.2 | 0 | Nodular Lymphoma. On Chlorambucil |
| 4 | M | 64 | 5.4 | 1 Lymphoid | Well differentiated lymphocytic lymphoma. On Chlorambucil. |
| 5 | M | 55 | 9.5 | 0 | |
| 6 | M | 60 | 3.8 | 0 | Nodular Lymphocytic Lymphoma. |
| 7 | M | 39 | 10.0 | 1 Lymphoid | Nodular Lymphocytic Lymphoma. |
| 8 | F | 51 | 4.5 | 0 | |
| 9 | F | 52 | 3.7 | 0 | |
| 10 | M | 78 | 8.1 | 2 Lymphoid | Lymphocytic Lymphoma. No treatment. |
| 11 | F | 26 | 6.7 | 2 Lymphoid | |

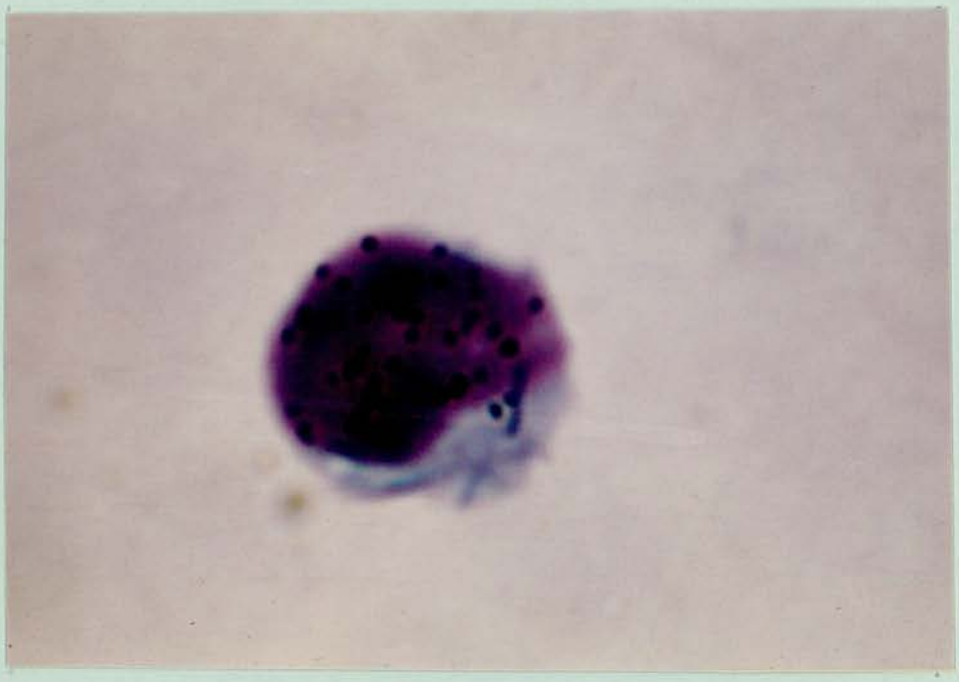


FIG. 17

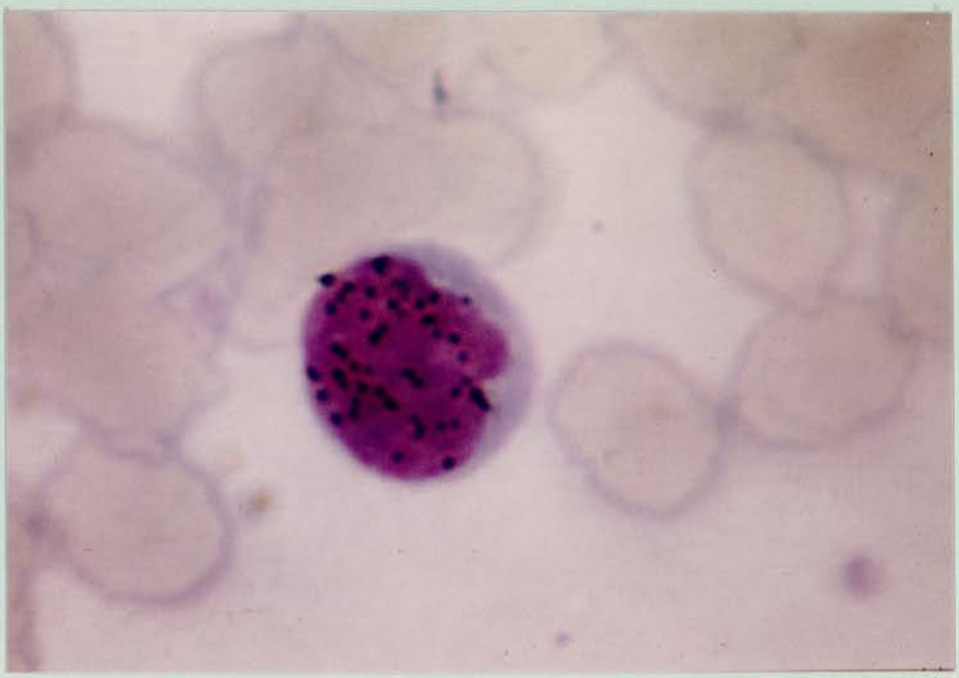


FIG. 18

Fig. 19 HODGKIN'S DISEASE

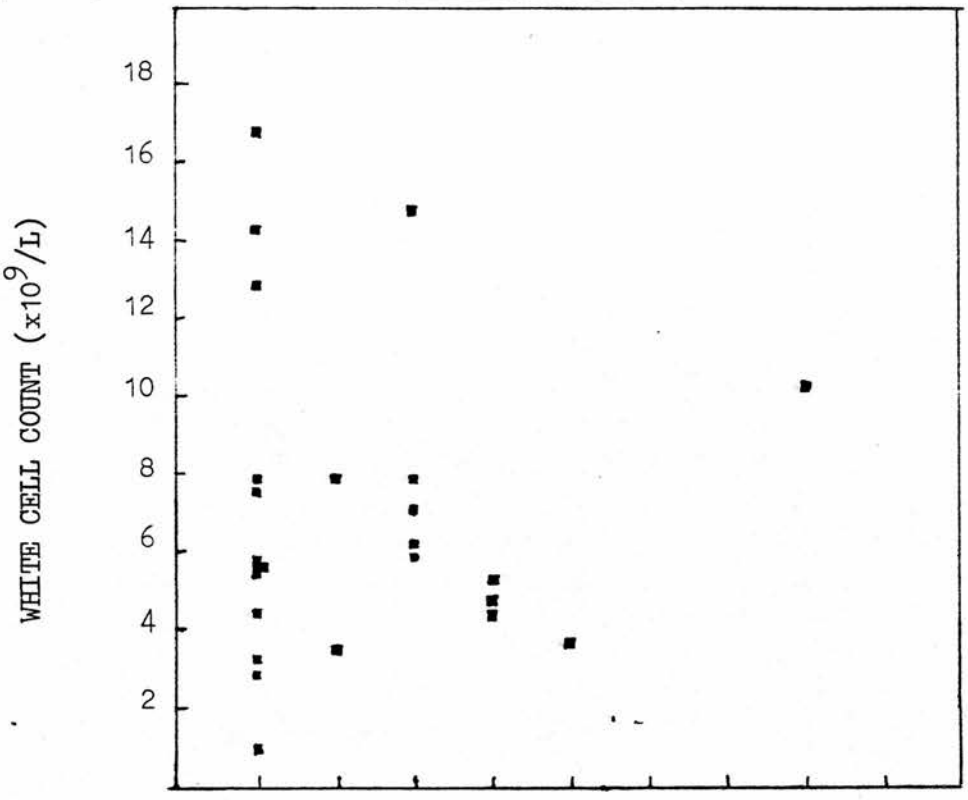
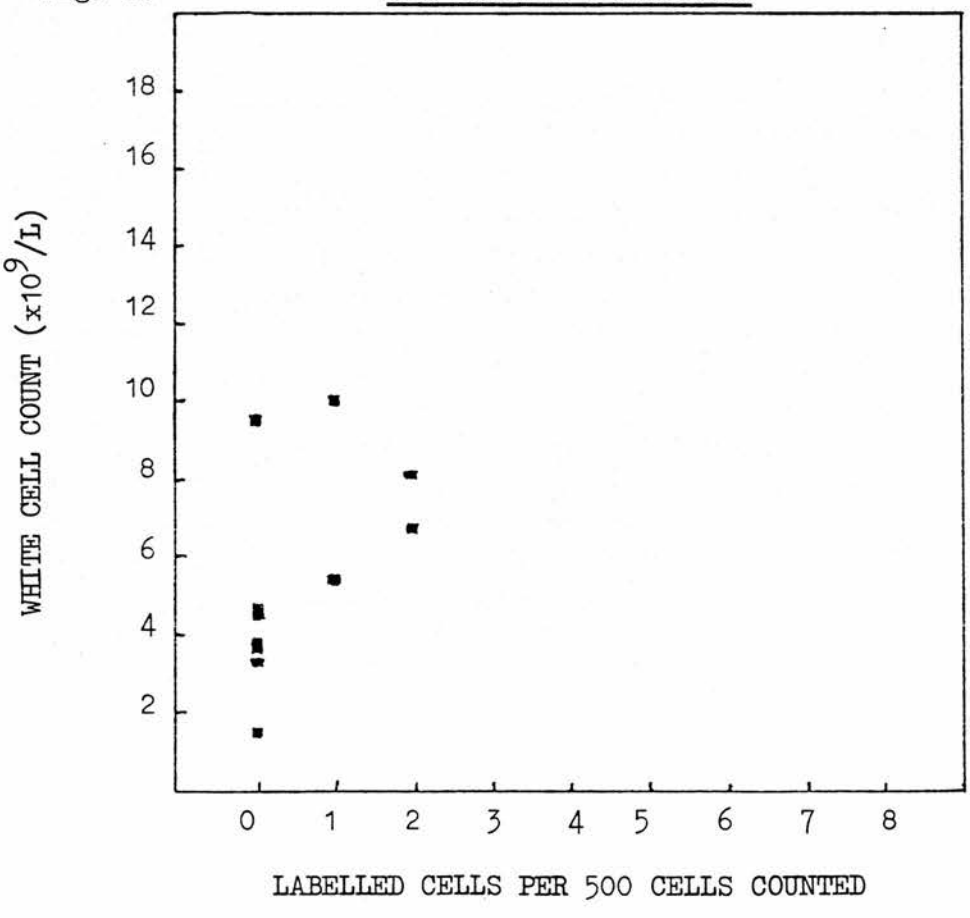



Fig. 20 NON-HODGKIN'S LYMPHOMA




Figs. 19 and 20 show the relationship of white cell count and labelled cells.

## HODGKIN'S DISEASE

### DISCUSSION

The number of cells in spontaneous DNA synthesis in this study was greatly increased as compared to the control group reported earlier. The finding is in agreement with Crowther et al (1967) and (1969). Virtually all of the cells incorporating $^{3}$H-TdR were large lymphoid cells as described by these workers and also by Halie et al (1974). (Fig. 17).

As can be seen from table VII, it is mainly those patients with an apparent recurrence of the disease who display an increase in the number of cells in DNA synthesis in the peripheral blood. Case no. 8 had the highest number of labelled cells recorded and following treatment, these numbers were reduced to near normal levels.

Crowther et al (1969) could not prove that there was a correlation between the stage of the disease and the percentage of these cells in the peripheral blood but did find a definite correlation as to the activity of the disease and their presence. This can be confirmed by the observations of this present study where response to therapy has not been entirely successful.

The presence of these cells may be due to a recurrence or dissemination of the disease or on the other hand may be a marrow response to chemotherapy or radiotherapy. The most likely and attractive explanation of the increased DNA synthesis in the lymphoid cells is that of an immunological reaction in response to the presence of the tumour. Since the DNA synthesizing cells are apparently reduced in remission of the disease, this may indicate a reduction in the tumour mass and thus a reduction in the resultant immunological response.

Therefore the presence of these cells should not be implicated in the dissemination of the disease but may rather be an indication of the disease activity whether before or during treatment.

In a recent study using liquid scintillation counting techniques involving low density lymphocytes from patients with Hodgkin's Disease, de Pauw et al (1980) observed a correlation between spontaneous DNA synthesis and the stage of the disease. This finding does not entirely contradict the work of Crowther et al (1969) since the disease activity within the various staging groups of Hodgkin's Disease reflects the amount of the tumour mass which in turn correlates with the level of spontaneous $^{3}$H-TdR uptake. The notion that the increased DNA synthesis in the lymphoid cells is the result of an immunological response to the Hodgkin's Disease process is further supported in the work of de Pauw et al (1980).

## NON-HODGKIN'S LYMPHOMA (NHL)

### DISCUSSION

Garret et al (1977) and (1979) recorded that abnormal lymphocytes do occur in the circulating blood of many untreated lymphomas at a relatively early stage in the disease.

The present study was undertaken to detect any cells in the peripheral blood of NHL patients which might be in DNA synthesis and to compare any findings with the conclusions of these workers which may have a bearing on the prognosis of the disease. The circulating blood lymphocytes in NHL were not always of obvious abnormal morphology, by routine examination of a peripheral blood film, as compared to those in some cases of Hodgkin's disease. No marked increase in labelled cells was found and the labelling index range of 0 - 0.4% compared favourably with that of the control group studied earlier. The labelled cells were similarly lymphoid in nature. (Fig. 18).

No particular significance could be placed on the presence of these occasional cells in the peripheral blood of lymphoma patients and taking into account available clinical information, no pattern of results emerged.

Since NHL consists of a well defined heterogenous group, the particular study reported here can only be accepted as a limited sample of the various types of disorder. In view of this a more comprehensive autoradiographical study of new untreated lymphomas should be undertaken involving bone marrow as well as peripheral blood investigation.

The findings of increased numbers of DNA synthesizing cells may provide an indication as to the further dissemination of the disease and would be of obvious value with regard to prognosis.

## CHRONIC LYMPHOCYTIC LEUKAEMIA (CLL)

### RESULTS

A total of 36 specimens were obtained from 14 cases of chronic lymphocytic leukaemia (CLL). These consisted of 9 male and 5 female patients and they ranged in age from 56 - 86 years (mean 71 years).

Single specimens were obtained from 6 patients only and a series of specimens ranging from 2 to 6 were obtained from the remaining patients at varying times. As can be observed from table IX the patients were at various stages of treatment and management throughout the period of study. The mean total white cell count and lymphocyte count for all specimens examined was 62.8 x $10^9$/L and 56.9 x $10^9$/L respectively. Lymphocytes constituted a mean of 85% of the circulating white cells in all specimens investigated. Virtually all of the labelled cells appeared lymphoid in nature and were both larger (10 - 15$\mu$ in diameter) and had more abundant cytoplasm than is normally associated with the small mature appearing lymphocyte predominant in CLL. (Fig 21). No mitotic figures were seen in any preparation nor were any labelled smear cells seen.

## CHRONIC LYMPHOCYTIC LEUKAEMIA

TABLE IX

| CASE No. | AGE | SEX | DATE | WHITE CELL COUNT x $10^{-9}$/L | LYMPHOCYTE COUNT x $10^{-9}$/L | % | LABELLED CELLS PER 500 CELL COUNT | COMMENTS |
|---|---|---|---|---|---|---|---|---|
| 1 | 70 | M | 26. 5.78 | 9.7 | 7.0 | 73 | 0 | On Chlorambucil |
| 2 | 60 | M | 7. 6.79 | 19.0 | 15.5 | 82 | 0 | |
| 3 | 83 | F | 13. 6.79 | 35.4 | 30.8 | 88 | 0 | Not on treatment |
| " | " | " | 12.12.79 | 76.0 | 72.2 | 95 | 0 | Not on treatment |
| " | " | " | 13. 2.80 | 28.8 | 25.5 | 88 | 0 | On Cyclophosphamide and Prednisolone |
| " | " | " | 30. 4.80 | 37.8 | 32.1 | 87 | 0 | Not on treatment |
| 4 | 86 | F | 4. 7.79 | 4.2 | 3.6 | 87 | 0 | Not on treatment |
| " | " | " | 3.10.79 | 7.5 | 5.7 | 77 | 0 | Not on treatment |
| " | " | " | 26. 3.80 | 22.9 | 21.8 | 95 | 0 | Not on treatment. Lymphadenopathy recurring. |
| 5 | 56 | M | 5. 7.79 | 55.4 | 53.7 | 97 | 0 | Pre-transfusion |
| " | " | " | 18. 3.80 | 44.8 | 53.9 | 98 | 0 | |
| " | " | " | 24. 6.80 | 157.0 | 133.4 | 85 | 0 | |
| " | " | " | 14.10.80 | 28.2 | 20.6 | 76 | 0 | |

## CHRONIC LYMPHOCYTIC LEUKAEMIA

TABLE IX contd.

| CASE No. | AGE | SEX | DATE | WHITE CELL COUNT x $10^{-9}$/L | LYMPHOCYTE COUNT x $10^{-9}$/L | % | LABELLED CELLS PER 500 CELL COUNT | COMMENTS |
|---|---|---|---|---|---|---|---|---|
| 6 | 68 | M | 22. 8.79 | 176.0 | 172.4 | 98 | 0 | In relapse. Not on treatment. |
| " | " | " | 10.10.79 | 7.9 | 3.9 | 50 | 3 Lymphoid | On Prednisolone |
| " | " | " | 21.11.79 | 24.2 | 20.3 | 84 | 2 Lymphoid | Not on treatment |
| " | " | " | 13. 2.80 | 33.6 | 26.2 | 78 | 0 | On Chlorambucil |
| " | " | " | 30. 4.80 | 23.5 | 18.8 | 80 | 0 | On Cyclophosphamide and Prednisolone |
| 7 | 66 | M | 1.11.79 | 20.2 | 15.9 | 79 | 0 | |
| " | " | " | 8. 9.80 | 24.2 | 16.2 | 67 | 0 | |
| 8 | 61 | F | 5. 3.80 | 20.9 | 18.3 | 88 | 3 Lymphoid | |
| " | " | " | 14.10.80 | 2.6 | 2.3 | 91 | 0 | Post Transfusion |
| 9 | 86 | F | 30. 5.80 | 63.5 | 55.8 | 88 | 0 | |
| " | " | " | 27.10.80 | 137.0 | 123.3 | 90 | 0 | Post Transfusion |
| " | " | " | 30.10.80 | 152.0 | 144.4 | 95 | 0 | On Steroids |
| " | " | " | 31.10.80 | 191.0 | 168.0 | 88 | 0 | On Prednisolone |

## CHRONIC LYMPHOCYTIC LEUKAEMIA

Table IX contd.

| CASE No. | AGE | SEX | DATE | WHITE CELL COUNT x $10^{-9}$/L | LYMPHOCYTE COUNT x $10^{-9}$/L | % | LABELLED CELLS PER 500 CELL COUNT | COMMENTS |
|---|---|---|---|---|---|---|---|---|
| 10 | 73 | M | 21.10.80 | 159.0 | 141.5 | 85 | 0 | New case |
| " | " | " | 28.10.80 | 143.0 | 140.1 | 98 | 0 | Post transfusion |
| " | " | " | 29.10.80 | 140.0 | 137.2 | 98 | 0 | On Chlorambucil |
| " | " | " | 30.10.80 | 143.0 | 138.7 | 97 | 0 | On Chlorambucil |
| " | " | " | 31.10.80 | 147.0 | 126.4 | 86 | 0 | Post transfusion. On Chlorambucil. |
| " | " | " | 9.12.80 | 9.6 | 8.6 | 90 | 0 | Post transfusion |
| 11 | 80 | M | 21.10.80 | 8.8 | 6.8 | 78 | 1 Lymphoid | On Chlorambucil |
| 12 | 80 | F | 5.11.80 | 43.8 | 32.4 | 74 | 0 | New case. Post-op hernia. |
| 13 | 60 | M | 4. 9.80 | 58.1 | 50.0 | 90 | 0 | Lymphadenopathy, neck, groins, axillae. New case. |
| 14 | 69 | M | 14.10.80 | 7.0 | 6.2 | 89 | 0 | Post transfusion |

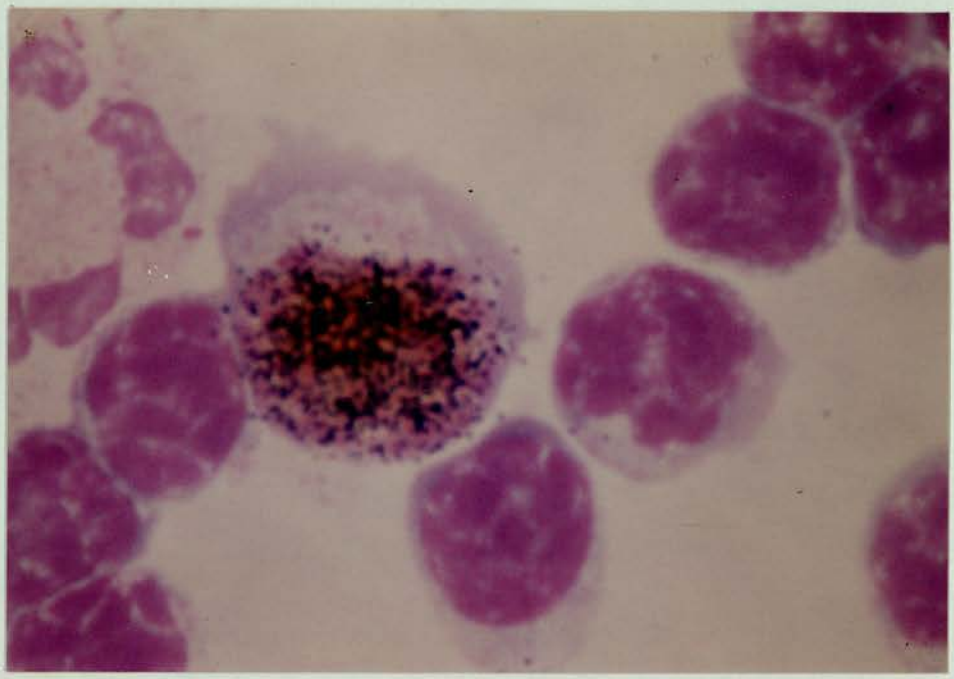


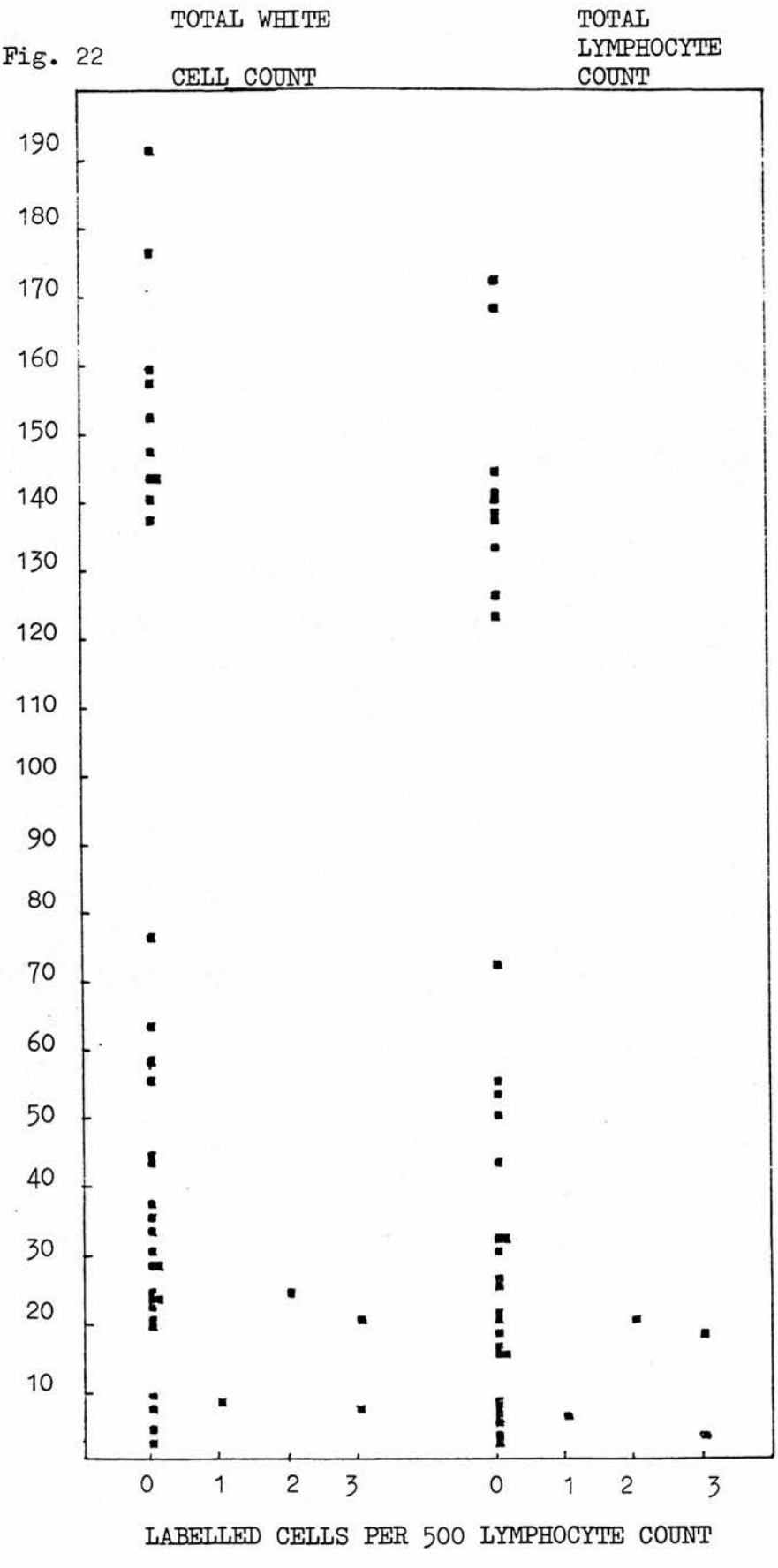
FIG. 21

Fig. 22 illustrates the comparison of the number of labelled cells to both the total white cell count and the total lymphocyte count.

Specimens were obtained from 3 new cases; nos. 10, 12 and 13 respectively at presentation, before any treatment had been commenced. In addition, samples from case no. 10 were studied successively over the first 5 days of treatment which included blood transfusion and chlorambucil chemotherapy. The specimens obtained from case nos. 3 and 6 on 13.2.80 were both incubated at $37^{\circ}C$ for 6 hours continuously after the addition of $^{3}H$-TdR. Films were fixed and made after each hour. Following the autoradiographical process, a total of 500 lymphocytes were counted in each film and the number of labelled cells recorded. No labelled cells were seen in any of the films from each of the new cases nor were any labelled cells seen in any of the films made from the specimens incubated from 1 to 6 hours. (Fig. 23).

Of the 36 samples examined, the L.I. ranged from 0 to 0.6% (mean = 0.25%). The labelled cells recorded were from specimens of which the highest white cell count was $24.2 \times 10^{9}/L$ and the lowest was $7.9 \times 10^{9}/L$. No labelled cells were seen in any of the remaining samples with total white cell counts reaching $190 \times 10^{9}/L$. This is also clearly illustrated in Fig. 22.

Fig. 22


TOTAL WHITE CELL COUNT
TOTAL LYMPHOCYTE COUNT
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0 1 2 3
0 1 2 3
LABELLED CELLS PER 500 LYMPHOCYTE COUNT

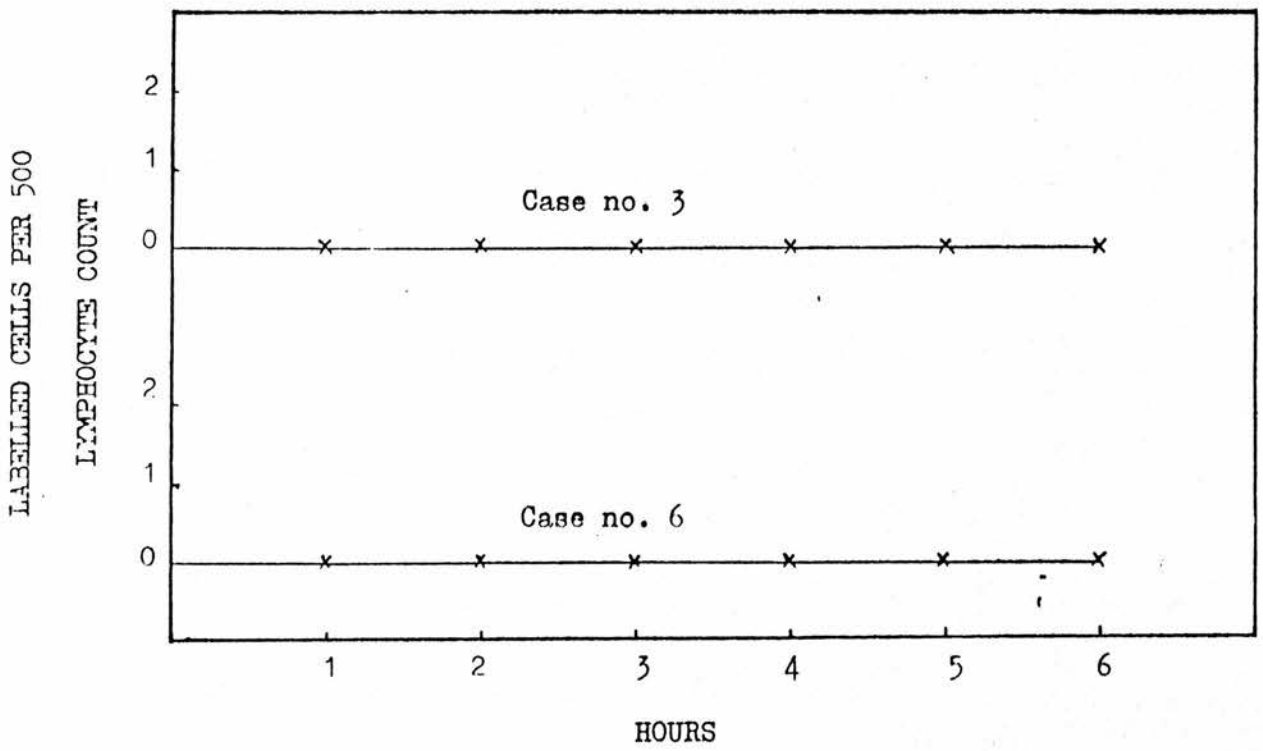



Fig. 23. Both specimens were incubated with $^{3}$H-TdR and sampled over 6 hours. No labelled cells were observed.

## CHRONIC LYMPHOCYTIC LEUKAEMIA

### DISCUSSION

The labelling index range of 0 - 0.6% found in the study is consistent with that of the in-vitro results of Rubini et al (1961) and Theml et al (1973).

The labelled cells (Fig. 21) did not have the appearance of the typical mature appearing small lymphocyte of CLL but were larger atypical mononuclear cells. Similar cells were present and unlabelled in other preparations examined. It is not known whether their presence has any prognostic significance. Although Rudders (1976) described two similar sub-populations of CLL cells and demonstrated that the patients in whom the CLL cells were of the atypical variety were more symptomatic and had greater organ infiltration, this could not be confirmed in this present study.

It is noticeable that in the majority of cases where the total white cell count exceeded 20 x $10^9$/L, no labelled cells were seen. In the in-vivo study of Zimmerman et al (1968) a possible explanation was provided for this occurrence. It was suggested that a decrease in the labelling indicated that the population consisted almost entirely of longer-lived and non proliferating cells and that this inevitably leads to organ enlargement, in this group, as the lymphocyte count rises. This organomegaly in CLL is the result of the progressive accumulation of these mature appearing and long-lived lymphocytes as has also been described by Dameshek (1967).

It is apparent that the large numbers of cells that will not label with $^3$H-TdR are in a prolonged Go phase of the cycle

or are end stage cells. The labelling index range together with a large increase in the actual numbers of lymphocytes means that despite the presence of a large number of cells that do not label, the actual number of cells in DNA synthesis at this given time is still quite high in CLL. From fig. 23 it is shown that no labelled cells were detected after 6 hours continuous incubation with $^{3}$H-TdR and although the in-vitro conditions may not have been conducive for DNA synthesis, it may have provided further evidence of the apparent inert nature of the lymphocytes in CLL.

Theml et al (1973) in an in-vivo study using autoradiography, produced results which were similar to Zimmerman et al (1968), in so far as they established the presence of a small short-lived proliferating population of lymphocytes and a larger long-lived non-proliferating population. Both of these studies used autoradiography following in-vivo injection of $^{3}$H-TdR.

Zimmerman et al (1968) combined this technique with liquid scintillation counting and this provided complimentary evidence in the study of CLL kinetics.

In the work of Lopez-Sandoval et al (1974) the in-vitro uptake of $^{3}$H-TdR was determined by liquid scintillation counting only without the application of autoradiographical techniques. The former method alone does not allow for the equally important information of cell population analysis obtained from cell identification which is provided by the latter technique. It would have been of further value therefore if autoradiography had been undertaken simultaneously in the study and the proliferating cell population

## ACUTE LEUKAEMIA

For the purpose of this study venous blood was obtained from 6 cases of acute lymphoblastic leukaemia (ALL) and 7 cases of acute myeloblastic leukaemia (AML). The ALL patients included 3 male adults, 2 male children and 1 female child and the AML patients included 4 male and 3 female adults. All specimens were processed autoradiographically and where the blast cell count permitted, 500 blast cells were counted including labelled cells and the result expressed as percentage labelling index (LI%).

Sizing of blast cells was performed using a microscope micrometer eyepiece. Blast cells with a diameter of 10 - 12 $\mu$ were considered small. Large blasts had a diameter of 13 $\mu$ or greater. From a blast cell count of 200 cells, measurements were made and the percentage of small and large blasts was estimated. Photographs of labelled blast cells in ALL and AML are shown in figs. 24, 25 and 26 respectively.

Films from an ALL specimen were made and fixed every 30 minutes while the specimen was incubated at 37°C for 4 hours with $^{3}$H-TdR. A similar experiment was carried out with 2 AML specimens at varying times during an incubation time of 20 hours. The LI% was determined from each film.

6 specimens were also obtained sequentially from the same AML patient over a period of 5 weeks from presentation, during therapy and prior to death. In addition, 2 specimens were obtained from another AML patient pre-treatment and 4 day post-treatment. The LI and cell diameters were determined as before.

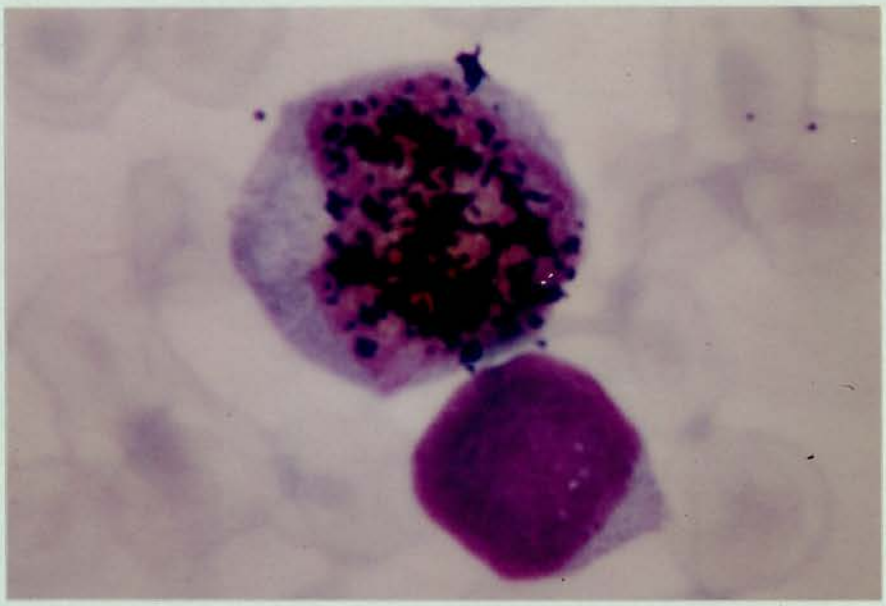


FIG. 24

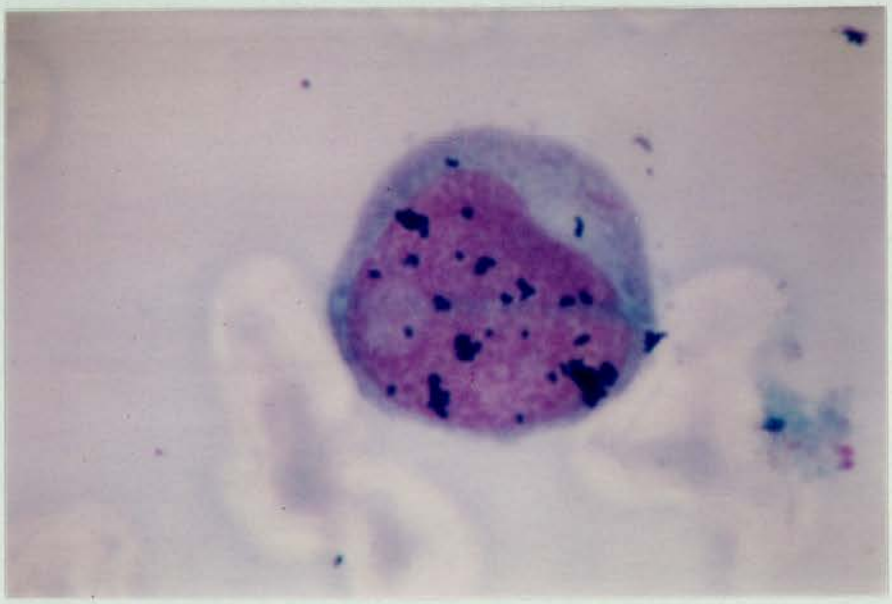


FIG. 25

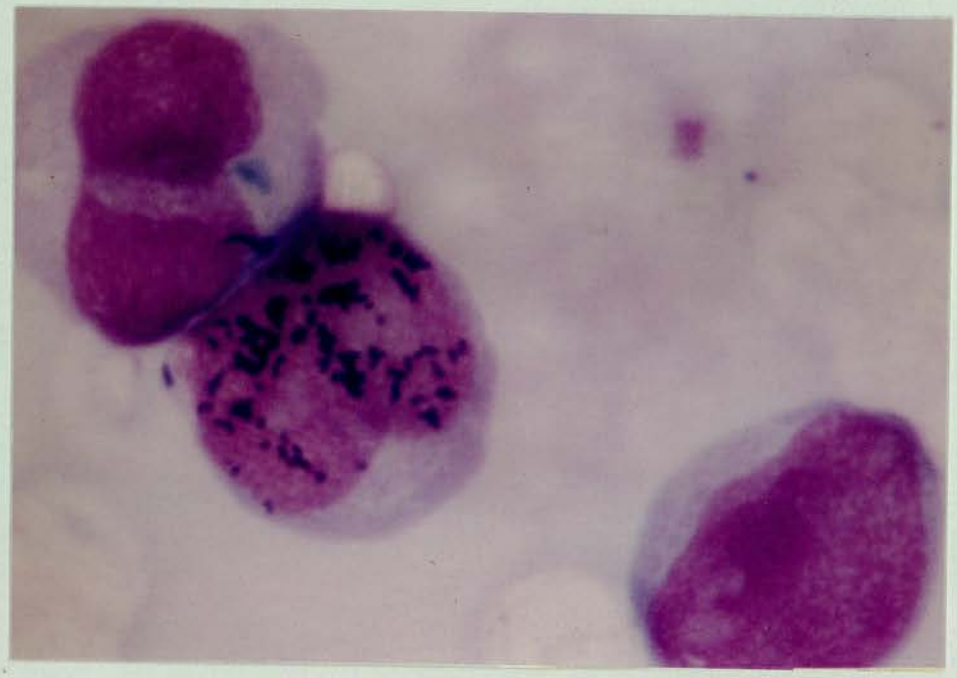


FIG. 26

## RESULTS

The results, including the percentage of blast cells, LI% and absolute numbers of blast cells in both ALL and AML specimens are summarised in tables X and XI. The labelling index range of ALL was 1.0% - 4.0% with a mean of 1.8% $\pm$ 1.1%. The labelling index range of AML specimens was 0.4% - 3.0% mean 1.7 $\pm$ 1.0%. No mitotic figures were seen in any preparation.

Table X ALL

| CASE No. | AGE | SEX | WHITE CELL COUNT $x10^{-9}/L$ | BLAST CELLS % | BLAST CELLS $x10^{-9}/L$ | LI % | LI $x10^{-9}/L$ | COMMENT |
|---|---|---|---|---|---|---|---|---|
| 1 | 56 | M | 110.0 | 85 | 93.5 | 1.0 | .935 | On Vincristine |
| 2 | 70 | M | 118.0 | 90 | 106.2 | 4.0 | 4.248 | Post transfusion |
| 3 | 72 | M | 59.1 | 88 | 52.0 | 1.0 | .520 | Post transfusion |
| 4 | 3 | F | 6.1 | 38 | 2.3 | 1.0 | .023 | At presentation |
| 5 | 5 | M | 8.3 | 15 | 1.2 | 1.0 | .012 | At presentation |
| 6 | 11 | M | 9.1 | 52 | 4.7 | 2.0 | .094 | |

Table XI AML

| CASE No. | AGE | SEX | WHITE CELL COUNT $x\ 10^{-9}/L$ | BLAST CELLS % | BLAST CELLS $x10^{-9}/L$ | LI % | LI $x10^{-9}/L$ | COMMENT |
|---|---|---|---|---|---|---|---|---|
| 1 | 76 | F | 1.6 | 32 | .512 | 1.0 | .005 | On treatment |
| 2 | 68 | M | 25.6 | 80 | 20.0 | 1.0 | .200 | |
| 3 | 54 | M | 15.7 | 56 | 8.7 | 3.0 | .261 | Pre-treatment |
| 4 | 54 | M | 7.0 | 25 | 1.7 | 2.0 | .034 | Post 4 day treatment |
| 5 | 71 | M | 14.2 | 60 | 8.5 | 2.0 | .170 | On Prednisolone |
| 6 | 72 | M | 6.4 | 30 | 1.9 | 0.4 | .007 | |
| 7 | 86 | F | 7.1 | 60 | 4.2 | 2.0 | .084 | |
| 8 | 49 | F | 8.9 | 35 | 3.1 | 2.0 | .062 | |

Figures 27 and 28 provide evidence that following intermittent sampling of both ALL case no. 2 and AML cases nos. 4 and 5 during prolonged incubation with $^{3}$H-TdR, the LI does not vary significantly. At the times of sampling the LI of ALL varied between 3.0% and 4.0% while that of AML was between 1.0% and 2.0%. (See over).

Fig. 27

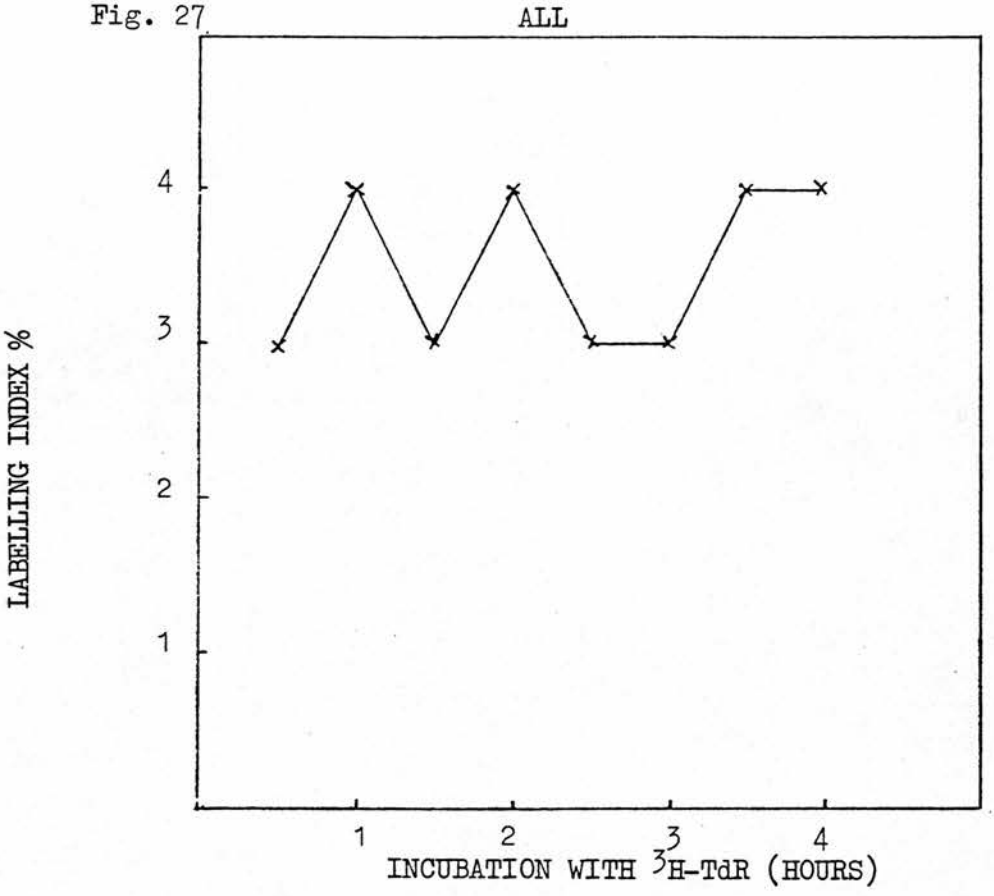

ALL
LABELLING INDEX %
4
3
2
1
1
2
3
4
INCUBATION WITH 3H-TdR (HOURS)

Fig. 28

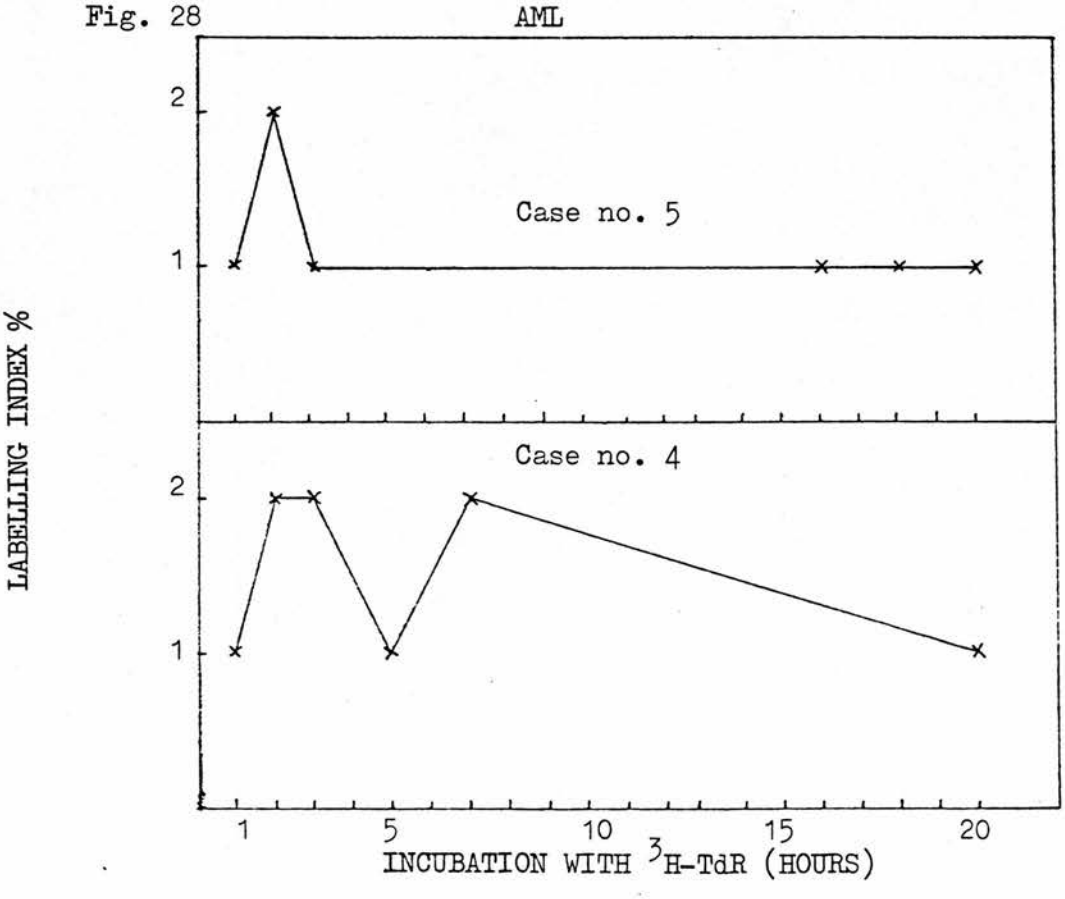

AML
LABELLING INDEX %
2
1
Case no. 5
Case no. 4
2
1
1
5
10
15
20
INCUBATION WITH 3H-TdR (HOURS)

TABLE XII

| SPECIMEN No. | DATE | WHITE CELL COUNT $x10^{-9}/L$ | BLAST CELLS % | BLAST CELLS $x10^{-9}/L$ | L.I. % | L.I. $x10^{-9}/L$ | COMMENT |
|---|---|---|---|---|---|---|---|
| 1 | 12.5.80 | 6.4 | 30 | 1.92 | 0.4 | .007 | Presentation |
| 2 | 22.5.80 | 21.0 | 28 | 5.88 | 1.0 | .058 | |
| 3 | 17.6.80 | 82.7 | 66 | 54.5 | 0.6 | .327 | On steroids |
| 4 | 23.6.80 | 108.2 | 60 | 64.9 | 0.4 | .259 | On steroids |
| 5 | 27.6.80 | 83.9 | 69 | 57.8 | 0.6 | .346 | On Busulphan |
| 6 | 30.6.80 | 137.2 | 76 | 104.2 | 1.0 | 1.042 | |

Table XII above depicts the results obtained from 6 consecutive specimens from the same AML patient over a period of time. During this time the total white cell count rose from 6.4 x $10^9$/L at presentation to 137.2 x $10^9$/L prior to death. The blast cells increased accordingly from 30% to 76% and produced a labelling index range 0.4% to 1.0% with a mean of 0.6%. The absolute numbers of blast cells and labelled blast cells are also shown.

Fig. 29 compares the gradual rise in the total white cell count with the comparable rise in the number of blast cells. From the results of table XII the total numbers of blast cells as compared to the minor fraction of the blast population which labelled in DNA synthesis is shown in fig. 30. It is important to notice the difference in scale of the labelled cells in this latter figure. This was altered in order to produce a simulation of the proportions of the blast cells to labelled cells, otherwise the labelled blast cells in the first 5 specimens would not appear if the same scale as that for the total blast count had been used. A true comparison of the labelled blasts can be seen in the column at specimen no. 6.

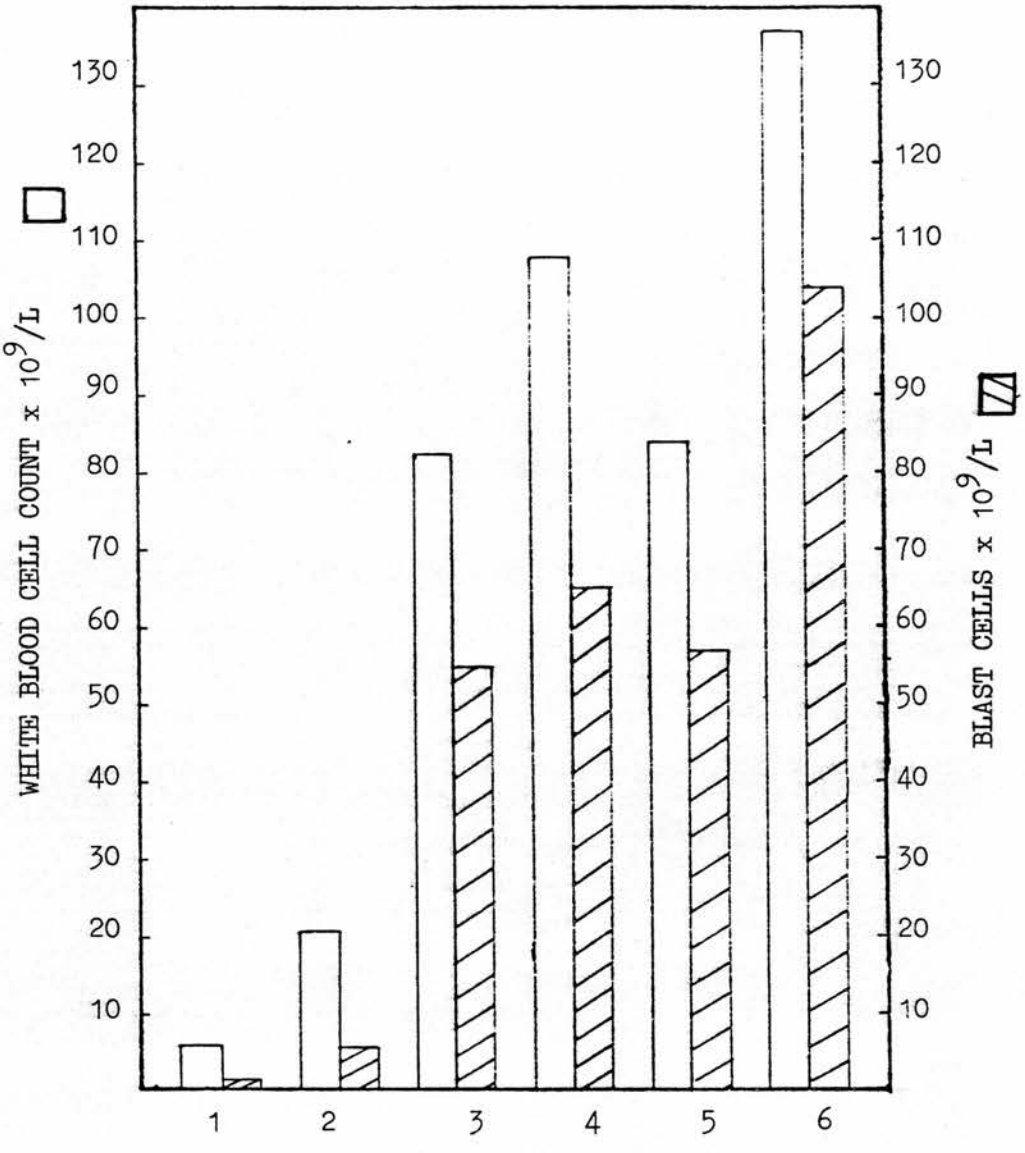



Fig. 29. Comparisons of white cell count with the rise in the number of blast cells in 6 successive samples of AML.

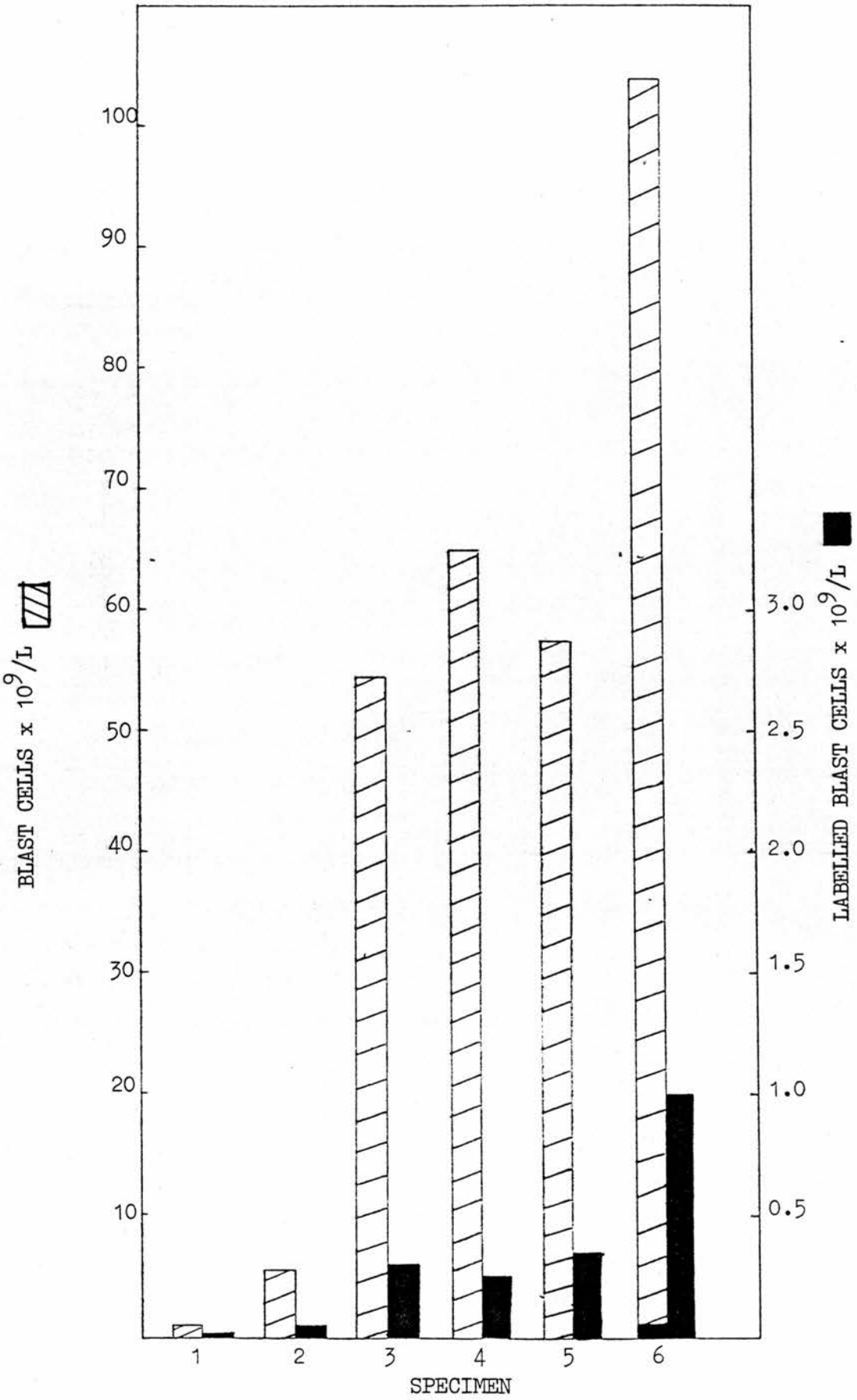



Fig. 30. Comparison of the total numbers of blast cells to the total numbers of labelled blast cells in 6 successive samples of AML.

In both ALL and AML together, no correlation was observed between the total white cell count and the LI% ($r = 0.33$) or the percentage of blast cells and the LI% ($r = 0.39$) respectively. Scattergrams depicting the individual values of total white cell counts against LI% and blast cells (%) against LI% for both ALL and AML are shown in figs. 31 and 32 respectively. No relationship can be observed between the variables. (See over).

The results of pre-treatment and post 4 day treatment with Daunorubicin, Cytosine-arabinoside and Prednisolone are included in table XI with case nos. 3 and 4 respectively. It was observed that both the total white cell counts and the percentage of blasts cells have been more than halved and the labelling index decreased from 3.0% to 2.0% reducing the actual number of labelled cells. Using a micrometer eyepiece the percentage of different blast cell diameters was determined in both pre and post treatment specimens after size estimation of 200 blast cells. The results are shown in table XIII and illustrated in fig. 33. In both cases, the labelled blast cells were found to measure 15$\mu$ in diameter.

Table XIII

| DIAMETER ( $\mu$ ) | 11 $\mu$ | 12 $\mu$ | 13 $\mu$ | 14 $\mu$ | 15 $\mu$ | 16 $\mu$ |
|---|---|---|---|---|---|---|
| Pre-treatment % | 0 | 22 | 28 | 36 | 14 | 0 |
| Post treatment % | 6 | 50 | 25 | 13 | 4 | 2 |

The proportion of large blast cells (13 - 16$\mu$ in diameter) was reduced from 78% to 44% and the small blasts (11 - 12$\mu$ in diameter) increased from 22% to 56% in pre and post treatment specimens respectively.

Fig. 31

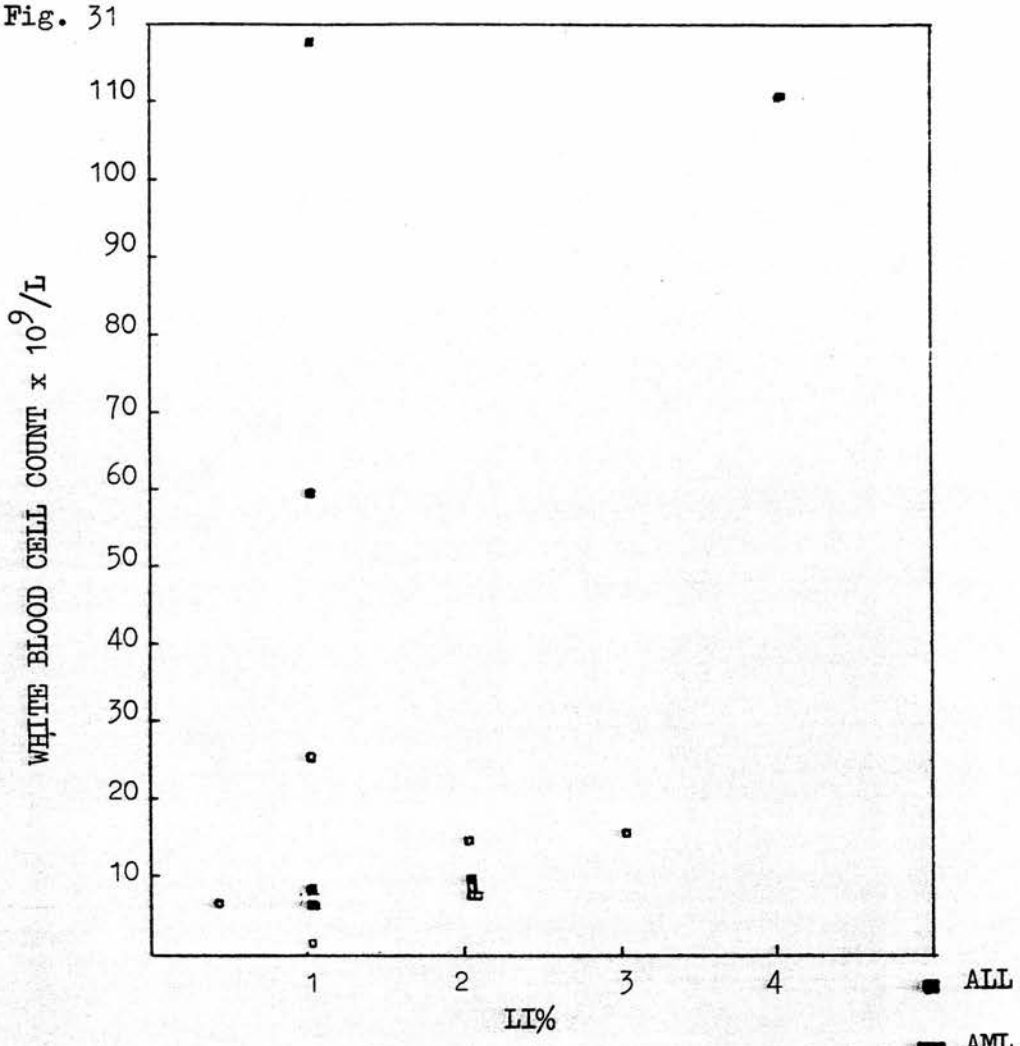
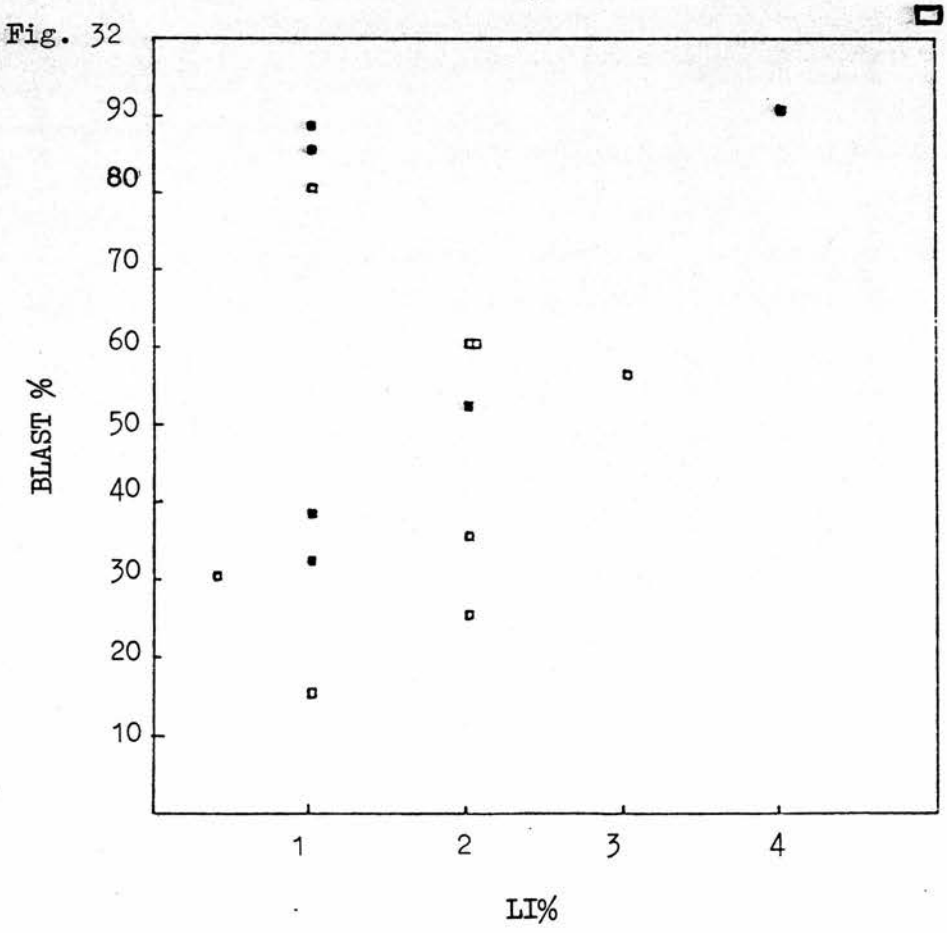

WHITE BLOOD CELL COUNT x $10^9$/L
110
100
90
80
70
60
50
40
30
20
10
1
2
3
4
LI%
ALL
AML

Fig. 32


BLAST %
90
80
70
60
50
40
30
20
10
1
2
3
4
LI%

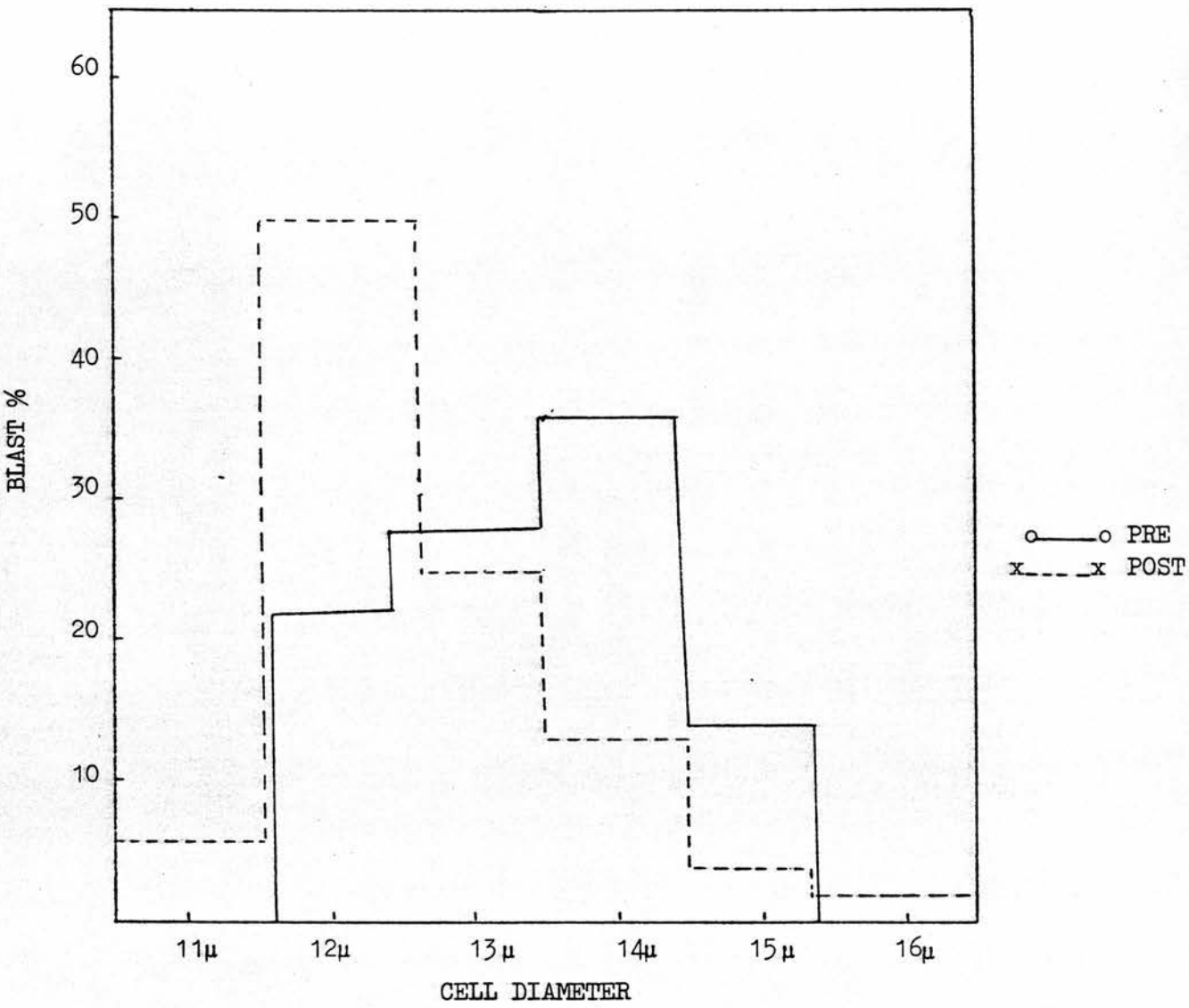



Fig. 33 depicts the comparison of the percentages of different blast cell diameters in pre and post treatment specimens from the results on table XIII.

Similar micrometer readings were taken of the cases of ALL and AML including measurements of the labelled blast cells. (Tables XIV and XV). In all cases of leukaemia irrespective of the proportion of large blasts to small or of the L.I. all labelled blasts were large with an average diameter of 14 μ. No labelled small blasts were recorded.

Table XIV (ALL)

| CASE No. | AGE | SEX | WHITE CELL COUNT $x10^{-9}/L$ | BLAST CELLS | | LABELLED BLASTS | |
|---|---|---|---|---|---|---|---|
| | | | | SMALL | LARGE | % | DIAMETER |
| 1 | 56 | M | 110.0 | 5% | 95% | 1.0 | 15 μ |
| 2 | 70 | M | 118.0 | 28% | 72% | 4.0 | 15 μ |
| 3 | 72 | M | 59.1 | 62% | 38% | 1.0 | 14 μ |
| 4 | 3 | F | 6.1 | 93% | 7% | 1.0 | 14 μ |
| 5 | 5 | M | 8.3 | 95% | 5% | 1.0 | 13 μ |

Table XV (AML)

| CASE No. | AGE | SEX | WHITE CELL COUNT $x10^{-9}/L$ | BLAST CELLS | | LABELLED BLASTS | |
|---|---|---|---|---|---|---|---|
| | | | | SMALL | LARGE | % | DIAMETER |
| 1 | 68 | M | 25.6 | 26% | 74% | 1.0 | 14 μ |
| 2 | 54 | M | 15.7 | 22% | 78% | 3.0 | 15 μ |
| 3 | 54 | M | 7.0 | 56% | 44% | 2.0 | 15 μ |
| 4 | 71 | M | 14.2 | 66% | 34% | 2.0 | 15 μ |
| 5 | 72 | M | 6.4 | 93% | 7% | 0.4 | 13 μ |
| 6 | 86 | F | 7.1 | 90% | 10% | 2.0 | 14 μ |

Although no clear correlation was discovered between the white cell count and the percentage of small and large blast cells, the figures ($r = 0.67$) suggest the possibility of a relationship which cannot be excluded.

Table XVI shows the findings of similar readings of the 6 successive specimens from the same AML patient during treatment and prior to death.

Table XVI

| SPECIMEN NO. | WHITE CELL COUNT $x10^{-9}/L$ | BLAST CELLS | | LABELLED BLASTS | | COMMENT |
|---|---|---|---|---|---|---|
| | | SMALL | LARGE | % | DIAMETER | |
| 1 | 6.4 | 95% | 5% | 0.4% | 13 μ | Presentation |
| 2 | 21.0 | 86% | 4% | 1.0% | 13 μ | |
| 3 | 82.7 | 96% | 4% | 0.6% | 13 μ | On Steroids |
| 4 | 108.2 | 97% | 3% | 0.4% | 13 μ | On Steroids |
| 5 | 83.9 | 96% | 4% | 0.6% | 13 μ | On Busulphan |
| 6 | 137.2 | 92% | 8% | 1.0% | 13 μ | |

From the outset, virtually all of the blast cells recorded were small and this predominance persisted throughout the study. The labelled blast cells were found to be just within the large blast range with an average of 13μ diameter.

## LEUKAEMIA

## DISCUSSION

The kinetics of acute leukaemia has been the subject of numerous reviews. Contrary to belief that the leukaemic blast cell population has a rapid and uncontrolled proliferation, Killman et al (1963), Rubini et al (1961) and Schmid et al (1966) produced evidence that generally leukaemic cells are not proliferating more rapidly than normal cells as reflected by the LI%. This fact is also apparent throughout this present study where small but similar labelling indices were found in the peripheral bloods of both ALL and AML.

It is clear from tables X and XI that while there is a variation in the LI of the blast cells in the blood, in general terms the results obtained did not indicate a rapidly proliferating blast cell compartment. There was no correlation between the total white cell count and the LI or the percentage of blast cells and the LI in both ALL and AML. It is observed from this that the kinetic pattern of leukaemic blast cells probably varies from one case to another, irrespective of the total white cell count and proportion of blast cells; and also differs in the same individual at different stages of the disease (Table XII). Early in this study it was established that prolonged incubation of leukaemic blood with $^{3}$H-TdR did not significantly alter the LI. (Figs. 27 and 28). This is in contrast to the in-vivo studies of bone marrow and blood by Killman et al (1963) and Gavosto et al (1967) who have shown that the LI increased considerably over a period of hours.

It is illustrated in figs. 29 and 30 that although the white cell count and thus the blast cell count is rising, the LI remains remarkably low. This would indicate that contrary to belief, the majority of blast cells in the peripheral blood of this patient with acute leukaemia are in a quiescent or non-proliferative state, similar to bone marrow blast cells as described by Cheung et al (1972). In addition, Killmann (1968) and Schumacher et al (1971) have shown that the labelling indices are lower in the blood than in the bone marrow. Although no bone marrow aspirates were available in this study the lower LI results in the blood as compared to the total blast population (Fig. 30) provides similar evidence that there is a substantial proportion of blast cells in the peripheral blood which are in a resting state or Go phase of the cycle. The most likely explanation for this finding was postulated by Mauer et al (1962) and Killmann (1965) and (1968). A preferential release of non-proliferating blast cells occurs from the marrow into the blood accumulating into a large pool of blast cells which therefore have a comparatively lower labelling index. Killmann (1965) also found that no correlation could be established between labelling indices of blast cells and the number of blast cells. In this present study, figs. 31 and 32 confirm that the LI in the peripheral blood of acute leukaemia is totally independent and unrelated to the total white cell count and proportion of blast cells.

The results in table 2 of pre and post 4 day treatment show the expected reductions in total white cell count, blast cells and labelling index. In both pre and post examinations the labelled blast cells in DNA synthesis were large. In

keeping with the work of Gavosto et al (1967) and Schumacher et al (1971) it was noted that in this entire study of acute leukaemia, labelling took place consistently in large blast cells with an absence of labelling in small blasts below 12μ in diameter. Consequently, the opinion was formed that the blast population in acute leukaemia could be divided into a proliferating and a non-proliferating stage.

Table XIII and Fig. 33 supply additional information as to the effects of chemotherapy. It was shown that the distribution of blast cell sizes had altered. It is evident that the large proliferating blast cells were reduced during the 4 day treatment and the numbers of apparently non-proliferating blast cells increased. The possible explanation of this observation is that the proliferating large blast cells have relatively short generation times and are more sensitive to chemotherapy which is directed primarily at proliferating cells. The small blast cells comprise of cells which have long generation times due to prolonged G1 phase and also Go cells. Therefore the smaller blast cell compartment is expanding, virtually unrestricted, due to the fact that one part of the population is proliferating slowly and the other, probably major part, is in a dormant Go phase. This explanation must apply equally to the bone marrow blast cells. The smaller blast cells in Go under the appropriate circumstances may resume proliferation, Gabutti et al (1969).

In most cases of ALL and AML (tables XIV and XV) it appeared that there may be a correlation, where the greater the rise in the total white cell count the greater is the proportion of large blasts and conversely the smaller the white cell count then the greater is the proportion of small blast cells. The correlation figure ($r = 0.67$) while not

totally conclusive is suggestive of such a relationship.

In studies of Murphy et al (1975) and Oster et al (1976) it was concluded that lymphoblast cell size (as measured in this study) in childhood ALL bone marrow was not a reliable prognostic indicator. While the blast cell sizes described here were from peripheral blood, nevertheless, it was noticeable that both the childhood cases of ALL examined in table XIV had blast cell populations which were predominantly small and this was also found in the work of Murphy et al (1975).

From table XVI it was obvious that a substantial proportion of blast cells, greater than 90%, are in some form of quiescent state.

The proportion of proliferating blast cells tends to be lowest when the initial diagnosis is made and higher values are found at relapse, Saunders et al (1967). The patient studied in table XVI never managed to achieve any kind of remission although no form of aggressive treatment was ever applied. Consequently, it can be observed that although the fraction of actively proliferating cells was as low as 1.0% and no sustained effort was made to contain this growth, the leukaemic blast cells proliferated slowly but inexorably towards their final peak. Therefore as the density of leukaemic mass increases, the number of cells entering a resting phase increases and the proportion of proliferating cells appears to fall. This is consistent with the findings of Mauer (1975).

Killmann (1972) concluded that the leukaemia blast cells are heterogenous as far as their kinetic properties are concerned. Quiescent cells may comprise Go cells together with cycling cells with a long and variable G1 generation time.

This explains, in part the absence of mitotic figures in this present study. By virtue of the fact that so few blast cells were undergoing DNA synthesis and because mitosis occupies a small fraction of the generation time, the chances of observing mitotic figures was reduced.

Increased understanding of the growth characteristics of acute leukaemia has led to the development of greater numbers of cycle specific and cycle non-specific anti-leukaemia drugs. The latter group are used against that population of cells which are in Go or out of cycle. These quiescent cells have, by their nature, presented a serious obstacle to therapy since they can persist in the patient and provide a source for relapse. Treatment schedules have subsequently been designed to synchronise the cell cycle in the hope that Go cells would be drawn into proliferation. Manipulation of the mitotic cycle by the use of drugs and monitored by kinetic studies as described by Lampkin et al (1974) has achieved a measure of success.

## MULTIPLE MYELOMA

The blood of 5 multiple myeloma patients - 4 male and 1 female, was studied autoradiographically. Circulating plasma cells were present in each sample and were the only cells which were found to be in DNA synthesis.

Table XVII

| CASE | AGE | SEX | WHITE CELL COUNT x $10^{-9}$/L | LABELLED CELLS PER 500 CELL COUNT | COMMENT |
|---|---|---|---|---|---|
| 1 | 68 | F | 9.5 | 4 | Untreated |
| 2 | 59 | M | 5.2 | 1 | Untreated |
| 3 | 62 | M | 5.0 | 2 | |
| 4 | 51 | M | 7.1 | 1 | On Melphalan |
| 5 | 50 | M | 7.5 | 3 | On Melphalan |

A mean of 2.2 labelled cells per 500 cell count was found. The presence of plasma cells in the peripheral blood of patients with multiple myeloma is not a common occurrence. Heavy labelling of the plasma cells was a particular feature in all of these cases and the cells were identified by the eccentric position of the nucleus and the basophilia of the cytoplasm. (Fig. 34).

In contrast to the presence of blast cells in acute leukaemia, plasma cells are not generally found in the peripheral blood in myeloma and of the small numbers that were detected, all were labelled. This may be suggestive of an overspill from an expansive proliferating compartment in the bone marrow. The work of Durie et al (1977) and Drewinko et al (1981) has shown that the results of kinetic studies on bone marrow, following intravenous infusions of $^{3}$H-TdR, have important prognostic implications in the treatment of multiple myeloma, in particular the measurement of LI%.

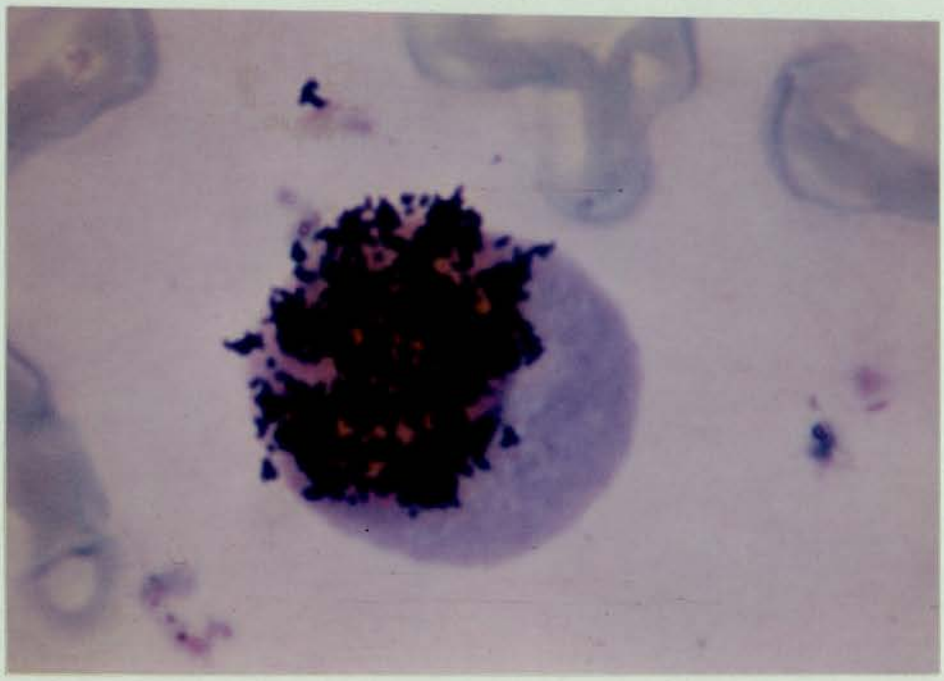


FIG. 34

## THE EFFECTS OF SURGICAL TRAUMA ON THE NUMBERS OF DNA-SYNTHESIZING CELLS IN THE PERIPHERAL BLOOD

There were two reasons for undertaking the study. The work of Philip et al (1980) had suggested that marked changes occurred in the concentration of circulating granulocyte precursor cells with a concomitant increase in the proportion synthesizing DNA, several days after surgery. From this information, it seemed apparent that this readily available group of patients could provide a source of samples in which DNA-synthesizing cells could be demonstrated by means of autoradiographic techniques.

Specimens were obtained from 10 female patients (age range 29-45 years) about to undergo an abdominal hysterectomy. The patients had normal haematological indices prior to operation and none required blood transfusion during the period of the study. Blood samples were taken from 5 of these patients before and 1 day after the operation; from 2 patients before and 3 days after the operation and from 3 patients before and 1 day and 3 days post operative respectively. The white cell counts and differential counts were recorded. Autoradiographical experiments were set up using Ilford K2 Emulsion with an exposure time of 5 days. Thereafter the films were developed, stained and examined for labelled cells with 500 cells counted in each preparation.

## RESULTS

No labelled cells were seen in any of the pre-operative or 1 day post operative specimens. One labelled cell was observed in a 3 day post operative specimen from case no. 7. This cell, 9 - 10 in diameter with a basophilic cytoplasm, had the appearance of a Türk or irritation cell type. (Fig. 35). No mitotic figures were seen in any of the preparations.

Similar changes in the total white cell counts, the neutrophil counts and the lymphocyte counts were demonstrated in virtually all subjects. (Tables XVIII and XIX). In each case a neutrophil leucocytosis occurred on the first day after surgery though there was considerable variation in the size of the individual response. By day 3, all cell numbers had passed the maximum reaction and were approaching the pre-operative levels and normal proportions to each other. This is demonstrated in figs. 36 and 37 respectively where the total white cell count and neutrophil count both reach their peak levels at day 1 and approach their normal levels at day 3. The lymphocyte count followed a similar pattern.

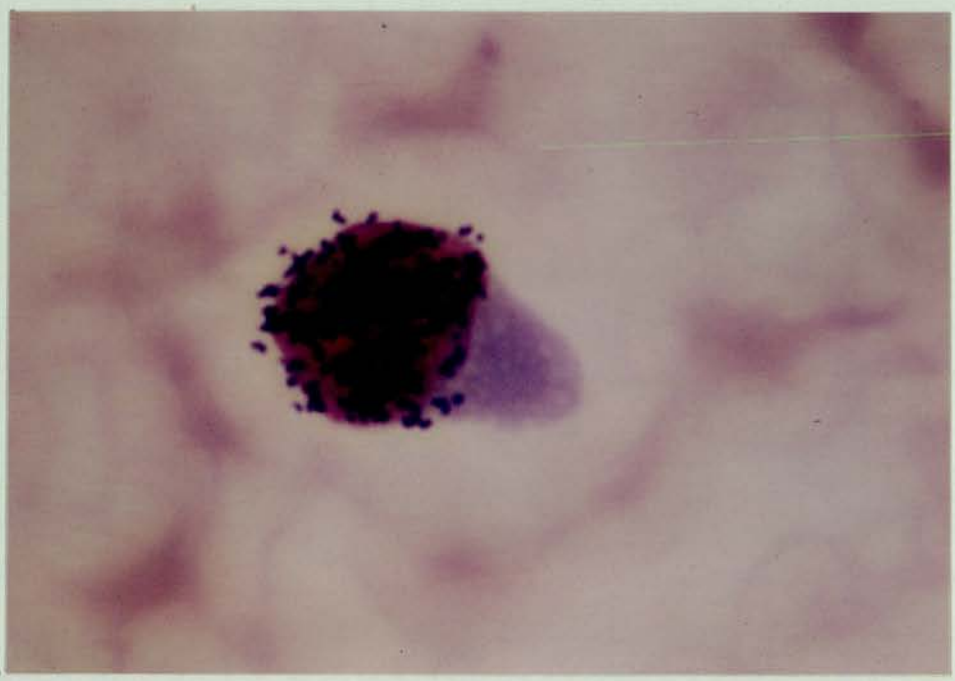


FIG. 35

Table XVIII

| CASE No. | AGE | PRE-OPERATIVE WHITE CELL COUNT x $10^{-9}$/L | 1 DAY POST OPERATIVE WHITE CELL COUNT x $10^{-9}$/L | 3 DAY POST OPERATIVE WHITE CELL COUNT x $10^{-9}$/L | LABELLED CELLS PER 500 CELLS COUNTED | | |
|---|---|---|---|---|---|---|---|
| | | | | | PRE-OPERATIVE | 1 DAY POST OPERATIVE | 3 DAY POST OPERATIVE |
| 1 | 29 | 9.1 | 14.7 | ND* | 0 | 0 | ND* |
| 2 | 44 | 9.5 | 14.9 | ND* | 0 | 0 | ND* |
| 3 | 43 | 9.5 | 18.1 | ND* | 0 | 0 | ND* |
| 4 | 45 | 6.6 | 16.8 | ND* | 0 | 0 | ND* |
| 5 | 39 | 4.4 | 17.7 | ND* | 0 | 0 | ND* |
| 6 | 45 | 5.7 | ND* | 9.1 | 0 | ND* | 0 |
| 7 | 40 | 5.5 | ND* | 6.1 | 0 | ND* | 1 |
| 8 | 34 | 5.3 | 20.1 | 9.7 | 0 | 0 | 0 |
| 9 | 45 | 6.9 | 18.0 | 11.2 | 0 | 0 | 0 |
| 10 | 34 | 9.9 | 15.4 | 10.8 | 0 | 0 | 0 |

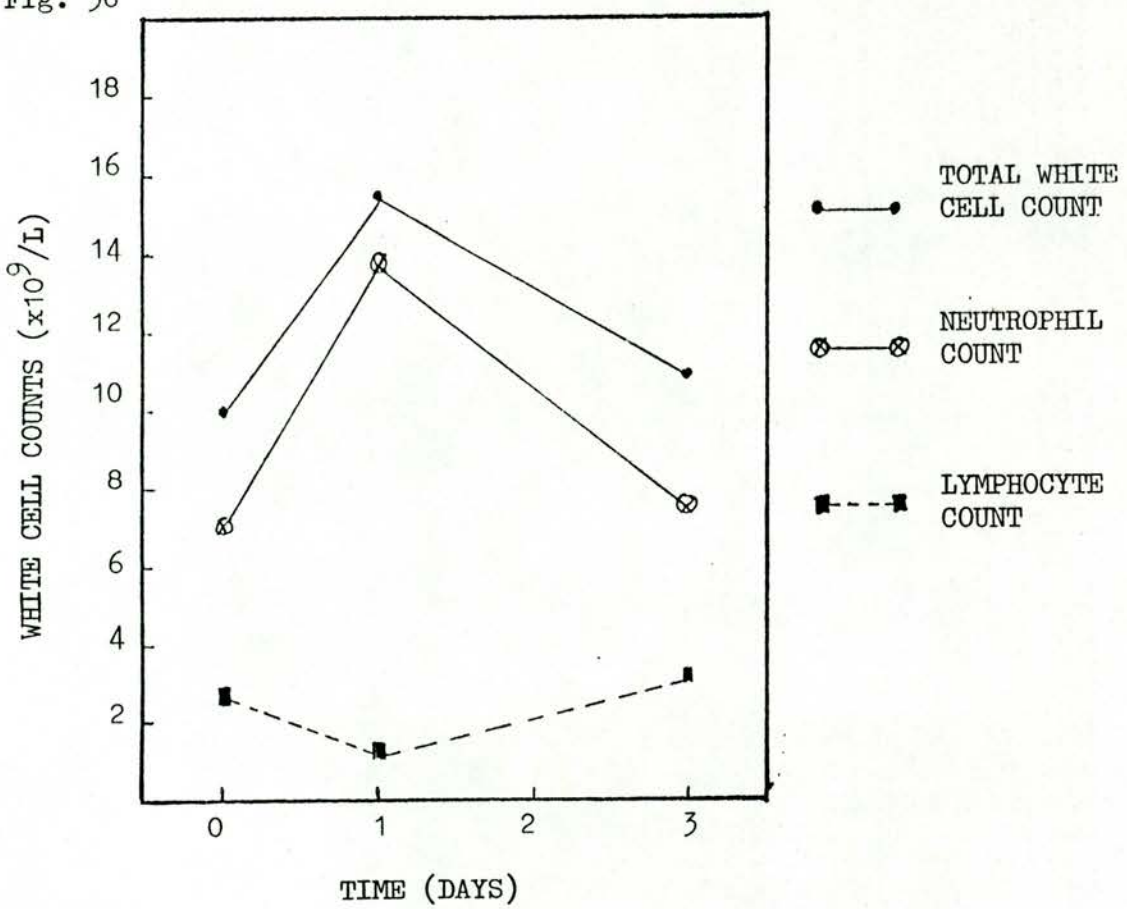
*ND - Not done

Table XIX

| CASE No. | AGE | PRE-OPERATIVE NEUTROPHIL COUNT x $10^{-9}$/L | 1 DAY POST OPERATIVE NEUTROPHIL COUNT x $10^{-9}$/L | 3 DAY POST OPERATIVE NEUTROPHIL COUNT x $10^{-9}$/L | PRE-OPERATIVE LYMPHOCYTE COUNT x $10^{-9}$/L | 1 DAY POST OPERATIVE LYMPHOCYTE COUNT x $10^{-9}$/L | 3 DAY POST OPERATIVE LYMPHOCYTE COUNT x $10^{-9}$/L |
|---|---|---|---|---|---|---|---|
| 1 | 29 | 5.4 | 10.2 | ND* | 3.5 | 3.8 | ND* |
| 2 | 44 | 7.7 | 12.5 | ND* | 1.6 | 1.9 | ND* |
| 3 | 43 | 5.4 | 15.0 | ND* | 3.8 | 2.1 | ND* |
| 4 | 45 | 4.1 | 13.2 | ND* | 2.8 | 3.1 | ND* |
| 5 | 39 | 2.4 | 14.8 | ND* | 1.8 | 2.3 | ND* |
| 6 | 45 | 3.3 | ND* | 7.1 | 2.2 | ND* | 1.6 |
| 7 | 40 | 3.2 | ND* | 3.3 | 1.9 | ND* | 2.3 |
| 8 | 34 | 3.0 | 17.0 | 6.1 | 2.1 | 2.0 | 3.2 |
| 9 | 45 | 3.9 | 14.9 | 8.6 | 2.6 | 2.8 | 2.2 |
| 10 | 34 | 7.0 | 13.7 | 7.4 | 2.7 | 1.2 | 3.1 |

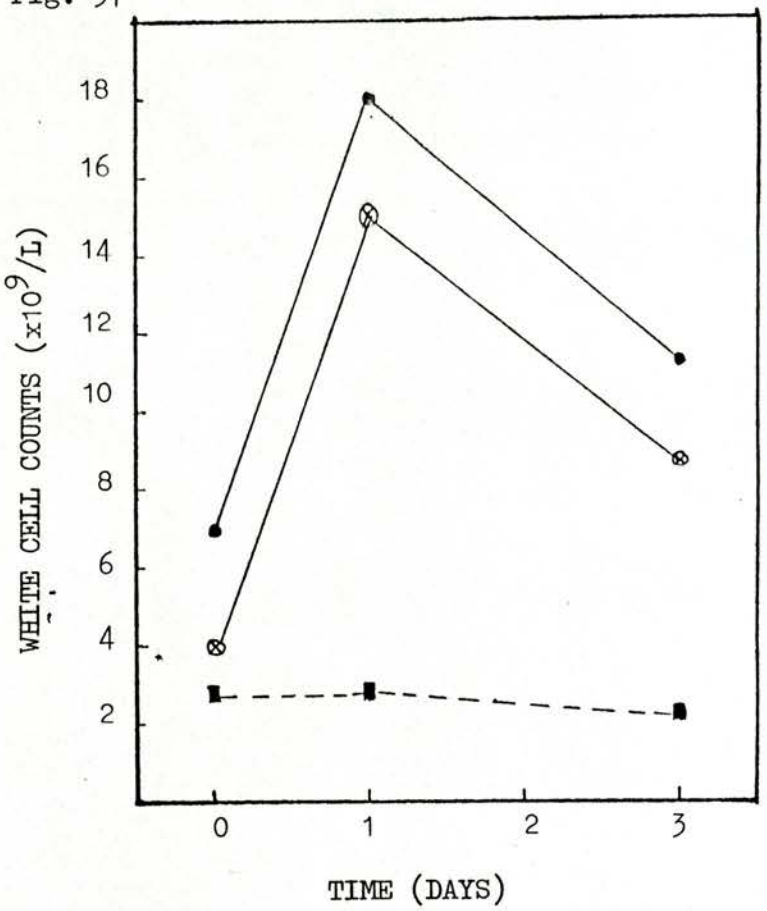
*ND - Not done

Fig. 36




Case no. 10

Fig. 37




Case no. 9

## DISCUSSION

Several studies concerning circulating granulocytic precursor cells (CFU-C) have been carried out using culture techniques, following chemotherapy in man, Richman et al (1976); Lohrann et al (1977) and following exercise and stress Barrett et sl (1978). Similar findings of increased numbers of these cells were reported in each case and this together with the work of Philip et al (1980) prompted the present investigation. The choice of the effects of surgical trauma as the subject of this study was because it offered the opportunity of a readily available group of patients providing a regular source of pre and post operative specimens in this laboratory.

Despite the fact that the proliferative rate of the circulating CFU-C is increased following surgical trauma, Philip et al (1980), no increase in DNA synthesizing cells was established in this present study. However, an explanation for this could be that only a small sample of cells were counted in each case whereas the culture techniques used required a concentration of cells to be induced to form discrete colonies in-vitro. Nevertheless, it may be that the surgical trauma was insufficient to produce any significant detectable increase in DNA synthesizing cells or that different techniques of cell separation and concentration should be used.

## MISCELLANEOUS GROUP

Venous blood from a group of patients with various disorders were studied autoradiographically for the presence of circulating DNA synthesizing cells after the incubation of blood with $^{3}$H-TdR. The patients included 3 with haematological disorders, two of which were iron deficiency anaemias and one pancytopenia; 2 patients with malignancies and 2 patients with infections. In addition, samples obtained from 5 patients with polycythaemia vera (PV), under treatment, were similarly examined.

## RESULTS

No labelled cells were seen in any of the films from the three haematological disorders. (Table XX). These included two severe iron deficiency anaemias and a case of pancytopenia. The differential cell count from each was within normal range and no abnormal cells were seen. Similar results were obtained from the malignant non-haematological disorders. An increase in the number of circulating DNA synthesizing cells was recorded where bacterial infection was diagnosed in case nos. 6 and 7. The labelled cells in case no. 6 included 4 myelocytes and 3 small atypical lymphocytes with deep basophilic cytoplasm. (Fig. 38). The labelled cells in case no. 7 were also atypical lymphocytes. In case nos. 8 to 12 (PV) no labelled cells were seen, with one exception, case no. 10 revealed 3 labelled lymphoid cells (fig. 39) approximately 12 - 14$\mu$ in diameter. No mitotic figures were seen in any preparation.

Table XX

| CASE No. | AGE | SEX | Hb g/dl | WHITE CELL COUNT x $10^{-9}$/L | LABELLED CELLS IN 500 CELL COUNT | COMMENT |
|---|---|---|---|---|---|---|
| 1 | 56 | F | 6.2 | 6.8 | 0 | Fe Deficiency Anaemia |
| 2 | 84 | F | 6.5 | 4.2 | 0 | Fe Deficiency Anaemia |
| 3 | 69 | F | 8.7 | 1.4 | 0 | Pancytopenia. Platelets 21 x $10^9$/L |
| 4 | 84 | F | 13.4 | 6.4 | 0 | Ca Ovaries. Diabetic. |
| 5 | 62 | M | 11.9 | 6.7 | 0 | Ca Colon. |
| 6 | 43 | M | 14.4 | 33.8 | 7 | Cellulitis, lymphangitis (R) chest. |
| 7 | 59 | F | 13.3 | 16.4 | 2 | Peri-anal abcess. Wound inf. Diabetic. |
| 8 | 62 | M | 14.2 | 6.7 | 0 | PV diagnosed 1978. Radiotherapy treatment. |
| 9 | 60 | F | 12.3 | 4.8 | 0 | PV diagnosed 1976. Long term Busulphan. |
| 10 | 78 | M | 18.4 | 8.0 | 3 | PV diagnosed 1963. $P^{32}$ injections. Node in (L) axilla. |
| 11 | 60 | M | 17.2 | 7.2 | 0 | PV. On radiotherapy. |
| 12 | 67 | M | 14.3 | 12.4 | 0 | PV. Post venesection. |

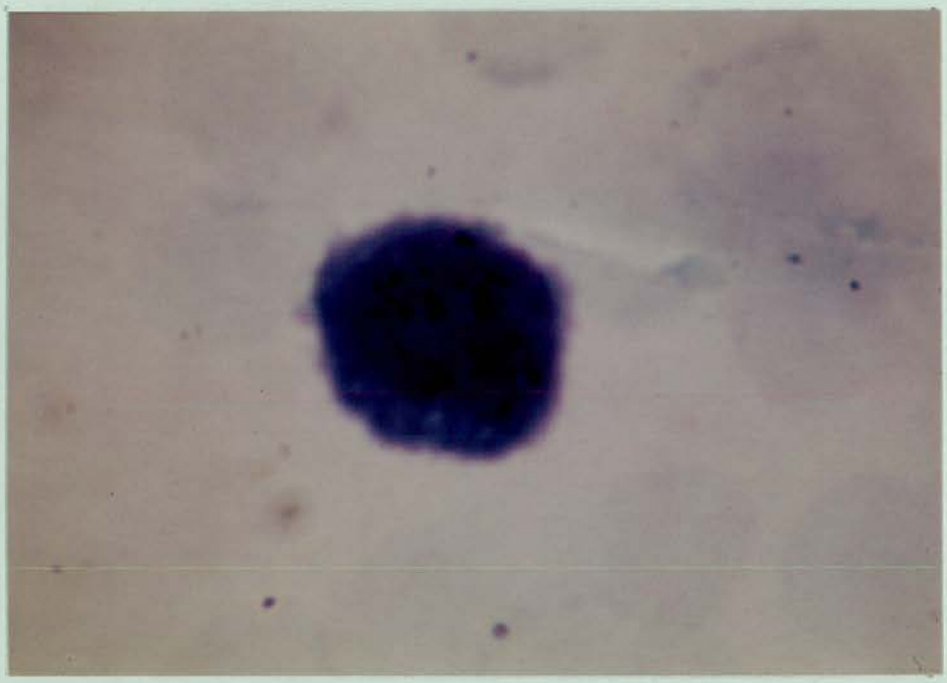


FIG. 38

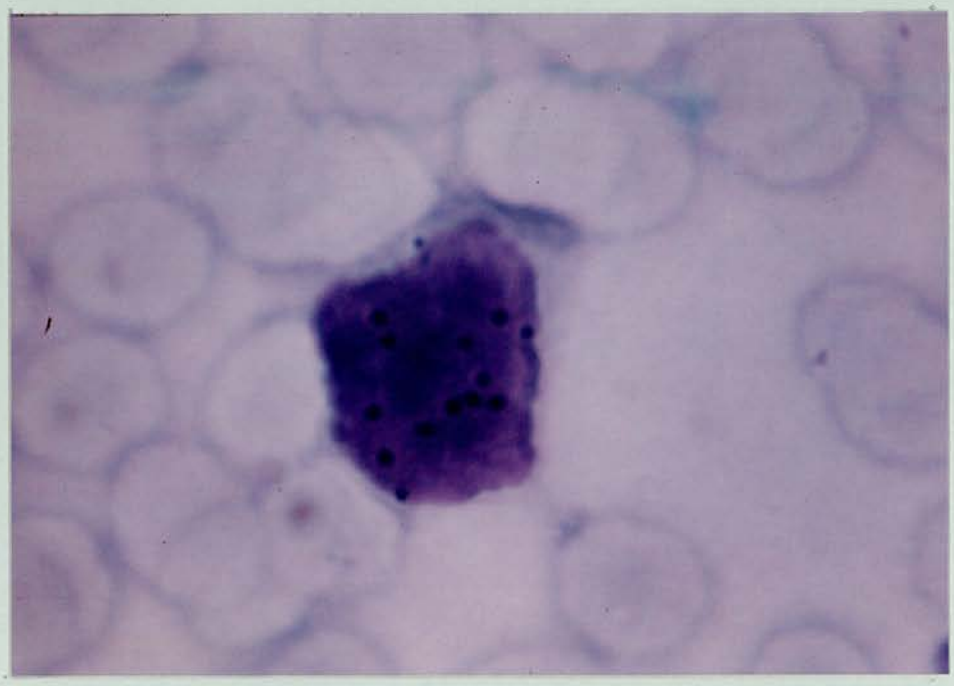


FIG. 39

## MISCELLANEOUS GROUP

### DISCUSSION

Rubini et al (1961) and Killmann (1968) reported increases in the number of DNA synthesizing cells in human blood in various haematological and non-haematological disorders. In comparison, no labelled cells were seen in similar conditions in this present study. It should be reported however, that differing techniques were used in these previous studies, in that buffy-coat preparations were examined and a greater number of cells were counted. Nevertheless, noticeable similarities were observed in the preparations from patients with infections. The cells that labelled were those normally associated with infection, namely myelocytes and atypical lymphocytes which resembled Türk cells. It is probable that these proliferating cells have an immunological function in response to the exposure of the infection. Killmann (1968) also considered that the cells are migrating in the circulation in order to stimulate further immune reaction leading to the inevitable development of antibody secreting plasma cells.

Among the PV patients it is interesting to note that only case no. 10 produced labelled lymphoid cells. Although the remaining patients were also on long term treatment no similar results were obtained. The cells did not have the appearance of those described in the previous immune response but were larger and less basophilic. The reason for their appearance in the circulation and their exact nature is unclear. Other PV patients were receiving treatment and no labelled cells were recorded although an increase in their numbers has been reported by Rubini et al (1961) following irradiation.

Polycythaemia vera is a myeloproliferative disorder characterised by the over-production of cells of differing haemopoietic origin. Generally however, the usual and most obvious abnormality occurs in the red cell series. From the appearance of these labelled cells it is unlikely that they belong to the erythroid series but it may be that they were reactive in some way to the malignant process. Furthermore, the finding of lymph node involvement in that particular patient may also explain their peripheral blood appearance as immunologically reactive.

In general, this miscellaneous group can be considered as a further control group. The presence of labelled cells in patients 6 and 7 has been explained as being part of the expected response to infection. The remainder, apart from case no. 10, have shown an absence of labelled cells in conditions of anaemia, pancytopenia or malignancy. The lack of DNA synthesizing cells in these disorders does not entirely exclude them but indicates that their numbers are comparatively small.

# FINAL DISCUSSION AND CONCLUSION

## FINAL DISCUSSION AND CONCLUSION

The number and possible significance of DNA synthesizing cells in the blood of patients with various disorders was studied autoradiographically after incubation in-vitro with $^{3}$H-TdR. In a control group of normal humans it was found that small numbers of DNA synthesizing cells were present in the peripheral blood. The labelled cells were classified as monocytes and large lymphocytes and it was postulated that these circulating cells were part of a normal transient population which may be increased under more demanding situations.

As described previously, the DNA synthesizing cells detected were present in varying numbers; in various cell types and with differing functions. Increased numbers of labelled cells were present in cord blood, Hodgkin's disease, infectious mononucleosis, acute leukaemia and multiple myeloma as compared to the normal control group. An increased concentration was also observed in bacterial infections. One sample from a case of polycythaemia had a moderate increase. The LI of CLL was only mildly raised, as compared to the control group but the absolute number of cells in DNA synthesis greatly exceeded that of normal due to differences in the total white cell counts.

Contrary to the findings of Philip et al (1980) which indicated a rise in CFUc, no increase in DNA synthesizing cells was observed in the peripheral blood of similar post surgical trauma patients. The explanation was given that perhaps the proliferating cells were so small in numbers that they went undetected by the techniques used.

In post-stained autoradiographs much of the cellular detail can be distorted or obscured due to the effects of

staining through the emulsion or the intensity of the grains. This was noted particularly in cord blood samples where differentiation of labelled erythroid precursors was difficult. A further problem in these and other samples was the presence of labelled cells which defied classification. Myelocytes also when present, tend to lose their granulation in the cytoplasm and the nuclear structure becomes unclear. Generally, the cells classified as lymphoid were readily identified. In Hodgkin's disease the labelled lymphoid cells resembled those described by Halie et al (1974). Similarly, labelled lymphoid cells resembling those described by Yoffey (1974) as transitional, were recognised in cord blood. The atypical lymphocytes of infectious mononucleosis were identified from their size staining and irregular morphological appearance but because of the post-staining effects and grain intensity as mentioned above, no effort was made to categorise them into particular types or groups as described by Schmid et al (1965). In acute leukaemia the blast cells were identified by their general uniformity of size and staining and in the case of AML, the occasional auer rod. In multiple myeloma a consistent feature was the very heavy labelling of the eccentric nucleus of the plasma cells which were identified by the deep blue basophilia of the cytoplasm.

The function of these DNA synthesizing cells in the blood is not generally clear. The study of Killmann (1968) suggested that the observed DNA synthesizing cells have an immunological function. This is probably true of the cells seen in infectious mononucleosis, infection and Hodgkin's disease in this present study. Harris (1973) indicated that the perinatal period was included with immunological response,

irradiation and anaemia, as a particular circumstance when DNA synthesizing cells increase in the peripheral blood. Of the investigations undertaken in this study, cord bloods were the only specimens which presented with a naturally increased proportion of DNA synthesizing cells. Transitional lymphocytes are a prominant feature of foetal blood and bone marrow. Yoffey et al (1961) and Yoffey (1974) in his study Bone marrow in Hypoxia and Rebound have suggested that these cells have a non-immunological function and a possible role as haemopoietic stem cells. Thus the foetal blood lymphoid cells in spontaneous DNA synthesis, together with the foetal CFUc, Prindull et al (1978), probably represent a mobile population of stem cells which has overspilled into the circulation. This occurs at a time when heavy demands are made on haemopoiesis such as in the period of rapid growth of the newborn infant.

Knudtzon et al (1974) and Richman et al (1976) advocate the harvesting of circulating CFUc from cord blood for use in bone marrow transplantation and as supportive treatment against marrow aplasia in patients receiving intensive treatment respectively. This is additional proof of the increased proliferative nature of probable stem cells in cord blood.

Of all the disorders studied, those of infectious mononucleosis (IM) and acute leukaemia provided the most striking results. Due to the self limiting nature of the disorder IM was notable for two reasons:-

1. the sharp increase in DNA synthesizing cells in the peripheral blood (mean LI = 8.0%) during the acute phase of the illness,
2. the diminishing number during the convalescent stage.

This demonstrates the remarkable proliferative capacity of the lymphocytes as part of the immunological response in this viral illness. In comparison, blast cells in acute leukaemia proliferate more slowly than normal cells due to a prolongation of the generation time. A fraction of the population is engaged in DNA synthesis and these are predominantly large blast cells. At any given time a proportion of leukaemic cells are out of cycle in the so-called resting compartment, (Go phase). These cells and those in cycle (or growth fraction) are in a balanced state in that a reduction of the growth fraction appears to stimulate those in the resting compartment to enter into the cycle. This has the effect of producing a variation in the proliferation of leukaemic blast cells.

As a result of similar findings of numerous workers of the variability of the leukaemic process, Saunders et al (1967) and the apparent sensitivity of the proliferating blast cells to chemotherapy, numerous attempts have been made to synchronise resting cell populations and manipulate mitotic cycles, Lampkin et al (1974). This renders the cells more susceptible to cell cycle-specific drugs. In contrast, in CLL the LI% is consistently low with only the occasional atypical lymphocyte being labelled and the mature small lymphocyte which is the predominant cell, remaining persistently unlabelled. It was concluded from this that ALL appears to be a disorder of gross accumulation of apparently inert small lymphocytes which are long-lived, Zimmermann et al (1968) and Schiffer (1968). As a result of these findings it would appear that kinetic studies using $^{3}$H-TdR labelling indices is of limited prognostic value in the management in CLL. On the other hand, the measurement of LI% is of prognostic significance and value

in the overall treatment in multiple myeloma, Durie et al (1977) and Drewinko et al (1981).

Cells which are detected in DNA synthesis can be assumed in most instances to proceed to mitosis. However, no mitotic figures were observed throughout the study. The mitotic index, where it can be measured, is not by itself informative. Many of the cells in mitosis are unable to be identified because the cellular detail is sometimes lost during the process. Furthermore, since mitosis normally occurs in minutes as compared to hours or days of the whole generation time, then it is not inconceivable that mitotic figures are not observed in many preparations especially from peripheral blood. Similarly, the measurement of the labelling index alone cannot describe completely the growth characteristics of a given population of cells. The kinetic measurement of the duration of each individual phase of the cell cycle and thus the generation time, forms the basis of the growth fraction in any cell population. In the case of leukaemia however, it does not include Go or out of cycle cells. Furthermore, it can be observed from this present study that the kinetic pattern of leukaemic blast cells varies from one patient to another and at different stages of the disease in the same patient. The use of auto-radiography and tritiated thymidine as a cell label has revealed valuable information of cell kinetics in many disorders particularly in acute leukaemia and especially in relation to drug therapy, Mauer (1975). It has been suggested by several investigations that there is a correlation between the initial LI% of the bone marrow leukaemic cells and the likelihood of achieving remission, Arlin et al (1978). However, Murphy et al (1977) found no evidence that the initial marrow

blast LI% was related to the prognosis of acute leukaemia in children as compared to the importance of the initial white cell count and the age of the child. Since a range or variation in generation times exists in leukaemic cells in the same patient, the 1 hour flash labelling technique as a single measurement is of limited value. Furthermore, since the in-vivo use of $^{3}$H-TdR in kinetic measurements such as the generation time, require the patient to be subjected to several marrow aspirations, it can seldom be justified on a routine basis.

Although cell kinetics have been of major importance in the development of new anti-leukaemic drugs as well as treatment schedules designed to synchronise cell division, the autoradiographical techniques are tedious and time consuming. Liquid emulsion techniques were preferred in this study as opposed to stripping film. The dipping process in liquid emulsion is quick and simple and adhesion to the specimen is improved. The emulsion layer can be thinner than that of stripping film offering better resolution and allowing easier and more effective staining. Liquid emulsion is also available in a range of sensitivities and grain sizes thus allowing for different experimental use. The drawbacks of the stripping film are concerned mainly with the handling technique. The process obviously involves more manipulation of the film in stripping from the glass support, floating out in the bath and picking up on the specimen slide. Inevitably higher background counts are possible and the time required for each emulsion to swell on the water surface extends the time of the experiment beyond that of any liquid emulsion technique. Nevertheless, the stripping film shelf-life of 6 months coupled with the fact

that it is available in a box of 12 plates allows autoradiography to be performed in any laboratory where only occasional experiments take place. On a small scale therefore, the stripping film technique when performed carefully can provide equally satisfactory information. The most important feature of autoradiography is that it allows the obvious visual examination of cells which are labelled and thus provides an insight into the actual proliferation, differentiation and probable organisation of cells in tissue. This is the main advantage over other techniques such as pulse counting using scintillation counters where a measurement is made of the total radioactivity present but no evidence is provided as to its actual location or distribution throughout the cellular population.

In recent years, the study of the mitotic cycle and cell proliferation has been considerably expanded since the development of flow cytophotometry analysis of cellular DNA content, Hillen et al (1975). Cells in G1 phase have a diploid (2n) DNA content, cells in G2 + M phase have a tetraploid (4n) DNA content and cells in S phase have a DNA content between 2n and 4n. The percentage of G1, S and G2 + M phase cells in a cell population can be determined on the basis of the measurement of this DNA content which is bound to specific fluorescent dyes. This technique provides precise information on the DNA content of large numbers of cells in a short time and this has obvious implications on the evaluation of drug response on the cell cycle. The main limitation of the technique as previously mentioned is that different cell populations are unable to be identified and this remains the major advantage of autoradiographical techniques.

In conclusion, the use of autoradiography with tritiated thymidine as a label, has provided much valuable information over many years leading to greater understanding of cell kinetics in both normal and abnormal cell populations. The technique has been particularly important and effective in the study of the tumour mass in acute leukaemia and myeloma where increased knowledge of their growth features has led to the subsequent design of important treatment programmes. Thus the selective use of cycle specific and cycle non-specific drugs had helped achieve more rapid and prolonged remissions. This is followed by more aggressive maintenance therapy which will also eliminate any non-proliferating cells that may induce a relapse.

Despite the obvious value derived from cell kinetic studies, the autoradiographical techniques used have proved tedious and time consuming. Several variables exist in the process and include the use and type of emulsion or stripping film, the incubation time and exposure time, the grain threshhold and the small numbers of cells counted in each investigation. Any deviation from these factors from one experiment to the next may inevitably lead to inconsistent results. The importance of cell kinetic measurement in the understanding and subsequent management and control of malignant cell population is self-evident. Despite all the apparent disadvantages mentioned and the laborious nature of the technique, autoradiography using stripping film or emulsion continues to be a valuable if limited research tool. The main advantage still at present remaining over more recent technology, is that different cell populations are readily identified and their relationships to each other can be recognised.

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## MATERIALS AND SUPPLIERS

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