

M. D.

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CLINICAL AND LABORATORY STUDIES
ON HUMAN LYMPHOMA

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ABSTRACT OF THESIS

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Title of Thesis ... Clinical and Laboratory Studies of Human Lymphoma

The thesis is in two parts. The first concerns a laboratory study of human lymphoma cells and their interactions with reticulo-endothelial cells. Electron microscopy and tissue culture studies indicated that macrophages and lymphocytes were in close contact with tumour cells but no cytotoxic effect was noted in the intact tissue. A time lapse film of lymph nodes in culture shows that when cells are teased out in a monolayer, the tumour cells become susceptible to direct attack by lymphocytes and tissue macrophages. Lymph node cultures of lymphoma liberate chemotactic factors to the supernatant which attract eosinophils, monocytes and leucocytes. The eosinophil chemotactic factor was found in high concentrations only in lymph nodes involved with Hodgkin's disease. Also in the supernatants were found two factors which depressed host cells. After incubation of supernatants with lymphocytes, their subsequent ability to transform was depressed and after incubation with normal monocytes, subsequent chemotaxis was inhibited. Peripheral blood monocytes from lymphoma patients were significantly less mobile than age-sex match controls in a chemotaxis assay.

The clinical part of the thesis describes firstly a search for space-time clusters in the occurrence of lymphomas in the South East of Scotland over a period of eleven years. Fourteen clusters were found from centralised computer records but only one of these survived closer scrutiny. Secondly a cohort of one hundred consecutive patients with lymphoma has been followed for two to seven years. Overall survival of 68% is considerably superior to retrospective studies which took place before 1970. Improvement in survival is due to rigorous staging, more appropriate radiotherapy and the advent of effective chemotherapy. Several serum parameters such as low albumin, low immunoglobulin M and low serum α_2 macroglobulin independently worsened the prognosis. This information taken with the findings of the laboratory studies suggest that immunodeficiency in lymphoma patients is accompanied by a poor prognosis and may be due to release of tumour specific factors. Such high risk patients should be managed by intensive immunological support including removal of such factors and restoration to high protein levels prior to or along with cytoreductive therapy.

PREFACE

The work contained in this thesis has been carried out by me over the last seven years in the University Departments of Pathology and Therapeutics, Edinburgh, and the University Department of Clinical Oncology, Glasgow. I am deeply grateful to Professor R.H. Girdwood for his clinical guidance and to Professor A.E. Stuart for teaching me many techniques, and to both for the generous facilities I have been offered.

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INTRODUCTION

The challenge of lymphoma celebrates its 150th anniversary this year. Although Cruickshank inadvertently described the first case in 1786 "in which the tracheo-bronchial lymphatic glands were affected with this morbid change to such extent as to cause fatal suffocation" lymphoma was not recognised as an entity until David Craigie's book was published in Edinburgh in 1828. Craigie was 4 years ahead of Hodgkin in his description and very little has been added to his thoughts on the disease until the last quarter of a century. Craigie recognised that the disease emanated in the lymph glands. He felt that the aetiology was possibly an inappropriate reaction to infection or inflammation and he recognised that this was a malignant disease. Over the next 125 years there was a proliferation of descriptive titles of the macroscopic and microscopic appearances of the lymphomas but a paucity of progress. In 1845, Craigie and Bennett in Edinburgh and Virchow in Europe independently described the first cases of leukaemia and linked them with disease of the spleen and lymphatic glands. Thereafter followed leucocythaemia lymphatica, pseudo-leukaemia, granuloma malignum, follicular lymphoma of Brill and later Simmers, retothele sarcome and clasmatocytic lymphoma.

Distinction by histology of Hodgkin's disease stemmed from Greenfield 1878, who like Craigie did not have the honour of being associated in name with his discovery. Instead, Sternberg 1898 and Dorothy Reed, 1902, were credited with the description of Hodgkin's giant cells. Later Gall and Mallory, 1942, tried to sort out the classification of non Hodgkin's lymphoma, and

Jackson and Parker, 1947 arranged the first semblance of order in the typing of Hodgkin's disease.

The last 25 years however, have seen considerable progress not only in more meaningful classification (Lukes and Butler, 1965, for Hodgkin's disease, and Rappaport et al, 1956, for other lymphomas), but also in the development of the concept of staging by radiology (Kinmonth, 1952) and surgery (Glatstein et al, 1970), the astonishing response to treatment by megavoltage radiotherapy (Peters, 1966) and quadruple chemotherapy (De Vita et al, 1973).

In the realms of immunology, the last 10 years has seen further challenges in lymphoma as the science of membrane markers has developed. From the time of Craigie till now little thought has been given to the nature of the cells involved in the malignant process, their origins or their eventual fate. Now it is clear that most non Hodgkin's lymphomas are derived from B lymphocytes which are characterised by their association with immunoglobulin (Proudhomme and Seligmann, 1973). T cell lymphomas do exist, as do a few histiocytic tumours. Most reticulum cell sarcomas are now known not to be histiocytic but lymphoblastic in origin (Lukes and Tindle, 1975).

Matters are a little more confused however in Hodgkin's disease. Reed-Sternberg cells have variously been acclaimed as B cells (Taylor, 1974), T cells (Biniaminov and Ramot, 1974) and histiocytes (Kadin, 1974). There is a well recognised deficiency of delayed-type hypersensitivity associated with some Hodgkin's patients which is shown by anergy and inefficient lymphocyte transformation (Aisenberg, 1962, Levy and Kaplan, 1974). At first this seemed to form evidence for the theory that the

disease arose as a result of impaired immunosurveillance. Now it is viewed as a result of immunological exhaustion following a "lymphocyte civil war" (De Vita, 1973) or graft versus host disease (Smithers, 1973). There have been data brought forward which shows evidence of a host response, raised immunoglobulin levels, and elevated T cell counts in Hodgkin's spleens (Kaur et al, 1974). Might lymphoma arise as Craigie hinted in 1828 from uncontrolled lymphocyte stimulation, perhaps due to a defective feedback mechanism which fails to switch off the stimulating control? The evidence from mice would support this theory (Islaker et al, 1975) as mice have a very high incidence of lymphoma in response to chronic stimulus of the reticulo-endothelial system. In man there is circumstantial evidence linking diseases such as Sjogren's syndrome, systemic lupus erythematosus and infectious mononucleosis with lymphoma (Gershwin and Steinberg, 1973, Rosdahl et al, 1974). Schwartz 1975, points out that "immunodeficient" renal transplant recipients who develop a high incidence of cancer, predominantly have a lymphoblastic lymphoma (which used to be called reticulum cell sarcoma). Antigenic stimulus might be the reason for the development of a similar histological type of lymphoma in a patient described by McVie et al, 1974, (appendix 9) who had polycythaemia vera for several years prior to development of malignant myelofibrosis and then lymphoma. On the other hand, one must provide proof of the antigen and most studies in this direction have been negative. A viral antigen is suggested by Spiegelman et al, 1973, and Chezzi et al, 1976, but definitive evidence is lacking.

Vianna et al, 1971, have found cases of Hodgkin's disease in clusters though the validity of their conclusions has been questioned by Smith and Pike, 1976. A genetic link has also been floated as a part solution to the problem of aetiology of Hodgkin's disease and lymphoma. Sibling studies (Grufferman et al, 1977, and Purtilo et al, 1977) are supported by findings of an increase in certain HLA antigens in these diseases (Falk and Osoba, 1971). A genetic predisposition to lymphoma may still, of course, be related to viral exposure. An autosomal recessive lymphoma of pigs for instance (Head et al, 1974), has been shown to contain reverse transcriptase for an RNA virus incorporated into the genetic material of the malignant cells (Todaro, G. personal communication). Furthermore, children who have combined immunodeficiency associated with a genetic fault governing adenosine deaminase synthesis have a predisposition to lymphoma.

The aspect of the relationship of viruses to lymphoma which is of most fascination is the similarity in immune defects associated with each. Viral infections are known to cause depression in lymphocyte transformation and energy although usually they are temporary (Kantor, 1975). It may be interjected here that "Reed-Sternberg" or Greenfield giant cells have been described in infectious mononucleosis as well as Hodgkin's disease (Lukes et al, 1969).

The present thesis is concerned with the possible relationships of immunity, lymphoma and infection. Attention has been turned to the interactions of malignant cells and host cells, in particular monocytes and macrophages, and the relevance of serum factors both defensive (such as immunoglobulins) and aggressive (such as immunosuppressive products of tumour cells). Whereas viral

infections, even mild influenza, can produce complete inhibition of monocyte function (Kleinerman et al, 1975) nothing is known of the effect of lymphoma on the monocyte.

Yoshida et al, 1975, have identified a macrophage inhibition factor (MIF) produced by kidney cells infected by simian virus 40. The virus stimulated MIF is identical in immunological and biochemical properties to the MIF synthesised when sensitised lymphocytes are exposed to specific antigen. This kind of preliminary evidence strongly supports a unified concept of immunity in which lymphocytes macrophages and their products (virally or immunologically induced) all take part, and which might be suppressed at any or all of those sites.

The monocyte and macrophage are now recognised to have important antitumour properties either direct (Keller, 1974) or by secretion of intermediary soluble factors (Alexander, 1972). Further, anergy in lymphoma which may accompany any histological types (Aisenberg, 1972) might be associated with primary macrophage dysfunction instead of a lymphocyte defect. Recall of previously recognised antigens is not as often impaired as is the ability to develop sensitisation to new antigens such as dinitrochlorobenzene; neither property relates to T, B or total lymphocyte numbers (Brown et al, 1967). Also recall of antigens such as streptokinase is dose related and is almost always positive in lymphoma patients if a sufficiently high concentration of antigen is used (Eltringham and Kaplan, 1975).

It would appear that T cell impairment at least in Hodgkin's disease, judged by depressed transformation by mitogens is almost completely reversed by carrying out the test in normal serum instead of autologous Hodgkin's serum (Trubowitz et al, 1966) or

in the presence of an anti-prostaglandin substance (Goodwin et al, 1977). Bjorkholm et al, 1976, used a mixed lymphocyte culture system and could not find significant depression of transformation of Hodgkin's patients' cells. This group, however, did find a total failure on the part of those Hodgkin's lymphocytes to stimulate blastogenesis in normal lymphocytes in the reverse experiment. They proposed that the likely explanation was the presence of blocking factor on the surface of lymphocytes, either produced by the tumour or by the cells themselves in an over enthusiastic response to the tumour. Immune complexes as discussed later might be a fitting candidate for such a blocking agent, or else substances such as sialic acid or orosomucoid which are common to all cell membranes, but are found in high concentrations in malignant disease (Israel and Edelstein, 1978).

The aetiology of such immune defects might also be the cause of lymphoma, or else the immune deficiency might lead to development of the malignancy. The third possible relationship is conversely that lymphoma tissue might lead to depressed immunity by release of factors such as the tumour associated peptide shown to cause anergy by Glasgow et al, 1974, or by direct invasion of the reticulo-endothelial system.

The thesis which follows has explored the above inter-relationships and has three broad aims; first to look for further associations of virus infection with lymphoma, second, to examine the interactions of the reticulo-endothelial system with invading tumour cells, and third to assess the relevance of simple immunological parameters to the management and prognosis of patients with lymphoma.

There are two broad divisions in the work, one laboratory and the other clinical. The first describes the study of the cells of the lymphoma lymph node, their products, and the efficiency of the monocyte system sampled from peripheral blood. The clinical section is in two parts, a retrospective, epidemiological and clinico-pathological study and a prospective clinical study. Patients included in the last group had serial measurements of blood counts, and serum albumin, immunoglobulins, α_2 macroglobulins and bacterial antibodies. Careful clinical and pathological staging and re-assessment of disease status was carried out and the data used to find prognostic features of value in prediction of infective complications and overall response to therapy.

A video cassette which shows a film of the interactions of lymphoma cells in culture accompanies the written text of the thesis.

METHODS

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- b) Electron microscopy
- c) Cell culture
- d) Time-Lapse cine photomicrography
- e) Fluorescence studies
- f) Supernatants from cultures
 - i leucocyte chemotactic factors
 - ii monocyte chemotactic factors
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 - v mitogenic factor

2. Peripheral Blood Studies

- a) Phagocyte Function
 - i identification of monocytes
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B. CLINICAL STUDY

1. Retrospective Search for Clusters of Lymphoma
2. Prospective Study of Management of Lymphoma
 - a) Patients
 - b) Histology
 - c) Staging procedures
 - d) Treatment
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METHODS

A. LABORATORY

1. LYMPH NODE STUDIES(a) Lymph Node Samples

Lymph nodes were obtained fresh from the operating theatre and were bisected immediately and tissue dabs were made on clean glass slides; one half was taken for routine histology, a portion was sampled for electron microscopy and the remainder was immersed in medium 199 containing pooled AB serum (appendix 1).

(b) Electron Microscopy

Samples of lymph node which were taken for electron microscopy were diced into 2 mm cubes. They were then fixed in gluteraldehyde (T.A.A.B. laboratories) in Sorensen's phosphate buffer (pH 7.2) for 4 to 18 hours at 4°C either by immersion or by direct perfusion through a fine bore needle. These small cubes were then trimmed again, washed in 2 molar phosphate buffer for 2 hours at 4°C and transferred to 1% osmium tetroxide in veronal acetate buffer (pH 7.2) for 90 min at 4°C. The tissue was then washed three times in changes of 10% ethanol over 30 min and twice in propylene oxide over 40 min. Following this, impregnation in araldite was carried out in plastic troughs overnight and the next day they were imbedded in gelatin capsules containing fresh araldite. Ultra thin sections were cut and stained with 1% toluidine blue and the areas were selected for examination in the electron microscope. After trimming, pyramids were cut to copper grids, scanned and photographed.

(c) Cell Culture

Cells for culture were obtained by carefully dividing the tissue into small fragments with two scalpel blades under sterile conditions. Gentle homogenisation was then carried out with a glass hand homogeniser and the cell suspension obtained was passed through a sieve to remove fibrous debris. Cell counts were adjusted to 5×10^6 cells/ml and viability was assessed by trypan blue exclusion and phase contrast microscopy. Only preparations which contained more than 90% viable cells were subsequently cultured.

Cultures were set up in glass test tubes containing flying coverslips (20 x 5 mm). The cells were contained in 1 ml volumes of medium 199 with 50 units of penicillin and 5 μ g of streptomycin to which were added 10% lactalbumin (appendix 2), or 10% pooled O or AB human serum. Fresh autologous serum and foetal calf serum were found to be toxic to human lymph node cultures. Tubes were stoppered and incubated at 37°C; supernatants were removed at days 1, 3 and 5 and replaced with identical culture medium. Supernatants thus obtained were centrifuged to remove particulate material, pooled and dialysed for 24 hours against phosphate buffered saline then lyophilised. Prior to use in assay systems the material was reconstituted either in distilled water or medium 199 to a standard protein concentration which was measured by the Folin-Coicalteau method.

In several instances, tiny (0.5 mm cubed) fragments of tumour were placed on flying coverslips and each covered by a drop of human plasma which was then allowed to clot. Cells would then be filmed as they moved outwards from the edge of the fragment over a period of 1 to 2 weeks. These cultures were not used for

fluorescence studies nor were their supernatants analysed.

Flying coverslips and tissue dabs on glass slides were air dried for fluorescent studies or fixed in methanol and stained with Giemsa.

An attempt was made to visualise the cultured cells by electron microscopy. After washing cultures thoroughly, cells were either scraped off the coverslip gently with a razor blade or exposed to Tween 80 detergent for 1 or 2 seconds. Neither process was very successful, as cells were inevitably damaged. After removal, they were centrifuged into a pellet and fixed in gluteraldehyde as above.

(d) Time Lapse Cine Photomicrography

Fresh tissue was available from an occasional large lymph node biopsy for direct study by phase contrast microscopy. Preparations of cells were made as above and concentrations of 1,000 cells per ml of medium 199 containing 20% pooled AB serum were placed in special air tight glass chambers. These chambers were then put in position on the warm stage of a Wild M-40 inverted microscope. Illumination of the cells in their culture chamber was provided by a quartz-halide lamp controlled by an external magnetic shutter. Time lapse cine films were then taken from a Bolex H-16 camera attached at one side of the microscope. The shutter on the camera was attached to a separate electric motor mounted outside the camera; x20 and x 40 phase objective lenses were used and UV and heat filters employed while filming. The electric motor for the camera shutter and the magnetic shutter on the microscope lamp were automated and controlled from an electronic unit with a manual override. The manual control was required for focussing in the few seconds between each exposure. The cine film used was Kodak Tri-X reversal in 100 feet lengths.

Editing of these films was carried out by hand and then run at 24 feet per second. Several single frames were enlarged for use throughout the film and for inclusion in the text. The film was then transferred to video tape with the help of the University of Glasgow Television unit, and a sound track was superimposed.

(e) Fluorescence Studies

Samples of sera from patients who had undergone lymph node biopsy along with control normal sera and sera from students suffering from infectious mononucleosis, were used in direct fluorescent studies. The gamma globulin fraction from serum samples was labelled with FITC in the standard manner of Nairn 1976. After purification of the conjugates and dialysis against phosphate buffered saline, the protein concentrations were standardised and the samples absorbed several times with rat liver powder. Direct fluorescent experiments were then carried out by applying the FITC labelled sera directly to tissue dabs or to glass coverslips on which lymph node cells had been cultured. After incubation at 37°C for 30 min the slides were studied by a phase contrast fluorescent microscope. In addition, non-specific FITC labelled polyvalent anti-immunoglobulin (Boehringerwerk) was applied in a similar manner to assess the staining of cultured cells and tissue preparations for immunoglobulin content.

(f) Supernatants from Cultures

(i) Leucocyte chemotactic factors

Chemotaxis of human peripheral blood eosinophils and neutrophils was assessed by using a modified Boyden chemotaxis method (Kay, 1970). A simple chemotaxis chamber (Fig. 1A) was used consisting of a sawn off syringe barrel with a Nucleopore filter ("Wallabs", San Rafael, California) pore size 2-5 microns sealing

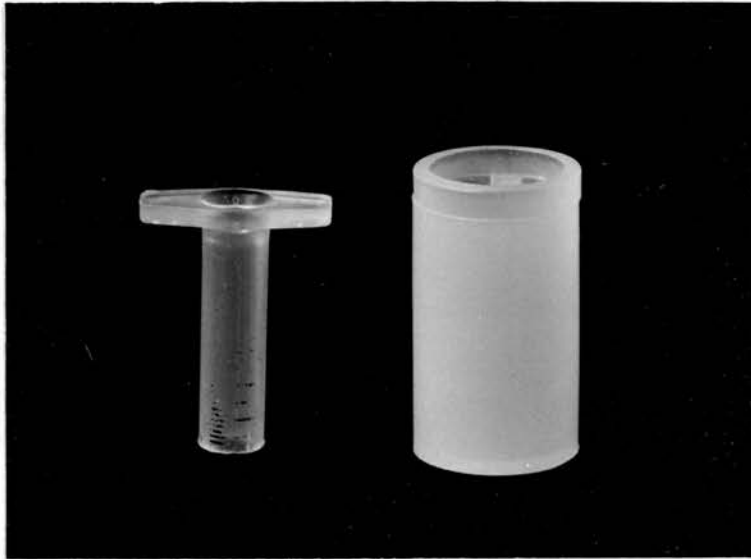


Fig. 1A Simple chemotaxis chamber consisting of sawn off syringe barrel on the left and plastic pot on the right

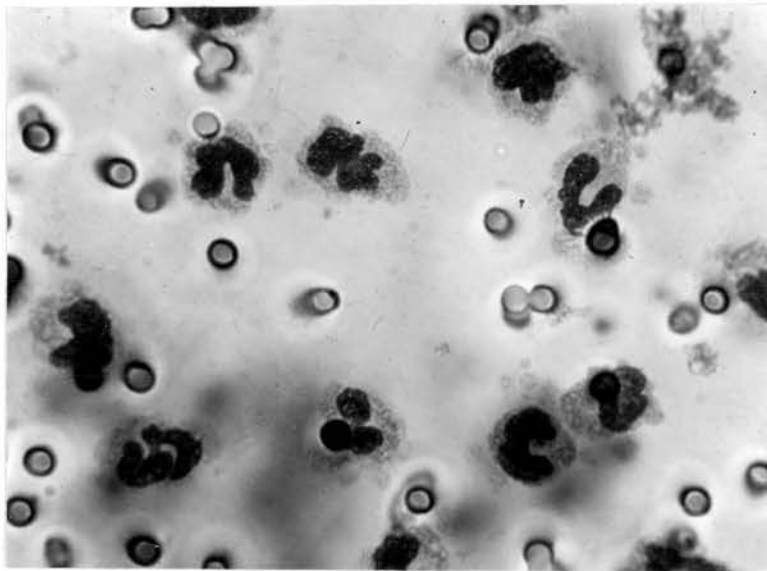


Fig. 1B Nucleopore filter showing the pores in the membrane and neutrophils which have moved through them (Giemsa x 500)

the sawn off end. The supernatant to be tested was placed at various dilutions in a small beaker and the syringe barrel was filled with normal leucocytes adjusted to a standard concentration of 1,000,000 cells per ml. Leucocytes were obtained from normal volunteers as follows:

ten ml of blood was drawn into a syringe containing 100 units of preservative-free heparin and then mixed with 3 ml of 10% dextran. The mixture was allowed to sediment for 40 min at 37°C and the buffy coat cells were used for the experiment after a cell count had been carried out in a Neubauer chamber. Samples rich in eosinophils were obtained from normal individuals suffering from allergies or in one instance, a ward sister who had a 50% eosinophilia of unknown aetiology. Cells were allowed to migrate through the Nucleopore filter at room temperature for 30 min. The membrane was then detached from the syringe barrel, washed, fixed in methanol and stained with a 5% Giemsa stain. The number of neutrophils and eosinophils in ten high power fields was counted and each assay was done in triplicate. The perforated filter is shown in Fig. 1B.

(ii) Monocyte chemotactic factor

The supernatants from lymph node cultures were tested for their ability to attract normal peripheral blood monocytes through a Millipore filter in a similar system to that described above. A more sophisticated chemotactic chamber was used however (Fig.2). Area B was filled with the chemotactic factor under test from the limb on the right hand side. Area A was filled synchronously with normal peripheral blood monocytes. Twenty ml of venous blood was withdrawn on each occasion from the author into a sterile plastic syringe containing 200 units of preservative-free heparin.

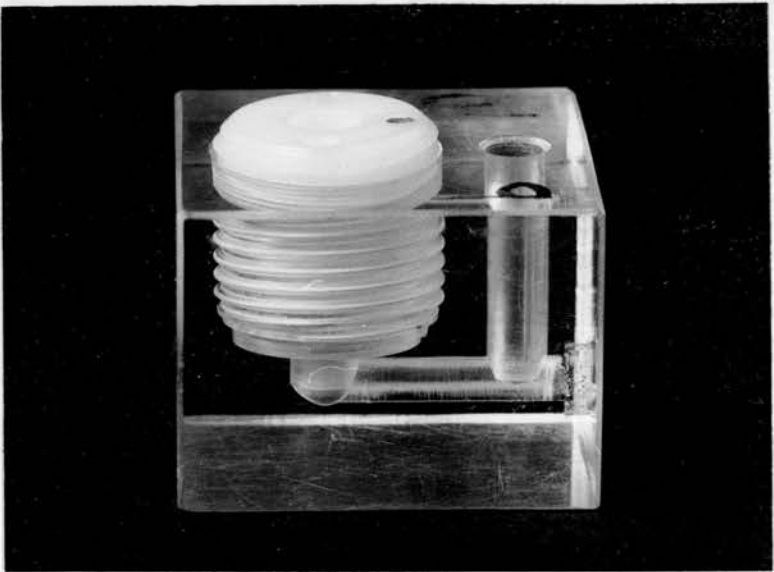
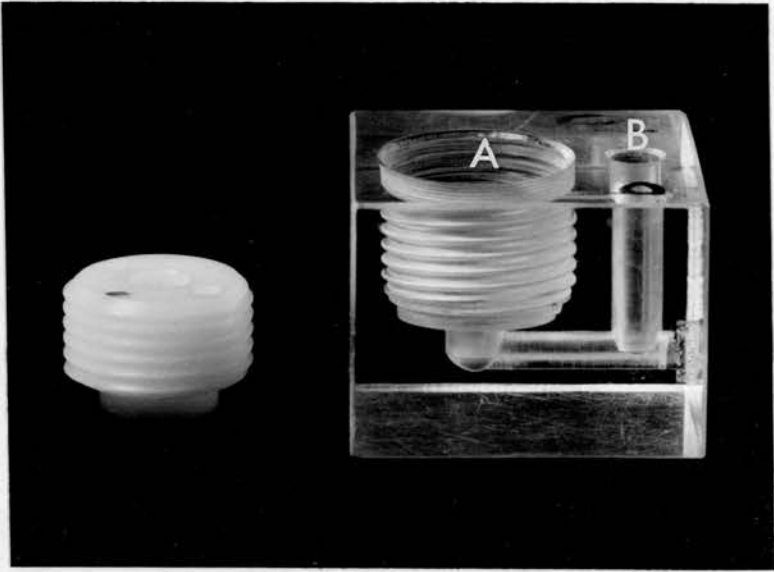


Fig. 2 Modified Boyden chemotaxis chamber

Mononuclear cells were separated as follows:

each blood sample was placed in a plastic 100 ml volumetric flask and diluted with 60 ml of sterile saline. The cylinder was closed with parafilm and the blood mixed by inversion. A 3 ml aliquot of Ficoll-Triosil solution (appendix 3) was placed in each of 8 plastic conical centrifuge tubes and 10 ml of the diluted blood was carefully layered over each 3 ml sample. All tubes were centrifuged at 400 g for 30 min and examined for a discrete layer of mononuclear cells. These layers were carefully removed using a Pasteur pipette and pooled in pairs in 4 sterile plastic tubes. The pools were washed once in medium 199 containing heparin and all cell deposits were pooled into one tube. The second wash was then carried out in medium 199 containing heparin and the final deposit resuspended in medium 199 containing no heparin. The cells were counted by haemocytometry using 2% acetic acid tinged with crystal violet and the final count adjusted by dilution to 0.5×10^6 monocytes/ml by addition of medium 199. During the total cell count by haemocytometry the cells were allowed to settle for 15 min, a technique which allows the monocytes to flatten out slightly, rendering them more easily enumerated. A Millipore filter with a pore size of 8 microns was used instead of the Nucleopore filter and one was inserted into the support mesh in Area A of each chemotactic chamber. It was then fixed into position by insertion of the screw which was tightened by a screw holder. The filter area exposed to mononuclear cells was similar to the area of the lumen of the screw insert.

Chambers were incubated in a moist environment for 2 hours at 37°C , both areas of the chamber were then emptied simultaneously employing Pasteur pipettes. The chamber must be tilted to remove

trapped fluid from the horizontal part of the Area B by allowing a bubble of air to displace it. The filters were removed after extraction of the screw insert and transferred using Millipore forceps into absolute ethanol for 10 min. Staining procedure was as follows:

absolute ethanol for 10 min, 90% ethanol 2-5 min, 75% ethanol 2-5 min, 50% ethanol 2-5 min, distilled water 2-5 min, Mayer's haematoxylin 5 min, tap water 10-30 min, 50% ethanol 2-5 min, 75% ethanol 2-5 min, 90% ethanol 2-5 min, absolute ethanol 2-5 min, xylol overnight. After clearance in xylol membranes were placed on a 3 inch x 1 inch microscope slide and mounted in Depex.

Examination of the membranes was carried out within 12 hours of each test as gradual opacification took place within the membrane. A Watson 60 microscope with hilus base was used and a x 40 dry objective lens. Each circular area was scanned initially with the x 10 objective to locate the centre of the field and to ensure that the cell population was evenly distributed and not clumped. Membranes with clumps of cells on them were discarded. With the x 40 objective the fine adjustment Vernier scale was set to zero, and using the fine adjustment focus, the upper border of the membranes was detected. The distance from the upper edge through the membrane to the "leading front" was measured on the fine adjustment Vernier scale. The "leading front" was defined as the point beyond which no monocytes were seen, but at which two monocytes were clearly in focus. The travel between the initial and the second reading on the Vernier was noted and ten readings were taken on each filter. A second observer carried out similar readings on the first 100 tests until it was clear that there was good reproducibility in the method.

The mean of ten readings from each filter were calculated with their standard deviations. Each test was carried out in duplicate.

(iii) Monocyte chemotaxis inhibitors

Lymph node supernatants

Several attempts were made to study the supernatants from lymph node cultures for the presence of macrophage inhibition factor. For this test, guinea pig macrophages were inserted into a capillary tube, one end of which was sealed. The ability to inhibit spread of macrophages out of the capillary tube into the surrounding culture medium is then measured. It proved extremely difficult to achieve reproducibility in this test. Particularly on no occasion was it possible to demonstrate macrophage inhibition factor in the positive control using PPD as the antigen. The technique was therefore abandoned and in its place the monocyte chemotactic assay was used in a different way.

Normal peripheral blood monocytes were incubated in a 50% dilution of each lymph node supernatant for 1 hour at 37°C. The cells were then washed three times and then resuspended in concentrations used for chemotaxis in the above experiments. As a positive known chemo-attractant, casein (appendix 4) was used at two concentrations, 0.5 mg/ml and 1 µg/ml. The distance of migration of cells pre-incubated in lymph node supernatants was then compared with cells which had not been so treated. In this way, it was hoped to demonstrate the presence or absence of factors present in the supernatants which might bind to monocytes during incubation and impede their progress through the Millipore filter.

Pleural Fluid

A sample of pleural fluid containing recognisable Reed-Sternberg cells was obtained from a 30 year old female patient with nodular sclerosing Hodgkin's disease which at post mortem was shown to involve both pleura and lungs. This fluid was centrifuged to remove debris and cells and the supernatant was added either neat or diluted to 50% to parallel cultures of normal peripheral blood monocytes and incubated for 60 min at 37°C. The washed cells were tested on nine occasions for chemotactic ability using nine different monocyte samples obtained from age-matched normal volunteers. On each occasion 0.5 mg/ml casein was used as a standard chemo-attractant and duplicate cultures of normal monocytes incubated in medium 199 alone were used as a positive control.

(iv) Skin reactive factor

Supernatants obtained from lymph node cultures were tested either neat or diluted 50% or 25% with phosphate buffered saline. An injection of 0.1 ml of test substance was injected subcutaneously into the shaved skin of a guinea pig abdomen. In each experiment 0.1 ml of phosphate buffered saline was used as a control substance. The guinea pig abdomens were inspected at daily intervals for the presence of erythema and/or induration.

(v) Mitogenic factor

Aliquots of 25 ml of heparinised normal blood were diluted 25% with phosphate buffered saline and layered on to Ficoll-Hypaque. A band of cells, predominantly lymphocytes, was achieved after centrifugation for 30 min and this band was removed with a Pasteur pipette and washed three times in medium 199. Aliquots of 200,000 lymphocytes were incubated in separate microtitre wells in 5%

carbon dioxide and 95% air in triplicate. Six different supernatants from lymph node cultures of lymphoma were added in a concentration of 25%, 50% or 75% to samples of lymphocytes. Six wells were left unstimulated and to 18 other wells PHA was added in 3 different concentrations, 0.43 $\mu\text{g/ml}$, 1.66 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$. This was done twice in triplicate. After incubation of the cultures for 72 hours they were pulsed with 1 μCi of tritiated thymidine with a specific activity of 27 Ci/mmol . Four hours later they were harvested with trichloroacetic acid then washed in methanol and after the addition of scintillation fluid, counted in a liquid scintillation counter with a counting efficiency of 50.5%.

2. PERIPHERAL BLOOD STUDIES

(a) Phagocyte Function

(i) Identification of monocytes

Samples of peripheral blood from patients with lymphomas and matched controls were obtained by venesection. The heparinised samples were treated in Ficoll-Trisil as described above in the section on monocyte chemotaxis. In addition to counting monocytes in a Neubauer chamber, non-specific esterase (Van Hoofe and Hers, 1968) and peroxidase stains (appendix 5) were carried out to ensure the correct identity and enumeration of monocytes.

(ii) Monocyte chemotaxis

Monocytes obtained as above were placed in the modified chamber (see above) for monocyte chemotaxis. Concentrations of 0.5 million cells per ml were placed in Area A and the chemo-attractant, casein at concentrations of 0.5 mg/ml and 1 mg/ml were placed in Area B below the Millipore filter (pore size 5-8 microns). Duplicate chambers were incubated for 2 hours at 37°C in a moist environment and then filters detached and prepared for cell counting as before. The leading front was used to assess the distance through the Millipore filter which lymphoma patients' monocytes had travelled compared to the distance travelled by age/sex-matched controls. Results were expressed as the distance travelled by control minus distance travelled by patient over distance travelled by control multiplied by one hundred.

(iii) Monocyte phagocytosis and bactericidal capacity

Twenty millilitre samples of heparinised blood were taken from lymphoma patients and controls and monocytes were separated

as above. Cells were then subjected to short term culture on glass coverslips during which bacteria were added and the rate of phagocytosis and eventual bactericidal capacity of cells was calculated by serial sampling.

Serum

Cells were cultured at a cell count of 1×10^6 mononuclear cells in 30% human pooled AB serum. The pool from three donors was inactivated and stored in aliquots at -40° . Autologous serum was attempted in several instances but was usually impracticable as it required additional blood samples from the patient. Foetal bovine serum, activated and inactivated and new born calf serum were found to be inferior to human serum as judged by viability of macrophages studied at 12, 24, 48 and 72 hours in culture.

Culture Conditions

One millilitre of a suspension of 1×10^6 cells in 30% serum was added to a 4 ml plastic tube; a circular glass coverslip was attached by plasticine to the lid of the tube and sterilised prior to use. The tube containing mononuclear cells was sealed with its lid and inverted so that cells settled on the glass coverslip on the upper surface of the lid. Cells were cultured for 22 hours at 37°C in a 5% CO_2 , 95% air incubator. After this time the tubes could be restored to the upright position and the glass coverslip removed for staining by Giemsa, non-specific esterase or peroxidase stains. Occasionally coverslips were broken but after practice they could be removed without breakage or without disturbance of the monolayer which had formed overnight.

Bacteria

Staphylococcus aureus was found to be the most appropriate strain of bacteria for use in these experiments. It was easy to culture,

replicated predictably, and stained well even when inside cells, Several strains of *Escherichia coli* were used but it was found in these experiments that phagocytosis was not reproducible and was often very poor even using normal cells. A standard staphylococcus number 92 was subcultured in neutral broth, one loopful of bacteria to 15 ml of broth, and cultured at 37°C for 20 hours, the bacteria were then spun at 2100 g for 10 min and the broth discarded. The bacteria were resuspended in normal phosphate buffered saline with heparin (concentration 10 units/ml), to prevent clumping. They were then respun at 2100 g for a further 10 min and resuspended after the supernatant was discarded, in 15 ml of HEPES buffer. Five ml of this solution was then diluted with approximately 15 ml of HEPES buffer to achieve a concentration of bacteria between 15 and 20 million/ml. This was judged optically.

Phagocytosis

Staphylococcus aureus was prepared as above and made into a solution containing 10% AB serum; a control solution containing HEPES buffer and 10% AB serum without bacteria was also prepared. Sterile glass tubes filled with 1.8 ml of 0.9% sodium chloride were arranged so that dilutions of bacteria could be achieved rapidly, and agar plates were labelled with times and dilutions. Cell cultures on coverslips were removed after 22 hours of incubation and the supernatant containing non-glass adherent cells discarded. The glass adherent cells were shown to be predominantly monocytes by peroxidase and non-specific esterase staining. A random coverslip was selected to assess viability of cells in culture by trypan blue staining and another coverslip was stained by Giemsa stain for reference counts. A suspension of 1.5 ml bacteria containing 0.75 ml

of bacteria and 0.75 ml of Hepes and 10% AB serum was added to each tube and to three tubes without cells on the coverslip. The tubes were re-incubated at 37°C and the bacteria were allowed to precipitate on to the coverslip to facilitate phagocytosis by the macrophages growing there. Phagocytosis was measured at 5, 10, 15, 20 and 30 min of incubation. The coverslip was removed from each sample and stained with Leishman's to confirm phagocytosis and to assess the number of bacteria per cell. The supernatant was subcultured as follows:

samples of 0.2 ml were taken from each tube and gently shaken to allow bacteria to be evenly resuspended in the Hepes. The samples were then diluted in 1.8 ml of normal saline in sterile glass tubes to final dilutions of 10^{-4} and 10^{-5} . Aliquots of 0.1 ml of both dilutions were cultured separately on mannitol salt agar plates, in duplicate. A zero sample of the bacterial suspension was taken from the blank tubes containing no cells and subcultured in the same way.

Bactericidal killing

After 20 min of phagocytosis duplicate coverslip cultures were interrupted by discarding the supernatant followed by addition of 1.5 ml of distilled water with bovine serum albumin. The distilled water was found to be extremely effective at lysing cells and the bovine serum albumin protected the bacteria. The tubes were then well mixed and the coverslip shaken to release all cells and their intracellular bacteria. Supernatant samples of 0.2 ml from each tube were taken and diluted as before to 10^{-3} and 10^{-2} . Samples of those dilutions were plated on mannitol salt agar and incubated for 48 hours at 37°C. The rate of killing of bacteria was

determined by incubation of duplicate cultures for 40 min beyond the time taken to test phagocytosis (20 min). Thus the total time of culture was 60 min with the first supernatants being discarded at 20 min, and replaced by 1.5 ml of HEPES with 10% human AB serum. The coverslips were sampled at 10, 20 and 40 min after phagocytosis had taken place. The supernatants were discarded as before and the cells lysed in distilled water to release intracellular bacteria. These were counted by subculture on mannitol salt agar. Colonies of bacteria were counted at 24 hours and 48 hours after incubation at 37°C.

(b) Serum Proteins

(i) Immunelectrophoresis

A pilot study of 31 patients with lymphoma was carried out to gain an estimate of the abnormalities of immunelectrophoresis (Grabar and Williams, 1953) of their sera. The technique was carried out on agar gel as follows:

Buffer Solution

Stock buffer pH 8.6

Sodium bicarbonate 50 gm

Sodium acetate 52 gm

N.HCl 32.1 ml

Distilled water to 5,000 ml

Working Buffer

Thirty ml of the above diluted with 70 ml distilled water;
or 50 ml with 50 ml distilled water.

Agar

Oxoid Janagar No.2 was used; 1.5 gm agar was dissolved in 98.5 ml of working buffer solution. This 1.5% mixture was

most satisfactory when melted in an oven at 120°C for 20 min. A very clear molten solution was obtained.

Pouring of slides

Using a 5 ml glass pipette which had been warmed in an oven at 56°C molten, 1.5% agar was carefully poured onto the slide; 2 ml agar was poured onto a microscope slide 1 inch x 3 inch. This gave a depth of 1.5 mm.

Cutting required pattern

When the agar was completely solidified the required pattern of holes and troughs was cut with a template. The holes for antigens were cleaned out, but the troughs were not cut out until after electrophoresis.

Tank for electrophoresis

The tank designed by Dr. J.J. Kohn and manufactured by Shandon Ltd. was used.

Electrophoresis

A constant current of 1.5 amps per slide (of 1 inch width) was used and a voltage of between 90-120 volts was maintained. The antigen in the wells was stained lightly with bromophenol blue. The passage of the albumin fraction could then be observed. After approximately 115 min, separation was at its optimum for precipitation of clearly defined lines. The agar strips were lifted out of the troughs and the antiserum was added. The slides were left in a cool damp atmosphere overnight.

Drying and staining of slides

The slides showing a precipitate were placed in a diluted buffer solution and this solution was constantly changed to remove excess protein. Photographs could then be taken using indirect lighting against a dark ground. Filter paper strips slightly

larger than the slides were carefully placed on top of the agar. After the agar had dried to a clear film, the filter paper was removed. Any excess was removed by rubbing lightly in distilled water.

Staining

The stain found to give the best results for both photography and observing with a lens was amido-black:

0.2 gm	amido-black (G.T. Gurr)
95 ml	distilled water
5 ml	glacial acetic acid
1 gm	mercuric chloride

Staining of the precipitation lines took approximately 40 seconds. Surplus stain was washed off with 5% acetic acid solution. Any bad staining due to unremoved excess antiserum was successfully removed by placing filter paper soaked in buffer on the stained area.

Antisera

Boeringwerke commercial rabbit antisera against human IgG, IgM, IgA, IgD, α_2 macroglobulin, kappa chain and lambda chain were used singly against each test serum.

(ii) Immunoglobulin quantitation

Immunoglobulins A, G and M were estimated serially from the sera of one hundred patients described below. The standard technique of radioimmuno diffusion (Mancini et al, 1965) was used employing Hyland commercial antiserum. Kits are available for this technique but due to the considerable expense it was decided to make a copy of the kit which could be used for ten times the number of estimations. Difco purified agar was melted and 8 ml was allowed to form a layer in a mould measuring 4 inch x 3 inch. Hyland

commercial goat anti-human immunoglobulin antiserum was mixed with agar at 56°C and the dilution used for anti IgG and anti IgA antisera was 5% in agar and for anti IgM 2.5% dilution. The agar and incorporated anti-immunoglobulin were then refrigerated at 4°C for 2 hours and 35 holes were punched in the agar using a suitable template. The agar was allowed to sit overnight before use. Using standard sera added to the wells the radii of diffusion of the immunoglobulin/anti-immunoglobulin in precipitates were plotted to obtain a standard curve. Test sera were added to the wells concurrently and the levels of immunoglobulin obtained from the standard curves. Normal values for this method were obtained by studying over a thousand samples from normal volunteers and convalescent patients and these values were in concurrence with the ranges quoted by Hyland for their antisera.

(iii) Escherichia coli antibodies

A haemagglutination test for the presence of *Escherichia coli* antibodies was adapted from Webster et al, 1974, as follows:

Preparation of *Escherichia coli* antigen

The following six *Escherichia coli* were seeded on nutrient agar plates:

- a. 01. K1.H7
- b. 02. K1.H4
- c. 04. K3.H5
- d. 06. K2ac.H1
- e. 014.K14.H4
- f. 075.K2.H5

Following incubation at 37°C overnight the organisms were harvested in saline and adjusted to a concentration of 1.2×10^8 /ml using a Hilger Spekker absorption meter. Over 20 ml of each organism was prepared.

Each organism suspension was then treated as follows:

1. Placed in a boiling bath for 2 hours
2. Centrifuged at 5000 x g for 30 min
3. Twenty ml of each supernate was pooled, mixed, distributed in 5 ml aliquots, and stored at -20°C.

Coating of human group 0 cells with bacterial antigen pool

Standard group 0 red cells were obtained from the Regional Blood Transfusion Laboratories, Edinburgh Royal Infirmary.

1. Three ml of the group 0 cells were washed three times with saline in a graduated centrifuge tube.
2. The final cell deposit was adjusted to 50% P.C.V. by addition of saline.
3. Of this suspension 0.2 ml was added to 10 ml of pooled bacterial antigen (i.e. 1% suspension).
4. The mixture was incubated at 37°C for 0.5 hours in a water-bath, and washed three times in saline to remove excess bacterial antigen.
5. To the final deposit 20 ml saline was added to give a 0.5% cell suspension

Test Procedure (preparation of serum)

- a. Test sera were inactivated at 56°C for 0.5 hours.
- b. Two 0.1 ml aliquots of each test serum were placed into tubes marked A and B.
- c. To A 0.1 ml phosphate buffer, pH 7.4 was added.

- d. To B was added 0.1 ml phosphate buffer, pH. 7.4 containing 0.1 mol/l of 2 mercaptoethanol.
- e. Tubes A and B were incubated in a waterbath for 5 hours at 37°C.

Titration of serum (using Takatsky microtitre equipment)

Dilutions of A and B were prepared as follows:

- a. 0.05 ml saline was placed into 9 wells in each row.
- b. Into the first well of one row was added 0.05 ml of sample A.
- c. 0.05 ml of sample B was added to the first well of the second row.
- d. Double dilutions from the first well throughout the 9 wells were then made for each row.
- e. 0.05 ml of coated O cell suspension (0.5%) was added to all wells to give final dilutions of 1/8 to 1/2048.
- f. Coated cells alone were incubated in a separate well as an autoagglutination control.
- g. Trays were incubated at 37°C for 3 hours followed by overnight at 4°C and the results were recorded on an appropriate assay forms.

(iv) Alpha 2 macroglobulin

These determinations were kindly undertaken by Dr. A. Tuastall. Antisera to human and mouse α_2 M were produced in New Zealand white rabbits by injection of purified preparations of α_2 M. The human α_2 M was isolated by repeated zonal ultracentrifugation of Cohn fraction 30 kindly donated by Ortho Pharmaceuticals (Raritan, New Jersey). On days 0 and 21, the α_2 M (2 mg in Freund's complete adjuvant) was given intramuscularly into three or four sites and a third injection (2 mg in alum) was given intraperitoneally on day 42. The rabbits were bled on day 9 and at 3 to 4 week intervals thereafter

without further challenge. The antisera were rendered monospecific for α_2 M by absorption. The anti-human α_2 M was absorbed with the low molecular weight ultracentrifuge fraction of Cohn 30.

Serum α_2 M was measured by a standard Mancini gel diffusion method. Standard antigen in 4 different concentrations was included on every plate, standard for the human measurement was a human α_2 M reference obtained from Meloy Laboratories Inc., (Springfield, Virginia, U.S.A.). Human measurements are given in milligrammes of α_2 M/100 ml. Normal samples were obtained from healthy donors aged 18 to 70 years from the Blood Transfusion Service, Royal Infirmary, Edinburgh. Approximately 20 samples per decade for each sex were tested.

B. CLINICAL

1. RETROSPECTIVE SEARCH FOR CLUSTERS OF LYMPHOMA

Cases of lymphoma which occurred in Edinburgh between the years 1961 and 1971 were grouped according to the address of each patient and the diagnosis (broadly categorised according to the international coding system - lymphosarcoma, reticulum cell sarcoma or Hodgkin's disease). With the help of the Common Services Agency of the Scottish Home and Health Department, a computer print-out was obtained of the relevant patients consisting of their identification number, age, sex, diagnosis, year of diagnosis and the electoral ward in which the patient resided. At that time there were twenty-three electoral wards of fairly static population size between 9,000 and 37,000. The computer print-out was scanned by hand to eliminate duplicated cases and then this information was programmed using the EMAS system on the University of Edinburgh computer service. The incidence of each type of lymphoma, lymphosarcoma, reticulum cell sarcoma or Hodgkin's disease in each ward each year was then calculated by the computer from the numbers of cases and from the population statistics obtained at the relevant census. The computer then calculated average values for incidence of each disease against time and plotted this information for each ward and for each year. Superimposed on graphs the computer plotted the actual incidence and this information was scanned by eye to determine peaks of incidence of any disease. Seven such peaks for Hodgkin's disease, four for reticulum cell sarcoma and three for lymphosarcoma were found. These patients were then identified by number and their case notes retrieved from the relevant hospitals. One hundred and seven patients were

involved and from the case notes, the diagnosis and date of diagnosis were checked along with the electoral ward and details of family history and social history which may have been relevant. The address of each patient was plotted on a map of Edinburgh so that further clusters across ward boundaries could be detected by eye. It was felt that confirmation of the data might have been achieved by either a death certificate study or a cancer registry study. A pilot study of death certificates however, showed that these were not accurately filled in for most patients with lymphomas, the cause of death being put down to infection. Unfortunately, access to cancer registry material proved difficult as at that time these data were not published.

A detailed clinical and pathological retrospective study was carried out of six hundred patients treated in the University of Edinburgh Department of Radiotherapy over the years 1959 to 1972. This was a large study and involved collaboration of the Departments of Pathology, Radiotherapy and Therapeutics, all in the University of Edinburgh. The author's contribution to the study consisted only of design of computer format, the programming of data retrieval and analysis of the results. The case records were retrieved and studied by Professor Duncan, Dr. Gordon Ritchie and Dr. Ann Grant, all of the Department of Radiotherapy. Histological sections from pathological specimens from each patient were studied independently by Dr. Angus Stuart and Dr. Robert MacAulay of the Department of Pathology, University of Edinburgh. The results of this study therefore will only be referred to in brief and used as some comparative data for the prospective study reported below.

2. PROSPECTIVE STUDY

(a) Patients

One hundred consecutive patients under the care of a Professorial Medical Unit were studied prospectively. Two of these patients presented with a relapse of lymphoma having been treated originally 10 years previously. The remaining patients presented for the first time. Clinical and basic laboratory data were recorded for computerisation before treatment started, at 2 to 3 months after the start of treatment, and then at 6 to 9 months when primary treatment had been completed for the majority of patients. The patients were then followed to obtain data on time of relapse and the time of death.

Details gathered about each patient included sex and date of birth, history of sweating and fever, weight loss, frequent infections and malignancy other than lymphoma. The site of palpable nodes, liver, spleen and extra-nodal manifestations were noted and after staging procedures (see below) accurate histological staging was achieved.

(b) Histology

Diagnosis of lymphoma was usually attained by biopsy of lymph node. On one occasion a marrow aspirate gave the diagnosis and in several instances where the gut was involved, the diagnosis was first made after laparotomy. All lymph node samples and specimens attained during the staging process such as liver biopsies and spleen were reviewed by one pathologist with a special interest in lymphoma (Dr. A.E. Stuart). Spleens removed at staging laparotomy were fixed in formalin and then cut on a bacon slicer in 2 mm sections. These sections were then laid out and studied with a hand glass for foci

of tumour. Suspicious areas were sampled and processed for paraffin sections. The histological classification (appendix 6) for Hodgkin's disease was the Luke's modification of the Rye classification (1966). The histological classification (appendix 7) of the non Hodgkin's lymphomas was by Rappaport (1956).

(c) Staging Procedures

All patients had a chest x-ray with liver function tests including serum proteins, full blood count and urinalysis. Unless the patients were clinically stage IV (appendix 8) that is involvement with lung, marrow, gut or skin at the time of presentation, bipedal lymphangiography was carried out. Patients who were shown by lymphangiography to have disease in the para-aortic nodes in addition to other groups of nodes on the contra-lateral side of the diaphragm in the presence of systemic symptoms (sweating, weight loss or fever) were not subjected to staging laparotomy but did have marrow biopsy carried out. Hodgkin's patients who were staged clinically either I, II or IIIA (A infers lack of systemic symptoms) were subjected to staging laparotomy. This operation consisted of wedge biopsies of both lobes of liver, splenectomy and removal of splenic lymph nodes, sampling of coeliac axis nodes, para-aortic nodes, iliac nodes and bone marrow. In pre-menopausal women, ovaries were moved laterally or into the midline behind the uterus, so that they would be less likely to be involved in radiation fields should this treatment be subsequently performed. Metal clips were left as markers at critical sites such as splenic pedicle, ovaries, and coeliac nodes.

Patients with non Hodgkin's lymphoma on initial biopsy proceeded from lymphangiography to marrow biopsy with a Jamshidi

trephine needle. If this was negative, closed liver biopsy was carried out in most instances. Two patients had open liver biopsy carried out under direct view with a laparoscope. If liver biopsy was negative, patients went on to staging laparotomy as for Hodgkin's disease. In addition to laparotomy to stage patients, in several instances it was necessary to perform a laparotomy to remove a large spleen because of hypersplenism.

(d) Treatment

Surgical treatment was limited to splenectomy for large spleens as mentioned above. Radiotherapy either to local glands but usually by a mantle field or inverted Y field was applied to patients with stage I or II disease. Patients who had stage IIIA disease were treated with total nodal irradiation occasionally followed by chemotherapy. All radiation treatment was undertaken by Dr. Gordon Ritchie, Department of Radiotherapy, Western General Hospital, Edinburgh. Patients who had stage IIIB disease (i.e. with the presence of sweating or weight loss) plus patients with stage IV disease were treated with chemotherapy. The chemotherapy consisted of, in the case of Hodgkin's disease, mustine, vinblastine, procarbazine and prednisolone given for a fortnight every 6 weeks for 6 courses. In the case of non Hodgkin's lymphoma, lymphocytic type, cyclophosphamide, vincristine, and prednisolone were given 3 weekly until remission was achieved and then 4 further courses were given. Certain patients either elderly or because of poor cardiac or renal function were given chlorambucil alone or with prednisolone, on an intermittent basis for 2 weeks every month. Patients with diffuse histiocytic lymphoma or lymphocytic lymphoma that had failed on first line treatment were given cyclophosphamide

vincristine, prednisolone and adriamycin 3 weekly for a minimum of 6 courses. All chemotherapy was administered on an out-patient basis by the author.

Throughout treatment, complications of treatment were noted such as myelosuppression, bleeding or the presence of infections. At each time of data recording, the patient's disease state was assessed clinically and histologically where appropriate and the patient was judged either not yet in remission, in remission, in relapse or if dead, dead due to disease or dead while in remission. No patient was lost to follow-up. The minimum time of follow-up was 22 months for two patients, the remaining ninety-eight were followed up for a minimum of 2 years and mean of 3.5 years. When patients relapsed the site of relapse and treatment of relapse were recorded.

Data Processing

The above data relating to patients on treatment and after treatment were transferred to computer cards and stored on a University of Edinburgh computer using the EMAS system. Survival figures were then achieved by subtraction of the date of first treatment from the date of death and the time to relapse was recorded in similar way. Laboratory data relating to these patients included results of full blood count, ESR, serum proteins, immunoglobulins, Escherichia coli antibodies, and α_2 macroglobulin levels. These were computerised and analysed for trends and patterns relating to each disease subtype.

RESULTS

A. LABORATORY STUDY

1. Lymph Node Studies

- a) Lymph node samples
- b) Electron microscopy
 - i identity of cell types
 - ii tumour-host cell interactions
 - iii C-type particles
- c) Cell culture
- d) Time-lapse cine photomicrography
- e) Fluorescent studies
- f) Supernatants from cultures
 - i leucocyte chemotactic factors
 - ii monocyte chemotactic factors
 - iii monocyte chemotaxis inhibitors
 - iv skin reactive factor
 - v mitogenic factor

2. Peripheral Blood Studies

- a) Phagocyte Function
 - i identification of monocytes
 - ii monocyte chemotaxis
 - iii monocyte phagocytosis and bactericidal capacity
- b) Serum Proteins
 - i immunoelectrophoresis
 - ii immunoglobulin quantitation
 - iii E. coli antibodies
 - iv Alpha 2 macroglobulin

B. CLINICAL STUDY

1. Retrospective Search for Clusters of Lymphoma
2. Prospective Study of Management of Lymphoma
 - a) Patients
 - b) Histology
 - c) Staging procedures
 - d) Treatment

RESULTS

A. LABORATORY

1. LYMPH NODE STUDIES

(a) Lymph Node Samples

Twenty-three lymph nodes were studied by electron microscopy and sixty-one studied in culture.

(b) Electron Microscopy

(i) Identity of cell types

The purpose of the ultra-structural study was firstly to identify malignant cells and to study their interaction with host reticulo-endothelial cells. A second intention was to search for evidence of virus particles within the cells. In Hodgkin's disease and histiocytic lymphoma (reticulum cell sarcoma), it was relatively easy to find large atypical cells on thin section (Fig. 3) which could then be focussed in more depth. The large nucleoli typical of the Hodgkin's Reed-Sternberg cell have a dense appearance on electron microscopy broken up by light dots. Many multinucleate cells were seen as in the first example (Fig. 4) frequently apposed to macrophages and fibres of collagen. The nuclei were irregular in shape and frequently multiple. Cell cytoplasm showed some rather empty mitochondria (Fig. 5) and occasional strands of endoplasmic reticulum. Active mitochondria and Golgi apparatus were rarely seen in the twenty-three nodes studied. The large multinucleate cells of histiocytic lymphoma were frequently more bizarre than those of Hodgkin's disease but other single nucleated forms were seen (Fig. 6, 7). These nuclei frequently had more than one nucleolus and their cytoplasm again, though bulky, showed little evidence of

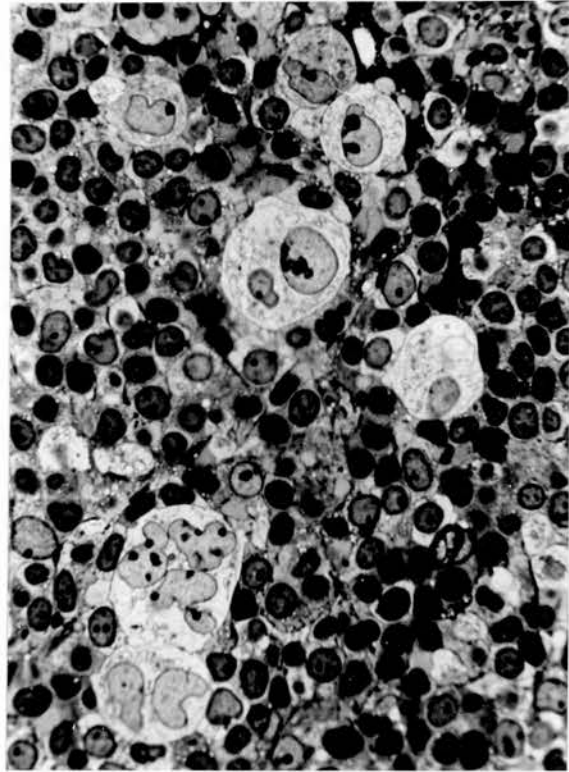


Fig. 3 Thin section of Hodgkin's lymph node with several multinucleated cells, lymphocytes and dendritic macrophages (Toluidine-blue x 600)

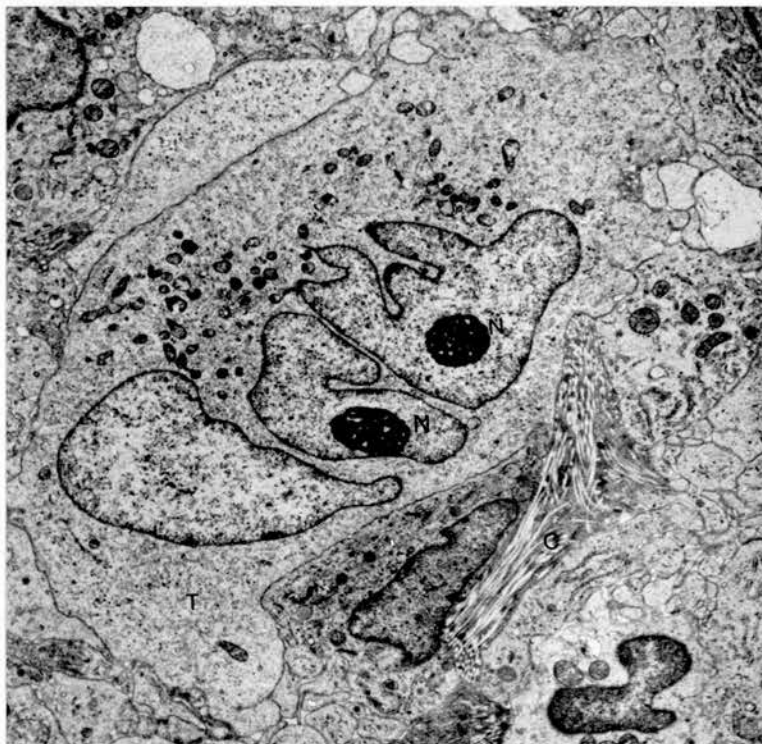


Fig. 4 Electron microscopy of large Hodgkin cell (T) with nucleoli (N) and surrounding collagen (C) (x 1000)

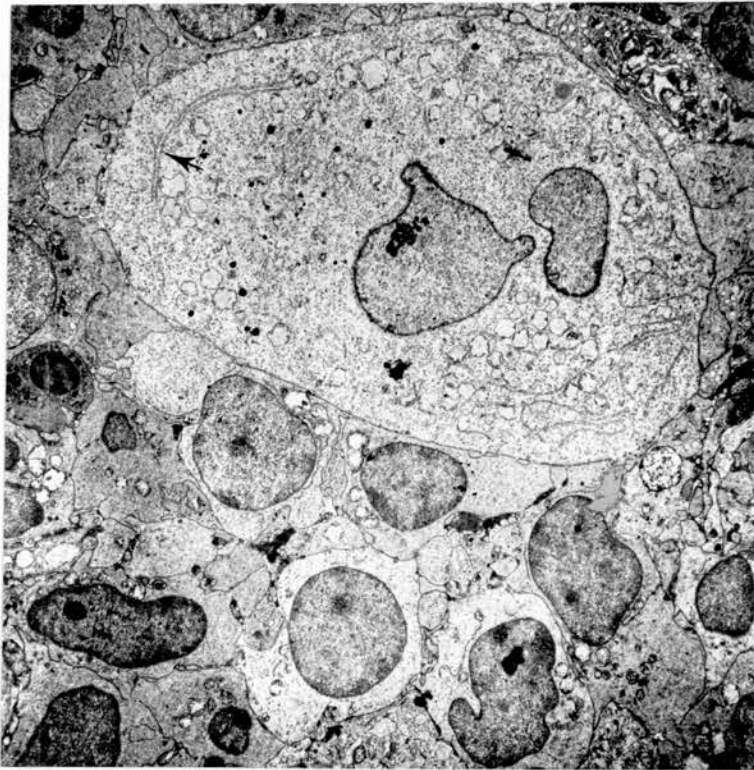


Fig. 5 Electron microscopy cell with poor mitochondria and strands of endoplasmic reticulum (arrowed) (x 800)



Fig. 6 Electron microscopy of giant cell of histiocytic lymphoma (x 1200)

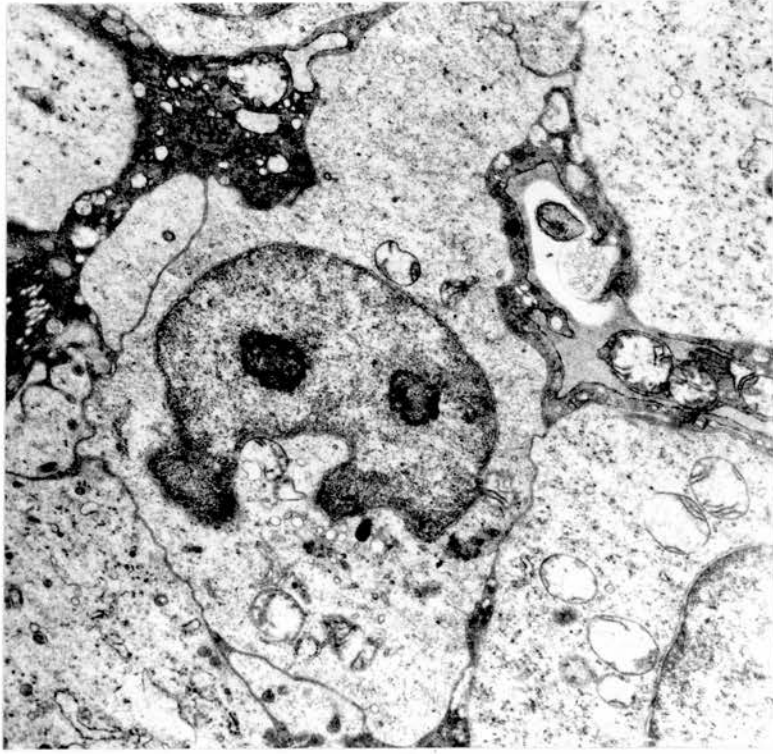


Fig. 7 Electron microscopy of single nucleated cell in histiocytic lymphoma (x 800)

active functional equipment. Although the name histiocytic lymphoma has been applied to the light microscopy of six of the twenty-three nodes studied, it was not possible to be certain that the malignant cells were necessarily of histiocytic origin. Phagocytic vacuoles for instance, were never demonstrated. It would require specific stains for peroxidase or non-specific esterase to prove the histiocytic origin of these cells. Seven lymphocytic lymphoma nodes were studied, all of them diffuse in nature. Characteristically these abnormal lymphocytes were of B-cell origin by their immune function and showed variable differentiation. There are three cells shown in Fig. 8, all of which have prominent nucleoli and are probably lymphoblasts. There are clefts in all three nuclei and indeed one of the nuclei looks as if it has a signet ring appearance. The nuclear cytoplasmic ratio is much higher than in the previous giant cells and indeed the lymphocytes are about a third of their size. Mitochondria are sparse and those that are present have very poorly formed or absent cristae. There are few ribosomes in the majority of these lymphoblastic cells. Occasionally binucleate forms were seen (Fig. 9) although these are occasionally found in normal lymph nodes. Normal host cells are seen in all the lymphomatous nodes despite the gross lack of architecture and normal form of the nodes when seen on conventional histology. Figure 10 shows an eosinophil leucocyte, a plasma cell, a monocyte and two lymphocytes apposed to a small vessel probably a venule from one of the samples of Hodgkin's disease. Indeed it was common to see large tumour cells surrounded by normal host cells. In the case shown in Fig. 11 these are lymphocytes which not only can surround the cell but even encroach on its borders (as is shown in Fig. 12).

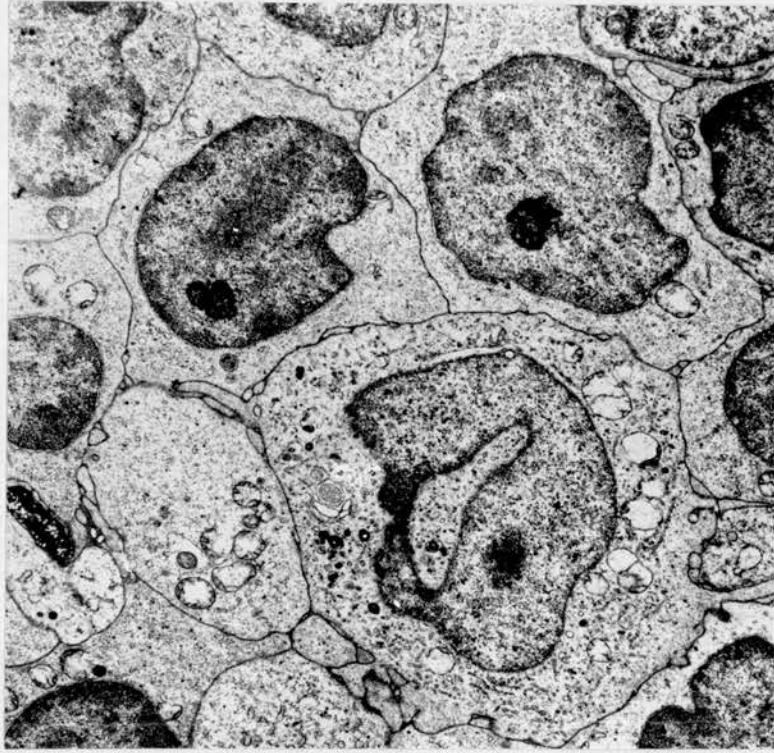


Fig. 8 Electron microscopy of three lymphoblasts
from lymphocytic lymphoma (x 5000)



Fig. 9 Electron microscopy of binucleated lymphocyte (x 10000)



Fig. 10 Electron microscopy of a venule (V) surrounded by an eosinophil (E) a plasma cell (P), a monocyte (M) and lymphocytes (L) (x 3000)

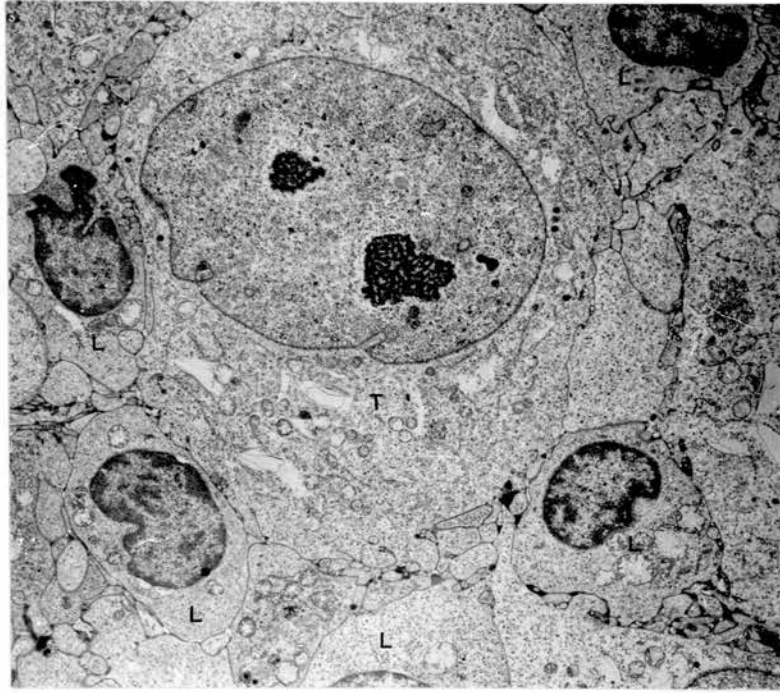


Fig. 11 Electron microscopy of unhealthy tumour cell (T) surrounded by lymphocytes (L) (x 3000)





Fig. 12 Electron microscopy of lymphocyte (L)
apparently prodding into a tumour cell
(T) (x 8000)

(ii) Tumour-host cell interactions

Macrophages were found throughout the lymph nodes studied. They tended to be apposed very tightly to tumour cells and because of their long and flat shape it was often difficult to obtain photographs of complete cells in one plane. There are clearly at least two forms that a macrophage can take in a lymph node. Figures 13 and 14 show perfused samples of a Hodgkin's lymph node in which several macrophages with numerous finger-like projections from their peripheries are clustered together. They have low nuclear cytoplasmic ratios and the cytoplasm of these cells is teeming with ribosomes, phagosomes, mitochondria and other particles. The nuclei have commonly an electron dense fringe with paler centres. The other variety of macrophage shown in these two frames may be a dendritic macrophage described by Stuart (1970). These are characteristically flat cells with very long projections which are thin, almost telescopic, and which intercalate with other identical processes from neighbouring cells forming a thin web-like back cloth to the more obviously highlighted conventional rounded cells. A high power view of the junction site of two dendritic processes is shown in Fig. 15 (this is a magnification of the area labelled J Fig. 14). It is quite conceivable that cytoplasmic organelles could be passed between macrophages through such junctions or else information despatched across the gap in some other form so that communication could take place throughout this network of reticulo-endothelial cells. Both dendritic macrophages and conventional macrophages did seem to come into close proximity to tumour cells though despite scrutiny of several hundred blocks no area was found in which there was a break in continuity of the plasma membrane

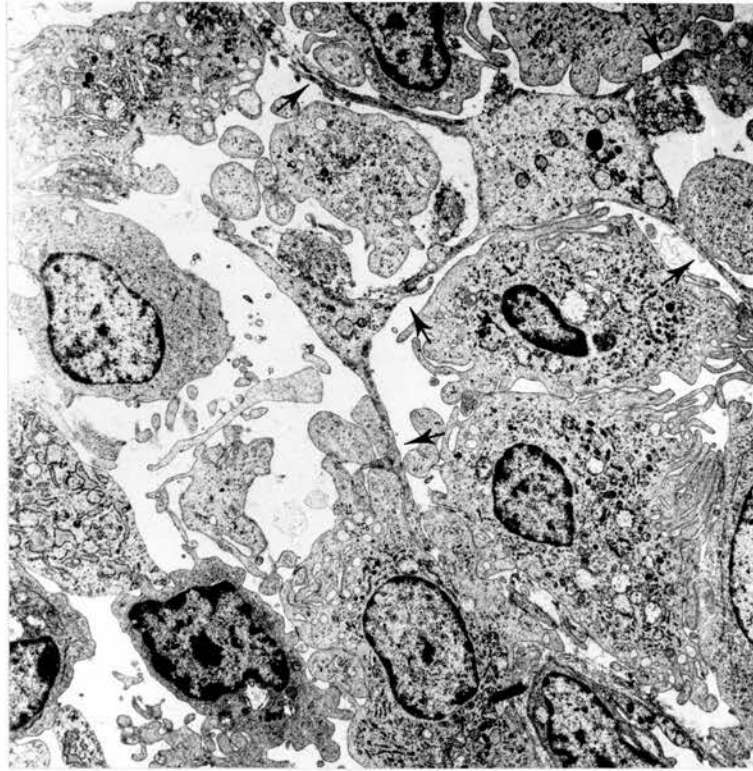


Fig. 13 Electron microscopy of perfused lymphoma node pointing out the finger-like projections of dendritic macrophages (x 3000)

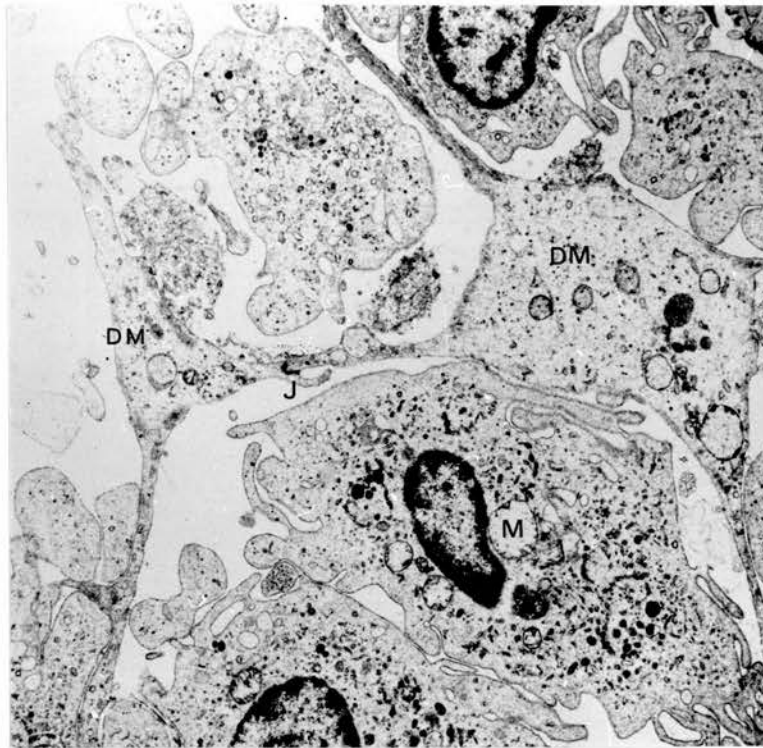


Fig. 14 Electron microscopy of dendritic macrophages (DM) adjacent to a conventional macrophage (M). "J" marks a junction site of dendrites enlarged in Fig. 15 (x 5000)

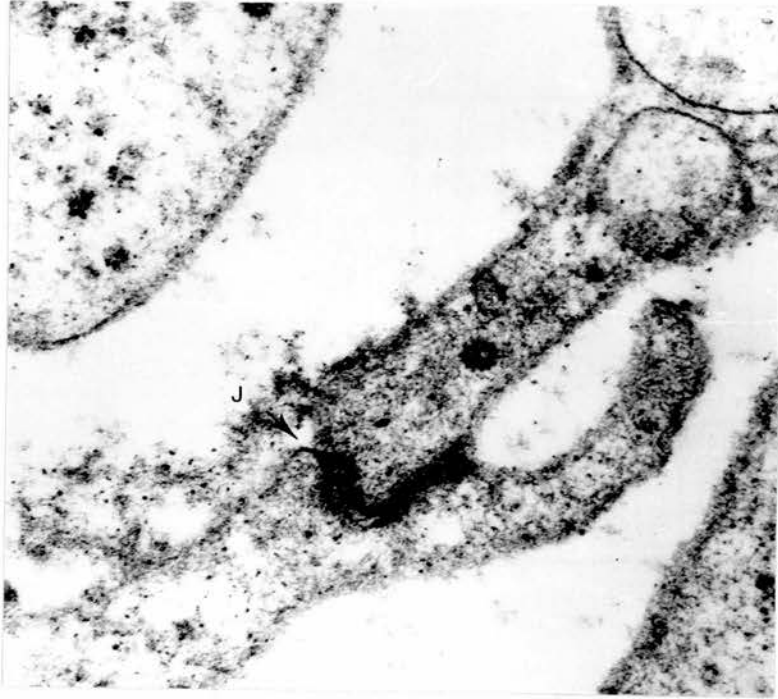


Fig. 15 Electron microscopy of junction site of neighbouring macrophages' dendritic processes. Enlarged from Fig. 14 (x 50000)

of either macrophage or tumour cell. Nor did tumour cells seem to be embarrassed or dying. Figure 16 shows a long dendritic process from one cell coming into contact with a tumour cell which is also being attended by the fronds of a neighbouring tissue macrophage. Figure 17 gives an example of two kinds of contacts between the host cell and the tumour cell. On the one hand occasional point contacts are made and on the other, long flat junctions are seen. It seems in the latter case that the macrophage may be feeling out the surface markings of the tumour cell perhaps in an attempt to detect or to recognise the cell. The possible end result of this confrontation is shown in Figure 18 where an exceptionally long dendritic process from a macrophage has almost completely engulfed the tumour cell. This kind of contact was not observed in the majority of lymph nodes because they were not fixed by perfusion. As a result the cells were tightly opposed to one another and on sectioning it was impossible to tell the origin of many of the processes and cytoplasmic projections which were in contact with target tumour cells. However, in half of the nodes studied macrophages which had engulfed other cellular material (not necessarily of tumour origin) were observed. Two examples (Fig. 19 and 20) were shown. From this kind of study it is impossible to tell whether actual aggression on the part of the host cells, lymphocytes and macrophages, is taking place, though circumstantial evidence is presented. Even the cell culture work (to be described) did not, because of its static nature, provide other than confirmation of the electron microscopy appearances. It was therefore important that the time lapse cine films were made to assess the dynamic nature of these interactions.

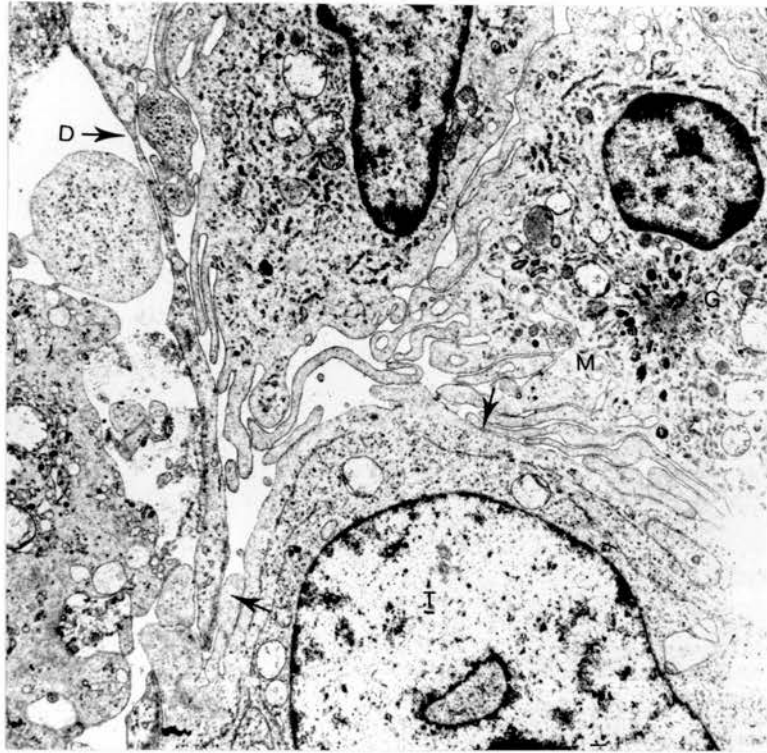


Fig. 16 Electron microscopy of tumour cell (T) in contact with fronds of macrophage (M) and dendritic process (D). "G" is an active Golgi apparatus (x 5000)

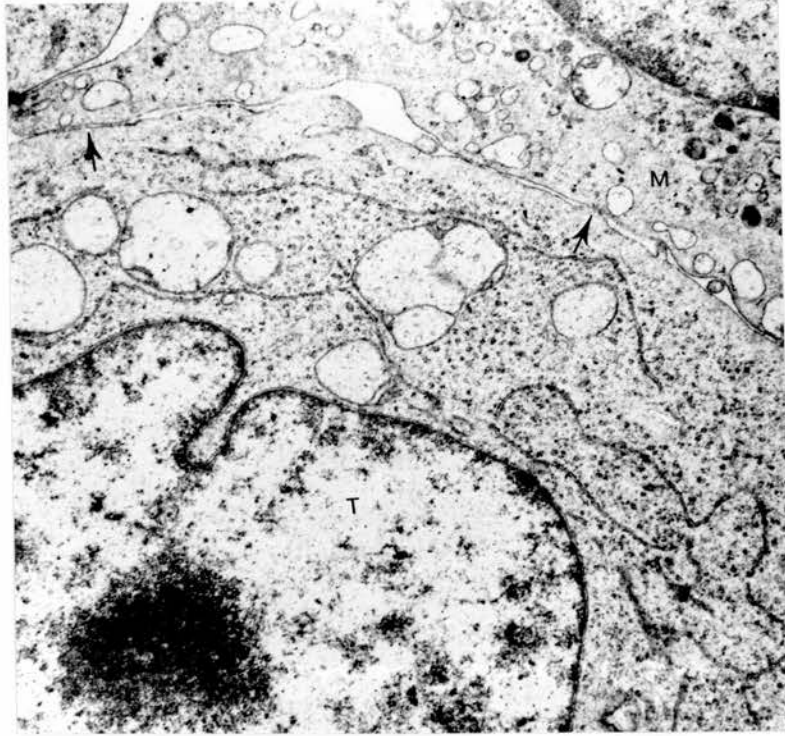


Fig. 17 Electron microscopy of tumour cell (T) making flat contact (arrowed) and point contact (M) with a macrophage (x 11000)

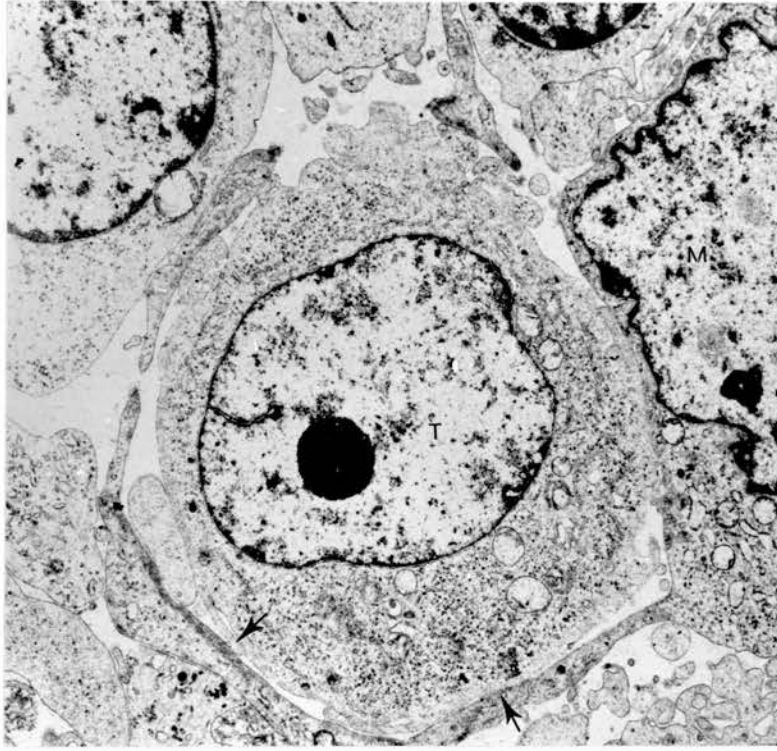


Fig. 18 Electron microscopy of tumour cell (T) almost engulfed by dendritic process (arrowed) from a macrophage (M) (x 5000)

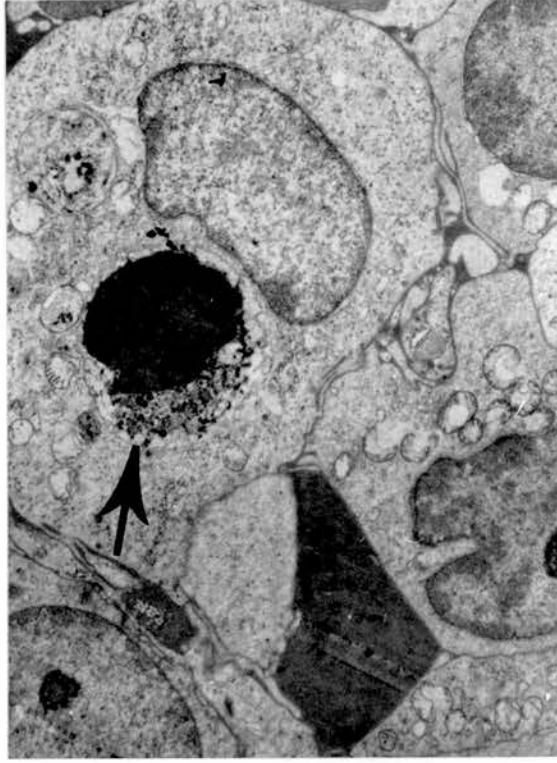


Fig. 19 Electron microscopy of cellular material (arrowed) ingested by a macrophage (x 5000)

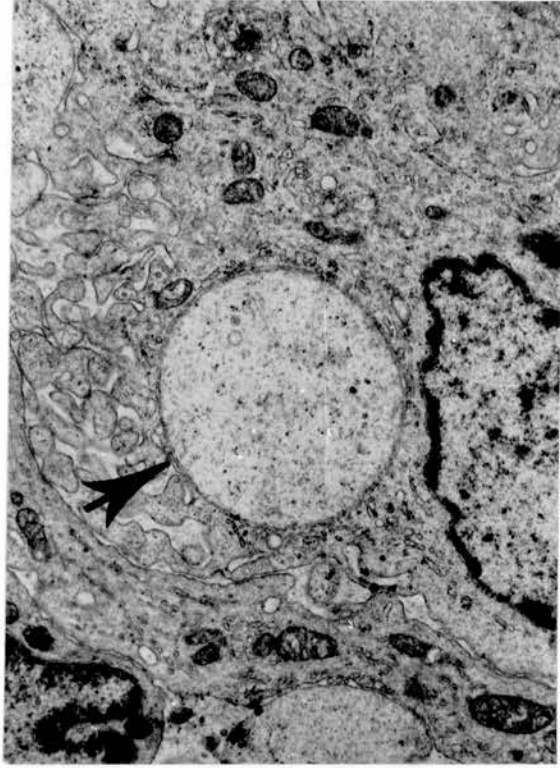


Fig. 20 Electron microscopy of cytoplasm from a dead cell (arrowed) inside a macrophage (x 5000)

(iii) C-Type particles

The electron microscope, however, excels in providing fine detail of cells and virus particles can only be seen at this dimension. Six out of twenty-three lymph nodes which were scrutinised showed the presence of structures resembling C-type virus particles. These have a characteristic size, a dense nucleus or core, and are surrounded by a double outer membrane. Figures 21 and 22 show examples of such particles in comparison to neighbouring mitochondria and show the variability of the size of particles themselves. It is possible that these are not large particles but are part of a tubular system which is particularly well developed in these tumour cells. Figure 23 shows such a series of tubules which on the other hand might be interpreted as a group of budding viruses. In two patients who had Hodgkin's disease cells showed curious nuclear structures. In the first (Fig. 24 and 25) a characteristically abnormal nucleolated nucleus is shown and a projection from the point of the nucleus arrowed gave the impression of formation of a circular structure. Figure 26 shows an equally bizarre double nucleus with the characteristic speckled nucleolus adjacent to what in cytoplasm would be termed a phagosome; it appears to be an invagination of the nuclear membrane containing large lumps of electron dense material which might be cytoplasmic intruders or hypothetically might represent clumps of virus particles.

An attempt was made to study cultured cells which might have developed larger numbers of virus particles. The average number of virus particles in tumour cells in the six nodes which were cultured was eight. Figure 27 shows a sample of the cells obtained from the washings of a flying coverslip. Most of the



Fig. 21 Electron microscopy of C type particle (arrows) compared in size to a mitochondria (M) from a lymphoma node (x 40000)

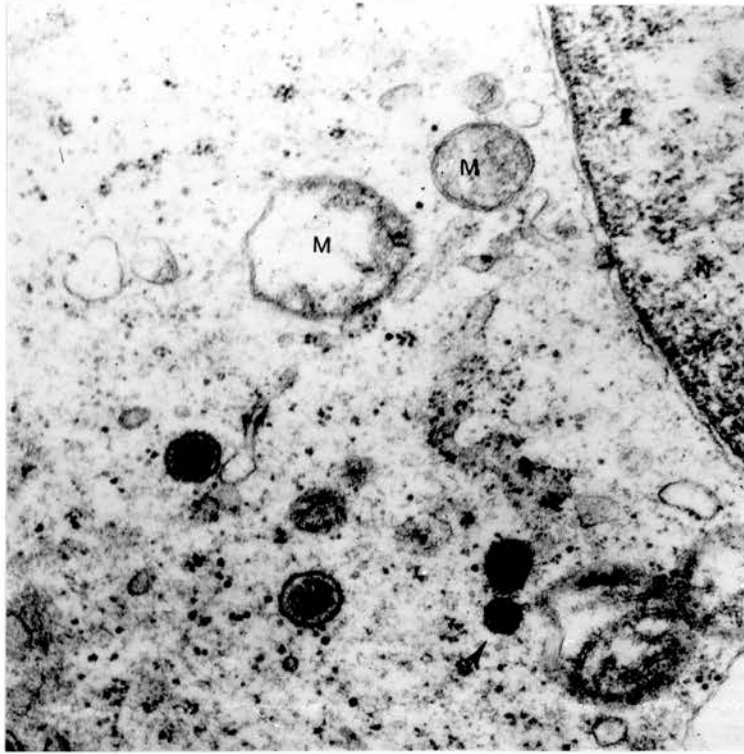


Fig. 22 Electron microscopy of particles and poorly formed mitochondria (M) (x 40000)

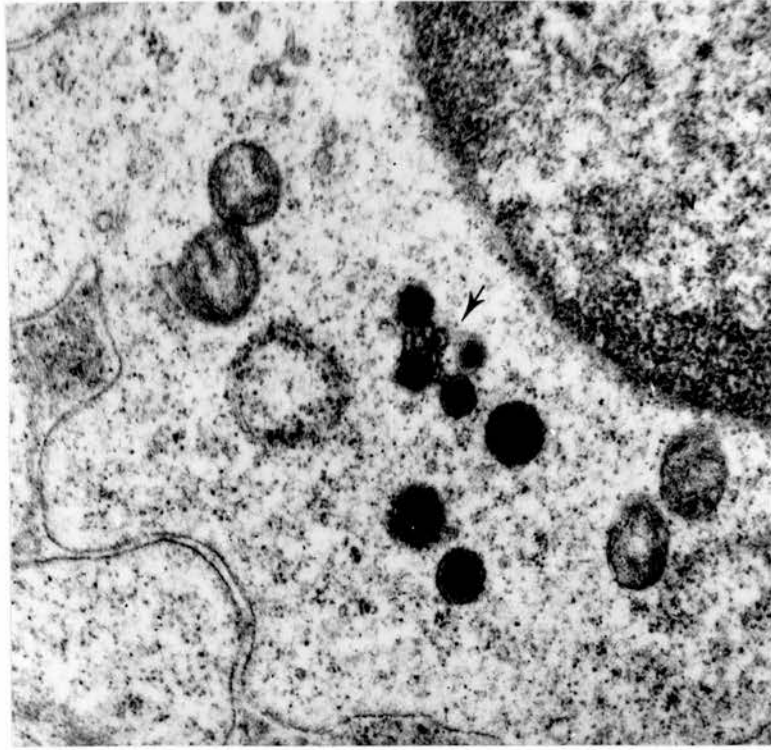


Fig. 23 Electron microscopy of tubules which might represent budding particles (arrowed) (x 40000)

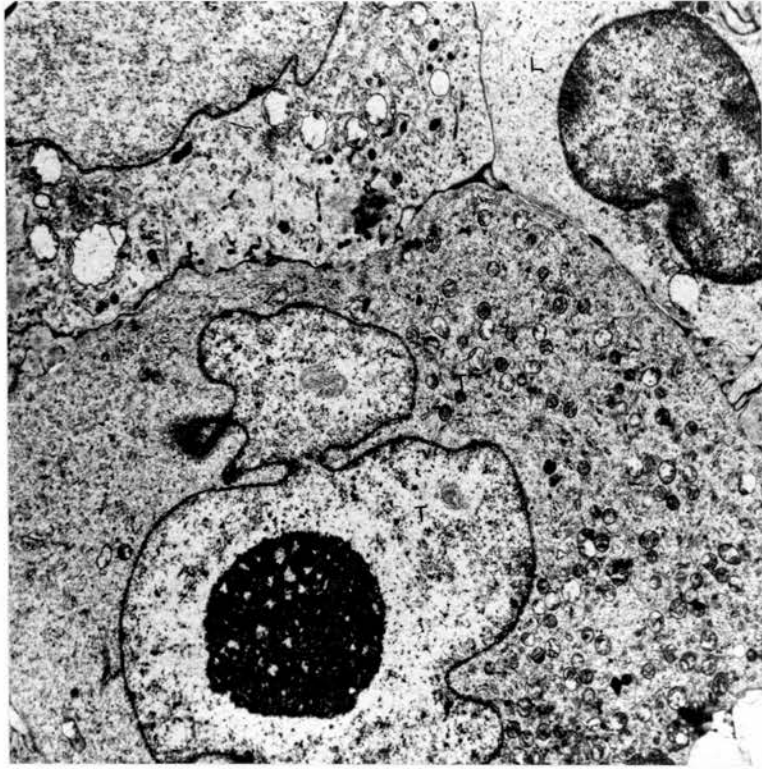


Fig. 24 Electron microscopy of tumour cell from histiocytic lymphoma (x 4000)

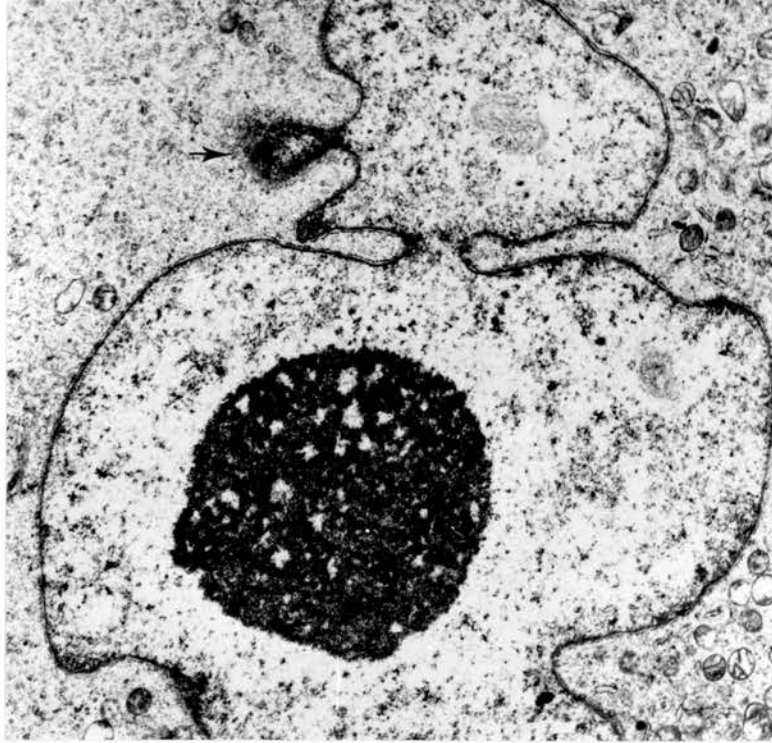


Fig. 25 Electron microscopy of previous cell (Fig. 24) to show nuclear bud (arrowed) and white speckled nucleolus (x 8000)

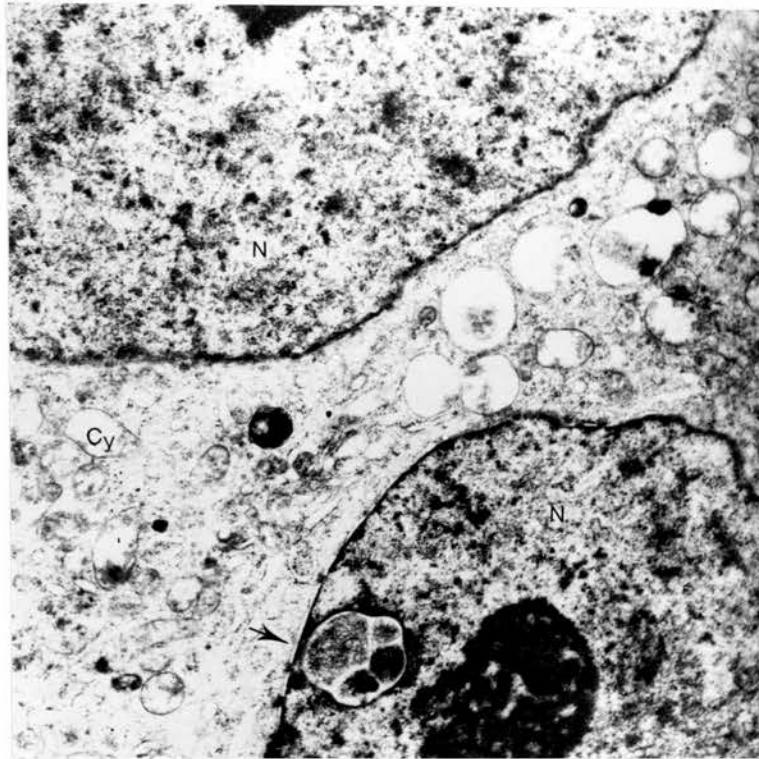


Fig. 26 Electron microscopy of twin nuclei (N) one of which has an inclusion "phagosome" in its midst (arrowed) (x 12000)

cells are lymphocytic in origin and have obviously not been sticking firmly to the glass; other cells are clearly dead and only the remnants of cell structure remain. In contrast, of those scraped from the coverslip, several established macrophages were shown (Fig. 28) clearly identified by phagocytic vacuoles labelled V which are filled presumably either with lipid or cytoplasmic debris from dying cells. Cells which have been detached from the coverslip were usually macrophages and tumour cells. Figure 29 however, is an exception. It shows a rosette of lymphocytes clustered round a tumour cell. This coverslip was from a patient who had histiocytic lymphoma and the cells were harvested at day 5 of the culture. Numerous small dense granules were seen in the area of the Golgi zone which may or may not be virus particles. In only three cultures were good cells obtained and in only two of them were virus particles seen. The second is shown in Fig. 30 and this shows the same kind of electron dense particles at the periphery of the cytoplasm in a small cluster as were observed in the original node. No evidence of budding is seen and so no firm conclusions can be reached about the identity of these particles. Even if they were virus particles, there is a theoretical possibility that they were contaminants which appeared after culture of the lymph nodes had commenced.

In conclusion twenty-three lymphomatous nodes were studied along with two normal lymph nodes. A wide range of bizarre, large tumour cells were noted usually encircled by or close to host lymphocytes or monocytes. Other host cells such as plasma cells and eosinophils were present in small numbers except in Hodgkin's nodes where the latter were present in significant number.

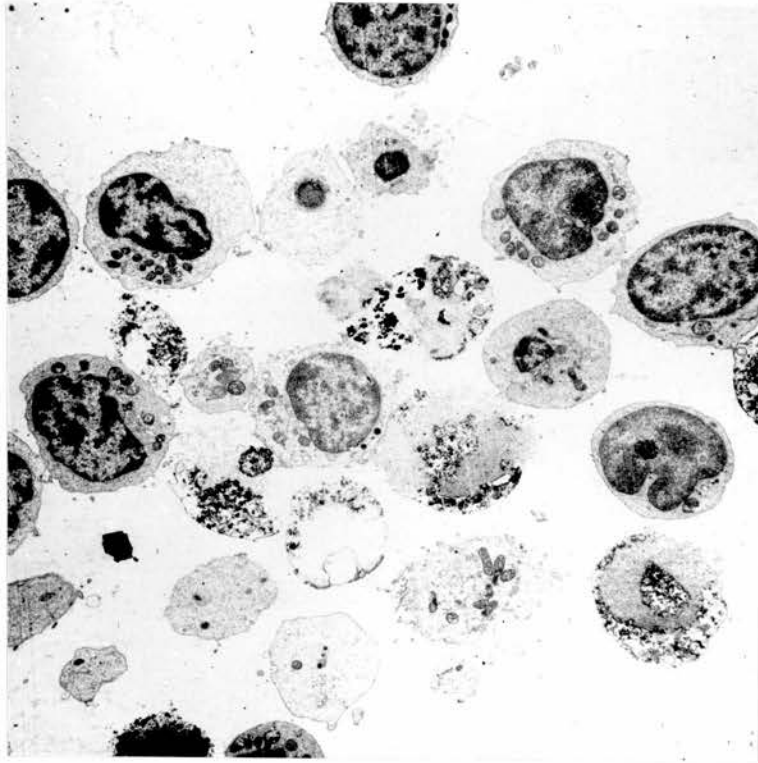


Fig. 27 Electron microscopy of cells from the washings of cultured lymphoma monolayers (x 1500)

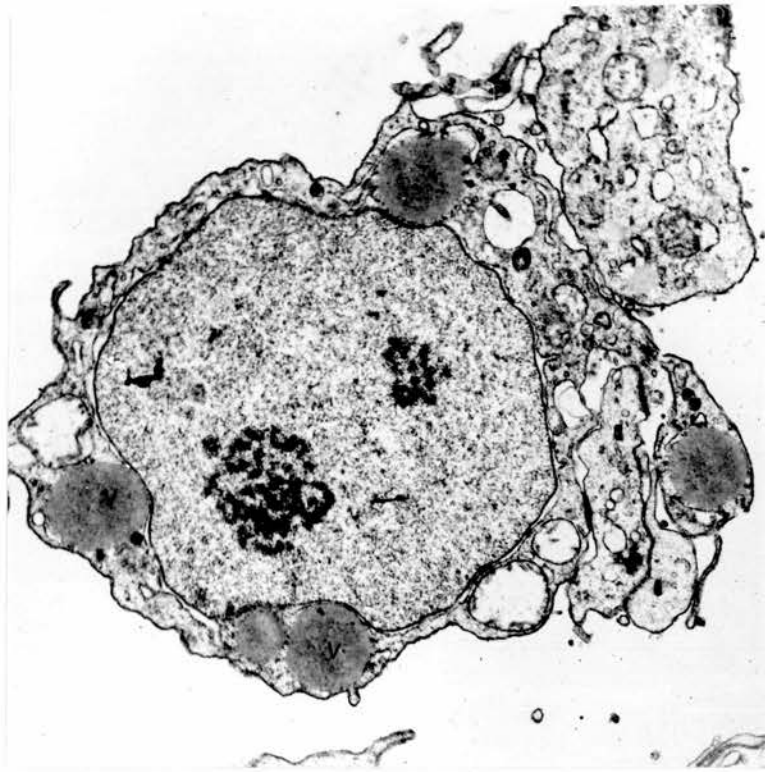


Fig. 28 Electron microscopy of macrophage cultured from a lymphoma monolayer showing phagocytic vacuoles (V) (x 8000)

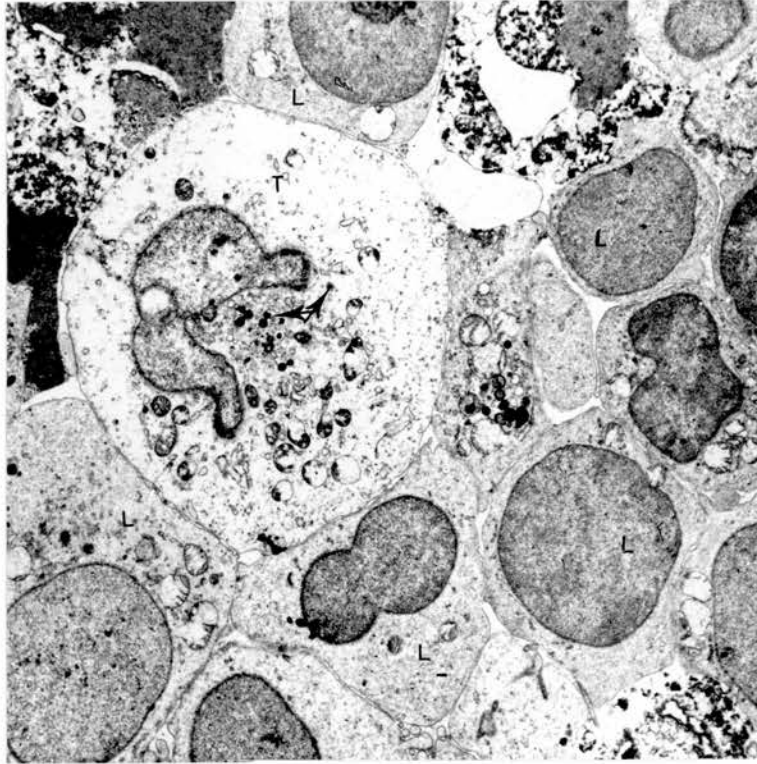


Fig. 29 Electron microscopy of a rosette of lymphocytes (L) around a tumour cell (T) with arrows to particle-like granules in the area of the Golgi zone (x 2000)

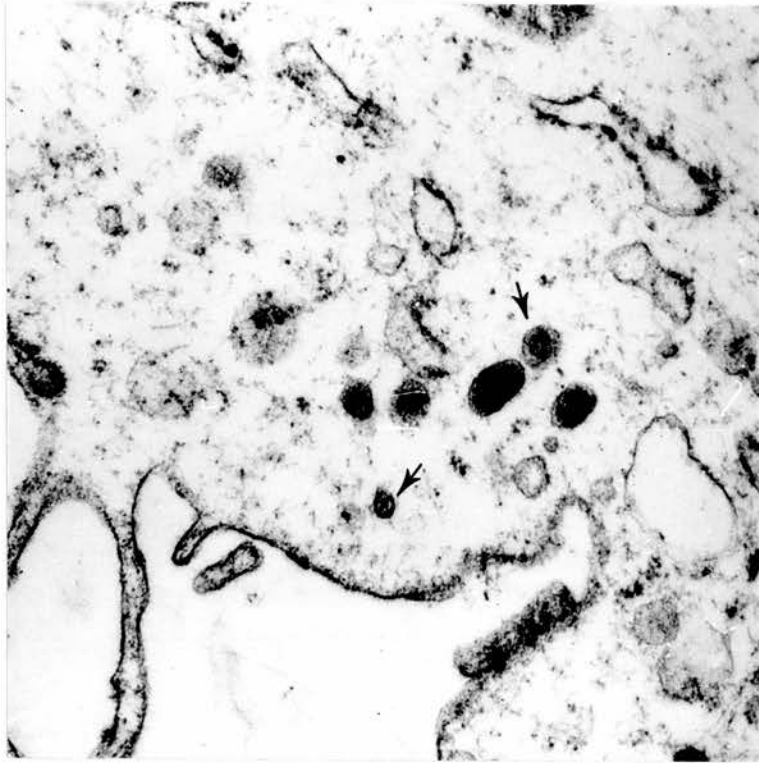


Fig. 30 Electron microscopy of cultured tumour cell showing poorly formed cytoplasmic particles resembling C type in size (x 40000)

Even in nodes whose architecture seemed to be demolished a network of dendritic macrophages was clearly seen provided the node had been fixed by perfusion with gluteraldehyde rather than immersed whole. These macrophages seem to intercalate with each other and on occasion were seen along with more conventional macrophages to phagocytose tumour cells. Dead cells were commonly seen in phagocytic vacuoles of macrophages but the origin of the ingested material was impossible to identify and similar cells have been reported in normal lymph nodes. Particles or structures resembling C-type particles in size and configuration were seen in several lymph nodes, usually in the cytoplasm. Nuclear abnormalities were seen in two cases of Hodgkin's disease and virus-like particles were also confirmed in cells which had been cultured 5 days prior to fixation for ultra-structural study.

(c) Cell Culture

Over a period of 18 months a total of 61 lymph nodes were cultured; 15 of these were normal and 8 were nodes replaced by secondary carcinoma. Fourteen of the lymph nodes were from Hodgkin's patients, 8 from patients with lymphocytic lymphoma and 16 were labelled histiocytic lymphoma. Of the total, 36 were successfully cultured for 3 days or more. Of the 38 lymphomas, only 12 were successfully cultured for longer than 5 days. The longest culture was 32 days which was derived from a patient with Hodgkin's disease. The purpose of the study however was in the main to provide short term culture of cells which could then be filmed dynamically with time-lapse cine, or used for the identification of tumour specific antibodies in the patient's serum. Cultures also provided supernatants for subsequent analysis.

It was initially hoped that it might be possible to label the cultured cells with a suitable tag such as chromium 51 in order to study cytotoxicity. In a pilot study of 6 cultures, however, it proved impossible to achieve reproducible uptake of chromium by the tumour cells so that consequent release experiments could not be carried out.

Tumour cells as seen in direct dab or cytospin preparations (Fig. 31 and 32) were clearly larger than the other cells present. They were round with dense central nuclei. Occasionally one or two nucleoli could be determined. These cells settled rather slowly on the coverslips and it is possible that some of them rolled off the edges thus providing negative cultures. Lymphocytes and monocytes settled within 6 hours (Fig. 33) and the tumour cells were seen to put out tiny thread-like projections by the end of the first day (Fig. 34). After 2 days in culture (Fig. 35 and 36) macrophages were clearly established and spreading out to reach one another and by the end of a week a confluent monolayer was seen in successful cultures (Fig. 37). It was possible to detect phagocytosis of tumour cells (Fig. 38 and 39) in only 6 of the cultures at the end of 5 days. These figures show that the aggressor cells are definitely macrophages because they contain phagocytic vacuoles and it is interesting to notice the paucity of lymphocytes. Lymphocytes had usually died by the end of the first week of cultures. At no time were dividing tumour cells seen and as previously mentioned, the longest surviving tumour cells were seen at 32 days.

The reasons for failure to grow more tumour cells for longer would appear to be multiple. Gram negative bacilli infected 5 out of 61 cultures. The tumour cells were particularly sensitive to change of pH and if medium 199 became too acid, the cells quickly

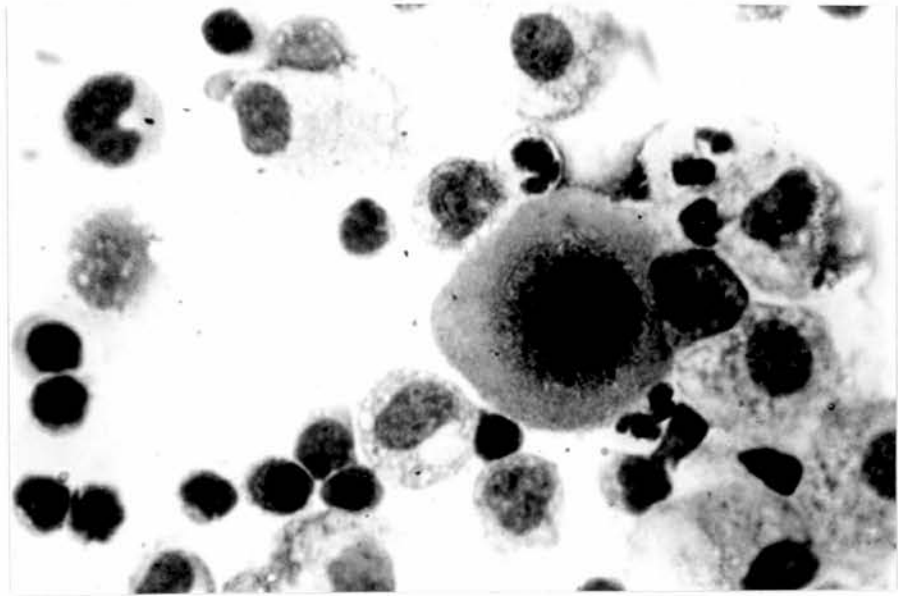


Fig. 31 Lymph node dab showing Hodgkin's giant cell, reactive lymphocytes and macrophages (Giemsa x 900)

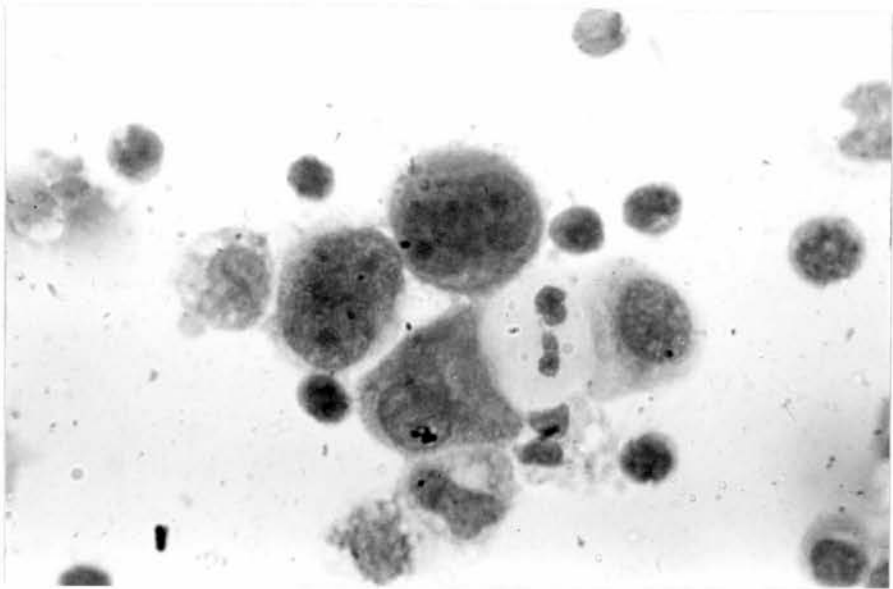


Fig. 32 Cytospin preparation of cell juice from a lymph node biopsy. Three binucleated cells are obvious plus two mononuclear tumour cells (Giemsa x 900)

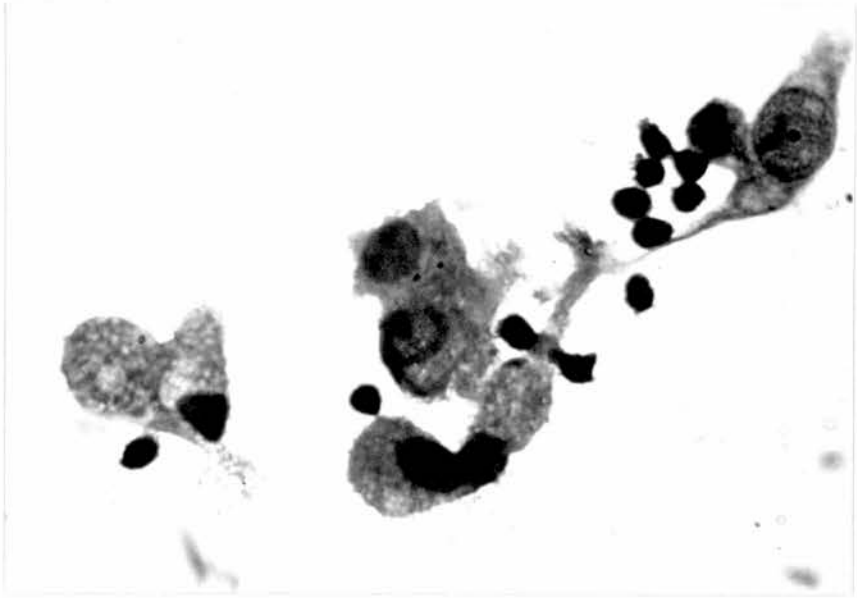


Fig. 33 Lymphocytes, monocytes and lymphoma cells after 6 hours in culture (Giemsa x 900)

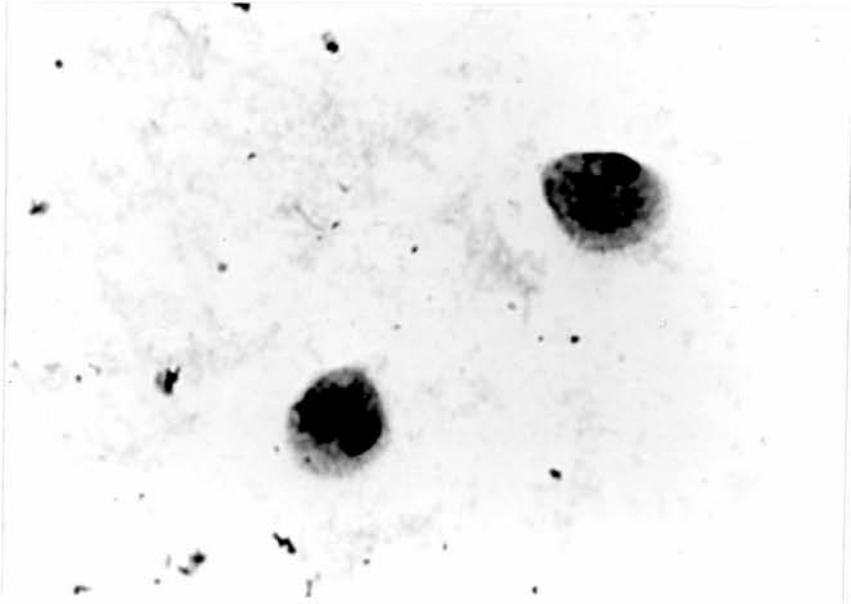


Fig. 34 Two tumour cells after one day in culture (Giemsa x 375)

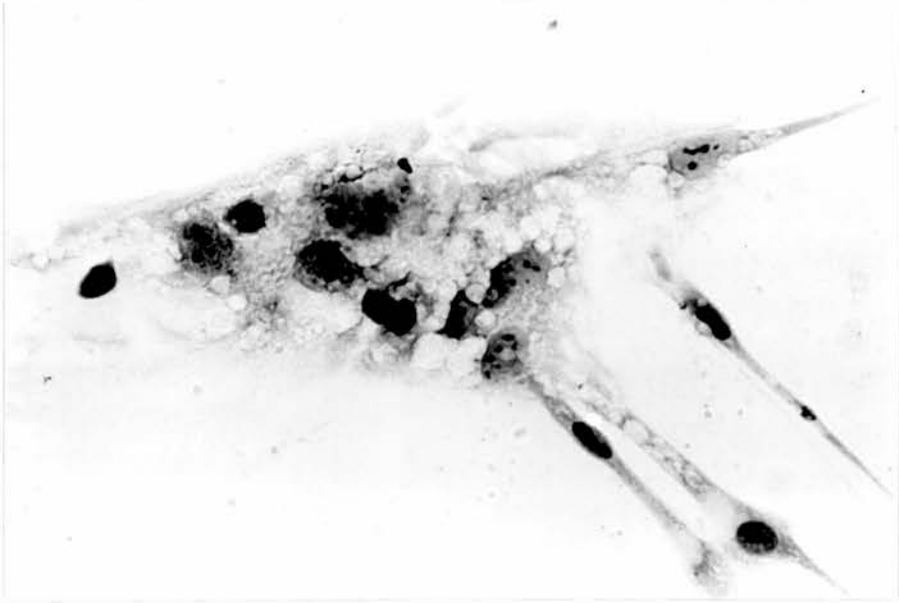


Fig. 35 Lymphoma cells in a 2 day culture showing flattened macrophages and several black dense lymphocytes (Giemsa x 375)



Fig. 36 High power view of macrophages and lymphocytes in a 2 day culture of lymphoma (Giemsa x 900)



Fig. 37 Confluent monolayer after one week in culture (Giemsa x 375)

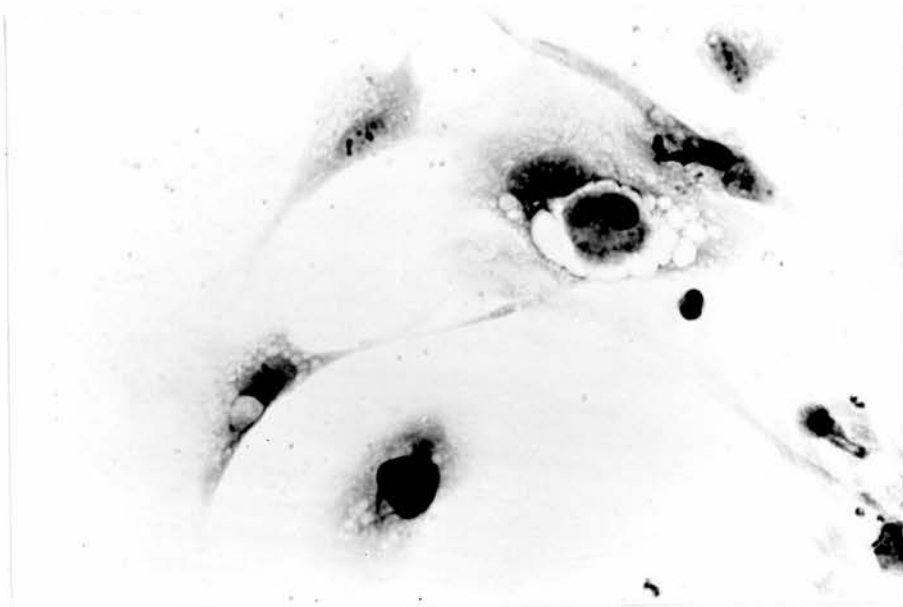


Fig. 38 Phagocytosis of a mononuclear tumour cell by a macrophage (Giemsa x 375)

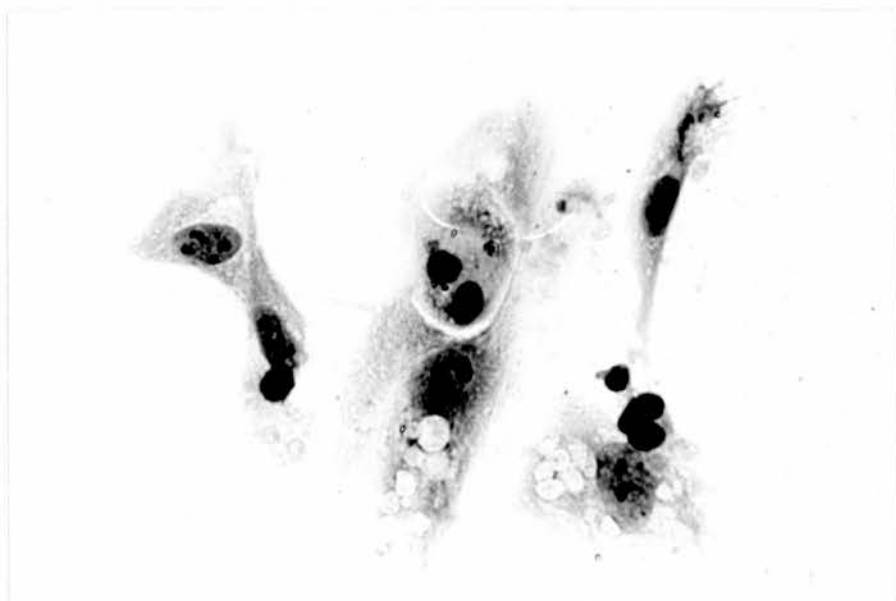


Fig. 39 Phagocytosis of a binucleate Reed-Sternberg cell by a macrophage in culture (Giemsa x 375)

died. Changing medium was not without risk also and several cultures were seen to die quickly after the change, for instance at day 3 or day 5. This may have been due to temperature difference or due to a physical effect of washing cells from the coverslip by the introduction of new medium, or else by the direct effect of serum in the medium. It was found that fresh autologous serum was toxic to cells and foetal new born calf serum or bovine serum did not provide optimal growth. Pooled human serum either AB or O, concentration 20%, was the optimal support medium though cells occasionally grew very well in lactalbumin. Lactalbumin was chosen with a view to the studies of supernatants. It was felt that when looking for chemotactic factors released by tumour cells in culture, it was very important to grow cells in a serum free medium to ensure that the factors were not derived from the serum; thus lactalbumin was chosen. One other toxic factor to cells was possibly a detergent left over from the glass washing process. When this was suspected, the rinsing procedure was doubled and this single step cut down the mortality of cultures by 50%. The final reason for failure of certain cultures to grow was that the growth of tumour cells was stifled by an overgrowth of fibroblasts. This was particularly obvious from two cultures of nodular sclerosing Hodgkin's patients where there was a large number of fibroblasts in the initial cell suspension. Viability of cells was tested by trypan blue exclusion, by electron microscopy appearances (see above) and by direct vision by phase contrast.

(d) Time Lapse Cine Photomicrography

A total of 3,200 feet of film was used in the study of 18 lymph node cultures. The resulting film which accompanies this thesis is approximately 30% of the initial length of film exposed.

The film was arranged to depict the different cell types seen in tissue culture. Particularly it shows the astonishing motility of lymphocytes which appear in conventional and ultra structural studies as rounded, rather static cells. Macrophages are seen in several forms, some with long dendritic processes (Fig. 40) others with flimsy hialoplasmic ruffled membranes (Fig. 41). The macrophages are seen to pinocytose small particles which form an outer halo around the nucleus and they are also seen to phagocytose larger particles such as lipid and cell debris. Tumour cells are seen in the film as rounded dense cells which appear to have whitish nuclei which reflect the light (Fig. 42 and 43). They are seen particularly in low power views to be the subject of much interest by passing lymphocytes. It is difficult to prove that these lymphocytes have positively been drawn towards the tumour cells but a definite impression of attraction is gained in the film (Fig. 44 and 45). Both lymphocytes and macrophages are seen to be aggressor cells at least in cell suspension in a culture vessel. The lymphocytes and macrophages themselves seem to interact (Fig. 46) and it is common to see lymphocytes crawling repetitively over and around the cytoplasmic surface of the macrophages. The interaction of lymphocytes and macrophages with a tumour cell invariably ended in its death (Fig. 47 and 48). Death was shown as a gradual rounding of the cell. The organelles within the cytoplasm of the tumour cell began to show Brownian movement. Eventually the nucleus or nuclei became displaced to one side and the whole cell became detached from the glass base of the culture vessel (Fig. 49). The circular transparent balloon of a cell was then seen floating with the current of the culture medium (Fig. 50). Occasionally cells actually exploded giving rise to the appearance of single nuclei

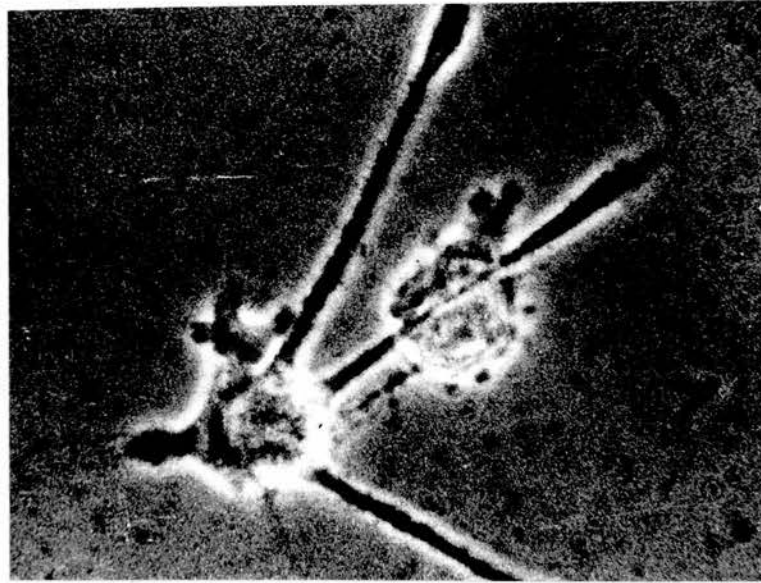


Fig. 40 Phase contrast (P.C.) of dendritic macrophage and a contiguous rounded tumour cell (x 800)

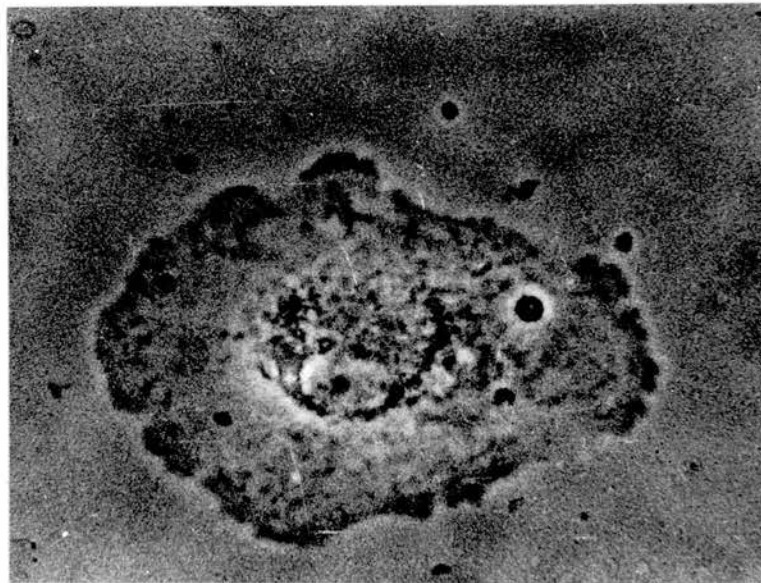


Fig. 41 Phase contrast (P.C.) of flat round macrophage with thin flimsy hialoplasmic membrane (x 800)



Fig. 42 Phase contrast of two white tumour cells, circular with halos plus attendant flat macrophage full of black granules (x 900)

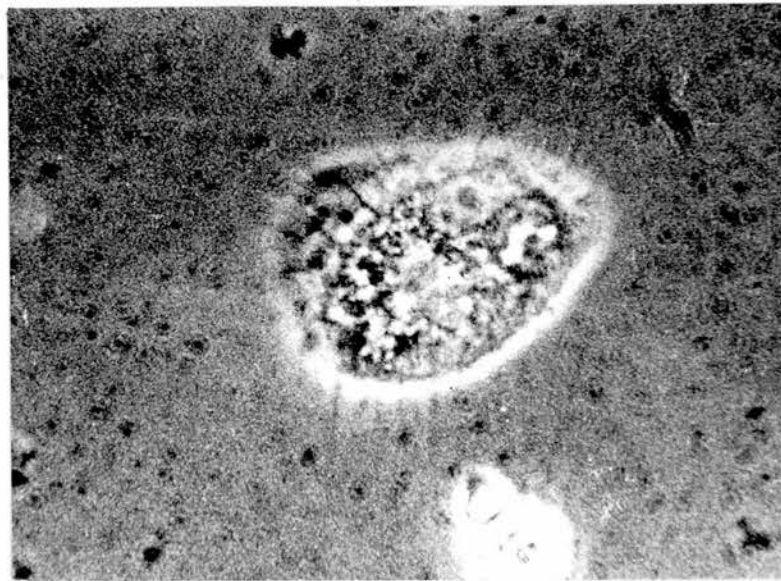


Fig. 43 Phase contrast of Reed-Sternberg cell showing numerous spikey projections and multiple nuclei (x 1000)

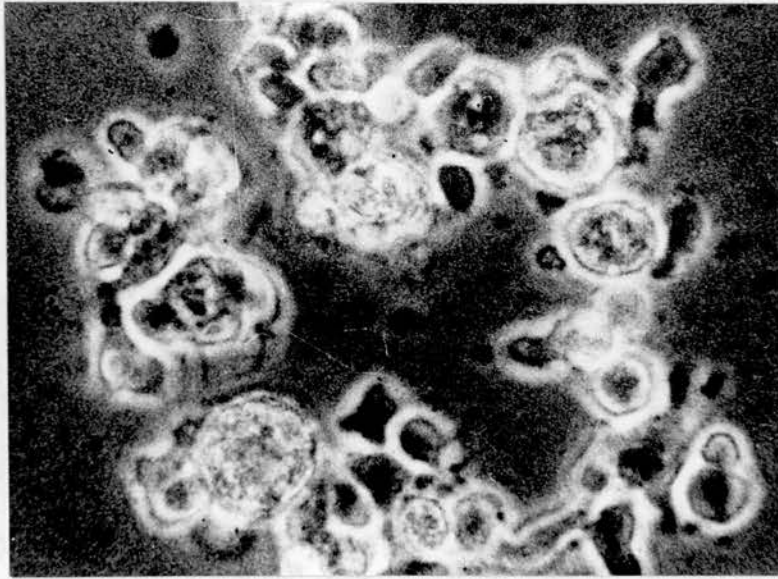


Fig. 44 Phase contrast of a ring of tumour cells each surrounded by a swarm of lymphocytes (x 400)

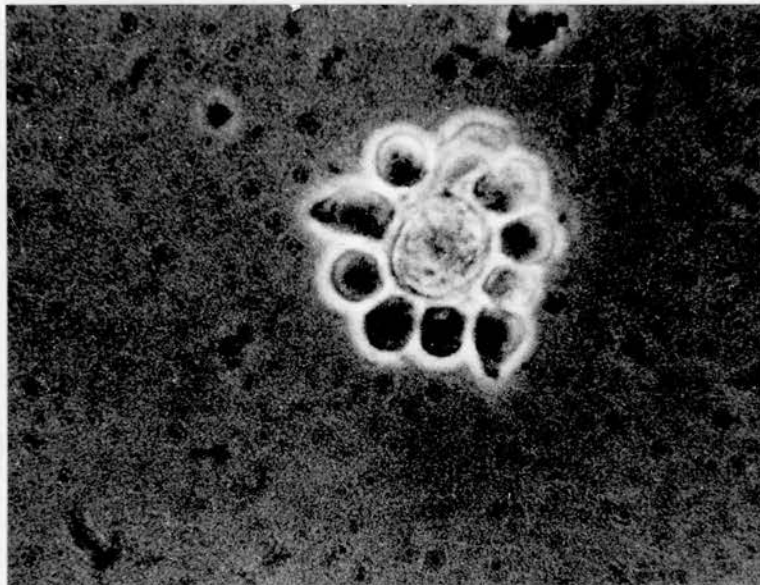


Fig. 45 Phase contrast of a rosette of lymphocytes encircling a tumour cell (x 400)

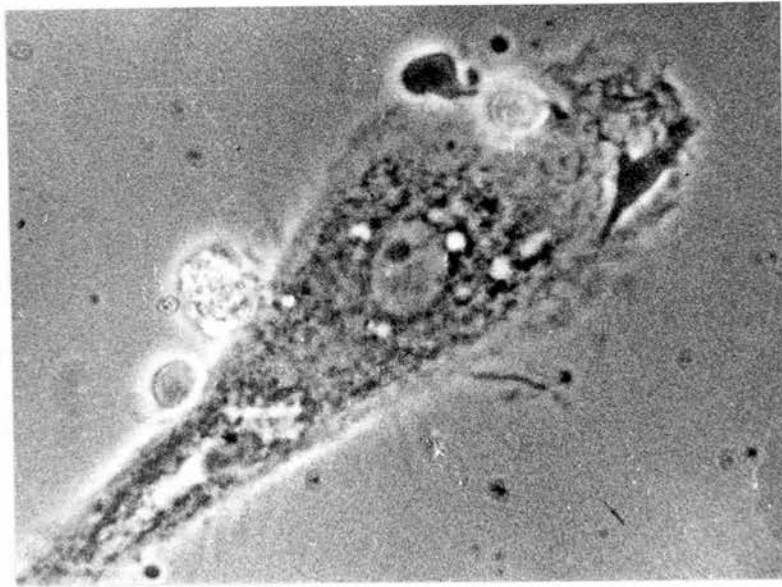


Fig. 46 Phase contrast of a macrophage being patrolled by two dark snail-like lymphocytes (x 500)

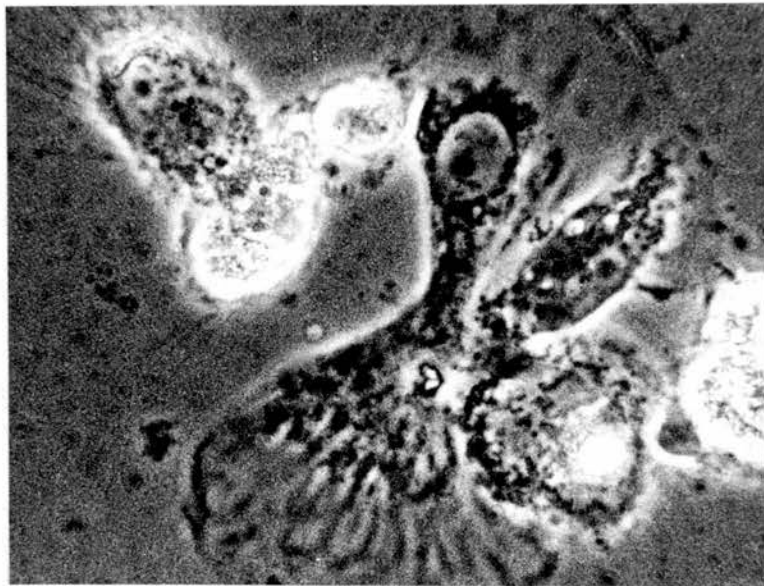


Fig. 47 Phase contrast of macrophages engulfing white rounded tumour cells (x 500)

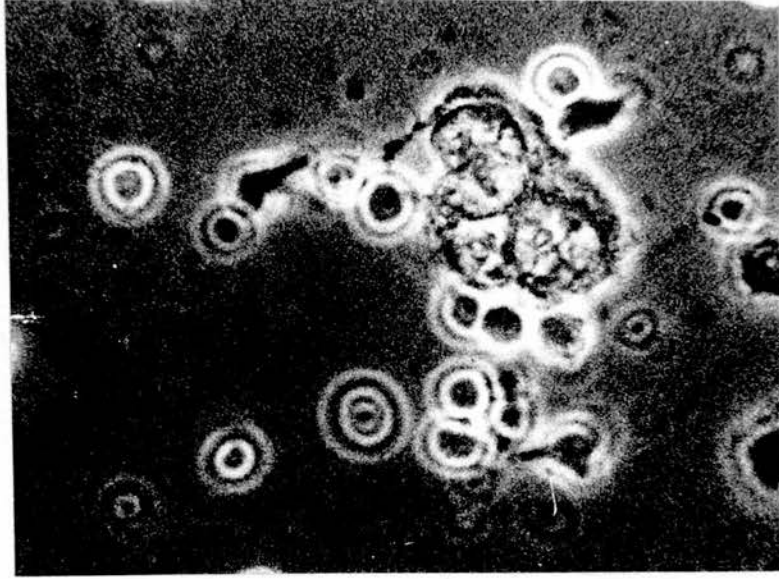


Fig. 48 Phase contrast of a dying multinucleated tumour cell surrounded by lymphocytes (x 400)

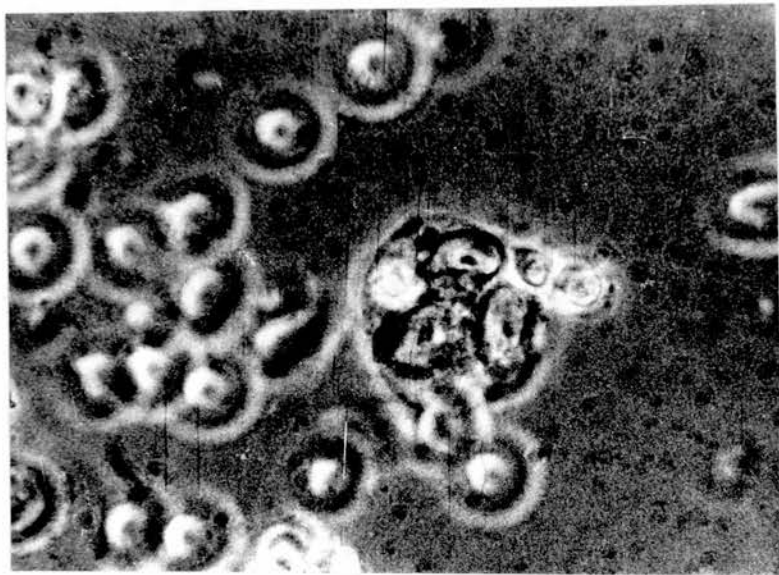


Fig. 49 Phase contrast of the cell in Fig. 48, detached and afloat in culture medium (x 400)

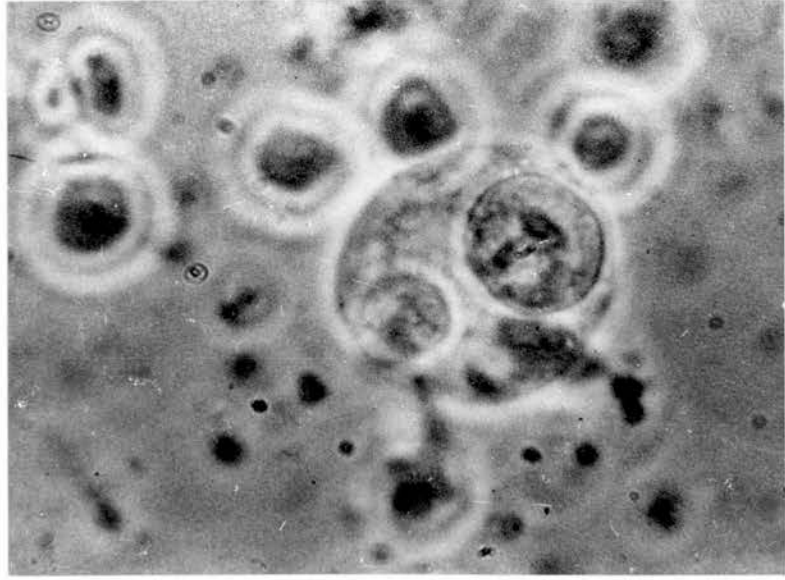


Fig. 50 Phase contrast of rounded dead tumour cell whose nucleus is displaced, and cytoplasmic granules lost (x 600).

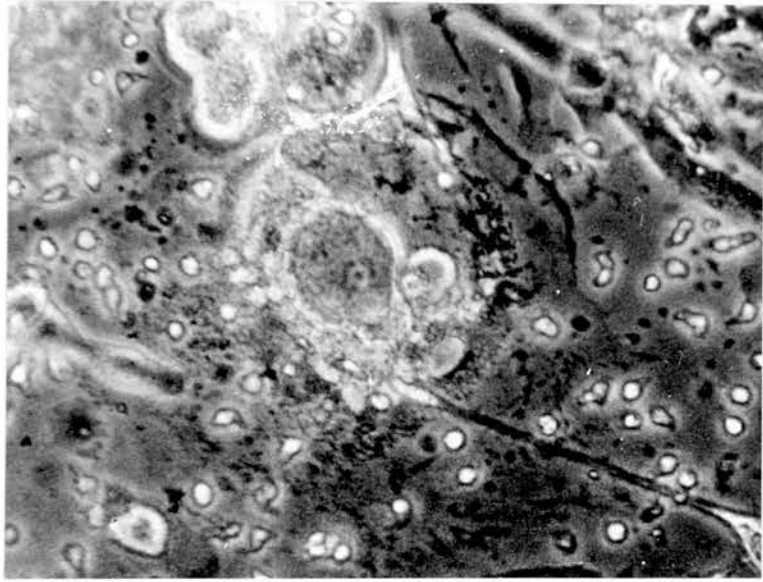


Fig. 51 Phase contrast of large tumour cell in a plasma clot culture surrounded by lymphocytes and several spindle shaped macrophages (x 400)

free in the supernatant of the culture. The eventual destiny of these nuclei was to be phagocytosed by scavenger macrophages. The entire film described the culture of either histiocytic lymphoma nodes or Hodgkin's nodes. It was impossible to depict host-tumour cell interaction in cultures of lymphocytic lymphoma nodes as the cells could not be identified because of the nature of the phase contrast microscopy. Thus the large cells of the other two lymphomas were obvious and readily observed. Even the short sequence of cells in a plasma clot culture allows identification of large tumour cells. In contrast to the preceding single cell suspension film, the large cells seem to survive in the organ culture within the plasma clot. This infers that tumour cells removed from the stroma of the surrounding tissue become more vulnerable to attack by host lymphocytes and macrophages (Fig. 51).

(e) Fluorescent Studies

Samples of serum were obtained from nine patients who had undergone lymph node biopsy and successful culture of the lymph node, 9 ABO matched control normal volunteers and 10 students recovering from infectious mononucleosis (the last specimens were kindly obtained by Dr. D.H. McVie, Student Health Service, Edinburgh University). After precipitation of the protein the gamma globulin fraction was labelled with FITC and its identity was checked by immuno-electrophoresis against human gamma globulin. These 28 samples were applied to lymph node dabs or glass coverslips containing cultured lymph node cells in duplicate. Each patient's lymph node culture cells were tested against his own labelled gamma globulin, one other labelled gamma globulin from another lymphoma patient and one ABO control. All lymph node cultures and

dabs were incubated with each infectious mononucleosis serum. The slides were studied under a fluorescent microscope and 25 large cells counted on each slide when possible. The results were uniformly negative with only occasional lymphocytes showing any marginal fluorescence.

Tissue dabs from 8 lymphoma lymph nodes all of which contained large cells were incubated fresh with non specific polyvalent antihuman-immunoglobulin. These cells were then studied under phase contrast, (Fig. 52 and 54) and the identical fields were then examined with fluorescent light (Fig. 53 and 55). Typical results are shown in these figures. The only fluorescence which was seen in the large cells consisted of very fine speckling throughout the nucleus with very poor staining of cytoplasm. In contrast several of the lymphocytes alongside have stained up brightly, presumably because they are B lymphocytes and contain immunoglobulin. Also present in Fig. 52 however are 4 round lymphocyte-like cells which do not stain in the fluorescent preparation. Presumably these are T cells. A formal study of the enumeration of B and T lymphocytes in lymphoma lymph nodes was being carried out in the same laboratory by Dr. J.A. Habeshaw and so no further work was done in this direction by the author.

(f) Supernatants from Cultures

(i) Leucocyte chemotactic factors

Twelve lymph node supernatants were studied, 6 derived from cultures of Hodgkin's lymph nodes, 2 from lymphocytic lymphoma and 4 from nodes which showed reactive hyperplasia with no signs of malignancy. Neutrophil chemotaxis was noted towards all supernatants and the number of neutrophils attracted through the filter varied between 20 and 240. There was no difference between the different

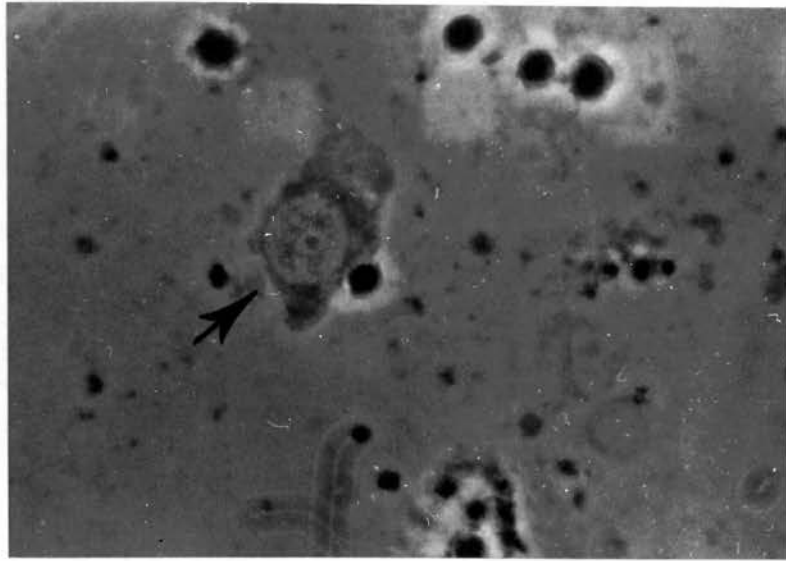


Fig. 52 Phase contrast of tumour cell (arrowed) and dark small lymphocytes (x 200)



Fig. 53 Fig. 52 photographed with fluorescent light showing speckling of the large cell's nucleus and several but not all of the lymphocyte membranes (x 200)

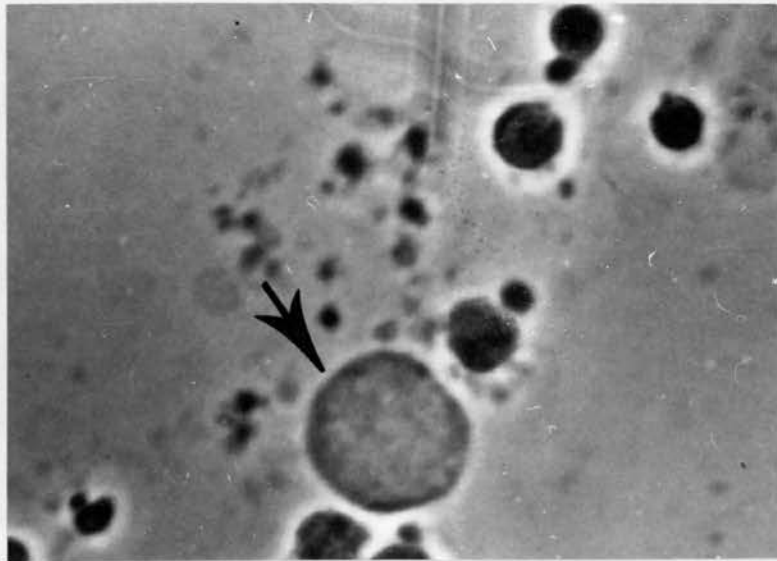


Fig. 54 Phase contrast of tumour cell plus lymphocytes adherent to it (x 300)



Fig. 55 Fluorescent version of Fig. 54 outlining good staining only in the lymphocyte membranes (x 300)

kinds of supernatants (Fig. 56). Significant eosinophil chemotaxis however was only noted in the supernatants deriving from the Hodgkin's lymph nodes. The variation was from 8 to 63 cells per high power field. When the relative proportion of eosinophils to neutrophils was calculated, an eosinophil chemotactic index was achieved. The equation used to derive this figure was as follows: eosinophil chemotactic count divided by neutrophil chemotactic count over the percentage of eosinophils in the cell suspension before migration divided by the percentage of neutrophils in the cell suspension before migration. In 5 out of 6 Hodgkin's nodes the eosinophil chemotactic index was higher than the highest index in the lymphocytic lymphoma nodes or the reactive hyperplasia nodes. This infers that eosinophils were selectively attracted towards some substances released by the Hodgkin's cells in culture. Figure 56 shows the eosinophil chemotactic index for each lymph node supernatant allied to the actual cell counts for each supernatant and the diagnosis is listed along the bottom. Also on the figure is the degree of eosinophilia seen on histological examination of a haematoxylin and eosin stained section from each node. Eosinophilia was arbitrarily scored from 0 to ++. In general there is a correlation between the eosinophil chemotactic index and the presence of eosinophils in the gland with two exceptions. Eosinophils were not seen in one of the Hodgkin's lymph nodes despite release of chemotactic factors and they were seen at a rating of 1+ in a reactive benign node. The neutrophil and eosinophil chemotactic activity was dose responded for each of the 12 supernatants by varying the protein concentration of the supernatant. A minimum of 3 points was used in each dose response experiment. A typical result from one of the Hodgkin's

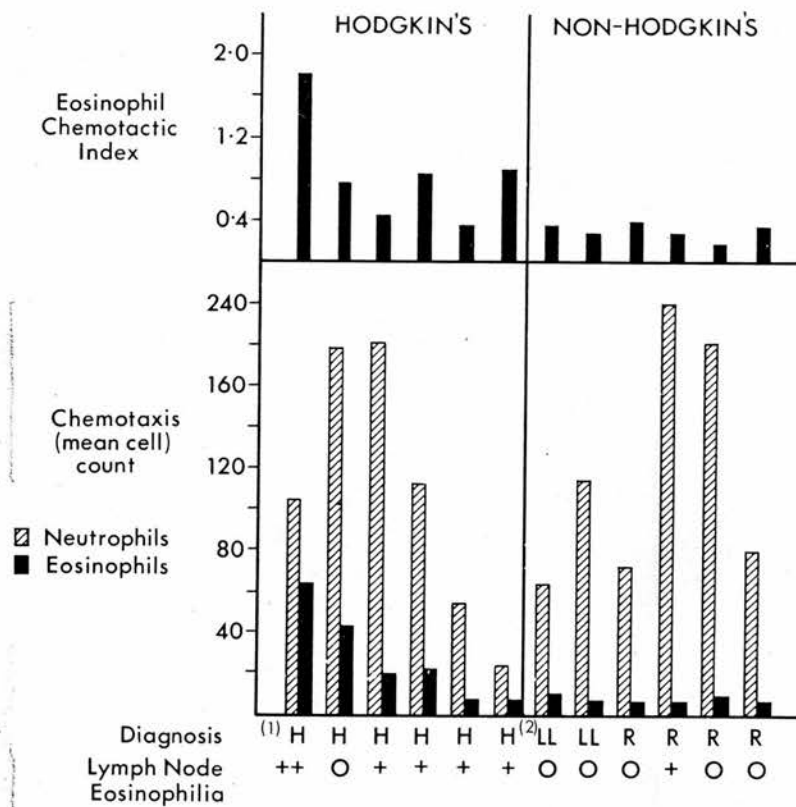


Fig. 56 Neutrophil and eosinophil chemotaxis and the eosinophil chemotactic index from Hodgkin's disease (H) and lymphosarcoma (LL) and reactive hyperplasia (R) cultured lymph node supernatants

CHEMOTAXIS
(mean cell count)

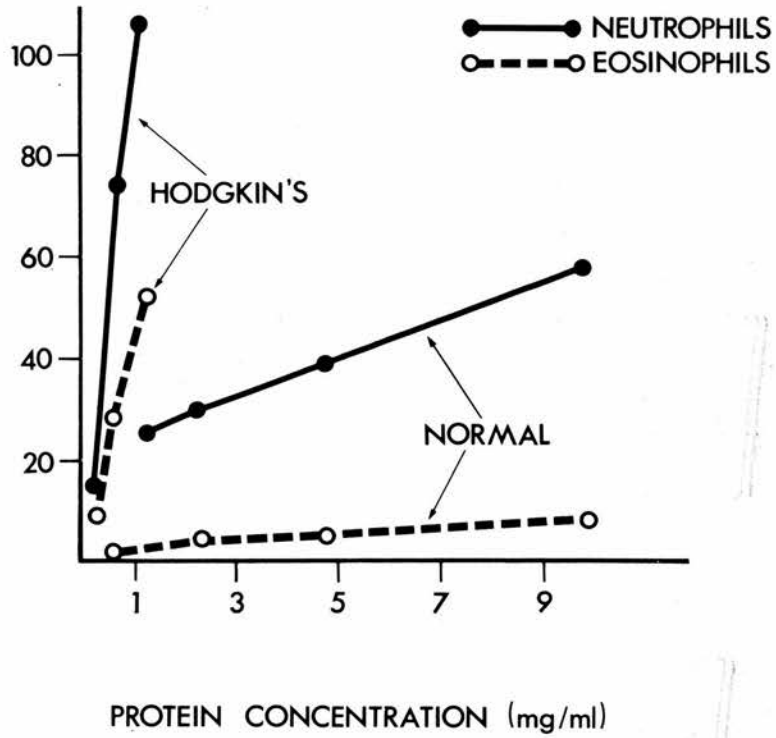


Fig. 57 Neutrophil and eosinophil chemotactic activity of a Hodgkin's and reactive cultured lymph node supernatant

supernatants and one of the reactive hyperplasia nodes is seen in Fig. 57. In all instances, a dose response effect was noted for both the eosinophil and the neutrophil cell counts. Further analysis of a concentrated pool of supernatants from 3 Hodgkin's lymph nodes was carried out by two research fellows working in the laboratory. Sephadex G50 chromatography consistently demonstrated 4 peaks of activity as confirmed by application in the eosinophil chemotaxis chamber. One of these peaks corresponded to eosinophil chemotactic factor of anaphylaxis previously described by Kay and Austin 1971, and Kay et al, 1971. A similar substance has been extracted from a sample of undifferentiated carcinoma of the lung associated with the peripheral blood eosinophilia (Wasserman et al, 1974). The results of this present study are reported by Kay, McVie et al, 1975 (appendix 9).

(ii) Monocyte chemotactic factors

Normal monocytes (the author's) were attracted through a Millipore filter towards 26 different supernatants of cultures derived from lymph nodes whose histology was as follows: 3 lymphocytic lymphoma, 6 diffuse histiocytic lymphoma, 11 Hodgkin's disease and 6 reactive hyperplasia. The results are shown in Fig. 58 where the distance migrated in microns for each supernatant is represented on the vertical scale. In only one instance, a supernatant from a diffuse histiocytic lymph node, was random migration alone seen to a distance of 11 microns. This distance (up to 15 microns) is the maximum that monocytes randomly moved in this experiment; in other words, when there was no chemo-attractant on the under surface of the Millipore filter. Also shown in this table are the control results obtained in the same experiments

**CHEMOTAXIS of MONOCYTES to
CASEIN compared to SUPERNATANTS
from LYMPH NODES**

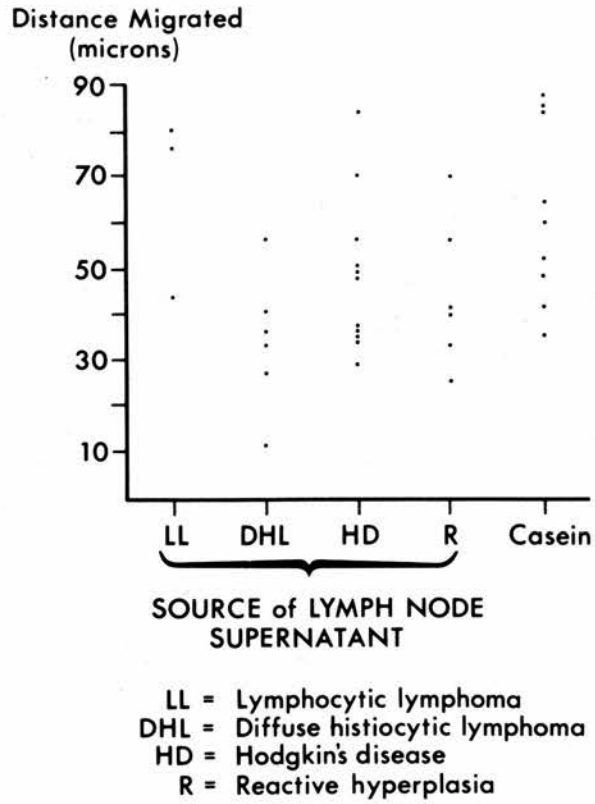


Fig. 58 Chemotaxis of monocytes towards lymphoma lymph node supernatants

when standard chemo-attractant 5% casein was used. It was found that casein though batched from a large stock was variable when tested sequentially using the same person's monocytes. Thus in these experiments two batches of casein were used. The first is represented in the lower 6 marks on the graph ranging from 35 to 54 microns. The second batch used a month later gave consistently higher results. The relevant results for the experiments described here are distances of 84, 85, and 87 which though remarkably reproducible vary considerably from the performance of the previous batch of casein. Nevertheless, with one exception noted above, all supernatants regardless of origin demonstrated chemotactic properties of the same order as casein.

(iii) Monocyte chemotaxis inhibitors

Lymph node supernatants

As it proved difficult to achieve reproducibility in the classical macrophage inhibition technique involving guinea pig macrophages in capillary tubes it was decided to look at a more direct effect of the lymph node supernatants on normal monocyte mobility. Accordingly the supernatants described above were incubated with the author's monocytes, prior to use in the identical chemotaxis assay. Casein 5%, was used as the chemo-attractant in all of these experiments and Fig.59 shows the same control column as the previous figure for normal cells unincubated attracted towards casein. The mean distances migrated bearing in mind the two batches of casein previously described was 65 microns for 8 experiments. The mean distance migrated of the 3 groups of lymph nodes diffuse histiocytic lymphoma, Hodgkin's disease and reactive hyperplasia were 36, 39, and 35 microns

CHEMOTAXIS of MONOCYTES
INCUBATED with SUPERNATANTS
towards CASEIN

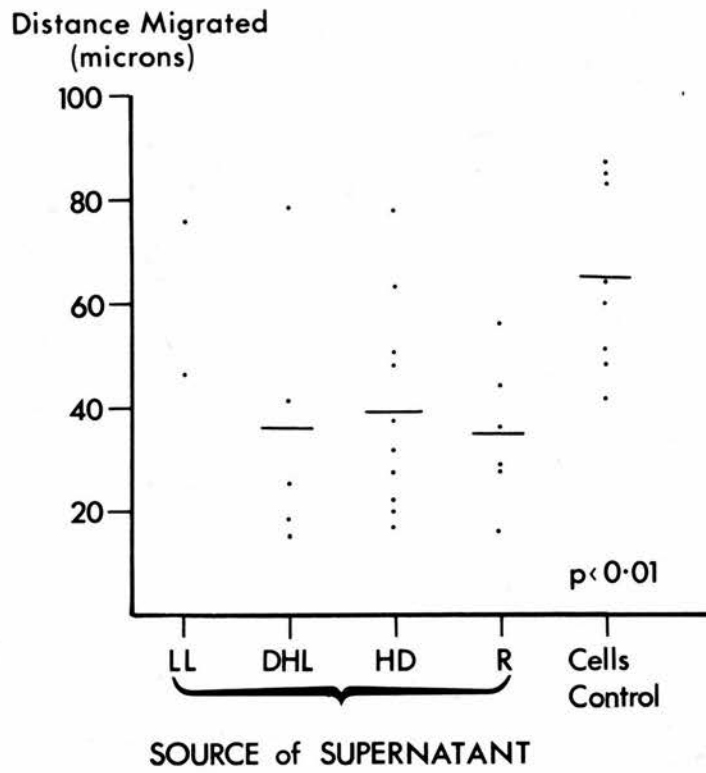


Fig. 59 Effect of incubation of normal monocytes in lymphoma node supernatants on chemotaxis (abbreviated as in Fig. 58)

respectively. There were only 2 supernatants which were technically satisfactory deriving from lymphocytic lymphoma nodes so no conclusion has been drawn from this group. The mean of the total group of lymph node supernatants was a distance of 36 microns. Using the standard error of the difference of the means, there is a p value < 0.01 between the distance travelled by the cells which were incubated in a lymph node supernatant compared to the unincubated cell control. All supernatants were used in this experiment at a protein concentration of 5 mg/ml. There was unfortunately not enough volume of supernatant to dose response the test or to go on further to analyse the chemical nature of the factor or factors which were interfering with monocyte migration through the filter. Fourteen of the supernatants showed greater than 10% inhibition of monocyte chemotaxis (Fig.60). The remaining 9 showed either no significant inhibition or else actual stimulation in 2 instances. Of the total group the mean inhibition of monocyte chemotaxis was 20%. There was no pattern according to the source of lymph node and the monocyte inhibition was as impressive using supernatants from benign nodes as that achieved with lymphoma supernatants.

Pleural Fluid

The result of incubation of 9 different samples of normal monocytes with 2 dilutions of pleural fluid derived from a patient with Hodgkin's disease is shown in Fig.61. There is a dose response effect in 3 of the 9 experiments between the 2 dilutions of pleural fluid and in 7 instances between the cells incubated with 0.5 dilution of pleural fluid and those incubated in medium 199. The significance of the difference in the last 2 readings is < 0.01 . The degree of

DEGREE of INHIBITION
of MONOCYTE CHEMOTAXIS
by LYMPH NODE SUPERNATANTS

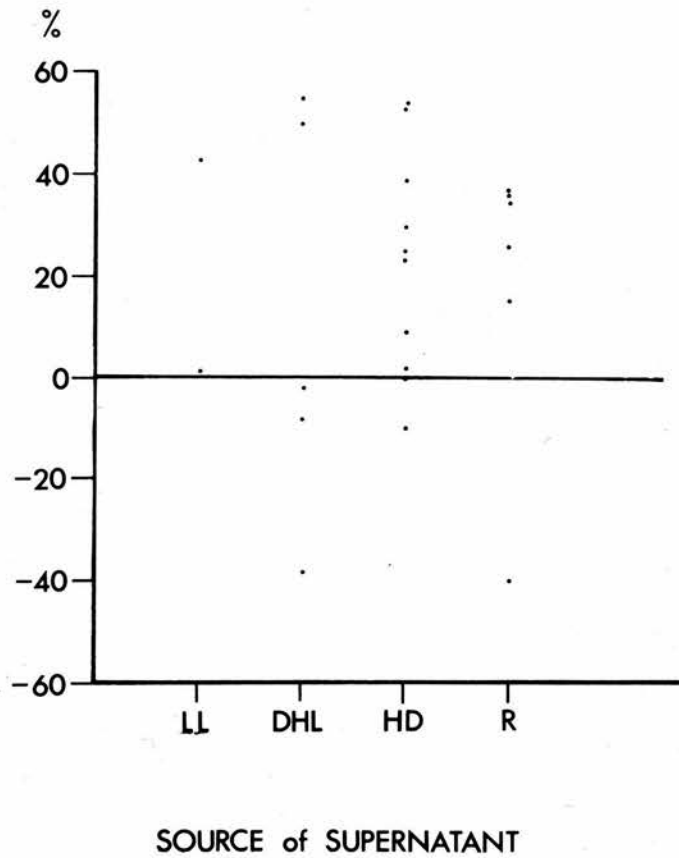


Fig. 60 Percentage inhibition of monocyte chemotaxis by lymphoma node supernatants

The EFFECT of INCUBATION
of NORMAL MONOCYTES with
DILUTIONS of HODGKIN'S PLEURAL FLUID
on CHEMOTAXIS

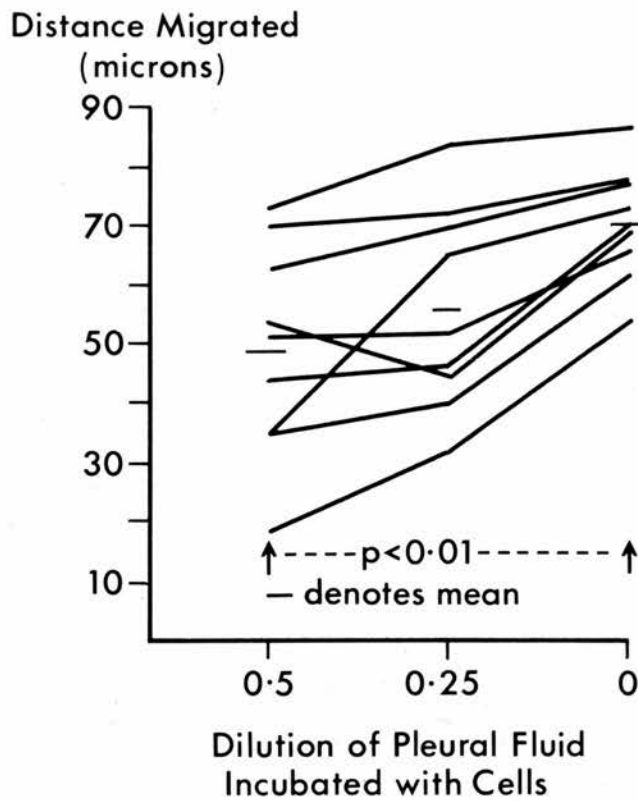


Fig. 61 Inhibition of monocyte chemotaxis by Hodgkin's pleural fluid

DEGREE of INHIBITION
of MONOCYTE CHEMOTAXIS
by TWO DILUTIONS of
HODGKIN'S PLEURAL FLUID

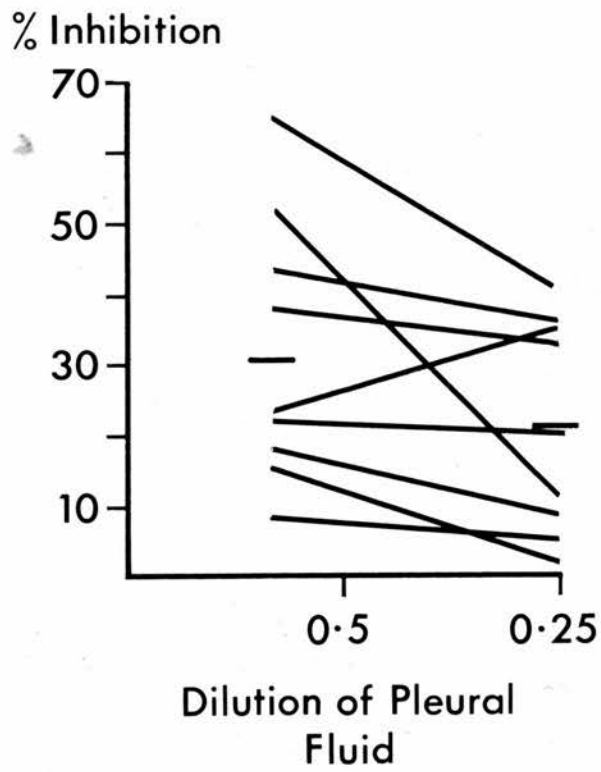


Fig. 62 Percentage inhibition of monocyte chemotaxis by Hodgkin's pleural fluid

inhibition in each culture can be calculated by subtracting the distance migrated by cells incubated in pleural fluid from the distance migrated by the control cells divided by the distance migrated by the control cells expressed as a percentage. Again there is a clear dose response effect in 3 instances (Fig.62) with a trend which is probably not significant in 5 others. The difference between the means is not significant. In 8 of the nodes however tested in dilution of 1 in 2, there is a suppression of monocyte chemotaxis greater than 10% of the normal value and in 6 there is a suppression greater than 20% of the normal value. This finding might explain the relative absence of monocytes in the cytopsin preparation of the pleural fluid despite the clear presence of numerous large atypical tumour cells, some characteristic of Reed-Sternberg cells.

(iv) Skin reactive factor

Six supernatants from lymph node cultures were analysed for skin reactive factors by injection into the skin of the guinea pig abdomen. The results of this experiment were entirely negative as no reaction was seen on injection of samples of supernatant apart from a mild erythema identical in degree and extent to that obtained by injection of phosphate buffered saline as a control. The supernatants were dose responded and even this made no difference up to the end of the experiment which was 5 days. The conclusion is that skin reactive factor was not present or was not active in supernatants derived from lymphoma lymph node cultures.

(v) Mitogenic factor

Phytohaemagglutinin produced up to a 30-fold increase in uptake of tritiated thymidine for cultures of unstimulated normal

lymphocytes (Fig.63). In the same experiment supernatants from 4 lymph nodes of Hodgkin's disease, one lymphocytic lymphoma and one reactive hyperplasia were mixed in 3 concentrations with aliquots of identical lymphocytes. Results are shown in Fig. 64. There is uniform absence of significant uptake of tritiated thymidine compared to cultures stimulated with any of the 3 doses of phytohaemagglutinin. Further, even the highest culture is approximately 3-fold less in counts per minute compared to the mean of 6 unstimulated samples. This suggests that not only is there not a mitogenic factor present in these supernatants but there may be a factor which is suppressing uptake of tritiated thymidine in unstimulated lymphocytes. There was enough supernatant from one of the Hodgkin's disease samples to investigate further a 50% dilution of the supernatants made and added either to unstimulated lymphocytes or to lymphocytes stimulated with the median dose of PHA (1.66 $\mu\text{g}/\text{ml}$). An almost identical result was obtained comparing the unstimulated lymphocytes alone to the unstimulated lymphocytes bathed in supernatant. In addition, however, the PHA response was reduced 20-fold by the addition of lymph node supernatant. This experiment was done in triplicate but unfortunately was not dose responded and so it is of questionable significance.

UPTAKE of TRITIATED THYMIDINE by
NORMAL and PHA STIMULATED LYMPHOCYTES

	PHA (0.42 $\mu\text{g/ml}$)	PHA (1.66 $\mu\text{g/ml}$)	PHA (5 $\mu\text{g/ml}$)
Unstimulated	3720	98,370	106,363
Counts per Minute	79,382	79,382	79,382

Fig. 63 PHA transformation of normal lymphocytes

UPTAKE of TRITIATED THYMIDINE by NORMAL LYMPHOCYTES
INCUBATED with LYMPH NODE SUPERNATANTS

Concentration of Supernatant	Source of Supernatant (counts / minute)					
	LL	HD	HD	HD	HD	R
25%	559	891	1062	469	1159	449
50%	327	197	243	154	517	276
75%	300	233	199	169	240	144

LL = Lymphocytic Lymphoma
 HD = Hodgkin's Disease
 R = Reactive Hyperplasia

Fig. 64 Failure of transformation of lymphocytes by incubation with lymphoma culture supernatants

2. PERIPHERAL BLOOD STUDIES

(a) Phagocyte Function

(i) Identification of monocytes

Two specific stains were used for identifying and enumerating monocytes in samples of peripheral blood from patients and controls. Both methods, one with the non-specific esterase and one for peroxidase were cumbersome and time consuming. They also involved expensive substrates and so were only carried out on a random basis to check that errors were not being made in the standard counts. An improvement in the standard counting technique using the Neubauer chamber was the incubation of the cells in the chamber for 10 to 20 min in a moist atmosphere. This was found to allow settling of the round cells on the glass and monocytic cells had either developed a halo after settling or were actually seen to flatten out. This technique was reliable compared with for instance, the peroxidase stain an example of which is shown in Fig.65. The black crystals tended to precipitate rather vigorously over cells and occasionally this obscured the view of each individual cell so that a cluster of cells might conceivably have been counted as one cell. Monocyte counts were carried out on a series of lymphoma patients receiving chemotherapy either before treatment, 3 days after treatment or 7 days after treatment. No significant depression of monocyte count was seen despite the regular observation of depression of polymorphonuclear leucocytes particularly in the 7 days after chemotherapy with cyclophosphamide or mustine. This is confirmation of a clinical impression held by the author and substantiated by several other oncology units. In

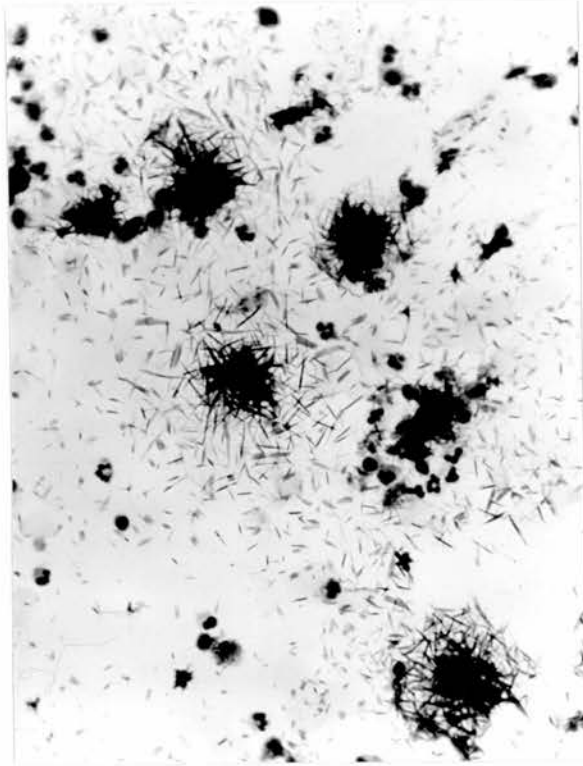


Fig. 65 Peroxidase stain shown by black crystals concentrated on monocytes. Neutrophils and lymphocytes are unstained (x 100)

thirty-one patients all of whom had lymphoma, monocyte counts were carried out on the monolayer obtained after centrifugation of 20 ml of venous blood through Ficoll-Triosil. The mean retrieval of monocytes identified as described above was 4.6 million compared to 4.4 million from 31 age/sex matched control donors. These findings were particularly interesting as the total mononuclear count was quite different in the two groups of individuals. The lymphoma patients had a significantly lower total mononuclear count at 14.8 million compared to 20.5 million for the normal donors ($p < 0.01$). The difference can be accounted for by lowered lymphocyte numbers in the patient group as these are the only other cells seen when venous blood is separated on Ficol Triosil. The contamination with polymorphonuclear leucocytes, red cells or platelets was less than 1%.

(ii) Monocyte chemotaxis

Twenty-two chemotaxis experiments were carried out before any treatment had started in fourteen patients with lymphoma. Either a dose of 0.5 mg or 1 mg of casein was used as a chemo-attractant and no experiment was considered valid unless a dose response effect was shown. Results were expressed as a percentage of the distance migrated by the patients' monocytes compared to the distance migrated by monocytes of an age/sex matched control converted to a percentage. The results of this group are shown in Fig.66. In 16 of the 22 experiments monocyte chemotaxis was depressed by greater than 20% in the patient compared to the control. The mean depression of the patient group was 35%. When the patients were divided according to pathological stage of disease the group with early disease (stages I and II) were nearer the normal than stages III and IV. Means in the two groups were

MONOCYTE CHEMOTAXIS of LYMPHOMA PATIENTS
by STAGE and TREATMENT
COMPARED to BRONCHIAL CARCINOMA
and CONTROL HEAVY SMOKERS

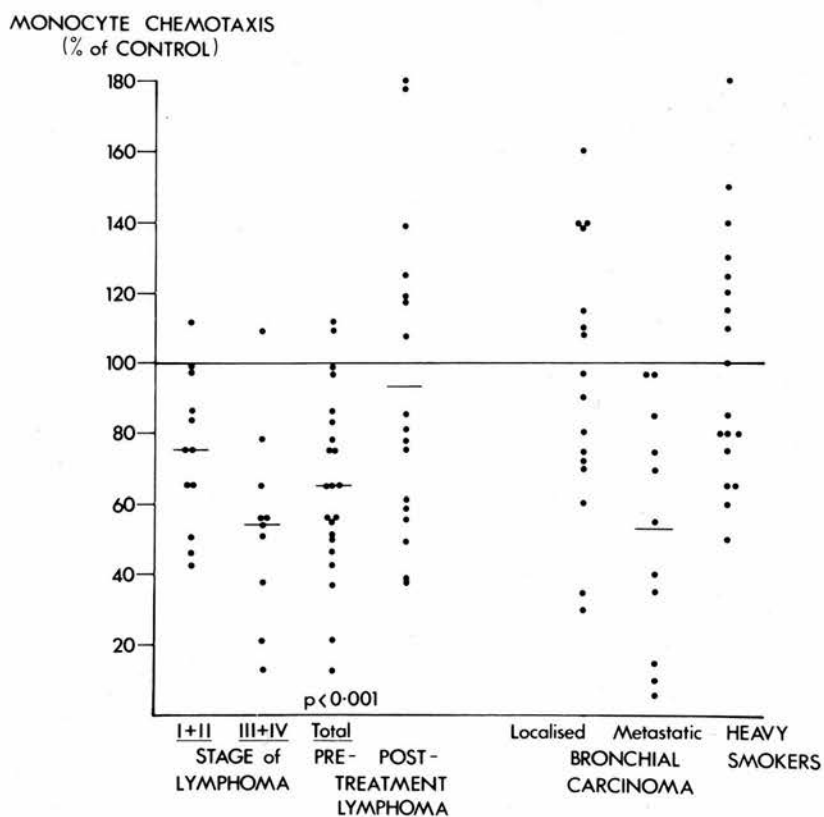


Fig. 66 Depression of monocyte chemotaxis in lymphoma patients and metastatic bronchial carcinoma

75% and 54% respectively. The presence or absence of symptoms such as sweating, fever or weight loss made no difference to the monocyte chemotaxis, nor did age or histology have any clear effect. Serum albumin was abnormally low in four patients but again this had no bearing on the mobility of patients' monocytes. Figure 66 also shows the effects of treatment on eleven of these either with radiotherapy or chemotherapy. The mean of 16 experiments was 93% of the control which indicates a depression from the ideal of 7% which is not significant. There is a considerable scatter in these results from marked stimulation of monocyte chemotaxis to marked depression. In general, chemotherapy had a more marked depressive effect on monocyte chemotaxis than radiotherapy. This was not consistent and could also be explained by the fact that chemotherapy was only given to patients with stages III and IV who as discussed above, started off with more abnormal monocyte chemotaxis. The association of depressed monocyte function and increased bulk of disease has been shown in a separate study (Kay and McVie, 1977) and results are shown briefly for comparison in Fig. 66. It can be seen that patients with metastatic bronchial carcinoma with one exception, have depressed monocyte chemotaxis compared to a scatter of results with patients who have bronchial carcinoma confined to the chest. Also shown in the diagram as a control group were nineteen heavy smokers who had no evidence of malignant disease. They showed no consistent pattern of monocyte function and the mean of the whole group was not significantly different from normal. Monocyte counts did not affect chemotaxis (Fig. 67).

In summary, monocyte chemotaxis is depressed in most patients who have lymphoma prior to treatment. The extent of abnormality

RELATIONSHIP of MONOCYTE COUNT
to MONOCYTE CHEMOTAXIS

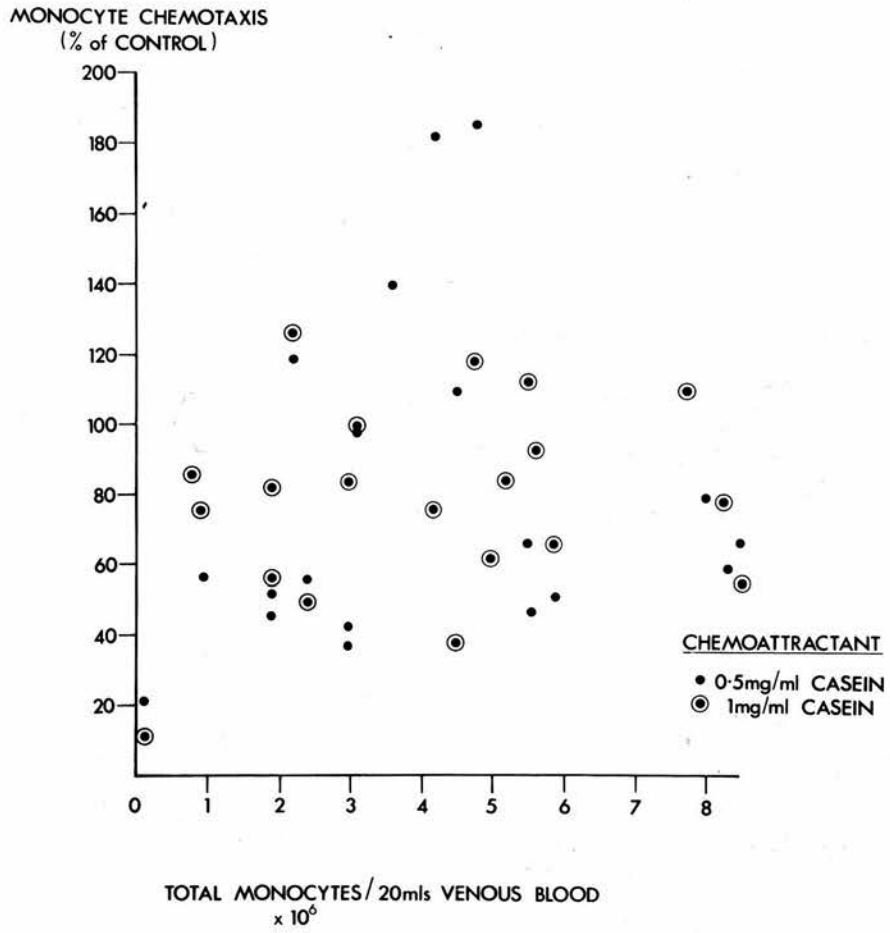


Fig. 67 Lack of relationship of monocyte numbers to chemotactic ability

related to stage of disease but not to histology, presence of symptoms or serum albumin. After treatment with radiotherapy or chemotherapy the results tended to become normal except in some patients who were still on chemotherapy. These results were of a similar order to those found in a parallel study of patients with localised and advanced bronchial carcinoma.

(iii) Monocyte phagocytosis and bactericidal capacity

Phagocytosis

The results of this section were communicated previously (McVie, Logan and Kay, 1976). Phagocytosis of *Staphylococcus aureus* was estimated after 20 min of incubation of the bacteria with patients' monocytes. Eighteen patients were studied each on several occasions. Prior to treatment of the patients' lymphoma, the mean percentage of *Staphylococcus aureus* phagocytosed from the surrounding supernatant was 36% as compared with a mean of 38% in the group of 16 control subjects. Phagocytosis tended to be inhibited when drugs were being administered (the combination most frequently used was cyclophosphamide, vincristine and prednisolone) but when patients were off treatment the mean percentage of bacteria phagocytosed returned to 35%. Figure 68 shows the progress of five patients who were studied serially either on chemotherapy or 2 weeks after the cessation of therapy. The rebound phenomenon is seen quite clearly in each patient.

Bactericidal capacity

Monocyte cultures from the above group of patients were studied in parallel for their capacity to kill phagocytosed bacteria. The percentage of bacteria killed was 89% in the group of lymphoma patients compared to 92% in the control group. This is quite an

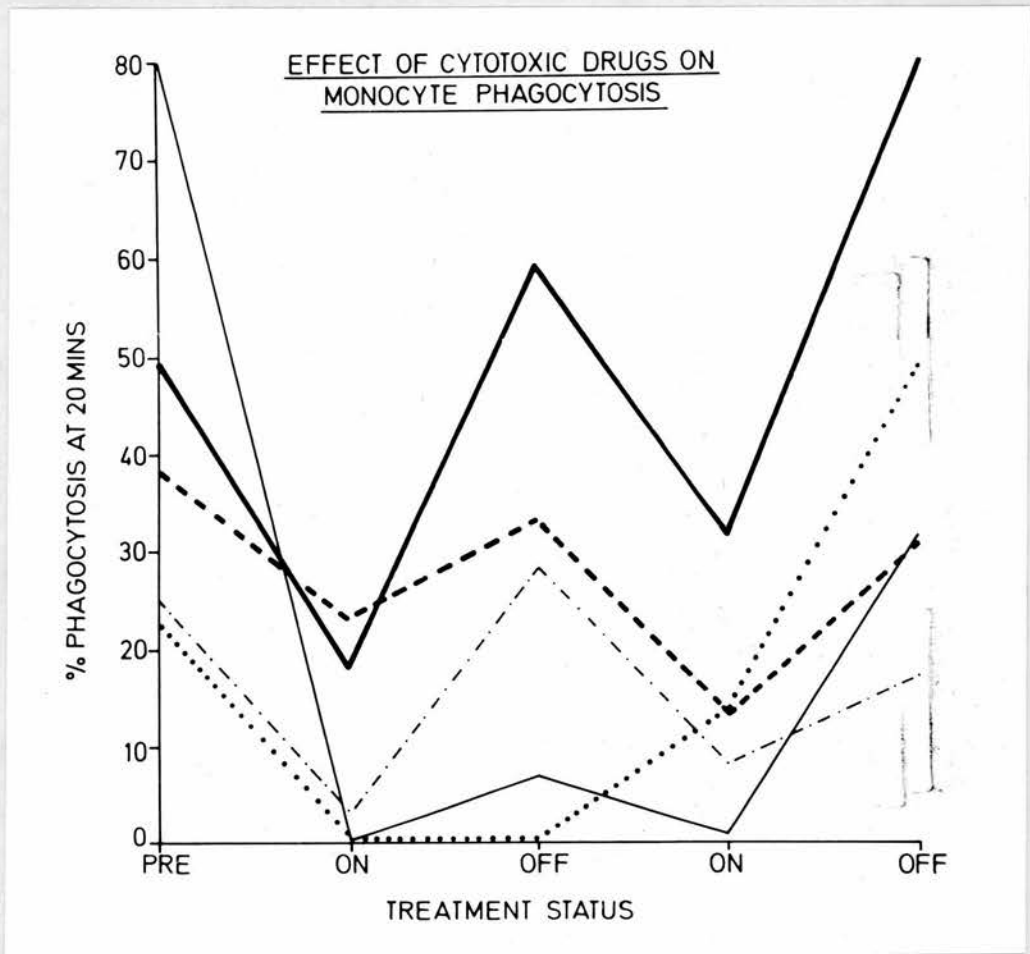


Fig. 68 Reversible depression of monocyte phagocytosis from lymphoma patients by drug treatment

insignificant difference and indicates that the mechanism for killing bacteria remains intact in patients with lymphoma irrespective of whether the patient is on chemotherapy or not. A different result however, was obtained when the rate of killing of bacteria was studied and the term bactericidal index was coined to describe the rate of killing over 40 min, 3 samples being studied through that time.

Values for the bactericidal index were extremely variable in the test group with four of the group not achieving any killing of bacteria within the first 40 min. On the other hand, five patients were significantly better than controls and they had a mean index of 80. The value for the control group was 45, and the mean of all patients studied was 23. The subsequent course of the five more efficient patients is shown in Fig. 69. It is quite clear that chemotherapy significantly reduces the bactericidal index in time. The only survivor of the five patients illustrated here was the one who had the highest bactericidal index after therapy. Three of the four who died, died from intercurrent infection.

Neither the degree of phagocytosis and cell killing, nor the bactericidal index was related to the total white count or monocyte count or the degree of monocyte chemotaxis when this was measured in parallel.

(b) Serum Proteins

(i) Immuno-electrophoresis

The serum samples from thirty-one patients with lymphoma were studied by immuno-electrophoresis. There were few gross abnormalities in the group though minor fluctuations in length and depth of precipitation lines were seen. Some attempt was made to

SERIAL MEASUREMENT of BACTERICIDAL INDEX
from START of CHEMOTHERAPY
in FIVE LYMPHOMA PATIENTS

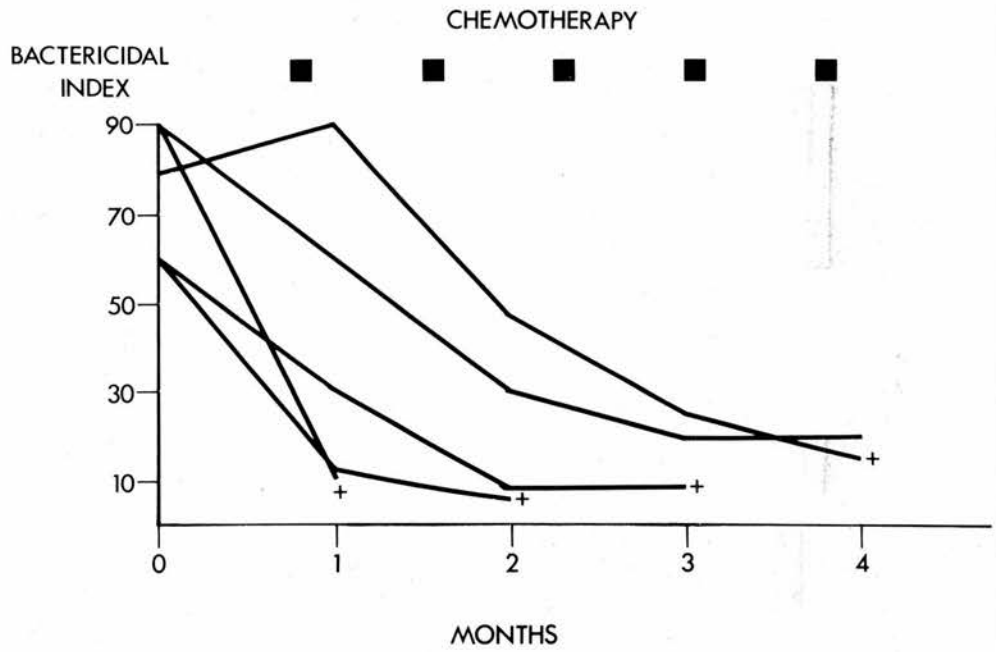


Fig. 69 Depression of monocyte bactericidal index in lymphoma patients on chemotherapy.

gauge an estimate of the quantitation of immunoglobulins by the distance of the precipitation arc from the trough. When the serum samples were examined retrospectively for their quantitative immunoglobulin levels by the Mancini immuno-diffusion method, the estimates on immunoelectrophoresis were found to be completely unreliable. No patient had a clear absence of immunoglobulins either IgA, IgG, IgM or IgD. All patients had α_2 macroglobulin detectable and only one patient with lymphocytic lymphoma had a decreased α_2 macroglobulin whereas one patient with lymphocytic lymphoma, two with diffuse histiocytic lymphoma and two with Hodgkin's disease had increased precipitation arcs.

Two patients had paraproteins clearly detected on immunoelectrophoresis. Both patients had lymphomas of stomach. The first was a diffuse poorly differentiated lymphocytic lymphoma occurring in a man of 49 years. In addition to primary lymphoma of the stomach he developed cervical lymph node involvement while receiving abdominal irradiation. He was treated with two courses of chemotherapy and died within 5 months of presentation. His immunoelectrophoresis pattern of his whole serum is shown in Fig.70. IgG has an abnormal kink in the arc as is shown when IgG anti-serum was used alone in Fig. 71, and this was shown to be made up of kappa light chains (Fig. 72). The other patient is described by Habeshaw, Hayward and McVie, 1975 (appendix 9). In summary, this man was shown to have a plasmacytoma of the stomach perhaps related to a gluten sensitive enteropathy which he had had for several years. The paraprotein was IgA and the level of this immunoglobulin dropped dramatically after gastrectomy for the gastric tumour. Later however, he was found to have a co-existing IgG secreting myeloma from which he died. Both

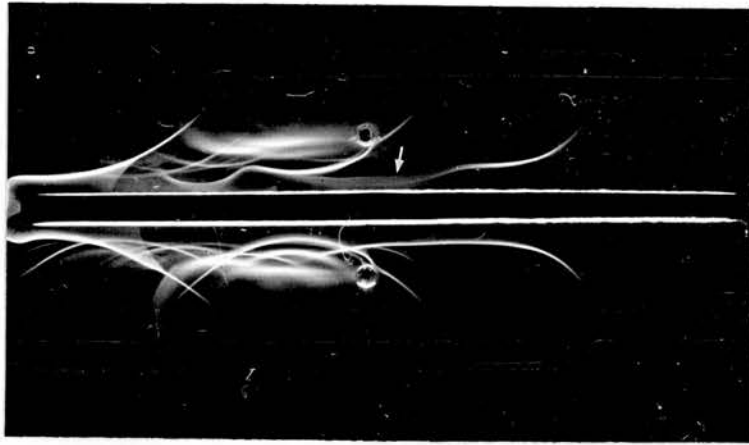


Fig. 70 Immunoelectrophoresis (IEP) of serum from a lymphoma patient with a paraprotein (arrowed)

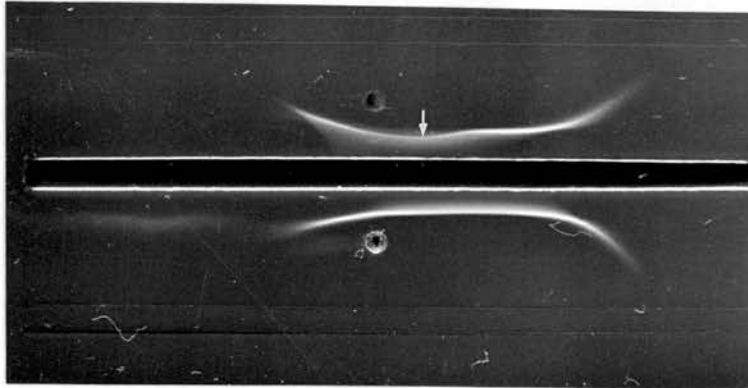


Fig. 71 Identification of paraprotein (Fig. 70) as IgG.

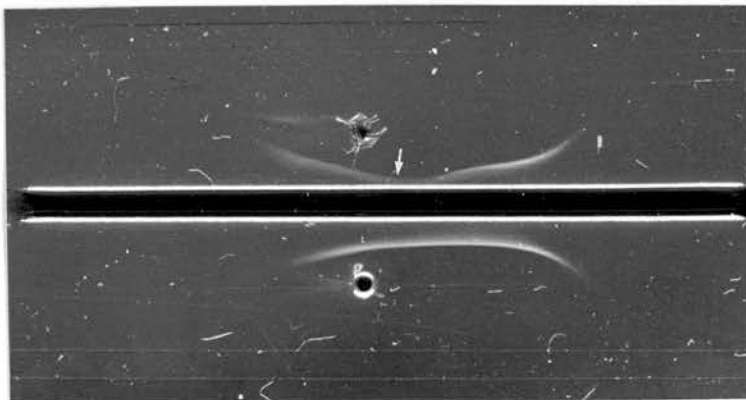


Fig. 72 Identification of light chain (Fig. 70) as kappa

kappa and lambda chains were noted in the urine and subsequent immunofluorescent staining of post mortem materials suggested that the kappa light chains were related to the IgG staining plasma cells in the bone marrow, whereas multiple metastatic lesions which were stained for IgA also stained for lambda chain.

For the majority of lymphoma patients electrophoresis was not a useful technique and was only subsequently employed when a paraprotein was indicated either by plasma protein electrophoresis or by suspicion on a raised immunoglobulin level.

Two patients with lympho-proliferative disease, father and son were studied by immunoelectrophoresis. The father had been known to have macroglobulinaemia for several years and his electrophoretic strip is shown in Fig.73. A large amount of IgM has shown, the arc almost extending into the edge of the trough compared to a trace present in the normal serum. This man's son presented at the age of 23 years with enlarged cervical lymph nodes which were shown on biopsy to be poorly differentiated nodular lymphocytic lymphoma. In contrast to his father's electrophoretic strip the son's pattern of immunoglobulins was entirely normal (Fig.74). The son had stage II disease and underwent radiotherapy and is alive and well 5 years later. The father remains on chemotherapy 15 years after presentation with a serum immunoglobulin M level which varies between 3 gm and 10 gm per litre.

(ii) Immunoglobulin quantitation

A normal range for the modified large scale immunoglobulin technique was achieved by studying a large number of samples from normal volunteers or convalescent patients. The values for the normal range quoted by Nordic (who supplied the

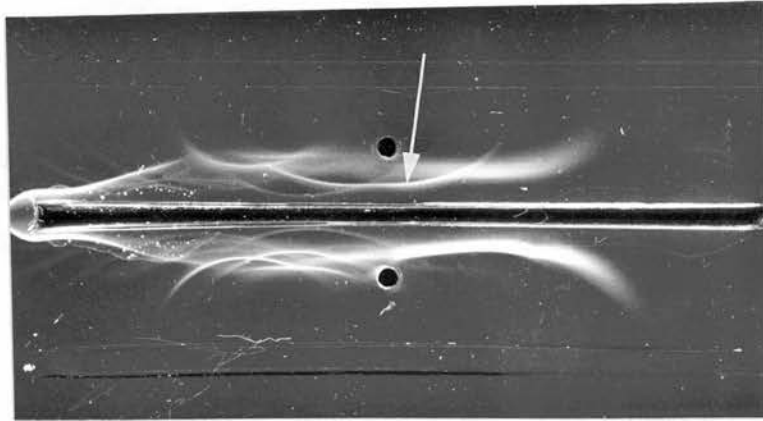


Fig. 73 Immunoelectrophoresis of serum from a patient with macroglobulinaemia. IgM is arrowed

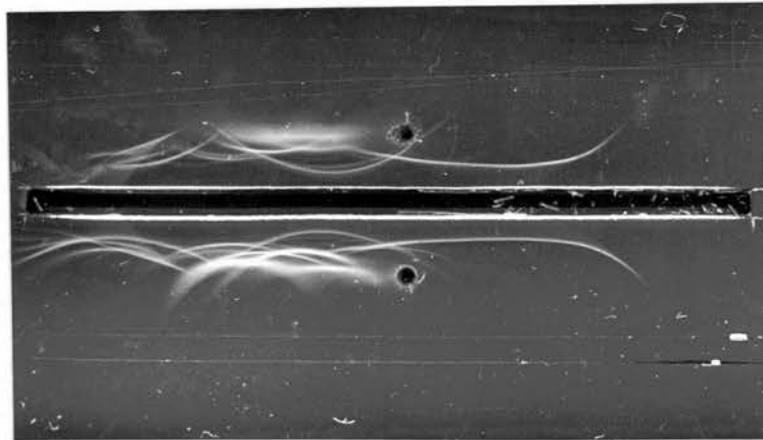


Fig. 74 Immunoelectrophoresis of serum from the son of patient in Fig. 73 who had lymphoma. Normal strip.

antiserum) were largely in agreement with those found in the study except at the lower level of the IgM range. The normal range found in our laboratory was IgG 700-1,675 mg%, IgA 90-315 mg%, IgM 45-250 mg% which agrees with Macris, 1976. Less than 5% of IgM levels had controls below 80 mg%. As will be described subsequently, this has proved a valuable cut off point when considering the prognosis of lymphoma patients in the prospective study. Thirty-nine patients had a level of IgM < 80 mg% before treatment and their 5 year survival was 59% compared to 74% for the remainder of the group.

(iii) Escherichia coli antibodies

The normal range for this test was established by measurement of Escherichia coli antibody titres in 180 normal individuals. There was no variation with respect to age or sex and the mean titre was 1 in 128 and 1.6% of the group had a titre less than 1 in 32. Parallel studies of the methanol resistant antibody revealed results which were in general parallel to the methanol sensitive antibody except that the mean titre was 1 in 16 and 25% of normal population had no titre of this antibody. When studying lymphoma patients subsequently using this assay, the methanol resistant antibody was of much less value because of the high numbers of normal controls who did not have the antibody. In contrast, the Escherichia coli methanol sensitive antibody was of some value in assessing the degree of immunodeficiency of lymphoma patients before and throughout treatment.

(iv) Alpha 2 macroglobulin

The normal range of α_2 macroglobulin in humans using the present assay was reported by Tunstall et al, 1975. It was found that the normal level was age dependent falling from a high

concentration in youth to the minimum in middle age gradually increasing again in old age. At all ages the mean was slightly higher in females than in males. Analysis of α_2 macroglobulin for a group of normal individuals aged 20 years was 380 mg% in males and 390 mg% in females. In a group in the fourth decade the means were 250 and 300 mg% and the sixth decade, 300 and 320 mg% respectively for males and females.

B. CLINICAL

1. RETROSPECTIVE SEARCH FOR CLUSTERS OF LYMPHOMA

The results of this study are reported by McVie and Bisset, 1978. A computer study of the incidence of lymphoma plotted against date of presentation and ward of residence of the patient suggested that there were 14 clusters of lymphoma throughout a period of 11 years. These clusters amounted to a total of 107 patients and 93 case notes were recovered for these patients (which represents a retrieval rate of 87%). After close scrutiny of the case notes, 49% were rejected due to inaccuracies either in electoral ward, classification of disease or because hospital registration numbers had been duplicated. Eight different patients from a single hospital were registered correctly by address but wrongly by international code number. The correct diagnosis from the case sheet was tuberculosis in each instance and the international code for this disease is 012 to 019 compared to 200.0 for lymphoma. At the time of study coding was carried out by the junior medical staff in each unit of this hospital and therefore it was an error presumably reproduced eight times by one member of staff.

Another source of error was multiple patient numbers for the same patient and the worst instance of this was in a hospital which uses the patient's date of birth as his identification number for the case sheet. A single patient was registered on six different occasions by that hospital with six different dates of birth. Four of his admissions had been in a single year. After exclusion of several cases on similar grounds, 13 clusters were dissolved. The remaining cluster occurred in two adjacent wards and consisted of 8 cases of Hodgkin's disease all of whom lived within a

radius of $1/3$ of a mile. The approximate population of this area is 10,000. From national incidence figures one would expect less than one patient to develop Hodgkin's disease from this population. As the cluster had occurred 10 years previously, most of the eight patients had died in the interim and therefore personal interviews were not possible. This limited any further investigation which might have established social contact, educational and other links. There have been three other pairs of cases in the author's subsequent practice. Two siblings presented with lymphoma of the gastrointestinal tract almost simultaneously. The details of this study are included in appendix 9 (Freedlander, Kissen and McVie, 1977). Another brother and sister presented within a year of one another with Hodgkin's disease, one patient in Canada and one patient in Scotland. These siblings had lived apart for 15 years and one of them, the Canadian, has died within a year of onset of the disease. Histological confirmation has not been obtained unfortunately due to the distance from the point of study but the physician's notes indicate that there seemed little doubt of the diagnosis. The Scottish sibling though understandably distressed by his sister's death remains in good health still receiving chemotherapy. The third pair concerned a father and son previously described in the section under immunoelectrophoresis. The son had lymphocytic lymphoma involving the cervical nodes and the father was a long standing case of macroglobulinaemia presenting with a lymph node enlargement and a high ESR.

An example of the degree of inaccuracy of the case listings of the Scottish Home and Health Department can be obtained from Fig. 77. The line marked A indicates the predicted incidence of

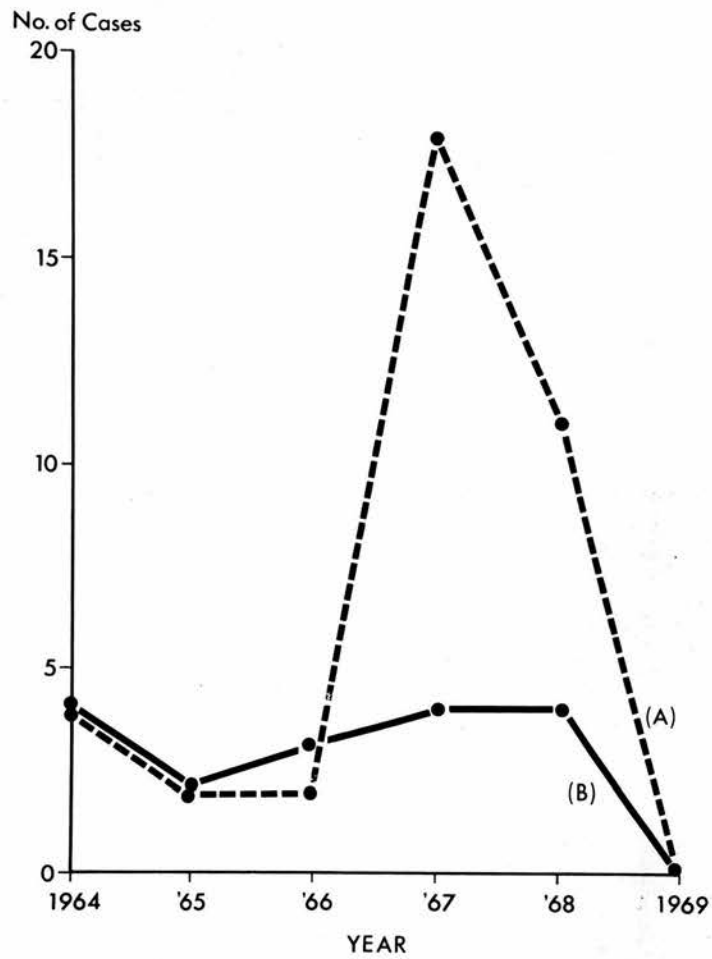


Figure 1

Case incidence of lymphosarcoma and reticulum cell sarcoma
in St Andrews/St Giles/George Square 1964-69

A From Scottish Home and Health Department case listings

B From patients' case notes

Fig. 75 Cluster data (apparent, A and real, B) for
lymphoma in Edinburgh 1967-1968

lymphoma cases compared to line B which gives the actual incidence obtained from the case note study. When the Home and Health Department listings are used on a population basis, for instance to look at age specific admission rates (Fig. 76) broad trends emerge which are in keeping with the other series in the literature (MacMahon, 1966). Thus Hodgkin's disease has an early peak in the twenties, a later peak in the forties and declines in instance throughout later life. This contrasts with non Hodgkin's lymphoma which slowly climbs from middle age to reach a peak in the sixth decade.

The use of data such as this is obviously preferable to chance discovery of sibling pairs and family pairs such as described above which could easily happen by coincidence. On the other hand, it is apparent that great care must be taken especially when working with bare computer data in the absence of recourse to either patients themselves or their case notes. Thirteen out of fourteen clusters were artefacts in the above study; the fourteenth was certainly a true cluster which withstood vigorous examination including histological review of the original biopsy slides. All the patients did have Hodgkin's disease according to an independent pathologist's opinion and the patient's general practitioners confirmed addresses though unfortunately could give little other information regarding possible social contact.

2. PROSPECTIVE STUDY

(a) Patients

A cohort of one hundred patients with histologically proven lymphoma formed the group for this analysis. Each patient presented over a period of 5 years to the University Department

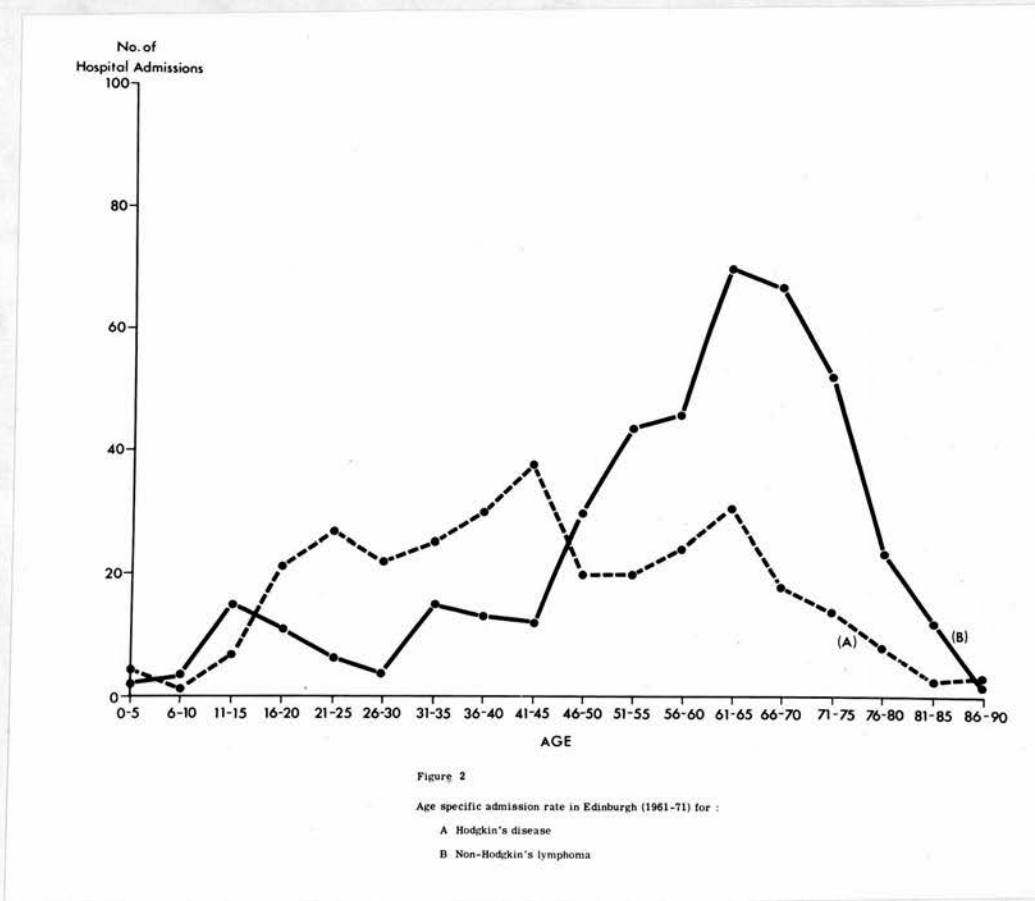


Fig. 76 Age specific admission rate for (A) Hodgkin's disease and (B) non Hodgkin's lymphoma in Edinburgh (1961-71).

of Therapeutics, Royal Infirmary, Edinburgh. The minimum follow-up time from the start of treatment is now 2 years and the mean follow-up time is almost 4 years. The ages and sex of the total group is shown in Fig.77. The range of ages is from 12 to 81 years and 56% of the group were males.

Almost all patients presented with the history of a lump either in the neck or the groin or the abdomen (Fig. 78). Nine patients were diagnosed at laparotomy to have lymphoma involving the gastrointestinal tract. These patients either presented with acute abdominal pain, vomiting or in one instance, symptoms suggestive of carcinoma of pancreas. Whereas Fig. 78 shows that five patients had involvement with skin and four had involvement of lung, all but two of these involvements were in association with peripheral lymph node disease. Two patients presented with skin rashes alone. Not shown in the table are two isolated cases of extra-nodal presentation. One male of 40 presented with insipient paraplegia due to an extra-dural histiocytic lymphoma compressing the spinal cord, and the other a 45 year old female presented with acute renal failure secondary to obstruction of both ureters. At laparotomy the hold-up was found to be due to involvement of the ureters with nodular sclerosing Hodgkin's disease.

A specific note was taken of symptoms which are associated with lymphoma and whose presence has been said to confer poorer prognosis on the patients. These are weight loss, fever or sweating, and patients with one or all of these symptoms have been annotated after the Rye classification as having stage B disease. Absence of these complaints is called stage A. Because of the laboratory interest in immuno-deficiency, two other points of interest were

AGE-SEX DISTRIBUTION OF TOTAL GROUP

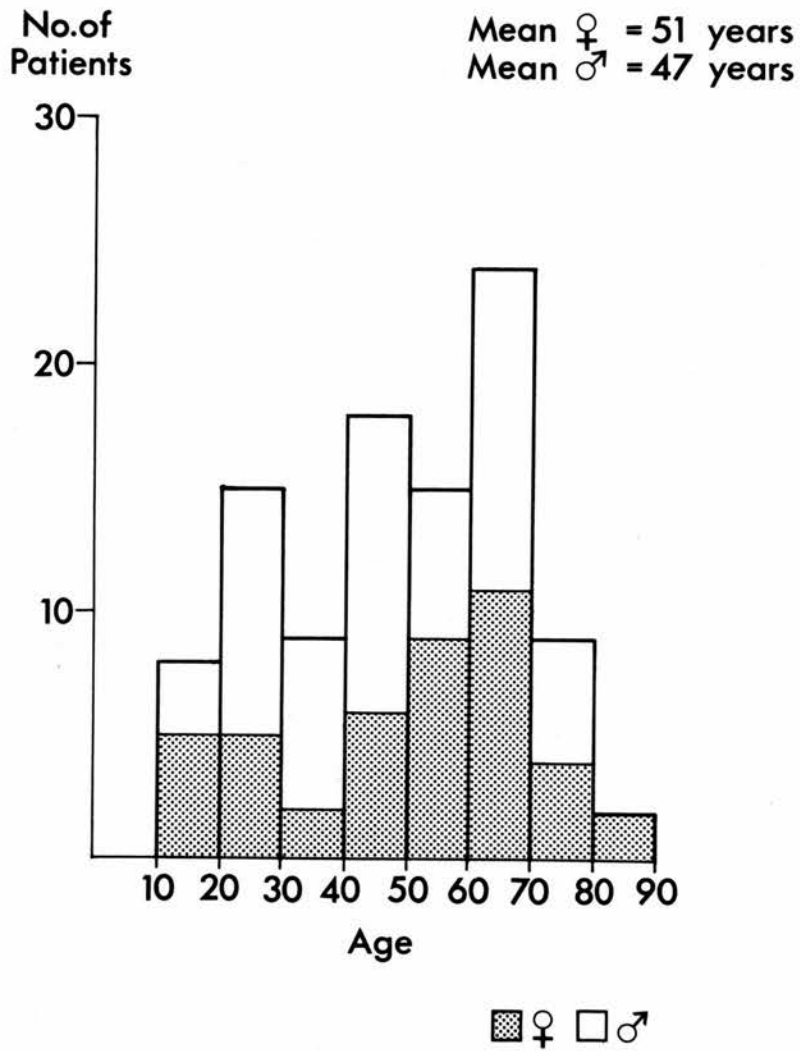


Fig. 77 Age and sex of lymphoma patients

SITES of LYMPHOMA at PRESENTATION

	<u>Number of Patients</u>
Cervical Nodes	61
Axillary Nodes	27
Mediastinal Nodes	20
Groin Nodes	36
Para-Aortic Nodes	37
Liver	23
Marrow	21
Gut	9
Skin	5
Lung	4

Fig. 78 Sites of lymphoma at presentation

noted. The first was a history of multiple infections and this was positive in fourteen patients. The nature of infections were mixed being either bacterial or viral. Three patients presented with Hodgkin's disease simultaneously with high Paul Bunnell titres and a history of contact with infectious mononucleosis. Three patients had had severe herpetic lesions and two farmers were suffering from brucellosis. A third farmer presented with a lump in the groin due to a kick by a cow. He had treated himself with some of the cow's antibiotics and initially the swelling had subsided. He was then kicked in exactly the same spot by another cow and on this occasion the swelling did not subside with antibiotics. The appearance of the groin mass was of antibiotic abscess with sterile pus in the centre. Histologically however it proved to be mixed cell Hodgkin's disease.

The other historical feature of note was that eleven patients out of the group had had previous malignant disease unrelated to lymphoma. Three women had had a breast removed for malignant disease up to 20 years earlier. One 49 year old woman had had a hypernephroma removed in 1949. Two men had excision of colonic lesions 4 and 6 years previously, and one 65 year old lady had had a rectosigmoid tumour removed 4 years prior to presenting with Hodgkin's disease of the skin. Despite going into complete remission from her Hodgkin's disease, this patient went on to die from widespread secondaries from rectal carcinoma 18 months after presentation with Hodgkin's disease. Two elderly men had co-existent carcinomas of prostate and two patients had squamous carcinomas of the skin which were probably of little significance.

There was no clear association of history of previous malignant disease and any particular histological type of lymphoma,

in contrast to a clear association of a history of recurrent infections. Table 79 shows that 12 of the 14 with multiple infections had diffuse non Hodgkin's lymphoma in contrast to 0 out of 10 with non Hodgkin's nodular lymphoma and 2 out of 40 in Hodgkin's disease. Recurrent infections were also associated with advanced disease (Fig.80). Nine of the cases occurred in stage IV and two in stage III.

A similar association with stage was noted with respect to a history of weight loss. Thirty-four patients presented with weight loss and twenty-four of these were in stages III or IV (Fig.81). When histology of this group was examined there was a fairly even split between diffuse non Hodgkin's lymphoma and Hodgkin's disease (Fig.82) although it is interesting to note that 4 out of the 10 nodular lymphomas also had a history of weight loss. Weight loss characteristically has carried a poor prognosis judging by other studies in the literature and indeed this was borne out in the present study from the 5 year actuarial survival figures. There is a statistically significant difference between the group who have a history of weight loss and those who do not, the percentage 5 year survival being 56 against 77 respectively (Fig.83). It is interesting to note that the same figure shows that there is no significant difference in the outcome of patients who complained of sweating or fever or those who had a history of other malignant disease. A history of previous infection however, although the numbers are small, was of considerable prognostic significance. The 5 year survival of this group of 14 patients was 43% compared to 74% for the remainder. Half of the group had weight loss in addition to infections which may bias that result. Of the total, one hundred patients, fifty-nine had

ASSOCIATION of RECURRENT INFECTIONS with HISTOLOGY

<u>Histology</u>	<u>Total Patients</u>	<u>No. \bar{c} Infections</u>	<u>%</u>
Hodgkin's Disease	40	2	5
Diffuse Non Hodgkin's	50	12	24
Nodular Non Hodgkin's	10	0	-

Fig. 79 Number of patients with recurrent infections by histology

ASSOCIATION of RECURRENT INFECTIONS with
STAGE of DISEASE

Stage	Total Patients	No. \bar{c} Infections	%
I	16	0	-
II	22	1	4.5
III	17	2	11.8
IV	38	9	23.7
(I + II) E	7	2	28.5
Total	100	14	14

Fig. 80 Number of patients with recurrent infections by stage of disease

ASSOCIATION of WEIGHT LOSS with STAGE of DISEASE

Stage	Total Patients	No. \bar{c} Weight Loss	%
I	16	2	12.5
II	22	5	22.7
III	17	8	47.1
IV	38	16	42.1
(I + II) E	7	3	42.8
Total	100	34	34

Fig81 Number of patients with weight loss by stage

ASSOCIATION of WEIGHT LOSS with HISTOLOGY

Histology	Total Patients	No. \bar{c} Weight Loss	%
Hodgkin's Disease	40	15	37.5
Diffuse Non Hodgkin's	50	15	30
Nodular Non Hodgkin's	10	4	40
Total	100	34	34

Fig. 82 Number of patients with weight loss by histology

INFLUENCE of HISTORY on 5 YEAR SURVIVAL

	<u>History</u>	<u>5 yr Survival %</u>	<u>Number</u>	<u>Standard Error</u>
Sweating or Fever	Present	68	25	9
	Absent	71	75	5
Weight Loss	Present	56	34	9
	Absent	77	66	5
Previous Cancer	Present	65	11	12
	Absent	71	89	5
Previous Infections	Present	43	14	13
	Absent	74	86	5

Fig. 83 Survival of patients according to history

neither infections nor weight loss.

(b) Histology

The lymphomas as previously discussed are a protean group and much subdivision either by histology or immunological markers or by sites of presentation (nodal versus extra-nodal) has been reported. Taking the simplest of histological classifications, the Luke's classification for Hodgkin's disease and the Rappaport classification for non Hodgkin's lymphoma, there are still 13 subdivisions in any study such as this. It follows therefore that meaningful clinical analysis of the significance of individual sub-types of lymphoma requires large numbers of patients. For this reason a retrospective study of over six hundred patients was carried out by the Departments of Pathology, Radiotherapy and Therapeutics, Edinburgh University, and brief mention will be made of the survival figures later. The incidence of individual sub-types of lymphoma are shown in Fig.84 alongside the equivalent figures for the present prospective study. The figures in brackets for both columns show the number of females in each histology. There is remarkable similarity between the sets of figures with only one apparent difference. The prospective study has a higher number of diffuse lymphocytic lymphomas compared to nodular lymphocytic lymphomas whereas the retrospective study has these in equal numbers. The principal interest of the sex distribution is that only fourteen out of forty patients with Hodgkin's disease are females. There is a striking absence of females in the mixed cell Hodgkin's group also and only nine out of nineteen of the nodular sclerosing Hodgkin's are females. This is in marked contrast to many published series where young females

COMPARISON of HISTOLOGY and SEX of
PROSPECTIVE GROUP with RETROSPECTIVE GROUP

	<u>Prospective %</u>	<u>Retrospective %</u>
<u>HODGKIN'S DISEASE</u>	40 (14)	37 (14)
Lymphocyte predominant	8 (3)	7 (2)
Lymphocyte depleted	2 (1)	1 (1)
Nodular sclerosis	19 (9)	15 (6)
Mixed cell	11 (1)	14 (5)
 <u>NON-HODGKIN'S LYMPHOMA</u>	 60 (30)	 63 (31)
Poorly differentiated diffuse lymphocytic	27 (11)	15 (5)
Diffuse histiocytic	13 (6)	18 (8)
Poorly differentiated nodular lymphocytic	7 (5)	15 (9)
Well differentiated diffuse lymphocytic	4 (2)	4 (2)
Mixed lymphocytic histiocytic	4 (3)	6 (3)
Well differentiated nodular lymphocytic	2 (1)	2 (1)
Intermediate differentiated lymphocytic	2 (1)	0
Undifferentiated	1 (1)	2 (2)
Plasmacytoid differentiation	0	1 (1)

* Females in Brackets

Fig. 84 Percentage of patients in present study by histology and sex compared to retrospective study.

HISTOLOGY of LYMPHOMA PATIENTS by STAGE of DISEASE

<u>HISTOLOGY</u>	<u>STAGE</u>					Total
	I	II	III	IV	(I + II) E	
Hodgkin's Disease (total)	12	14	7	4	3	40
Lymphocyte predominant	6	1	-	-	1	8
Lymphocyte depleted	-	1	-	1	-	2
Nodular sclerosis	5	7	3	2	2	19
Mixed Cell	1	5	4	1	-	11
Non Hodgkin's (total)	4	8	10	34	4	60
Poorly differentiated lymphocytic	2	3	7	20	2	34
Diffuse histiocytic	1	3	3	5	1	13
Well differentiated lymphocytic	1	1	-	4	-	6
Remainder	-	1	-	5	1	7
TOTAL	16	22	17	38	7	100

Fig. 85 Number of patients with differing histology by stage

comprise the majority of the group of nodular sclerosing Hodgkin's disease. Rather than subdivide each aspect of staging and subsequent course for the prospective group of patients, the groups have been clustered together, either as shown in previous tables into three categories, Hodgkin's disease, diffuse non Hodgkin's lymphoma and nodular non Hodgkin's lymphoma, or else the non Hodgkin's lymphomas have been bunched together into poorly differentiated lymphocytic tumours, diffuse histiocytic tumours and the rest. By doing this numbers for each group assume significance and there is some evidence from the retrospective study that the behaviour of the rarer types of non Hodgkin's lymphoma is not very different from the behaviour of the classical lymphocytic lymphomas.

An example of how large numbers can be split into almost meaningless subdivisions is shown in Table 85. This describes the number of patients at each stage and each histological type. There is very little that can be said from the small numbers in each box except that poorly differentiated lymphocytic lymphomas have a marked tendency to be advanced at the time of presentation (7 in stage III and 20 in stage IV out of 34) and nodular sclerosing Hodgkin's disease tend to be early (5 in stage I and 7 in stage II out of 19). Discussion of the methods of staging will follow below; the present table however gives an illustration of a possible reason for the superior survival of patients who have Hodgkin's disease compared to those with non Hodgkin's lymphoma. In this unselected cohort 60% of patients with Hodgkin's disease presented early (stages I and II) and 73% of patients with non Hodgkin's lymphoma had advanced disease (stages III or IV),

when they consulted their doctor. This provides valuable information about the natural history of the disease and justifies subdivision histologically at least into the two large categories.

(c) Staging Procedures

The terms "clinical" and "pathological" are used to describe the stage of lymphoma according to two different criteria. The clinical stage is arrived at after the physician has examined the patient, carried out a chest x-ray and routine blood analysis such as full blood count and liver function tests. The pathological stage is achieved after an attempt has been made to find disease in other areas and wherever possible to biopsy it and prove its presence by histology. The relevance of considering clinical stages at all in the context of this thesis is that comparisons can be made of results of the present prospective study with the retrospective study of patients treated between 1958 and 1972. During that 15 year period no other investigations were carried out for patients other than those described under the term "clinical" above. Treatment was therefore geared to the sites of disease which had been visualised by those rather limited methods. It is quite clear from the results of staging laparotomies for instance that several spleens which are impalpable to the clinician's hand were involved with tumour and conversely occasionally a palpable spleen was found to contain reactive tissue only. Further, liver function tests are of little value in predicting whether the liver is involved with lymphoma tissue or not. Radioisotope scans are of limited additional use unless there are large deposits of tumour greater than 2 cm across. Lymphoma tends to involve organs such as liver diffusely and therefore no abnormality is seen on the radioisotope scan.

The techniques which were used routinely with the present group of patients were lymphography, marrow biopsy, liver biopsy and staging laparotomy including splenectomy, wedge biopsy of both lobes of liver and sampling of all the abdominal lymph node groups. The progression through these techniques is described in the method section. Comparison between the numbers of patients in each stage according to the label "clinical" and "pathological" is shown in Fig. 86. It is clear that the majority of patients are in stages I and II by clinical judgement yet are converted into stages III and IV when further staging techniques have been applied. Figure 87 shows the individual progression from each clinical stage to each pathological stage. The only accurate staged clinical group are the extra-nodal group though one of the seven patients did not undergo staging laparotomy or biopsy. This was the patient who presented with spinal cord compression. He completely recovered after surgical decompression and after radiation to the back declined any further investigation or treatment. The most notable point arising from this figure is that not a single patient was over-staged clinically. Either the patient had been accurately staged clinically or else the stage increased. A total of forty-one patients were under-staged clinically and moved into a higher stage after one of the various techniques. The most dramatic shift was from stage III into stage IV, 17 out of 26 patients being wrongly labelled. Table 88 shows which techniques were most useful for detecting hidden disease. It can be seen for instance that 12 patients moved up a stage after lymphography, 9 after liver biopsy, 19 after marrow biopsy and surprisingly only 2 after splenectomy. One of these last patients had a positive lymphogram and cervical nodes rendering him stage III, and the spleen was the

COMPARISON of CLINICAL and
PATHOLOGICAL STAGES

<u>Stage</u>	<u>Clinical</u> (no. of patients)	<u>Pathological</u>
I	25	16
II	32	22
III	26	17
IV	10	38
IE & IIE	7	7

Fig. 86 Number of patients by clinical compared to pathological stage

CHANGE of PATIENTS' STAGING from
CLINICAL to PATHOLOGICAL

	<u>CLINICAL</u>		<u>PATHOLOGICAL</u>				<u>Total</u>
		I & II E	I	II	III	IV	
Stage I	-		16	5	2	2	25
II	-		-	17	6	9	32
III ^c	-		-	-	9	17	26
IV	-		-	-	-	10	10
I & II E	7		-	-	-	-	7
Total		7	16	22	17	38	100

Fig. 87 Number of patients who changed stage after pathological examination



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only positive organ biopsied in the abdomen apart from the para-aortic lymph node and therefore the patient only changed stage in as much as he went from III spleen negative to III spleen positive. This did not result in a change of treatment policy as the patient went on to receive total nodal irradiation. The histology of each group is shown and the outstanding feature is that non Hodgkin's lymphoma was present in 18 of the 19 marrow biopsies when there was no sign of it in any other organ than lymph nodes. Marrow biopsy using a Jamshidi needle is remarkably simple and free of morbidity. In addition, as just discussed, it produces a high yield of positive results in the non Hodgkin's lymphoma group and it should be considered an absolute prerequisite of management of this group of diseases. Its value in Hodgkin's disease is less certain in that spread to bone marrow tends to occur later and advanced disease is much more likely to be characterised by liver or lung lesions than marrow lesions. There are only 4 stage IV Hodgkin's patients in the present series and this is really too small a group for analysis. Lymphography was carried out on almost all patients in the series of 100. It was associated with morbidity in 10% consisting principally of pain at the injection site on the dorsum of the feet, reaction to the dye in one patient and dyspnoea in two patients. The technique was unsatisfactory due to rupture of lymphatics in two patients. In the majority of patients the dye remained in the para-aortic lymph nodes for greater than 9 months allowing monthly follow-up abdominal films to be taken without any added unpleasantness to the patient. The accuracy of lymphography confirmed by staging laparotomy was 95% in this series with only one false positive result and no false negative results.

In the discussion of liver biopsy one must separate two

EVALUATION of TECHNIQUES AGAINST CHANGE of STAGE

<u>No. of Patients Changing Stage</u>	<u>Technique</u>	<u>Histology</u>		
12	Lymphogram	Hodgkins	6	(NSH 2) (MCH 3) (LDH 1)
		Non Hodgkins Lymphoma	6	(PNLL 2) (PDLL 2) (DH 2)
9	Liver Biopsy (2 open)	Hodgkins	2	(MCH 1) (LDH 1)
		NHL	7	(PNLL 1) (PDLL 2) (DH 1) (IL 1) (ML 2)
2	Splenectomy	Hodgkins	2	(MCH 1) (LDH 1)
19	Marrow Biopsy	Hodgkins	1	(NSH 1)
		NHL	18	(PNLL 3) (PDLL 9) (WDLL 3) (DH 2) (IL 1)
TOTAL = 42				

Fig. 88 Number of patients who changed stage by staging techniques and by histology

techniques, wedge biopsy of liver which is part of the staging laparotomy and closed needle biopsy. Two patients who were converted into stage IV by open liver biopsy each had Hodgkin's disease and this formed part of the staging laparotomy. In the remaining 7 the diagnosis of non Hodgkin's lymphoma was confirmed in the liver by a needle biopsy. Whereas this is a relatively safe procedure in patients who have not had any treatment, it is considerably more hazardous if a patient has been on cytotoxic drugs. When the staging techniques therefore were repeated after completion of chemotherapy liver biopsies were usually carried out under direct vision via a laparoscope. This was done under general anaesthetic and should be carried out on day patients without any risk apart from that of the anaesthetic.

A staging laparotomy has been made more controversial by the reported incidence of complications related to splenectomy. Numerous studies report increased susceptibility to infection and increased morbidity after this operation. In children under the age of 6 it is rarely carried out because there is a 10% risk of mortality according to some series. There is unfortunately no other way to biopsy the spleen successfully other than splenectomy and subsequent examination as described earlier. It is of considerable interest therefore that splenectomy only altered the management in one patient out of 34 from a group of 100 consecutive lymphomas. This patient had a positive spleen with no other disease below the diaphragm. He had cervical nodes above the diaphragm and therefore would have been stage I; with positive spleen he was converted to stage III. This implied a poorer prognosis group and in addition to radiotherapy to the nodes above the diaphragm he went on to receive prophylactic radiation to the para-aortic nodes below the diaphragm.

In the present series, it must be said that splenectomy did not cause additional morbidity. The group of thirty-four patients spent a mean of 7 days in hospital; eight patients had minor post operative complications, either wound infections or transient pneumonic episodes; one patient developed a deep venous thrombosis in association with a very high platelet count of over one million per cmm. The remaining patients developed a mean platelet count of 500,000 per cmm by day 7 after operation. There were no unusual septicaemic episodes and the average delay to commencement of therapy was of the order of 2 weeks. There were no consistent alterations of immunoglobulin levels or antibody levels in the patients following splenectomy and their survival was not different from the patients who did not have splenectomy.

A single patient clearly had an inadequate staging laparotomy. A splenectomy was carried out successfully and has proved negative. Liver biopsy was negative. No coeliac axis or splenic lymph nodes were obtained. Samples which the surgeon thought were nodes turned out to be pieces of fat. The lymphogram in this patient was negative and she had a mantle radiation field for her stage II nodular sclerosing Hodgkin's disease. She relapsed very quickly after treatment developing a mass arising from coeliac axis nodes. There was one other practical problem which theoretically might have been of significance. A young male with stage II nodular sclerosing Hodgkin's disease had an apparently complete staging laparotomy and presented 2 years after achieving complete remission by radiotherapy with an episode of jaundice. On investigation, liver function tests were abnormal suggesting hepatitis but an isotope liver scan was carried out in any case. This showed that the patient had a normal liver but in addition he appeared to have uptake of radioisotope

over the area of the spleen. This was clearly defined and was later shown at laparoscopy to be due to a splenunculus which presumably had grown after splenectomy and might theoretically have contained Hodgkin's tissue. The patient's episode of hepatitis settled down, the aetiology was never shown and he remains in complete remission.

In an attempt to highlight the coeliac axis other than at laparotomy a group of twenty patients were given an injection of gallium preceding the lipiodol dye in their lymphogram. Gallium 67 given intravenously is excreted very quickly by liver into the gut. The gut uptake tends to obscure the area of interest, that is the coeliac axis nodes, and therefore it was felt if gallium were infused by a different route it might highlight the target area. The lipiodil in a normal lymphogram only outlines lymph nodes to the level of the upper border of the third lumbar vertebrae. Accordingly a 20 ml sample of the patient's blood was taken at the time of cannulation of the lymphatics for lymphography and after sedimentation of cells, the plasma protein fraction was incubated in sterile conditions with a diagnostic dose of gallium. Ten ml of this mixture was then infused slowly up the lymphatic cannulae prior to the lipiodil.

In only one patient was a positive result obtained. This patient went on to develop obstructive jaundice due to porta hepatitis lymphomatous nodes obstructing the common bile duct. Another patient however did show disease outside the abdomen after infusion of gallium. This patient presented with groin nodes and after the usual staging procedures was due to undergo abdominal lymph node irradiation for limited disease non Hodgkin's lymphoma. The gallium scan highlighted two hot areas in the nasopharynx which had been overlooked at the first ENT opinion. Biopsy of tissue

in this area showed the same histology as the first node sample from the groin. This patient then had local radiotherapy to the nasopharynx and remains in complete remission.

Laboratory Investigations

A series of parameters associated with lymphoma were measured from peripheral blood samples taken from each patient before treatment, at 2 months after the start of treatment, finally 6 to 9 months after the start of treatment or before death, where appropriate. In effect therefore the second sample reflected the immediate post radiotherapy changes and the second review sample reflected changes due to radiotherapy plus chemotherapy or chemotherapy alone. Also at 2 months, remission was assessed clinically and 51% of patients were in remission at that time irrespective of treatment. Changes in parameters therefore have been assessed for remission status at that time. The total mortality in the period of study was 34%. It has been possible to look retrospectively at the levels of various parameters for their value in prediction of eventual outcome, with relation to mortality and in the same light it has been possible to investigate the degree of change of each parameter between the pre-treatment period and the review periods.

The median and means of the haematological parameters, serum proteins, immunoglobulins, Escherichia coli antibody titres and α_2 macroglobulin are shown in Fig. 89. The range in each parameter is extremely wide for instance there were two patients with paraproteins of an IgM nature. The absolute levels at presentation were 1,800 mg% and 3,600 mg%. This accounts for the marked difference between the median and the mean for this immunoglobulin. Similarly one patient presented with an IgG paraprotein band of 2,075 mg% which skewed

PRE TREATMENT PARAMETERS of TOTAL GROUP

<u>Parameter</u>	<u>Range</u>	<u>Median</u>	<u>Mean</u>
Hb	6 - 17 G	12.5	12.5
WBC	2 - 43 x 10 ³ /cm	6.5	8.6
Neutrophils	1 - 22 x 10 ³ /cm	4	5.6
Lymphocytes	1 - 43 x 10 ³ /cm	1	2.8
Platelets	2 - 76 x 10 ⁴ /cm	21	24.4
ESR	3 - 149 mm/1st hr	29	41
Albumin	23 - 46 G/l	37	37
Globulin	16 - 50 G/l	30	30
IgG	231 - 2075 mg%	896	952
IgA	51 - 846 mg%	158	204
IgM	23 - 3600 mg%	92	167
E. coli. inverse titre	4 - 512	32	140
E. coli. (M.E.) inverse titre	4 - 512	8	36
α_2 Macroglobulin	166 - 624 mg%	317	338

Fig. 89 Range, median and mean of pre-treatment parameters of one hundred lymphoma patients

the mean for IgG. It can be seen that the median *Escherichia coli* inverse titre is 32 which is considerably lower than the median of the normal group previously mentioned (128). The methanol insensitive IgM antibody was not altered compared to the mean of the control group. Because of the age dependency of α_2 macroglobulin it is not possible to comment on the median or mean for the total group of one hundred patients but it is obvious that when age and sex are taken into consideration there is a considerable scatter of α_2 macroglobulin results and a significant number of patients had depressed levels. The clearest way of indicating the numbers of patients who had abnormal parameters is to define the lower limit of abnormality for each parameter and these results are shown in Fig. 90. It can be seen from the first column of this table that there is a significant percentage of patients who are abnormal for each parameter. The remaining two columns show the same figure for the first and second reviews. Thus the number of patients who have depressed white cell counts is almost trebled between time 0 and 6 to 9 months. This almost certainly is the result of chemotherapy and radiotherapy. Similarly there is a trend of depression of IgG, IgA and IgM. The α_2 macroglobulin on the other hand goes in the opposite direction with less patients at the end of treatment showing a low level which is arbitrarily taken as 325 mg%.

The stage of disease and histology were both related to abnormalities in haematology and serum protein (Fig. 91 and 92). For instance, no patient had a low white cell count in stage I (Fig. 91). Surprisingly however, there were patients who had depression of immunoglobulins in association with stage I disease and half of the group had low α_2 macroglobulin levels. The levels of IgG, IgA and α_2 macroglobulins were not significantly different

PERCENTAGE PATIENTS with ABNORMAL PARAMETERS THROUGHOUT TREATMENT

<u>Parameter</u>	<u>Pre Treatment (%)</u>	<u>First Review (%)</u>	<u>Second Review (%)</u>	
WBC	< 4000 /cmm	13	28	30
Neut	< 3000 /cmm	25	28	39
Lymphs	< 2000 /cmm	67	66	84
Albumin	< 35 G/l	32	46	44
Globulin	< 30 G/l	49	50	66
IgG	< 700 mg%	28	38	43
IgA	< 100 mg%	22	32	32
IgM	< 70 mg%	43	53	52
E. coli. titre	< 1 : 40	35	56	53
ME E. coli. titre	< 1 : 8	46	67	73
α_2 M	< 325 mg%	50	46	38

Fig.90 Percentage of patients who had abnormal parameters before treatment and at review

ASSOCIATION of ABNORMAL PARAMETERS with STAGE of DISEASE

Percentage of Patients with Abnormally Low Parameters

<u>Stage</u>	<u>WBC</u>	<u>Neut</u>	<u>Lymphs</u>	<u>IgG</u>	<u>IgA</u>	<u>IgM</u>	<u>E. coli</u>	<u>α_2M</u>
I	-	19	63	33	26	13	19	50
II	11	16	79	25	6	33	16	40
III	13	19	81	31	18	44	25	50
IV	19	38	65	29	19	69	54	57

Fig. 91 Percentage of patients with low parameters by stage.

RELATIONSHIP of HISTOLOGY to ABNORMAL PARAMETERS

Histology	Percentage Patients with Abnormal (low) Parameters										
	WBC	Neut.	Lymph.	Alb.	Glob.	IgG	IgA	IgM	E. coli	ME	α_2M
Hodgkin L.P.	00	25	50	13	00	25	25	13	25	25	25
LD + MC	8	16	85	31	16	00	16	46	31	46	16
NS	5	10	68	32	11	05	21	32	5	47	21
Non Hodgkin's											
D.H.	15	23	69	54	31	00	08	62	31	69	23
P.L.	21	44	65	26	21	12	29	68	41	35	15
WL, IL, M	17	25	66	42	42	00	17	33	50	25	17

Fig. 92 Percentage of patients with low parameters by histology

between stages I and IV. In contrast IgM and Escherichia coli antibody titres were progressively more abnormal as stage of disease increased from 1 to 4. The relationship of histology to abnormal parameters (Fig.92) shows that as a generality, patients with non Hodgkin's lymphoma had more chance of having abnormal white cell and neutrophil counts, depression of globulin levels, IgM and Escherichia coli antibody. Lymphopenia IgG, IgA and α_2 macroglobulin levels were not related to histology. The actual means of immunoglobulin and α_2 macroglobulin levels are shown in Fig.93 for each stage. This reiterates the point that IgM is the only globulin which is clearly related to stage, thus as more tumour is present, the IgM falls.

It would be reasonable to expect that cytotoxic drugs and radiation treatment would independently have an immunosuppressive effect. To separate this effect from the effect of advancing disease or else remitting disease the percentage change in immunoglobulins and α_2 macroglobulin levels were calculated in each patient who subsequently went into remission and in each patient who subsequently died. The percentage was calculated by comparing the first value for each parameter with the third which was either immediately before death or at 6 to 9 months. The figures are plotted with the means of each group in Fig.94 for the patients who are in remission and each immunoglobulin would seem to be depressed. There is no significant difference between the means of IgG and IgA and IgM.

In striking contrast there is no change in the mean of α_2 macroglobulin level that is to say, the α_2 macroglobulin did not change with treatment or time in patients who went into remission. When the same graph is examined for deceased patients exactly the

**ASSOCIATION of IMMUNOGLOBULINS and
 α_2 MACROGLOBULIN with STAGE of DISEASE**

<u>Stage</u>	<u>IgG</u>	<u>IgA</u>	<u>IgM</u>	<u>α_2M</u>
I	857	214	169	358
II	1044	187	151	346
III	1033	229	101	322
IV	901	204	90	330

Fig. 93 Mean immunoglobulin and α_2 macroglobulin levels by stage

% CHANGE in PARAMETER
of PATIENTS in REMISSION

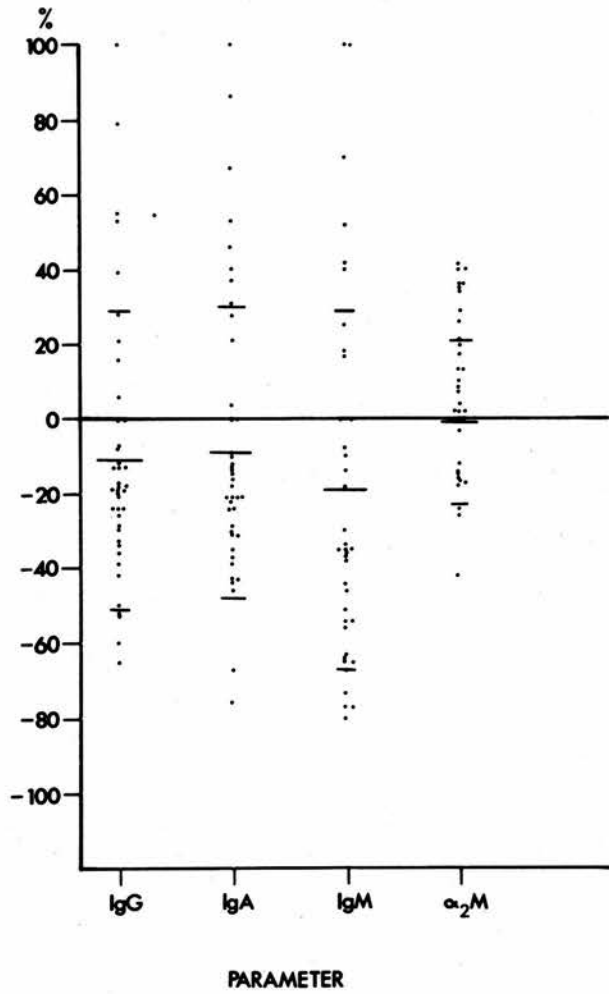


Fig. 94 Percentage change in immunoglobulins and α_2 macroglobulin through treatment for patients in remission

% CHANGE in PARAMETERS
of DECEASED PATIENTS

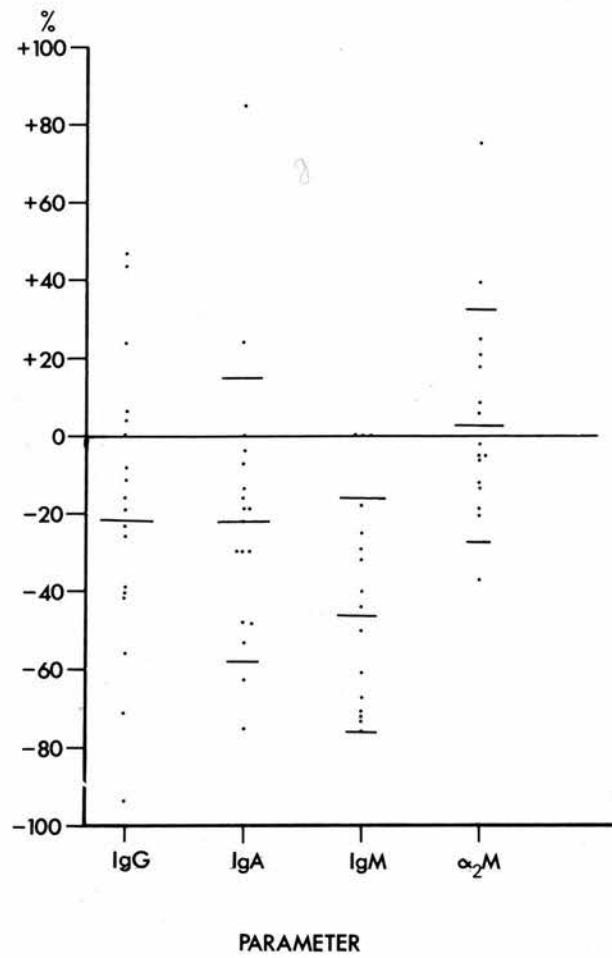


Fig. 95 Percentage change in immunoglobulins and α_2 macroglobulin through treatment for patients who died.

same can be said for α_2 macroglobulin (Fig.95). The depression of IgG and IgM would appear to be slightly more convincing in the deceased group but it is not nearly so dramatic as the fall in IgM. This is significant to the value of $p < 0.001$. Of the four proteins studied therefore, a change only in IgM gives any clear guidance as to eventual outcome for the patient. Depression of between 10 and 20% of levels of IgA and IgG can be expected due presumably to the effects of treatment but no prognostic significance can be attached to these two immunoglobulins. Alpha 2 macroglobulin levels seem remarkably stable throughout the course of each patient's illness. This is of considerable importance when it comes to the prediction of outcome from first pretreatment samples of immunoglobulins, antibodies and α_2 macroglobulin.

The means of these figures for the group of patients who eventually achieved remission and stayed in remission compared to those patients who died while under study is shown in Fig.96. It can be seen that the only pretreatment parameters of any value in predicting the eventual outcome for the patient, are albumin, IgM and α_2 macroglobulin. The low level of IgM in the deceased group might be related to the depression of IgM with progression of disease from stage I to stage IV as discussed previously in Fig.95. The figure for α_2 macroglobulin however is not related to stage or histology (see Fig.92 and 93) and just as discussed, does not alter with treatment. This therefore becomes a particularly vital difference in α_2 levels which will indicate with some degree of certainty at the time of presentation whether the patient has a good prognosis or not. The 5 year survival figures for patients with low IgM and α_2 macroglobulin levels were 59% and 57% (Fig.97 & 98)

VALUE of INDIVIDUAL PRE-TREATMENT PARAMETERS in
PREDICTION of OUTCOME

<u>Parameter</u>	<u>Remission</u> (mean of group)	<u>Dead</u> (mean of group)	<u>Significance</u>
Haemoglobin G/100 ml	13.0	12.1	N. S.
White cell count, /c. mm	7,700	9,600	N. S.
Neutrophil count, /c. mm	5,800	4,950	N. S.
Lymphocyte count, /c. mm	1.5	1.4	N. S.
Monocyte count, /c. mm	1,150	1,000	N. S.
Platelet count, /c. mm	245,000	222,000	N. S.
E. S. R. , mm/hour	36	49	N. S.
Albumin, G/l	37.5	33.8	P = 0.02
Globulin, G/l	31.1	28.5	N. S.
IgG mg%	973	953	N. S.
IgA mg%	201	233	N. S.
IgM mg%	145	106	P = 0.01
E. coli, inverse titre	153	125	N. S.
E. coli (ME), inverse titre	40	22	N. S.
α 2 macroglobulin, mg%	353	294	P = 0.01

Fig. 96 Mean of each parameter in groups of patients who remitted or died

ACTUARIAL SURVIVAL of LYMPHOMA PATIENTS
by INITIAL I.G.M. LEVEL

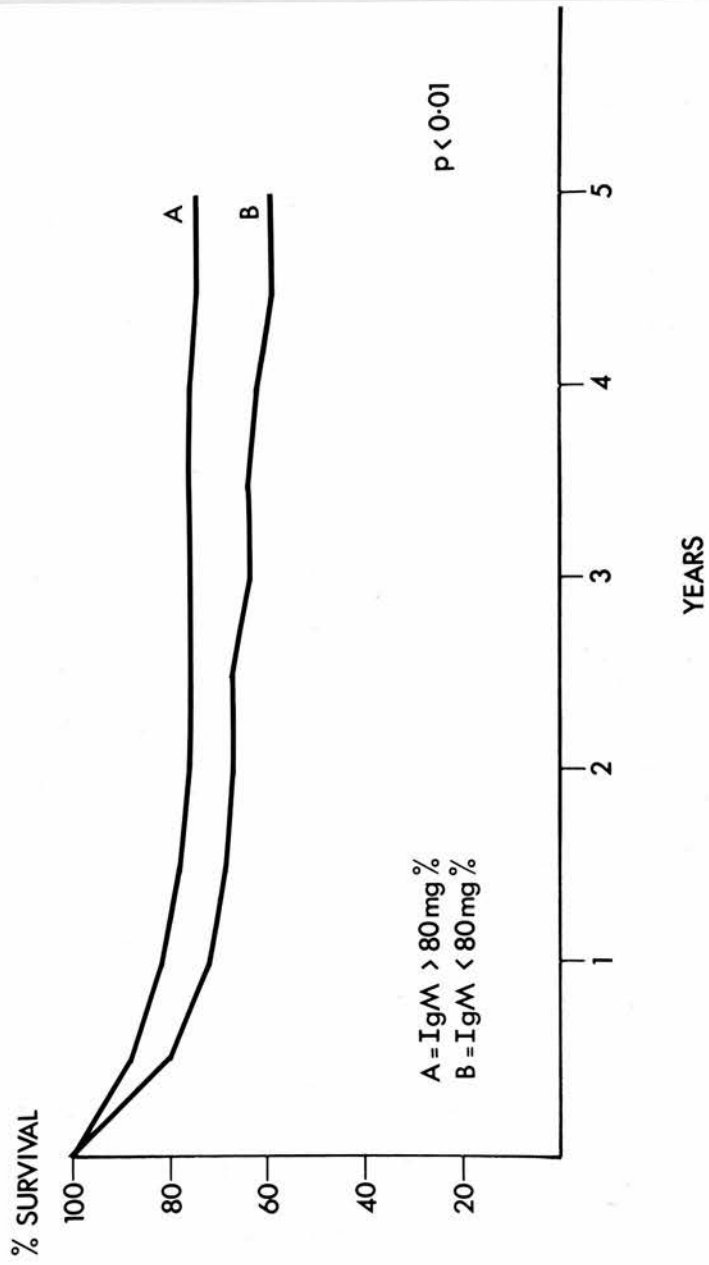


Fig. 97 Survival by IgM level

ACTUARIAL SURVIVAL of LYMPHOMA PATIENTS
by INITIAL ALPHA 2 MACROGLOBULIN LEVEL

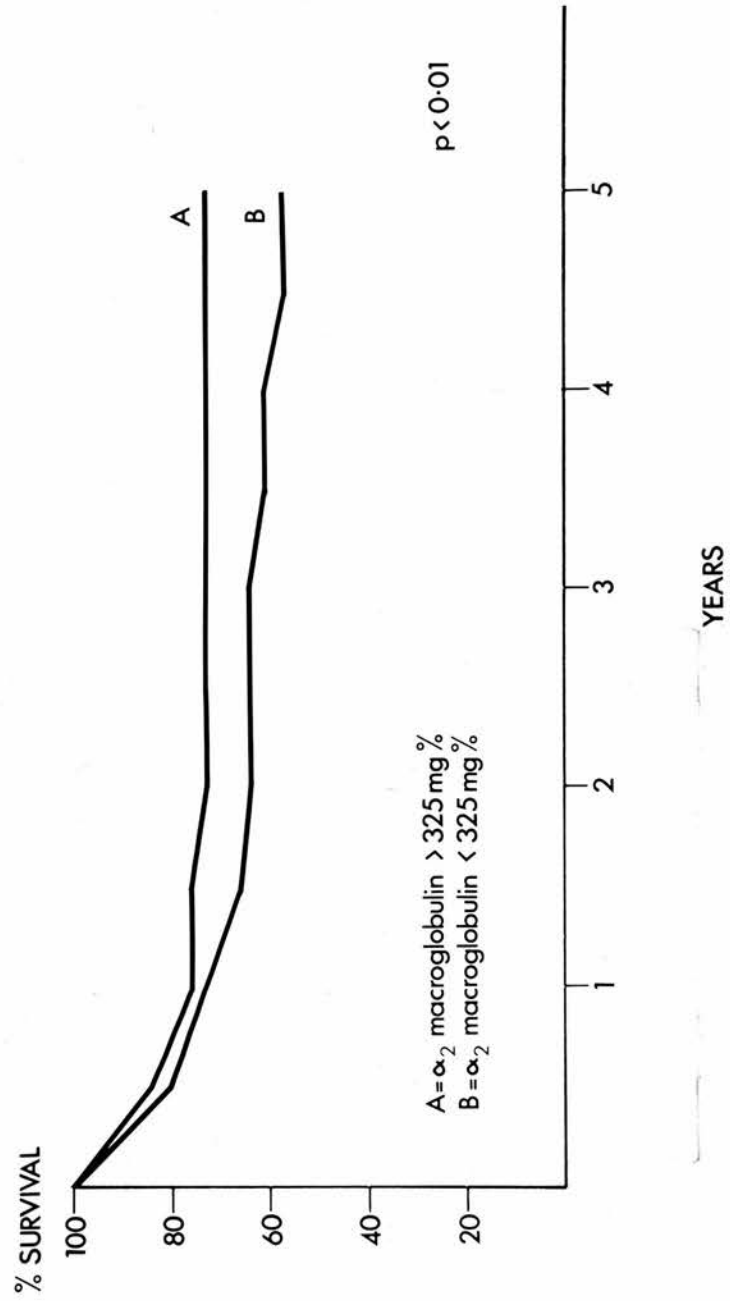


Fig. 98 Survival by α_2 macroglobulin level

respectively compared to the figures for the groups with normal levels, 74% and 70%. Both differences are significant ($P < 0.01$).

It might have been expected that haematological parameters would be of more value in this sense in that patients with marrow involved with tumour might not tolerate chemotherapy well and might have added complications in addition to having advanced disease. Whether marrow involvement predisposes to complications or not it clearly has no adverse effect on survival. Similarly of interest, the height of the ESR so long relied on as a monitor of the degree of illness is unreliable in this context. The figures for the immunoglobulins and α_2 macroglobulin are repeated in Fig. 99 along with the equivalent means of each group when studied at the two review points. The downward trend of IgG and IgA are again seen irrespective of disease state and the more dramatic fall in IgM is seen to be between the pretreatment samples and the first review. In contrast the *Escherichia coli* antibody continues to decline gradually throughout the entire period of review. The clear statistical difference between the α_2 macroglobulin levels for the good prognosis group and the bad prognosis group is maintained at each time of sampling.

Albumin and Globulin Levels

It is seen from Fig. 96 that the third parameter which predicted outcome for the patient was the pretreatment serum albumin. Protein levels have been compared with the stage of disease (Fig. 100). Although the majority of the group of 32 who had hypoalbuminaemia at presentation were in stages III and IV, it is interesting to speculate on the mechanism of this abnormality in seven patients with stages I and II disease and four with stages I and II

ALTERATIONS of PARAMETERS with TIME and EVENTUAL OUTCOME

Eventual Outcome	Pre-Treatment		First Review		Second Review	
	Remission	Died	Remission	Died	Remission	Died
<u>Parameter</u>						
IgG (mg%)	973	953	842	755	758	775
IgA (mg%)	201	233	168	174	143	159
IgM (mg%)	145	106	84	70	84	90
E. coli antibody (inverse titre)	153	125	115	91	74	73
ME E. coli antibody (inverse titre)	40	22	16	29	8	29
α_2M (mg%)	353	294	370	299	376	311

Fig. 99 Mean of several parameters pre-treatment and at each review according to remission status

extra-nodal disease. The serum albumin of course falls in liver disease, but only in 31% of the present group was the liver involved with lymphoma. There is an obvious association of hypoalbuminaemia as in malnutrition with loss of weight and as has been previously discussed, weight loss was a feature in a third of the patients with lymphoma. Of the group of patients who had hypoalbuminaemia only eighteen (56%) had concurrent weight loss (Fig. 101). This is important because for the sixteen patients who had hypoalbuminaemia and no weight loss the low albumin became an independent factor which was associated with a poor prognosis. It was discussed earlier (page 135) that half of the patients with weight loss also had a history of recurrent infections. In this group of fourteen patients who had infections before their presentation with lymphoma, obvious alterations of the albumin level or the globulin level were present in less than half.

The age and sex of patients presenting with hypoalbuminaemia is shown in Fig. 102. There is no significant difference between the mean ages of the sexes and although the means are high there are five patients who have hypoalbuminaemia and whose age is thirty or less. The alteration of albumin and globulin levels with time was plotted in graphic form in Fig. 103 and 104 respectively. The actual levels of protein are given for the pre-treatment sample and the second review sample. Patients were divided into three pairs of columns according to whether they had disease present but were alive, whether they were in remission or whether they went on to die. The albumin levels for the patients in remission at both sampling points tend to be in the normal range, indeed at second review there were only four patients who fell below the lower limit of normal. The figures for the group who were alive

ASSOCIATION of PROTEIN LEVELS with STAGE of DISEASE

	<u>STAGE</u>					
	<u>I</u>	<u>II</u>	<u>III</u>	<u>IV</u>	<u>(I + II) E</u>	<u>Total</u>
Albumin > 35 G/l	14	17	10	24	3	68
Albumin < 35 G/l	2	5	7	14	4	32
Globulin > 30 G/l	10	12	11	14	4	51
Globulin < 30 G/l	6	10	6	24	3	49

Fig. 100 Number of patients of each stage by pre-treatment levels of albumin and globulin

ASSOCIATION of WEIGHT LOSS with SERUM PROTEINS

<u>Protein</u>	<u>Total Patients</u>	<u>No. \bar{c} Weight Loss</u>	<u>%</u>
Albumin > 35 G/l	68	16	23.5
Albumin < 35 G/l	32	18	56.3
Globulin > 30 G/l	51	21	41.2
Globulin < 30 G/l	49	13	26.5

Fig. 101 Number of patients with weight loss by pre-treatment levels of albumin and globulin

**AGE and SEX of PATIENTS
with LOW ALBUMIN**

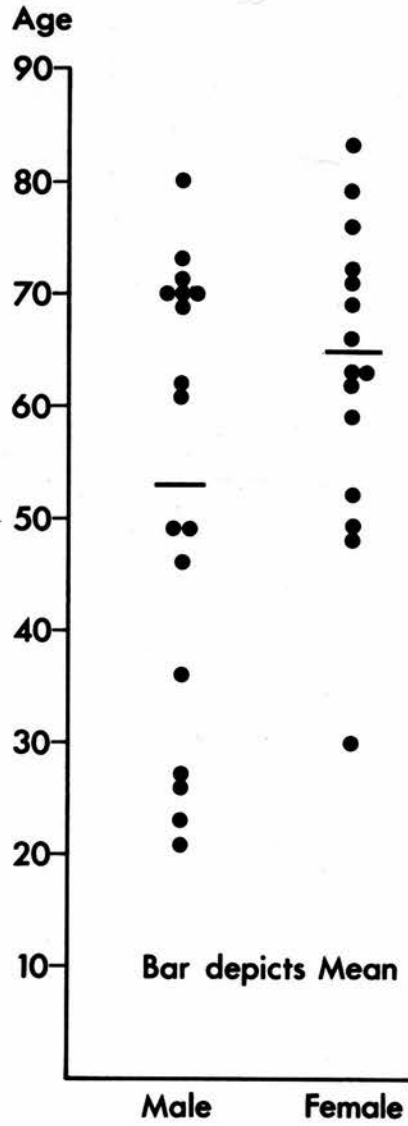


Fig. 102 Age and sex of patients with hypoalbuminaemia

Initial and Second Review Albumin Levels
against Disease Status

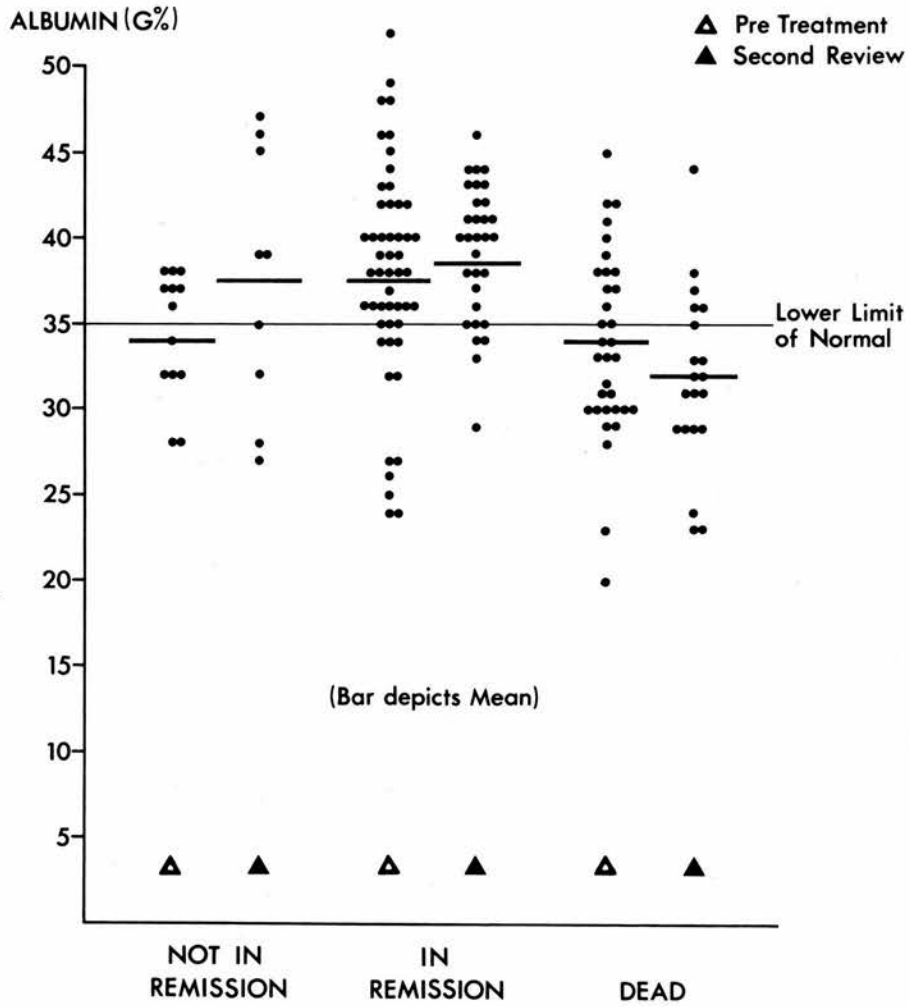


Fig. 103 Level of albumin before and after treatment by disease status

Initial and Second Review Globulin Levels
against Disease Status

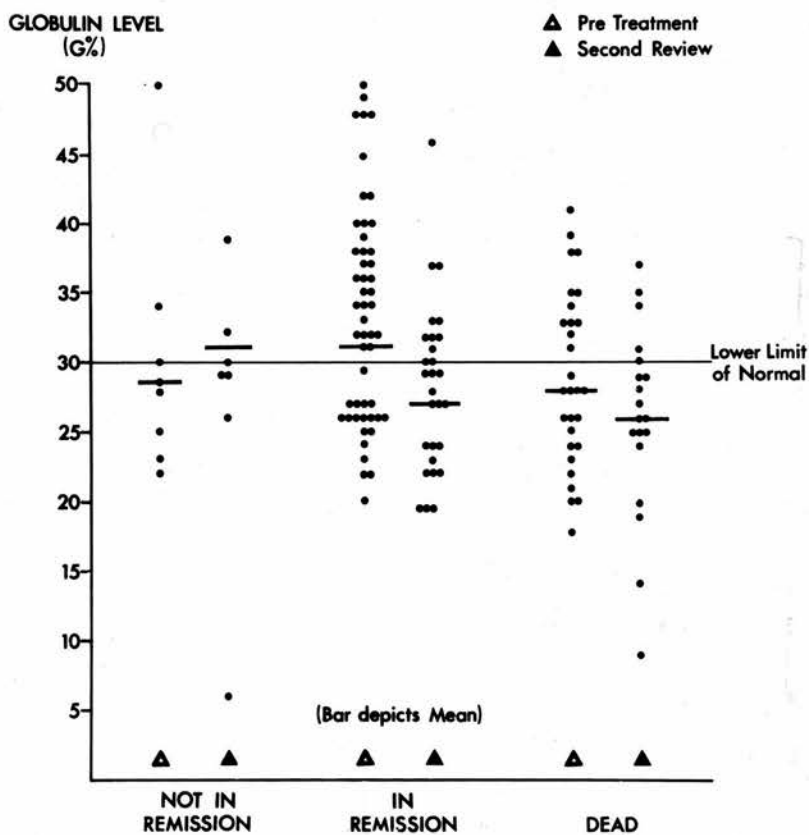


Fig. 104 Level of globulin before and after treatment by disease status

and not yet in remission are rather small but there seems to be a trend for the mean to rise from the pre-treatment level to the time of second review. There is a statistical difference between the remission group and the deceased group at both pre-treatment and second review. The equivalent graph for globulin levels (Fig. 104) indicates that the mean globulin level falls with treatment despite the clinical outcome. The only apparent difference is in the pre-treatment samples of the remission group and of the deceased group but this is not of significance.

The actuarial 5 year survival figures were obtained for four groups of patients (Fig. 105). The hypoalbuminaemia cohort had a significantly poorer survival rate, 56% compared to 76% with the patients with normal albumin levels. There are small standard errors in the groups and the P value is less than 0.01. The albumin level at presentation therefore seems an important measurement compared to the globulin level and a rise in serum albumin with treatment would indicate an improved prognosis whereas a drop from a sub-normal level of albumin indicates a dismal outlook.

Inter-relationship of parameters

All the parameters described above were studied for their inter-relationship by calculating the Pearson and the Spearman correlation co-efficients. Serum albumin was independent of any other parameter as was α_2 macroglobulin. The lymphocyte count was related to the total globulin level ($p = 0.002$) and total globulin was related to the IgM level ($p = 0.002$) and IgG ($p = 0.012$). Levels of IgG and IgA correlated and this bears out their similar trends described above ($p = 0.001$). There was a correlation between IgG and Escherichia coli IgG antibody titres and the Escherichia coli

INFLUENCE of SERUM PROTEIN LEVELS on
5 YEAR SURVIVAL

<u>Protein</u>	<u>5 yr Survival %</u>	<u>Number</u>	<u>Standard Error</u>
Albumin < 35 G/l	56	32	9
Albumin > 35 G/l	76	68	5
Globulin < 30 G/l	63	49	7
Globulin > 30 G/l	76	51	6

Fig. 105 5 year survival rates by initial protein levels

IgG antibody correlated as predicted from the normal distribution curves with the methanol sensitive Escherichia coli IgM antibody ($p = 0.001$). Pre-treatment levels of IgM, albumin and α_2 macroglobulin independently predicted survival for patients. As treatment progressed α_2 macroglobulin was of no further value, but a fall in albumin and/or a decrease in IgM accurately predicted ultimate failure of therapy. Of all parameters studied two factors were of use in prediction of remission status. The older a patient and the less his IgM level, the less was the chance of complete response to treatment. Low levels of albumin, IgA, α_2 macroglobulin or Escherichia coli IgM antibody were of less value independently in this respect though taken together, they too pinpointed with similar accuracy the patients least likely to succeed in terms of total response. Using standardised discriminant function coefficients the above group of features, or the first (age and IgM level) predicted with an accuracy of 75% eventual disease status.

(d) Treatment

The modern management of lymphoma depends critically on the stage of disease in individual patients. A limited field of radiotherapy was given to forty-seven patients in the present study, all of whom were staged I or II. Four patients who had stage III disease had total nodal irradiation. The remaining patients had chemotherapy either because of advanced pathological stage (III or IV), because of clinical suggestion of relapse after or during radiotherapy, or on several occasions as an adjuvant form of treatment in patients with stage I or II disease with poor prognosis histology. Thus twenty-three patients were treated with chlorambucil and prednisolone, thirty-nine patients with

cyclophosphamide, vincristine, prednisolone (five patients had adriamycin also) and twenty patients received mustine, vinblastine, procarbazine and prednisolone.

The clinical results of treatment are seen in Fig. 106 where the percentage of sites of involvement at each review are seen. The majority of patients who presented with disease involving lymph nodes have undergone remission of that disease. The least sensitive group of nodes would appear to be the para-aortic nodes where twenty out of thirty-seven patients had gone into remission by the first review and only one more by the second review. All but nine out of thirty-six patients with groin nodes had achieved remission by the first review but by the time of the second review eight patients had relapsed in that site. The results of treatment of liver and skin disease are far from encouraging and only a third of patients who had marrow involvement had negative bone marrow biopsies at the time of final review. These patients continued on some form of chemotherapy thereafter, but no further comment will be made about their eventual disease status. However survival has been documented and will be described according to first treatment option which is either radiotherapy, chemotherapy or a combination of both. A breakdown of each group into sub-divisions according to subsequent treatment is meaningless, due to the very small numbers involved. Crude survival is shown in Fig. 107.

It is the author's experience that many forms of malignant disease respond to therapy whether it is by irradiation or drugs within 2 to 3 months after initiation of treatment. This was thought to be an important time to reassess patients from two points of view, firstly, the effect of treatment on the tumour and secondly, the effect of treatment on the normal tissues of

CHANGES in SITES of INVOLVEMENT
AFTER TREATMENT

	<u>Pre Treatment</u>	<u>1st Review</u>	<u>2nd Review</u>
Cervical Nodes	61%	10%	13%
Axillary Nodes	27	10	11
Mediastinal Nodes	20	4	6
Groin Nodes	36	9	17
Para-Aortic Nodes	37	17	16
Liver	23	16	18
Marrow	21	16	14
Gut	9	4	5
Skin	5	5	4
Lung	4	1	6

Fig. 106 Effect of treatment on percentage of sites involved by lymphoma

ACTUARIAL SURVIVAL of LYMPHOMA PATIENTS
BY TREATMENT

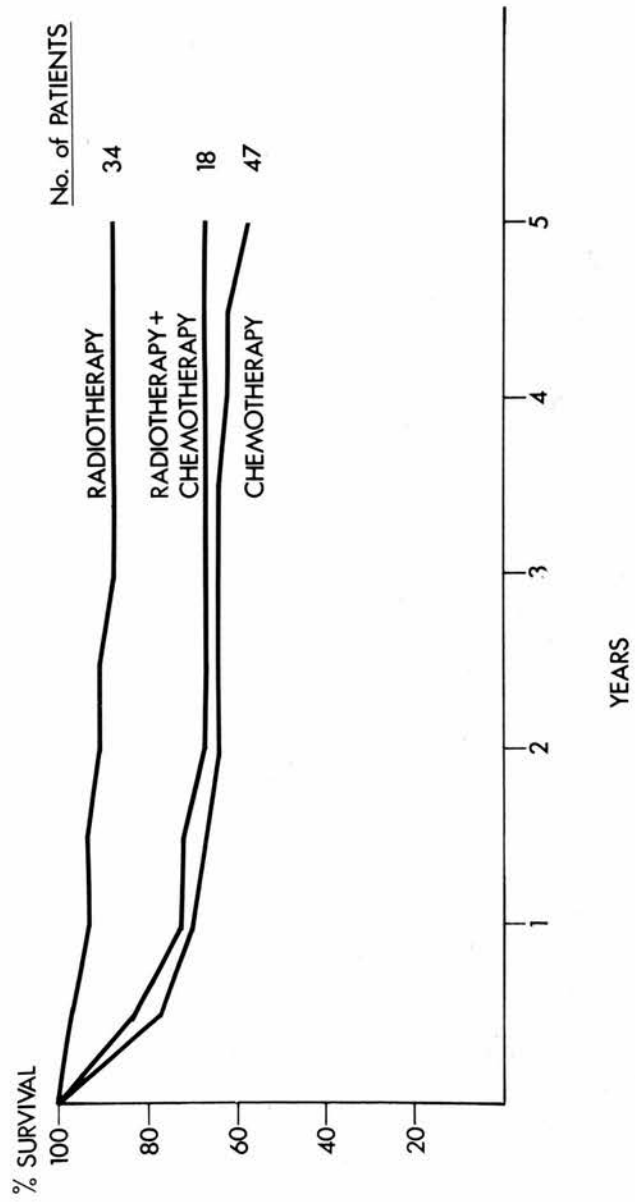


Fig. 107 5 year survival by initial treatment

the host, in other words, assessment of side effects. The complications of treatment are shown in Fig. 108 for each review period and it will be seen that they are considerable in number. Only three deaths had occurred at the two month period and each was associated with overwhelming infection. It is of considerable relevance however to look at the same figures for complications and to divide them according to the disease status of the patient. In Fig. 109 it can be seen that 72% of the complications occurred in patients who still had disease compared to 28% in patients who achieved remission. Further support for the notion that complications are associated with the presence of disease can be seen in Fig. 110 where the patients who died are studied. It can be seen that 65 of the total of 91 episodes of documented side effects occurred in the group who went on to die. It is felt that because of the high incidence of complications in patients who still have disease and who are not responding to treatment, a decision should be taken early on change of therapy. A priority must be to achieve remission as quickly as possible; if the first line of treatment fails the evidence would suggest that further persistence only leads to complications and it would seem logical to alter the treatment strategy.

Chemotherapy (Fig. 111) is certainly allied to the development of complications compared to radiotherapy alone. As explained earlier however, drugs were the treatment option for advanced disease and less of those patients achieved remission than the minimal disease group. The results of the study of monocyte function would explain increased susceptibility to infection after chemotherapy in addition to its recognised myelosuppressive consequences. Splenectomy would appear to have little adverse effect in this

COMPLICATIONS of TREATMENT

	<u>First Review</u>	<u>Second Review</u>
Anaemia	24	32
Bleeding	3	5
Viral Infection	9	11
Fungal Infection	3	5
Gram -ve Infection	10	15
Gram +ve Infection	9	14
Septicaemia	4	9
Death	3	12

Fig. 108 Number of complications at each review

CORRELATION of COMPLICATIONS with DISEASE STATUS

<u>Complication</u>	<u>Number of Patients</u>	
	<u>Disease Present</u>	<u>In Remission</u>
Anaemia	22	10
Bleeding	5	0
Virus Infection	9	2
Fungus Infection	4	1
Gram - ve Infection	10	5
Gram + ve Infection	11	3
Septicaemia	5	4
	<u>66 (72%)</u>	<u>25 (28%)</u>

Fig. 109 Number of patients suffering each complication by disease status

NUMBER of COMPLICATIONS OCCURRING in
PATIENTS WHO DIED

<u>Number of Complications</u>	<u>Number of Patients</u>
0	5
1	6
2	10
3	8
4	2
7	1
<u>Total</u>	<u>32</u>

Fig. 110 Number of deceased patients who suffered more than one complication

ASSOCIATION of COMPLICATIONS with TREATMENT INCLUDING SPLENECTOMY

TREATMENT ± SPLENECTOMY

Complication (Number of Patients)	Radiotherapy		Chemotherapy		Chemotherapy + Radiotherapy		Total Patients
	Splenectomy	None	Splenectomy	None	Splenectomy	None	
Anaemia	-	-	2	20	3	7	32
Viral Infection	2	-	-	8	-	1	11
Gram -ve Infection	-	2	-	8	3	2	15
Gram +ve Infection	-	-	1	8	1	4	14
Septicaemia	-	1	-	5	2	1	9
Death	-	-	-	7	1	4	12
Total Complications	2	3	3	56	10	19	93
Total Patients	21	13	7	40	6	12	99

Fig. 111 Number of patients suffering complications by initial treatment and by splenectomy

in this context, in contrast to the findings of other series. Only thirty-four patients are involved however, and this is a small number relative to those other studies. The importance of definition of response at two months can be seen in Fig. 112 where there is a very clear long term survival advantage for the group who had achieved complete response at that time. There was no significant difference between the group who had only achieved partial response and those who had not responded at all at the period of first review. The group in remission had the same sex ratio as the total group; thirty-one of them had stage I or II disease and fourteen of them stage III or IV disease. Only eleven of the total of forty patients with Hodgkin's disease had not achieved remission by the time of first review, in contrast to the non Hodgkin's group where thirty-nine out of sixty had failed to achieve a response. Of the forty-nine patients who were not in remission, three of them died at the time of first review and twenty-one further patients died throughout the period of study. This represents a 49% fatality rate compared to 16% for the patients who had achieved complete response.

There was a small group of fifteen late remitting patients, nine of whom had poorly differentiated lymphocytic lymphoma stage III or IV. This group are an obvious exception to the general principle of early response outlined above as this group's survival parallels that of the other complete responders. In every case, complete response was checked histologically by repeat marrow or liver biopsy where appropriate.

The overall complete response rates varied from 94% in stage I to 29% in stage IV (Fig. 113) and the survival percentages at

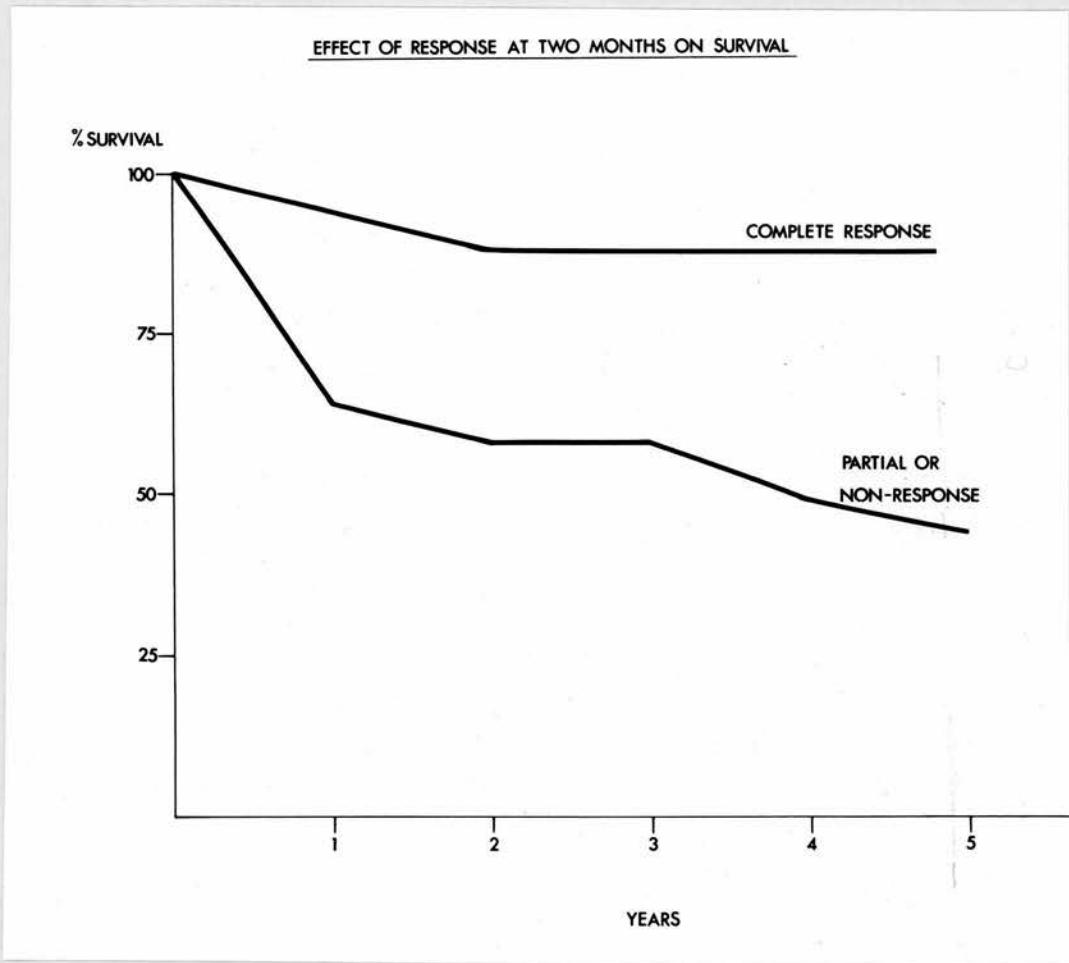


Fig. 112 5 year survival by remission status at 2 months

PATHOLOGICAL STAGE of
SURVIVING PATIENTS by DISEASE STATUS

Stage	Number of Patients (% in brackets)		
	Total	In Remission	Not in Remission
I	16	15 (94)	0
II	22	15 (68)	1 (5)
III	17	9 (53)	4 (24)
IV	38	11 (29)	8 (21)
(I + II) E	7	5 (71)	0
Total	100	55	13

Fig. 113 Number of surviving patients in each stage by disease status

5 years were 94%; stage I and 50% stage IV (Fig. 114). The actuarial survival curves (Fig. 115) demonstrate that there is little to choose between stages II and III though stage I varies from the remainder ($p < 0.05$) and stage IV varies from the remainder ($p < 0.05$). It must be remembered that while these curves are actuarial predictions they are accurate for all patients up to 2 years as this is the minimum follow-up and the mean follow-up is greater than 3 years. This survival pattern has been compared with that of a retrospective study of 650 cases (Fig. 116) whose histological composition was similar to the prospective study (page 141). There are clear differences between every stage at almost every time. One caveat however is that as these patients were studied retrospectively only a clinical stage could be assessed using the parameters described earlier. It has already been demonstrated that around 50% of patients increased stage with accurate histological assessment. If the prospective group are studied by survival and clinical stage rather than by pathological stage, the entire group with stage IV (clinical) are dead in 5 years and the majority of stage III are dead in 5 years. This does not differ significantly from the behaviour of the retrospective group. The prospective patients in stages I and II (clinical) however do considerably better than the retrospective group stages I and II, presumably due to improved radiotherapy and the use of chemotherapy in patients who failed to respond to radiotherapy.

Figure 117 shows the actuarial survival figures for the group of Hodgkin's patients and the group of non Hodgkin's patients. These results must be taken along with the previous graphs on pathological stage and survival because it may be remembered that

INFLUENCE of STAGE on FIVE YEAR
ACTUARIAL SURVIVAL

<u>Stage</u>	<u>5 yr Survival %</u>	<u>Number</u>	<u>Standard Error</u>
I	94	16	6
II	77	22	9
III	82	17	9
IV	50	38	8
(I + II) E	71	7	17

Fig.114 5 year survival rates by stage

ACTUARIAL SURVIVAL of LYMPHOMA PATIENTS by PATHOLOGICAL STAGE

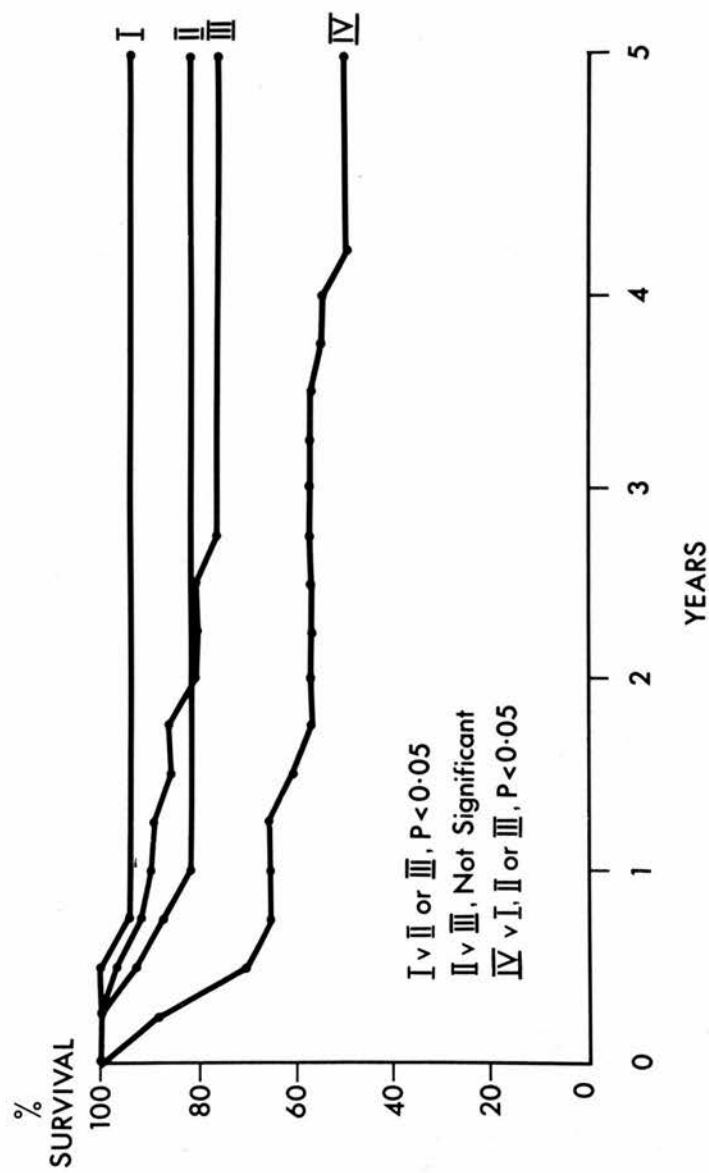


Fig. 115 5 year survival by stage of prospective group

SURVIVAL OF 650 CASES OF LYMPHOMA
1958-1972 by CLINICAL STAGE

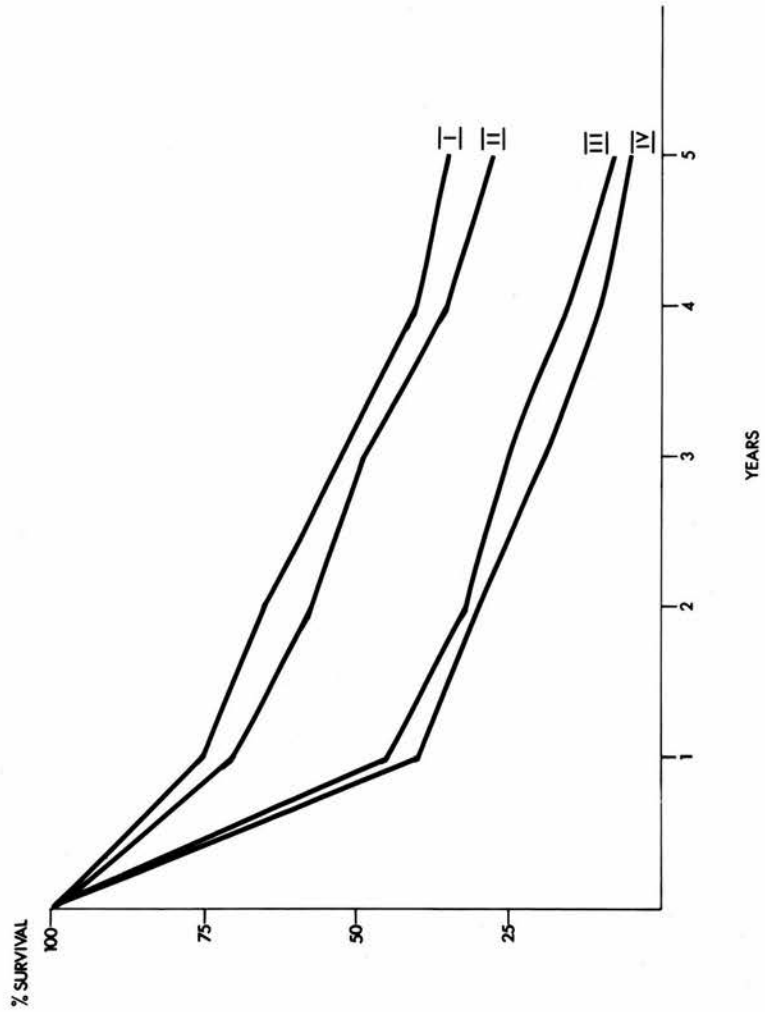


Fig. 116 5 year survival by clinical stage of retrospective group

ACTUARIAL SURVIVAL of LYMPHOMA PATIENTS by HISTOLOGY

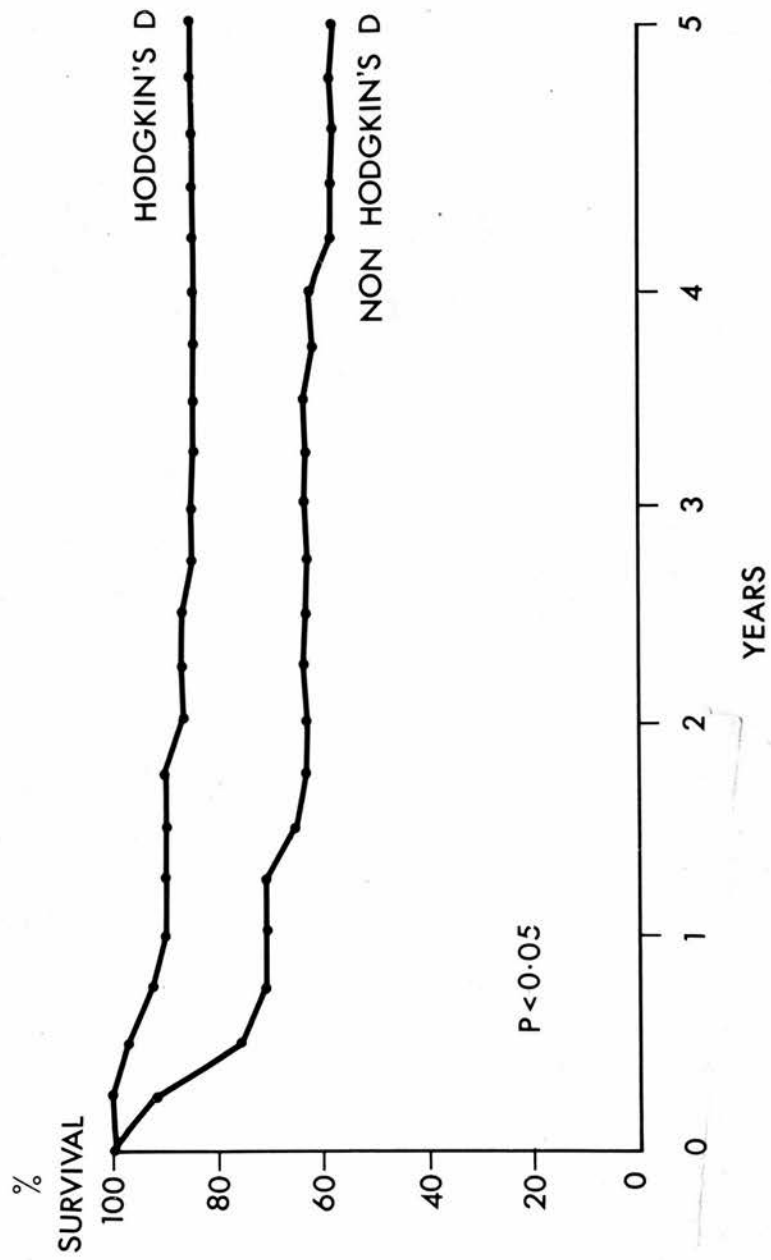


Fig. 117 5 year survival by histology of prospective group

SURVIVAL OF 650 CASES OF LYMPHOMA
1958-1972 by HISTOLOGY

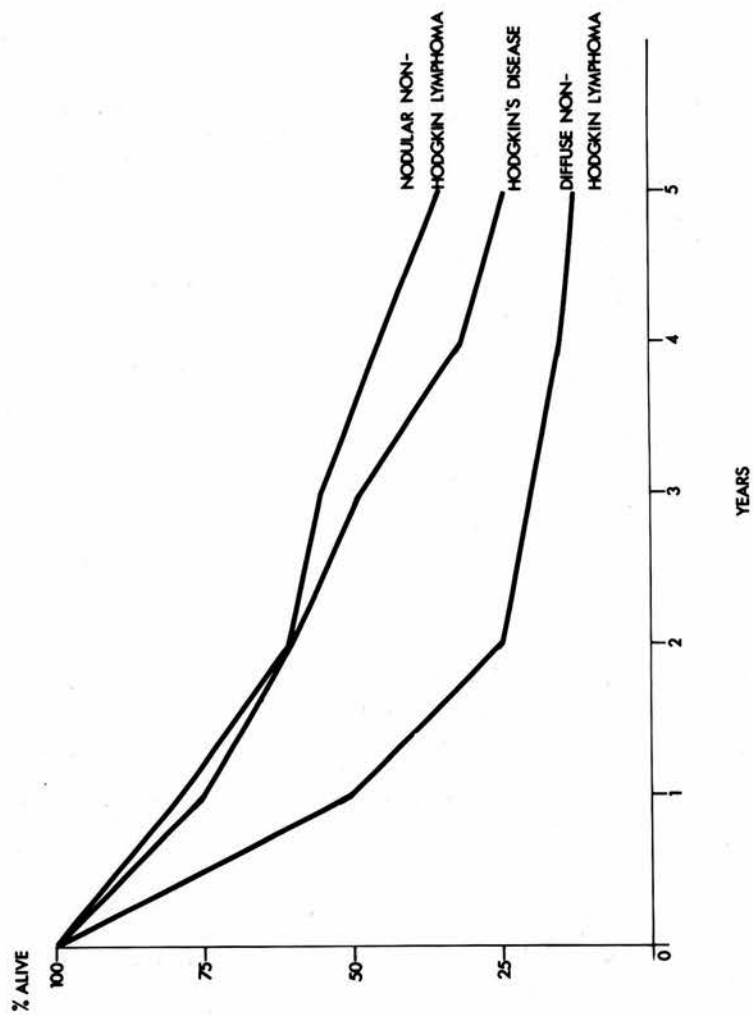


Fig. 118 5 year survival by histology of retrospective group

the majority of non Hodgkin's patients had advanced disease and the majority of Hodgkin's disease patients had early disease. The contrast between the prospective group and the retrospective group (Fig. 118) is again striking in favour of the prospective group. There should be no artefact due to histopathology typing as the same pathologist was responsible for staging the prospective and retrospective studies.

The survival of sub-types of histology is shown in Fig. 119. Patients with lymphocyte predominant Hodgkin's disease clearly survived longest though there are too few patients with lymphocyte depleted disease to make any comment about them. It is of interest that nodular sclerosis and mixed cell Hodgkin's patients seemed to do equally well. This is in contrast to other series in the literature. The similarity is maintained throughout the entire time of study as is shown by the survival graph in Fig. 120. This contrasts with the state of affairs with non Hodgkin's sub-types. Whereas the eventual outcome of the histologies poorly differentiated lymphocytic and diffuse histiocytic is not significantly dissimilar (Fig. 121), the survival graphs show that the patients with diffuse histiocytic disease died very early in fact most of the deaths occurred in the first year, and those who survived the first year tended to survive for 5 years. The patients with lymphocytic disease on the other hand appeared to be doing well over the first year of study but their numbers gradually dwindled as patients were followed up for longer periods.

One of the keystones of the Rappaport histological classification of non Hodgkin's lymphoma is the degree of nodularity of the biopsy. Only ten out of the total group of patients had nodular non Hodgkin's lymphoma. They would appear to be doing

INFLUENCE of HISTOLOGY on FIVE YEAR ACTUARIAL SURVIVAL

<u>Histology</u>	<u>5 yr Survival %</u>	<u>Number</u>	<u>Standard Error</u>
Hodgkin's Disease (total)	85	40	6
Lymphocyte predominant	100	9	-
Lymphocyte depleted	50	2	35
Nodular sclerosis	84	19	8
Mixed cell	82	11	12
Non Hodgkin's Lymphoma (total)	59	60	6
Poorly differentiated diffuse and nodular lymphocytic	64	34	8
Diffuse histiocytic	53	13	13
Remainder	58	13	14

Fig. 119 5 year survival rates by histological subtype

ACTUARIAL SURVIVAL of HODGKIN'S PATIENTS by HISTOLOGICAL SUB TYPE

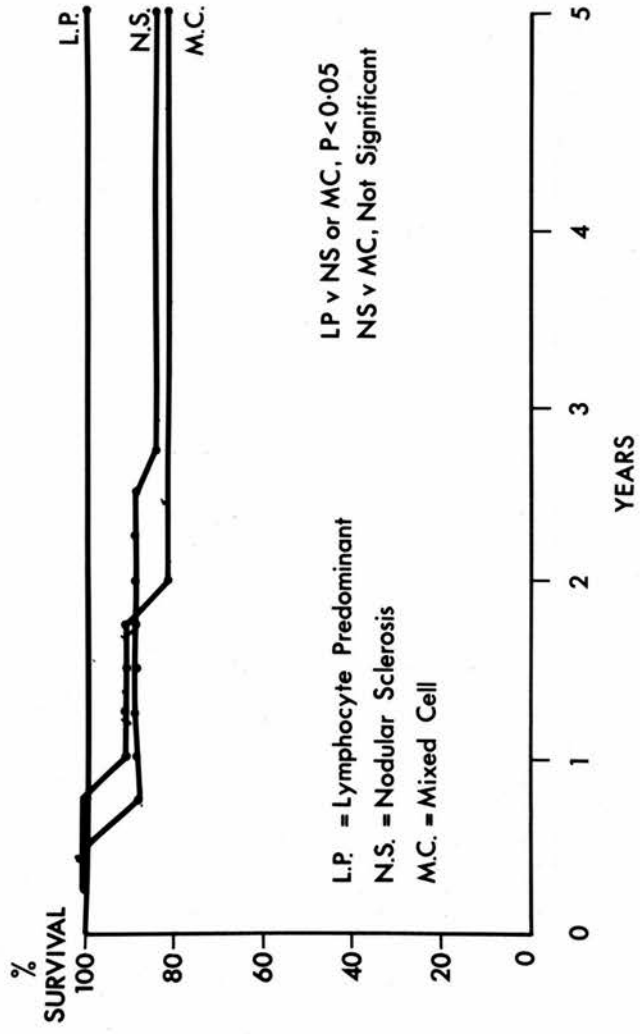


Fig. 120 5 year survival by subtype of Hodgkin's disease

ACTUARIAL SURVIVAL of NON-HODGKIN'S PATIENTS by HISTOLOGICAL SUB TYPE

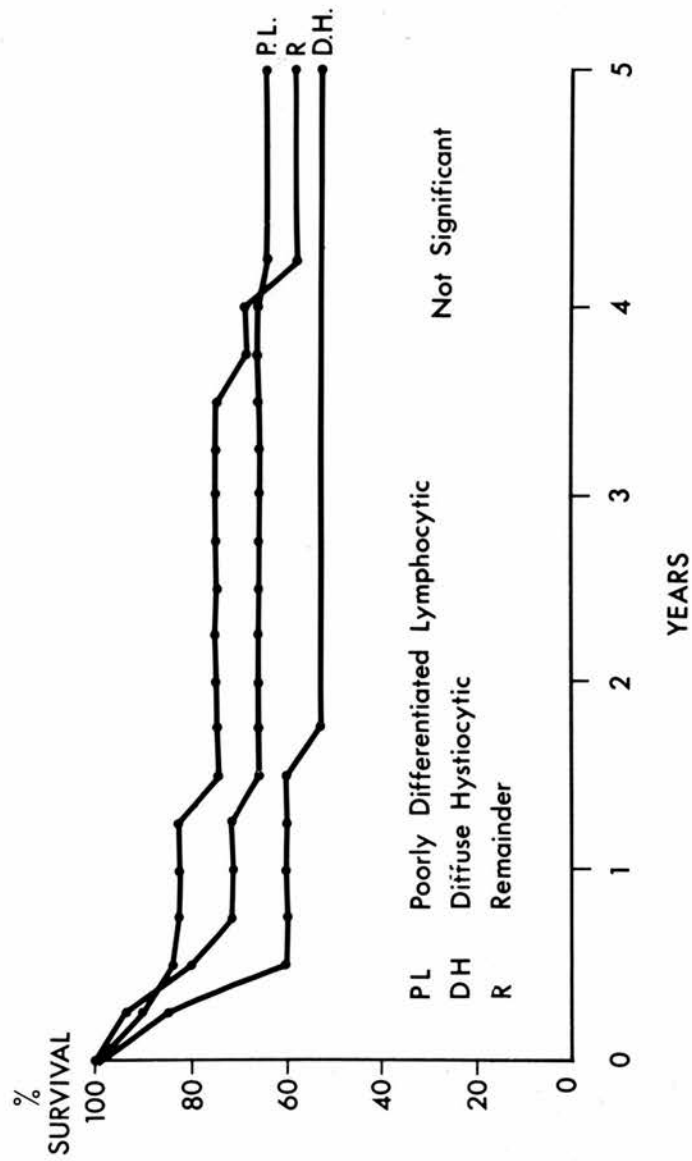


Fig. 121 5 year survival of non Hodgkin's lymphoma by histological sub-type

ACTUARIAL SURVIVAL of NON-HODGKINS PATIENTS by
NODULARITY of HISTOLOGY

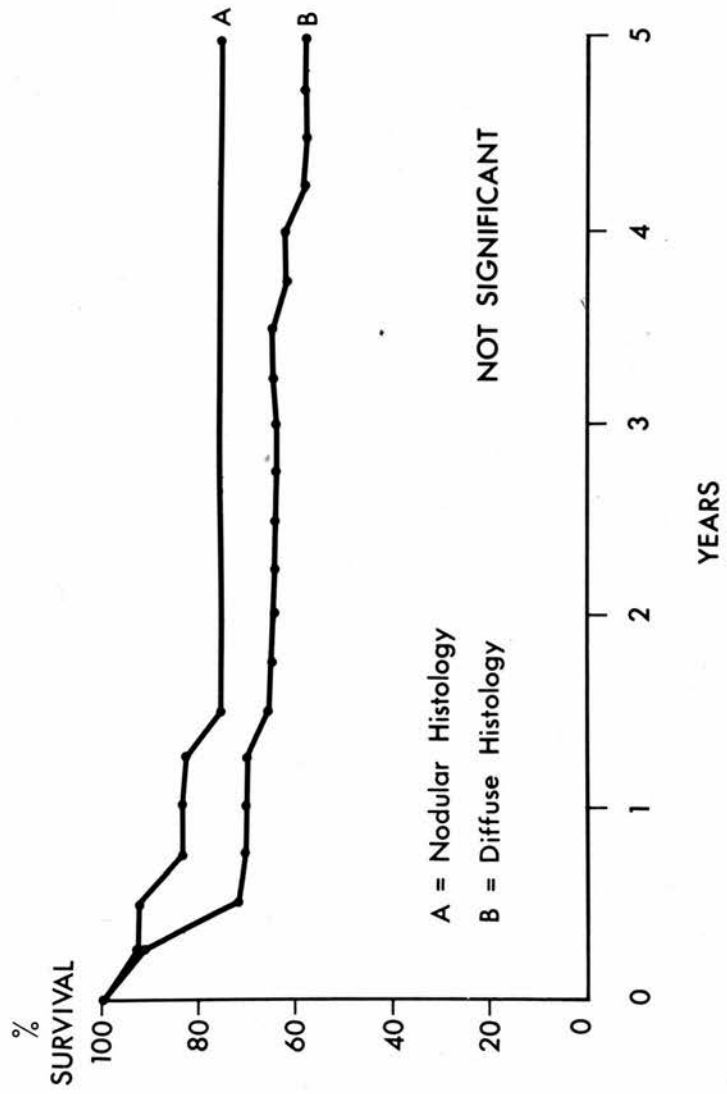


Fig. 122 5 year survival by nodularity of histology

better from the survival table (Fig. 122) than the diffuse group. The two curves however are not significantly different and more patients are obviously required to confirm the trend clearly suggested also in the retrospective study in favour of nodular disease (Fig. 118).

There is general acceptance that patients who have "B" symptoms i.e. weight loss, fever or sweating have a poorer prognosis regardless of stage or histology than those grouped "A" who do not have such symptoms at presentation. Earlier tables (Fig. 83) showed the 5 year actuarial survival percentages for patients who had these symptoms. Survival patterns (Fig. 123 and 124) show that whereas weight loss was clearly accompanied by a sharp increase in mortality in the first year compared to patients who did not have weight loss, this difference was not present with patients who had a history of either sweating or fever. The present study also indicated the importance of the history of infections and this is confirmed again by the pattern of survival (Fig. 125). Akin to the situation with weight loss, the majority of these deaths occurred in the first year, indeed the first six months.

Several pre-treatment parameters were shown to be of value in the outcome of disease. Survival figures for albumin and globulin are shown in Fig. 126 and 127; again the patients with a low albumin have died in the first year whereas low globulin levels did not affect survival. It is quite clear that further attention must be paid to the group of patients who have either a low albumin, a history of weight loss or a history of multiple infections. The critical period of intensive care of these patients appears to be the first 12 months and if they survive that time, they have the same survival chances as the remaining patients who had normal

ACTUARIAL SURVIVAL of LYMPHOMA PATIENTS by INITIAL WEIGHT LOSS

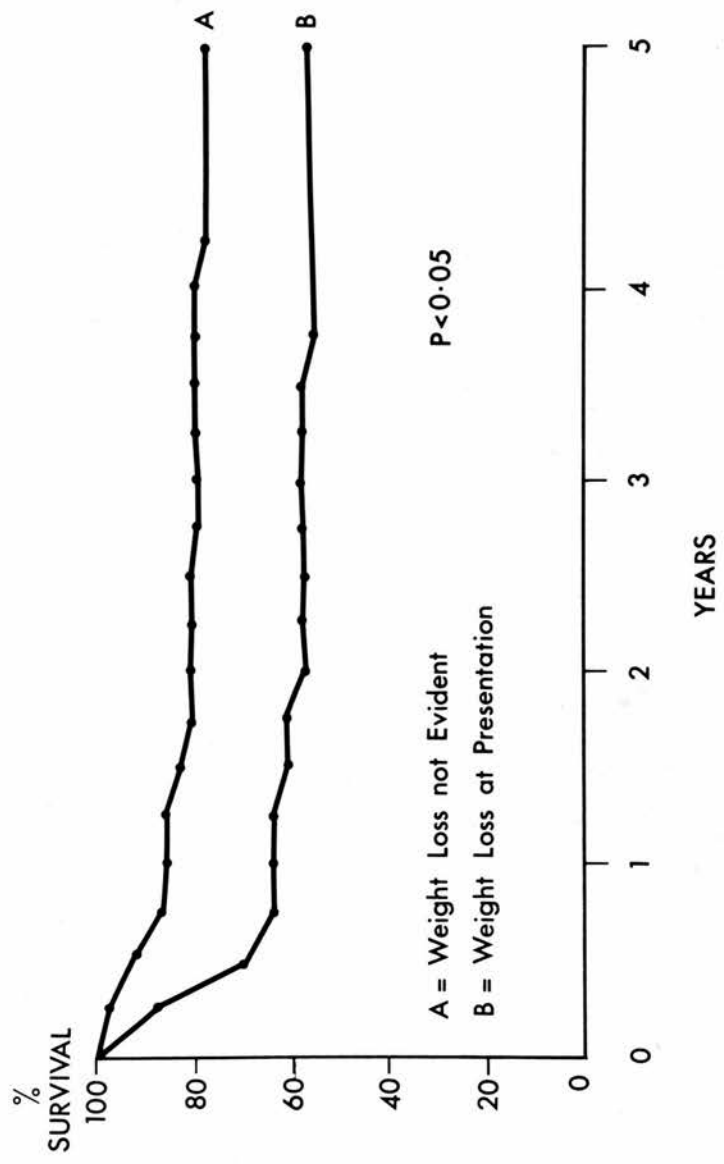


Fig. 123 5 year survival by initial weight loss

ACTUARIAL SURVIVAL of LYMPHOMA PATIENTS by HISTORY of SWEATING or FEVER

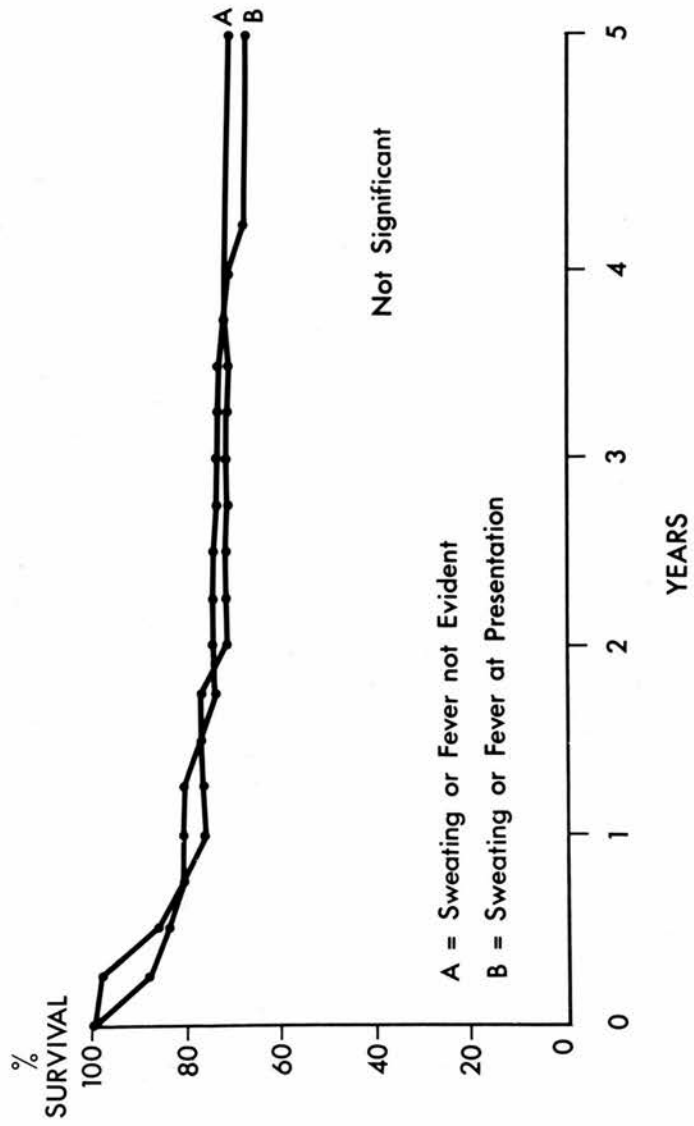


Fig. 124 5 year survival by initial fever or sweating

ACTUARIAL SURVIVAL of LYMPHOMA PATIENTS by HISTORY of MULTIPLE INFECTIONS

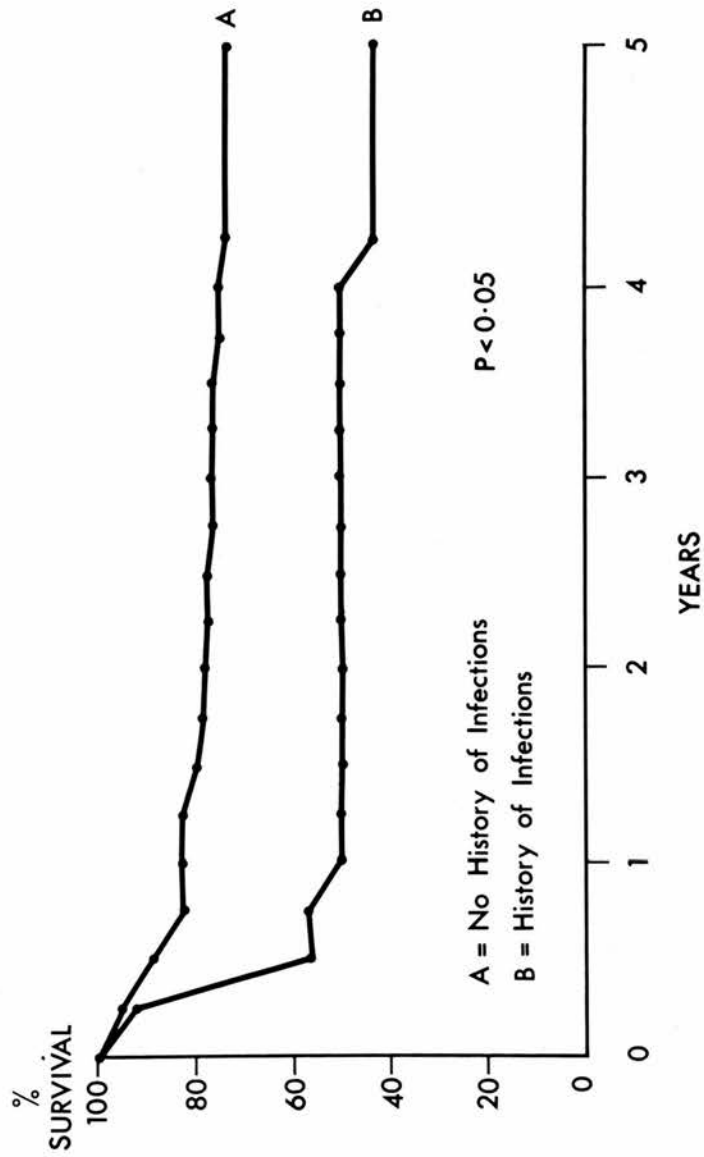


Fig. 125 5 year survival by multiple infections

ACTUARIAL SURVIVAL of LYMPHOMA PATIENTS by ALBUMIN LEVEL

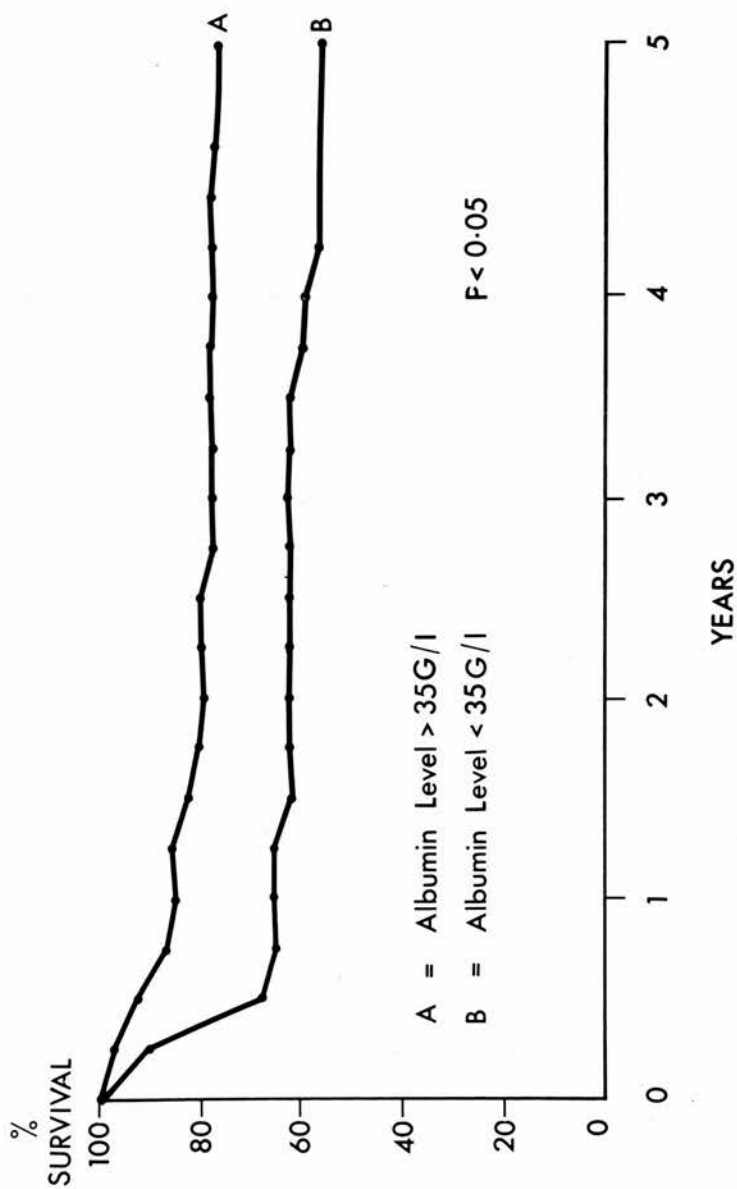


Fig. 126 5 year survival by initial albumin level

ACTUARIAL SURVIVAL of LYMPHOMA PATIENTS by GLOBULIN LEVEL

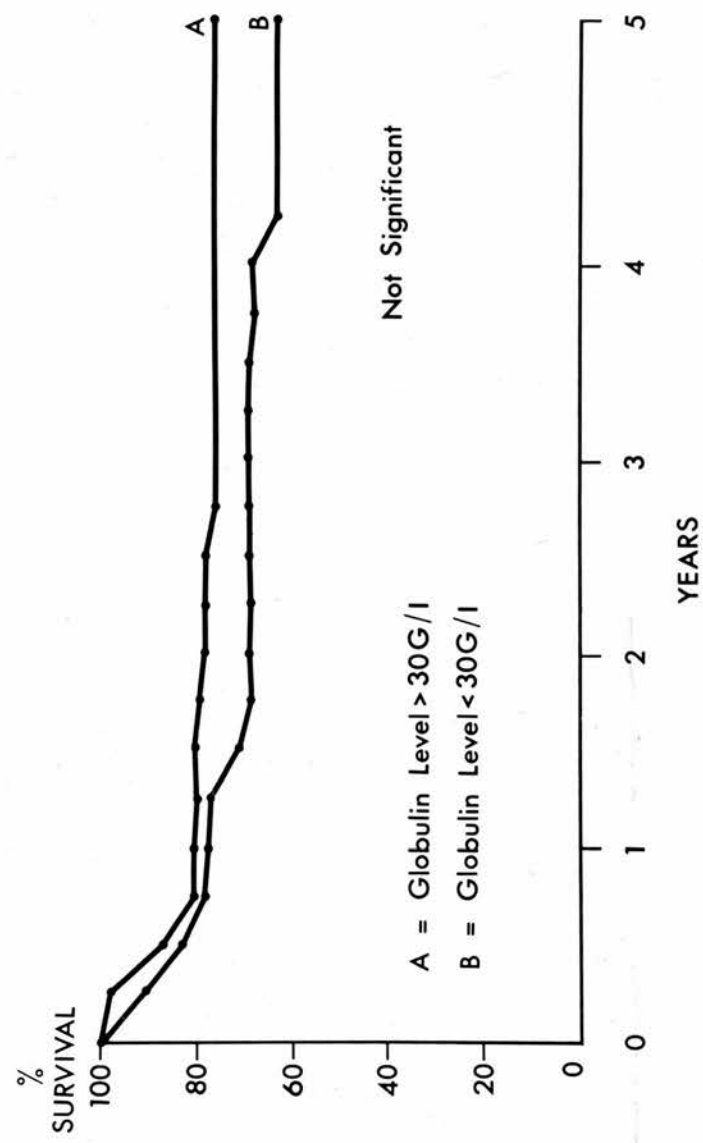


Fig. 127 5 year survival by initial globulin level

pre-treatment parameters.

The pre-treatment parameters can also be seen from Fig. 128 to assist in prediction of complications of treatment which as noted above were frequently seen in patients who did not achieve remission were on chemotherapy and who died of disease. A low haemoglobin not surprisingly predisposed to anaemia but also was positively correlated with predisposition to virus infection. A low neutrophil count at the start of treatment was of no value in predicting subsequent infection but was associated with development of anaemia presumably indicative of compromised marrow reserve. Various immunoglobulins can be seen to be useful in warning of pending infections and a low level of α_2 macroglobulin significantly correlated with development of gram positive infections and septicaemia.

Patients of both sexes who died tended to be older than the patients who stayed in remission (Fig. 129). The mean age of the deceased group is 64 compared to 45 for survivors. Thirteen of 68 survivors had not achieved remission, 8 of these were stage IV patients (Fig. 113) and the predominant histology was diffuse non Hodgkin's lymphoma (Fig. 130).

Several of the patients had gone into remission, relapsed and then achieved remission again by the time of review. Five patients relapsed in an area of disease which was previously irradiated, 10 relapsed in areas of bulk disease. One patient developed a psychosis in association with later brain involvement with lymphoma. He like three others was shown to have exfoliated lymphoma cells in the buffy coat of peripheral blood samples. Another of that group relapsed in the left testicle which has recently been recognised

PREDICTION OF TREATMENT COMPLICATIONS BY PRE-TREATMENT PARAMETERS

COMPLICATION	Mean of Pre-Treatment Parameters													
	Hb G %	WBC/ cmm	Neut/ cmm	Lymph/ cmm	Mono/ cmm	Plates/ cmm	ESR mm/hr	Albumin G/l	Globulin G/l	IgG mg %	IgA mg %	IgM mg %	E. coli inverse of litre	α_2 M mg %
Anaemia	11.5 (0.01)	7.7	4.3 (0.03)	3.4	1.0	194,000	48	34.6	29.6	926	202	110	137	322
Bleeding	13.0	8.6	7.2	1.3	0.5	293,000	51	30.4 (0.02)	28.4	1043	379	81	180	330
Virus Infection	11.1 (0.02)	11.6	5.3	6.0	1.0	203,000	29	37.1	32.2	1097	202	72	131	314
Fungal Infection	14.3	13.0	6.0	8.8	0.5	336,000	19	34.2	27.2	799	249	66	78	270
Gram - ve Infection	11.8	11.8	5.4	3.9	1.0	258,000	51	33.1	30.4 (0.03)	956	227	114	164	322
Gram + ve Infection	12.7	9.1	5.4	3.7	1.0	250,000	44	35.5	27.8 0.19	746 (0.02)	181	79 (0.38)	96	305 (0.14)
Septicaemia	12.1	8.8	6.5	1.8	1.0	182,000	41	33.4	27.8	681 (0.04)	188	100	98	295 (0.16)

Brackets Denote p Values

Fig. 128 Mean pre-treatment parameters of groups with complications

AGE and SEX of
DEAD PATIENTS

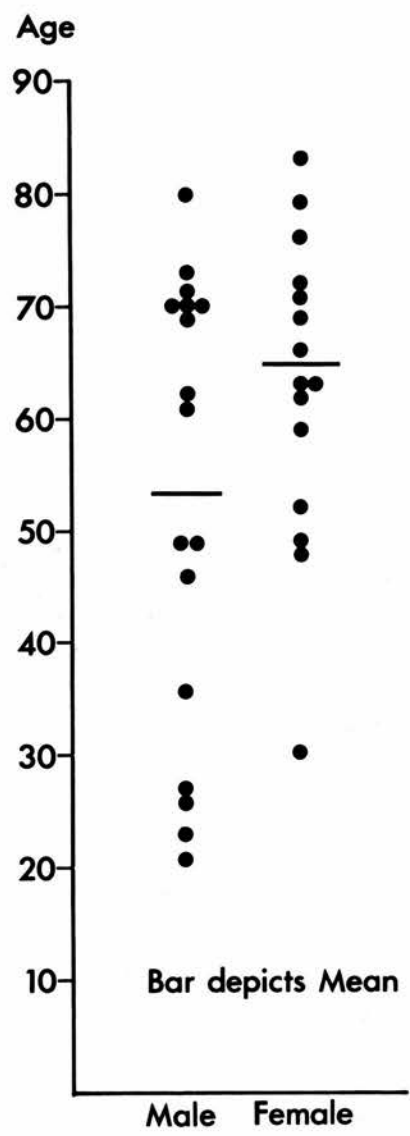


Fig. 129 Age and sex of deceased patients

HISTOLOGY of SURVIVING PATIENTS by DISEASE STATUS

HISTOLOGY	Numbers of Patients (% in brackets)		
	Total	In Remission	Not in Remission
Hodgkin's (Total)	40	30 (75)	4 (10)
Lymphocyte predominant	8	8 (100)	0
Lymphocyte depleted	2	1 (50)	0
Nodular sclerosis	19	13 (68)	3 (16)
Mixed cell	11	8 (73)	1 (9)
Non-Hodgkin's (Total)	60	25 (42)	9 (15)
Poorly differentiated lymphocytic	34	15 (44)	6 (18)
Diffuse histiocytic	13	6 (46)	1 (8)
Remainder	13	5 (42)	2 (17)
Nodular lymphoma	10	7 (70)	1 (10)
Diffuse lymphoma	50	18 (36)	8 (16)

Fig. 130 Number of surviving patients with each histology by remission status

as a "sanctuary site" like brain for leukaemic cells in childhood lymphoblastic leukaemia. Three patients relapsed in lung and one in the left pleura. The lung lesions were all nodular sclerosing Hodgkin's disease and have proved relatively resistant to chemotherapy; the pleural lesion (mixed cell Hodgkin's) was quickly resolved with quadruple drug therapy consisting of mustine, vinblastine, procarbazine and prednisolone.

The time interval from the start of treatment to time of relapse is shown alongside the pattern of overall survival in Fig. 131; whereas sixteen patients out of thirty-two died in the first 6 months, there were few deaths beyond 2 years. The relapse dates are slightly later as fourteen patients had relapsed in a year and there was a steady trickle of relapses throughout the 5 year period of follow-up. Treatment of relapse as mentioned above was nearly always by chemotherapy although six patients who relapsed outside previously radiated areas were treated with local irradiation.

TIME to RELAPSE COMPARED to LENGTH of SURVIVAL

Interval from Start of Treatment (months)	Number of Patients	
	Relapsed	Dead
1 - 6	7	16
7 - 12	7	5
13 - 18	4	3
19 - 24	3	2
25 - 36	2	1
36 - 48	2	2
48 - 60	3	1
7 - 61	1	2

Fig. 131 Number of patients who relapsed or died in 6 month intervals from start of treatment

DISCUSSION

1. Clusters
2. C-type particles
3. Tumour-host cell interactions
4. Immune deficiency
 - a) Defective monocyte function
 - b) Depression of immunoglobulins and E. coli antibodies
 - c) Paraproteins
 - d) Depression of alpha 2 macroglobulins
 - e) Hypoalbuminaemia
 - f) Tumour associated factors
 - g) Association of infections and second malignancies
5. Patterns of disease spread
6. Improved survival
7. Analysis of failure
8. Implications for therapy

DISCUSSION

The aetiology and pathogenesis of lymphomas in man are largely obscure. Case cluster studies point either to infective or genetic causation. For instance in Burkitt's lymphoma early epidemiology studies led to the implication of the EB virus by the demonstration of the antibody in serum of patients and the antigen on the cell membranes of the lymphoma cells. How a virus might cause any malignant disease whether in man or in animals however is still far from clear. One theory is that the virus becomes incorporated into the genome of host cells and thereby directs or misdirects protein synthesis and subsequent growth control of the cell. Another postulate is that the viral infection brings about a defect of immune surveillance which then fails to detect and destroy abnormal cells which spontaneously occur due to genetic errors.

The present work has demonstrated that whereas many clusters of lymphomas are artefacts true clusters do exist; electron microscope studies of lymph node tissue suggested the presence of C-type particles which may be related to previous viral infection and furthermore several defects of the host response both cellular and humoral have been detected.

1. Clusters

The data analysed from the Scottish Health Services records gives confirmation of the epidemiological reports in the literature (Doll et al, 1970). In particular the bimodal curve of incidence of Hodgkin's disease first noticed by MacMahon in 1966 was confirmed. MacMahon's theory that the two peaks of Hodgkin's disease represented two different diseases has been disputed by Smithers (1973) who

points out that patients who are successfully treated after presenting in youth with good prognosis histology (nodular sclerosis or lymphocytic predominant Hodgkin's disease) have in several institutions developed recurrence or relapse of Hodgkin's disease which is, on further biopsy, found to be either mixed cell or lymphocyte depleted Hodgkin's. Both of these sub-types carry a poor prognosis and are commoner in the older age group of Hodgkin's patients.

The clusters which were dissolved in the present study after close scrutiny are presented in response to a plea for negative epidemiological studies (Smith and Pike, 1976). They also serve as a warning to epidemiologists using data derived from similar types of registries. The majority of cluster reports in the literature are anecdotal and uncontrolled. The first was Vianna et al, 1971, who reported an epidemic of Hodgkin's disease in a High School in Albany. The same group (Vianna et al, 1974) produced figures to show an increase in mortality from Hodgkin's disease amongst physicians. A British study (Smith et al, 1974) followed a year later by an American study (Matanoski et al, 1975) have clearly shown the contrary. Indeed it is surprising to find from their figures that physicians have a degree of protection from Hodgkin's disease. These data have been interpreted by some immunologists as circumstantial evidence for the development of immunity in these doctors to whatever antigen is associated with Hodgkin's disease. A parallel may exist in melanoma where doctors involved with affected patients developed significant titres of antibodies to melanoma associated antigens (Mackie, R., 1977).

There are numerous sibling studies in the recent literature. Mention was made earlier of three pairs of patients in the author's personal experience. A father and son had macroglobulinaemia and lymphocytic lymphoma respectively, a brother and sister both developed Hodgkin's disease in middle age while living apart and a brother and sister presented almost simultaneously with non Hodgkin's lymphoma of the bowel (Freedlander, Kissen and McVie, 1977). Lymphoma of the small bowel has been reported before in one family (Maurer et al, 1976); four boys in a family were affected in this instance. One of them had low levels of immunoglobulins at the time of presentation but apart from this and a transient depression of delayed hypersensitivity skin tests no immunological defect was discovered. In the pair of patients with gut lymphoma in the present thesis, immunoglobulins were normal and so were delayed hypersensitivity skin tests. Staining of lymphoma tissue samples for immuno-peroxidase failed to detect immunoglobulins; chromosomal analyses of white blood cells were normal. The only abnormality was that both patients had a significantly high IgG titre to EB virus. The sister had never left Scotland but the brother had been told that he had suffered from malaria while abroad 30 years earlier.

In this context it is interesting that Grufferman in a retrospectively matched sibling pair study discovered 51 pairs of siblings (Grufferman et al, 1977) who overall showed a two-fold increase in the risk of Hodgkin's disease provided the siblings were of the same sex. The inference from this study was that closer contact is usual between siblings of similar sex for instance sharing a bed or bedroom. The two sibling studies in the present study however were of dissimilar sex.

A large family is reported by Purtilo et al, 1977 where three outcomes from EB virus infection appear to have taken place. Either infectious mononucleosis ensued or else agammaglobulinaemia or malignant non Hodgkin's lymphoma of B cell type. Three other families have been reported to have similar exceptional susceptibility to EB virus and in each it has been shown to be inherited as an X linked recessive. This is of interest as several other genetically determined immune deficiency diseases have been shown to be X linked. They include congenital agammaglobulinaemia, Wiskott-Aldrich syndrome, hypermacroglobulin immunodeficiency and the majority of severe combined immunodeficiency diseases. This unusual group of families who have a genetic susceptibility to EB virus is obviously not entirely relevant to the majority of lymphomas in adults. It has been shown however that whereas T cells in cord blood permit EB virus replication (Thorley-Lawson et al, 1977) adult T cells suppress EB infection. It is possible that T cell inhibition in adult life or late adolescence by some other means might permit uncontrolled EB virus replication such as might be encountered in a routinely innocuous bout of infectious mononucleosis.

2. C-Type Particles

The electron microscopy appearances of C-type particles presented earlier suggest that in a number of human lymphomas there has been either a recent or concurrent viral infection. This is confirmation of Spiegelman's evidence for tumour viruses in human lymphomas, 1975. Similar particles were also described by Stewart et al, 1969, in lymph node samples from Hodgkin's disease. Dorfman et al, 1973, only studied five lymph nodes with lymphoma and did not find C-type particles but did notice abnormal tubular arrays and dense nuclear

blebs identical to those described in Fig. 25. Uzman et al, 1971, confirmed these tubular structures in electron microscopy studies in human tumour cells and considered that they were associated with production of immunoglobulin. Molecular biologists have confirmed the presence of viral reverse transcriptase in human lymphoma tissue (Chezzi et al, 1976). They found complexes of this reverse transcriptase bound to high molecular weight RNA which infers that previous viral infection has certainly happened and that the viral genome is incorporated in the host cell DNA.

The suggestion that tumour cells might actually produce immunoglobulin or some other factor would be of relevance in explaining the lack of host efficiency in killing the tumour cells within the lymph nodes studied.

The electron microscopy appearances of several of the large tumour cells indicated that although mitochondria were not particularly healthy looking (several of them had distorted cristae), this might have been due to staining or a fixation artefact and what is important is that they were present at all. Well developed Golgi apparatus was rarely seen but strands of endoplasmic reticulum were evident suggesting that the basic apparatus for protein assembly is present in these cells.

3. Tumour Host Cell Interaction

Apposition of lymphocytes, eosinophils, plasma cells and tissue macrophages with tumour cells has clearly been demonstrated in this thesis. Identical observations have been made by Archibald and Frenster 1973, who believed that the tightness of apposition was related to the degree of cytotoxicity of the tumour cell. This group did not study perfused tissues and it is possible that their apposition data

were skewed because of retraction of tissue after first immersion in fixative. On the other hand it is not inconceivable that some degree of tumour cell killing is carried out in lymphoma lymph nodes. Certainly cell debris was noted in several of the macrophages in the ultra structural study.

In tissue culture there seems little doubt that aggression takes place. Evidence is seen in the time lapse cine film where lymphocytes and macrophages together and singly seem to possess the ability to attack and eventually to kill target tumour cells. Chemotactic factors have been isolated from the supernatants of the lymph node cultures and these would seem to be an important part of the host response to the presence of tumour cells. Raised immunoglobulin levels were seen in the peripheral blood and many patients with lymphoma maintained the ability to produce specific antibodies for instance to *Escherichia coli*. It is therefore unlikely that with the tests available immuno-paresis due to some factor such as a viral infection which has subsequently resulted in the emergence of a malignant disease will be confirmed. Evidence for a defect in the host response however has been shown in that peripheral blood monocyte chemotaxis, phagocytic ability and bactericidal capacity have all been shown to be decreased in individual patients. Similarly a factor which may well be non-specific which has depressed spontaneous lymphocyte transformation and a substance which inhibits normal monocytes from responding to a chemotactic stimulus have been shown in the supernatants of lymph node culture and in a pleural effusion associated with Hodgkin's disease of the pleura. It is unlikely that even these defects of immunity are related to the aetiology of lymphoma. Firstly, some

of the defects were more pronounced with increased mass of tumour and secondly, the factors found to depress lymphocytes and monocytes were not specific as they were also found in lymph node supernatants from reactive hyperplasia. It is more likely that the defect in monocyte chemotaxis at least is associated with release of inhibitory substance from the tumour.

Koren et al, 1973, have noticed in animal systems that only immune lymphocytes in tissue culture with tumour cells attached to the tumour cells whereas non-immune lymphocytes pass over the tumour cells, as assessed by time lapse cine photomicrography, and pay no further attention to them. Attachment of lymphocytes to tumour target cells is not only specific but inevitably results in lysis of the tumour cells. Optimum adhesion requires magnesium and calcium ions and soon after contact, the plasma membrane of tumour cells becomes increasingly permeable to small molecules suggesting that the subsequent disruption of tumour cells may be osmotic due to loss of potassium and gaining of sodium, calcium and water. This would explain the Brownian movements of intracellular particles seen in the film of cell death. The way that the lymphocytes bring about increase in membrane permeability is not known. It is clear however that whereas adhesion of lymphocyte plasma membranes disrupted from the nuclei to tumour cell plasma membrane will occur followed occasionally by cell lysis (Ferluga and Allison, 1975), lysosomal components of lymphocytes have no effect whatever. The optimum tumour cell kill is achieved using intact living lymphocytes.

Adherence can be diminished by cytochalasin B or inhibitors of oxidative phosphorylation but these do not prevent death of the tumour cells taking place after adherence of lymphocyte to

tumour cell has taken place. This last effect of the plasma membrane can be irreversibly inhibited with plasma membrane proteinase (Becker and Henson, 1973).

There is no evidence as yet that the same specificity of lymphocyte for tumour cell exists in the human situation but it is certainly conceivable that after attachment of lymphocytes and perhaps macrophages has taken place, death of the tumour cell comes about in a similar fashion. It is interesting that Sellin et al, 1971, have shown the transfer of fluorescein from H_2 target cells in the mouse to H_2 lymphocytes across gap junctions and if this is so in humans, then it is almost certain that there must be some inhibitory factor either on the membrane of the tumour cell or in the gap between tumour cells and lymphocytes and macrophages, which prevents contact taking place.

Monolayers of macrophages kill lymphoma cells after direct contact in the animal systems under three conditions (Alexander 1972). Firstly, the macrophages derived from the peritoneal cavity of mice immunised against the tumour are effective; secondly, the normal peritoneal macrophages from normal mice will kill if pre-incubated along with spleen cells from hyper-immune mice; and thirdly, normal peritoneal macrophages can be made effective when "armed" by supernatant factor which is obtained when lymphoid cells are cultured with specific tumour antigen from the mouse. Why therefore when macrophages are known to be in lymphoma nodes and they can be seen to be effective along with lymphocytes in a tissue culture situation, are they ineffective in the intact lymph node? That they are so is evident from the progressive growth of tumour in patients with lymphoma and indeed even in the tissue culture situation when the plasma clot culture was used containing an

undisturbed fragment of tissue. Time lapse cine film did not show any killing of tumour cells in this arena.

4. Immune Deficiency

Mention was made in the introduction of defects of cell mediated immunity particularly obvious in Hodgkin's disease, but also occasionally reported with advanced non Hodgkin's lymphoma. Almost without exception these defects have been shown in the advanced disease and no abnormality has been demonstrated in early disease. The principal evidence for immune deficiency from this thesis is:

- (a) Defective monocyte function
- (b) Depression of immunoglobulins and Escherichia coli antibodies.
- (c) Paraproteins
- (d) Depression of α_2 macroglobulin
- (e) Hypoalbuminaemia
- (f) Tumour associated factors
- (g) Association of infections and second malignancies.

(a) Defective monocyte function

It is clearly shown that monocyte chemotaxis is depressed in relation to advancing stage of disease irrespective of histology, age, sex or albumin level. As was inferred from Fig.66 a similar defect of chemotaxis has been found by Kay and McVie, 1977, in patients with bronchial carcinoma, in patients with genitourinary neoplasms, Hausman et al, 1975, and malignant melanoma, Rubin et al, 1976. In these and other studies (Snyderman et al, 1975) advanced disease caused maximum impairment of monocyte function and it was rare to find any abnormality in early disease.

It is therefore appropriate to assume that this deficiency is related to tumour mass and it is possible that it is due to release of an inhibitor from the neoplasms (Norman and Sorkin, 1976). Such an inhibitor has been described by Snyderman and Pike, 1976, in an assay using peritoneal macrophages from mice transplanted with neoplasm. They describe and partially identify the protein with a molecular weight of 6,000 - 10,000.

Incubation of normal monocytes with pleural fluid derived from a patient with Hodgkin's disease has been shown above to produce inhibition of chemotaxis which in three instances was clearly dose responded implying that a factor was being diluted out. The pleural fluid was not chemotactic in itself in contrast to the lymph node supernatants which were also tested. It was already mentioned that the pleural fluid cytopsin preparation showed numerous tumour cells and a relative paucity of reacting host cells. It must be emphasised that the factors which have been demonstrated need not be immunologically specific. No evidence has been produced to suggest that these factors are lymphokines i.e. that they are produced by interaction of sensitised lymphocytes, macrophages and antigens.

Monocyte phagocytosis and bactericidal capacity are extremely difficult to quantitate. The results presented here show wide variations for the normal group of controls and this is due to difficulty in standardising the number of bacteria used and the crude methods available for enumerating bacteria on plates at the end of a phagocytosis or bactericidal experiment. The figure for phagocytosis of *Staphylococcus aureus* is almost identical in the control group and the lymphoma patients before treatment. The result of note in the phagocytosis experiments was that chemotherapy

pulses drastically reduced the ability of monocytes to phagocytose. Clearly this was a temporary measure because in almost every instance the percentage of bacteria phagocytosed had returned to within the normal range prior to the next course of chemotherapy (usually a matter of 2 weeks).

The percentage of bacteria killed over the first 20 min of study was 90% in patients and controls. This implies that killing of bacteria occurs either at the time of phagocytosis or very shortly afterwards. The rate of killing of the remaining 10% of organisms was studied over 40 min and here again there was a wide scatter of results; whereas four patients did not achieve any further killing of bacteria in that time period, five patients achieved a rate of killing twice as fast as the mean for the control group. These five patients were studied serially throughout the course of therapy with drugs and without exception the rate of killing was depressed by treatment. Cline, 1973, first reported on a microbicidal defect in two patients out of nine with lymphoma whom he studied. These two patients along with eight out of ten patients with myelomonocytic leukaemia, phagocytosed *Listeria monocytogenes*, *Escherichia coli*, *Proteus vulgaris* or *Staphylococcus aureus* as well as controls. They did not however show efficient killing of the bacteria once they had been ingested. Similar results were found with *Candida albicans* as the organism in eleven patients with acute myeloid leukaemia and ten patients with chronic granulocytic leukaemia (Goldman and Th'ng, 1973): whereas *Candida* were ingested normally, killing was impaired. Later work by Territo and Cline, 1977, emphasised the importance when using *Candida* species, to use *Candida pseudotropicalis* in parallel to

Candida albicans. *Albicans* requires myeloperoxidase for effective killing but this enzyme is lost when the monocyte matures into a tissue macrophage. *Candida pseudotropicalis* on the other hand is killed by a mechanism which is independent of myeloperoxidase and therefore will predict more accurately the efficiency of the tissue component of the monocyte macrophage system. Territo and Cline concur with the findings of this thesis with respect to the variation of rate of killings of monocytes. They found that a more reproducible method which also allowed phagocytosis and killing to take place faster was simply to spin down the monocytes and bacteria into a pellet thereby bringing the cells and bacteria closer together. They offered no improvement however on the problems of quantitation of bacteria other than by the standard plating out technique. A previous paper, Cline 1973, suggested that incorporation of tritiated thymidine by intracellular bacteria was a useful index of phagocytosis. A second label however is required after the phagocytosis reaction has been stopped to determine the viability of the intracellular organisms. This technique is not reproducible because of the small numbers of bacteria involved and the non specific factors which might upset the uptake of bacteria. Further there is inevitable agglutination from time to time of bacteria on the outside of phagocytes and unless antibiotics are used in the medium these will be counted erroneously. Antibiotics were used routinely in the present experiments as it was shown that the time of culture was too short for the drugs to penetrate the monocytes thereby affecting intracellular killing efficiency.

Few studies have been undertaken in the intact patient principally due to methodological problems. Sheagren et al, 1967, however managed to measure the clearance of I-125 labelled

aggregates of human serum albumin in patients with Hodgkin's disease. The clearance of the labelled albumin was normal in all patients though was different in early Hodgkin's disease from late Hodgkin's disease. The later the disease stage i.e. the more advanced it was, the more rapidly was the label cleared. Similarly after remission was achieved by radiation or chemotherapy, several patients then showed deceleration of the clearance rate. It was suggested that the only value from this study was to confirm that phagocytosis was intact in a general sense and that an increased rate of clearance of a particular colloid indicated advanced disease and therefore was of some value in staging.

Corticosteroids are known to have several effects on the immune system. Van der Meer et al, 1976, could find no change in the functions of polymorphs incubated in pharmacological doses of glucocorticoids. In a group of volunteers however, Reinhart et al, 1975, showed a reduction of killing of *Staphylococcus aureus* by monocytes. Killing of *Candida tropicalis* was depressed in four out of fifteen patients for a period of 48 hours after the last dose of prednisolone. There was no alteration of chemotaxis, phagocytosis or hexose monophosphate shunt response to phagocytosis. There has been little work on the effect of other cytotoxic agents on monocyte function except in as much as it is known that drugs which affect microtubular function such as vincristine or vinblastine inhibit monocyte movement and therefore monocyte chemotaxis (McVie, 1977).

The role of the macrophage has never been questioned in the context of infection. It has long been known to be the key cell associated with bacteria, such as brucella, listeria and mycobacteria, tuberculosis and leprae. It appears to compensate for

the neutrophil when it is absent. Kay et al, 1975B, for instance, showed that in a patient with absence of neutrophils there was a compensatory rise in monocyte count and totally intact monocyte function. It has been known for some time that monocyte numbers are not depressed in patients with lymphoma unlike lymphocyte counts and the present study demonstrates that monocyte numbers do not decrease even after chemotherapy with combinations of cytotoxic drugs. It is important however always to consider function rather than be content with numbers alone; when chemotaxis, phagocytosis and bactericidal ability are assessed, considerable insight is gained into the possible reasons for the high incidence of inter-current infection in lymphoma patients. In the clinical part of the study it will be remembered that several patients presented a history of recurrent infections including infectious mononucleosis, and brucellosis. They had a significantly high mortality during the first year. There were numerous episodes of infection too in the subsequent course of patients' management though most of these were not fatal. The predominant organisms were *Escherichia coli* and *Staphylococcus aureus* though it is significant that one episode of septicaemia due to *Listeria monocytogenes* occurred in a patient whose monocyte function was significantly depressed both in chemotaxis and bactericidal ability. Normal activated macrophages should release a soluble factor which "sterilises" *Listeria monocytogenes* (Middlerbrook et al, 1974). Unfortunately this patient's bactericidal function was not tested with listeria as this was not available in the laboratory at the time but the defect shown in the killing of *Staphylococcus aureus* would probably have been exaggerated for listeria.

It is not yet possible to devise reproducible tests of monocyte or macrophage tumouricidal ability using the human system but this is clearly an important objective as it is increasingly obvious that macrophages have a direct cytotoxic role against tumour cells (Lohmann-Matthes et al, 1973) and in addition, cooperate with lymphocytes in producing lymphokines and indeed produce macrophage derived anti-tumour factors de novo. The macrophage arming factor (Alexander, 1972) for instance is a cytophilic antibody which has been shown to act as a kind of opsonin which will coat target cells and possibly micro-organisms rendering them more easily killed and phagocytosed. In the animal systems non-specific i.e. non immunological factors have been isolated along with specific immune factors previously mentioned in the above discussion (Alexander 1972). There is evidence now that α_2 macroglobulin acts as a non-specific cytophilic substance in exactly this role. It has been shown to coat large particles such as cells and to render them more easily phagocytosed by monocytes (Black and Gregoriadas, 1976).

The difficulty of dissecting the effects of blocking factors or unblocking factors and opsonins such as α_2 macroglobulin in the integral tumour are considerable. Their action, positive or negative may only be effective over a short range such as the gap between host cell and tumour cell. Evidence that the host macrophages have any function at all has only been obtained from studies which rely on dissection and processing of tumours in vitro. A compromise approach such as Wood and Gillespie, 1975, have used may yield further results. They demonstrated variable numbers of macrophages in immune solid tumours and showed that suspensions

rich in macrophages showed little propensity for metastasis on re-injection into suitable donors. Suspensions depleted of macrophages either naturally or by adherence on glass caused markedly aggressive tumours on transplantation. Survival figures for the two groups of transplanted animals were significantly different ($p = < 0.05$) in favour of the macrophage rich transplants. There can be little doubt of the effect of the macrophages, though their anti-tumour mechanisms are for the most part obscure.

(b) Depression of immunoglobulins and Escherichia coli antibodies

The immunoglobulins which were detected were IgG, IgA and IgM. Levels of IgD and IgE were difficult and expensive to measure at the outset of this study because of unavailability of appropriate antiserum. Nevertheless there are reports that IgD and IgE are elevated in malignant disease including lymphomas and mycosis fungoides (Corte et al, 1977, Zachariae et al, 1975, Jacobs et al, 1972). It need not be emphasised that the other immunoglobulins particularly IgG and IgM have a critical role as humoral antibodies against micro-organisms and possibly also tumour cells. Both antibodies bind complement particularly IgM and receptors for IgM have recently been demonstrated on the surface of Hodgkin's tumour cells (Habeshaw et al, 1976). Further, as discussed in the introduction the majority of lymphocytic lymphomas have clearly been shown to be of B cell origin and to carry immunoglobulin receptors for IgG.

A single molecule of IgM can lyse target leukaemia or lymphoma cells in the presence of complement in tissue culture experiments; two molecules of IgG side by side are required to perform the same trick (Allison, 1972). IgG 1 and IgG 3 fix complement well in

this situation compared to IgG 2 and IgG 4 which fix complement poorly. Similar effects of immunoglobulins in lysing tumour cells are difficult to demonstrate in vivo because of the problems in constructing an appropriate model; however, this is yet another mechanism whereby the findings in this communication might be explained viz the relative immunity of tumour cells to lysis in intact tissue compared to their ready killing in tissue culture.

It is clear therefore that at least on theoretical grounds normal levels of immunoglobulins would be an ideal situation for the intact immune state. The results above indicated that for one hundred patients the mean of each immunoglobulin type was within the normal range. Around 30% of the entire group irrespective of stage of disease had abnormally low immunoglobulins, IgG and IgA. Thirteen per cent of patients in stage I had a low IgM compared to 69% of the patients in stage IV. Throughout the course of study levels of IgG and IgA fell by a mean of 10% throughout treatment and IgM by 20% in the group of patients who achieved remission. Of the patients who went on to die an increased number developed depression of IgG and IgA with a mean of 20% depression whereas a startling 45% depression of IgM was evident in this group. It must be emphasised that these changes are irrespective of the initial treatment level, and as many of the patients who died started off before treatment with low levels of IgM because of their advanced state of disease the fall of IgM by 45% was catastrophic.

Hancock et al, 1977, reported similar trends in patients with Hodgkin's disease who were followed up over a similar time period. They reported that IgG and IgM levels fell further in splenectomised patients but this was not confirmed by the work of this thesis.

Indeed splenectomy in the patients reported here produced no significant immediate falls in immunoglobulin levels and did not predispose patients to further complications or bias the prognosis after treatment. Levels of IgG were more likely to be depressed in Hodgkin's disease particularly lymphocyte predominant Hodgkin's disease which it may be remembered is said to carry a good prognosis and indeed no patient has died in that group. On the other hand deficiency of IgA was common throughout all histologies and deficiency of IgM was particularly marked in non Hodgkin's lymphoma compared to Hodgkin's disease. There are no equivalent reports of immunoglobulin levels in untreated non Hodgkin's lymphoma patients though there are numerous communications of varied immunoglobulin levels in large numbers of lymphoma patients on treatment. Hypogammaglobulinemia however, has been reported in one instance before the development of Hodgkin's disease (Hoffbrand, 1974). Goldman and Hobbs, 1967, noticed that 24 out of 50 patients with Hodgkin's disease had reduced IgM levels, IgA levels tended to be low but IgG levels were normal or raised. Unfortunately Hobbs' patients were not staged accurately nor were they assessed pretreatment.

IgG synthesis has been shown in normal spleens and in Hodgkin's spleens in tissue culture (Longmire et al, 1975). The spleens from Hodgkin's patients synthesised over five times as much IgG as normal spleens on the same conditions. If this is a host response by the spleen then perhaps splenectomy is not entirely in the patient's interest.

Measurement of haemagglutinating antibodies to pools of *Escherichia coli* were first carried out in immuno-suppressed patients by Webster et al, 1974. They studied six patients who

were suffering from recurrent chest infections and who despite normal serum IgG levels had absence of *Escherichia coli* antibodies. Five of the six patients went on to regular gamma globulin injections and improved. Webster et al, did not establish a clear normal range of antibodies as they limited their normal group to 34. They claim however that from that small group no patient had a titre less than 1 in 64. This is in general accord with the much larger series reported here. They found that 70% of normal subjects had an IgM antibody i.e. it was destroyed by mercapto-ethanol. This is a higher figure than for the present series. As discussed previously the IgM antibody was of less value in detecting immuno-deficiency and monitoring patient progress in the lymphoma study because of the high percentage of normal individuals who lack the antibody. It seems from the correlation data in any case that the behaviour of *Escherichia coli*, IgG antibody and IgM antibody is paralleled and therefore measurement of and comment on the first IgG antibody probably suffices. Graham Pole et al, 1975, used Webster's assay in a study of the immune status of children with malignant disease. They did not publish a normal range for the antibody but over half of their patients (14 out of 20) gave a haemagglutinin titres of less than 1 in 64. This corresponds well with the present figure where the median of one hundred patients with lymphoma was 1 in 32 compared to a median for the control population of 1 in 128. The mean titre for both IgG and IgM antibodies declined with time and with the effect of treatment. Half of the patients in stage IV lymphoma had abnormal *Escherichia coli* titres compared to 20% of those in stage I and, like IgM, the principal histology involved

was non Hodgkin's lymphoma. Indeed within the group of non Hodgkin's lymphoma the IgM Escherichia coli antibody differs from the IgG antibody only in the group of diffuse histiocytic lymphomas. Of these lymphomas, 70% had an abnormal or absent IgM antibody compared to 30% who did not have a significant titre of IgG antibody. Neither Escherichia coli antibody, IgG or IgM was significantly lower in the group of patients who developed infective complications which concurs with Graham Pole's findings in the children's study. This infers that the Escherichia coli antibody though useful in providing additional information about the ability to synthesise particular humoral antibody in relation to the stage of disease, does not give any additional information about predilection to infection.

Weitzman et al, 1977, showed that specific antibody against haemophilus influenzae type B was significantly reduced in patients receiving radiotherapy and chemotherapy together. One treatment modality alone did not alter the titres significantly. Untreated patients had normal values and splenectomy did not cause any abnormality. A small group of five patients with Hodgkin's disease is reported by Sullivan et al, 1978. This group formed part of a large study of patients undergoing splenectomy for a variety of reasons. Four of the five patients with Hodgkin's disease did not respond to immunisation with bacteriophage or a pneumococcal polysaccharide antigen. The results for the other patients with splenectomy were varied but tended not to be as obvious as the immuno-deficiency demonstrated in Hodgkin's disease. This is a small group however and few conclusions can be taken from it. Jones et al, 1976, carried out studies on antibody production to the same bacteriophage used by Sullivan (ϕ X174) in volunteers.

In their group of studies there was only one man who showed a significant decrease of secondary response to bacteriophage out of a group of thirteen in total. This man went on to develop nodular sclerosing Hodgkin's disease 4 years later. This chance discovery is one of the few pieces of evidence which might suggest that patients who are destined to develop lymphoma such as Hodgkin's disease may be immuno-deficient during the "incubation period". Further studies along these lines would have to be carried out in large populations in a prospective fashion.

(c) Paraproteins

Paraproteins are classically associated with multiple myeloma. As previously mentioned however, many of the non Hodgkin's lymphomas are B cell tumours which differ from multiple myeloma only in their degree of differentiation. It is not surprising therefore that occasionally paraproteins were seen in association with non Hodgkin's lymphomas. Two patients were detected by immuno-electrophoresis and indeed this is the most useful clinical application of the technique. Paraprotein marks appear in the precipitin lines as a dense bulge lying towards the antibody trough in the area in the immunoglobulin arcs. The paraprotein occasionally intersects the normal immunoglobulin precipitin lines and may display a varying degree of cross reaction with it. On the other hand, the paraprotein may be completely independent of other immunoglobulin arcs and not in contact with them. Lastly, as was shown in one of the examples earlier the paraprotein may have an undulating appearance with some prozone effect due to antigen excess.

It is probably a coincidence that both of these patients had lymphomas of stomach; whereas the first man had a relatively

straight forward poorly differentiated lymphocytic lymphoma which showed areas of plasmacytoid differentiation, the second man described who had a coexisting myeloma of a different class was really quite unusual. Clinically it would appear that his lymphoma of bowel which on histology was clearly a plasmacytoma might have been related to a previous history of gluten-sensitive enteropathy. One of the first presenting symptoms this man complained of was weight loss and the immediate assumption of the clinician in charge was that he had stopped taking his gluten free diet. On enquiry however, it was clear that he had stuck rigidly to his diet and further investigation disclosed the malignancy. Patients with myeloma are well known to develop acute myelomonocytic leukaemia but this is nearly always related to chemotherapy with alkylating agents. Two plasma cell malignancies in the same patient is very unusual. Hefferman, 1947, and Ruland 1954, reported primary gastric plasmacytomas followed by a skeletal plasma cell tumour. No immunological data are available however in these two patients. Mediterranean lymphoma (Rappaport et al, 1972) and Waldenstrom's disease of the stomach (Froget et al, 1972) have frequently been associated with abnormal paraproteins either IgM or IgA and of course the classical description of Mediterranean lymphoma includes the accompaniment of alpha chains (Seligman et al, 1971). The patient described here had IgG kappa light chains along with IgA lambda light chains. His kidney showed the features of myeloma but amyloid was not shown. It is assumed that the presence of myeloma in the kidney was responsible for the terminal episode in this man's illness which took the form of an antidiuretic hormone resistant polyuria with hypoalbuminaemia.

Three further patients in the study of one hundred consecutive lymphomas were shown to have paraproteins, two of them IgM and one of them IgG. None of these was associated with gastrointestinal disease and only in one was plasmacytoid differentiation noted in some parts of the lymph node biopsy. In none of these patients did the paraproteins serve as a valuable marker of tumour mass and the fluctuations were quite independent of the remission status judged clinically by palpation of nodes, liver and spleen. Alexian 1975 and Macris 1976, both described IgM paraproteins in non Hodgkin's lymphoma, histiocytic lymphoma and lymphocytic lymphoma respectively. The principal importance of the finding of paraprotein is that it is frequently accompanied by depression of other immunoglobulins in a similar manner to the classical multiple myeloma rendering the patient at increased risk from bacterial infection. The above patients however had no infection problems.

(d) Depression of Alpha 2 macroglobulin

A pilot study of α_2 macroglobulin levels in lymphoma was carried out by Tunstall et al, 1975. They failed to show any consistent trends in the small groups studied; for instance, half of the patients with Hodgkin's disease had elevated levels, all the patients with lymphocytic lymphoma had depressed levels while six patients with histiocytic lymphoma had elevated levels. Half of a small group of myelomas had low levels and the other half high levels. The present larger study shows that approximately a quarter of the patients in every histology have depressed levels of α_2 macroglobulin and taking into account age and sex, levels did not depend on stage of disease and levels did not change throughout treatment regardless of whether the patient died or

achieved remission. Alpha 2 macroglobulin however does assist quite considerably in predicting the course of disease. Of forty-four patients who had a level below 325 mg% (taken as an arbitrary cut off point) twenty went on to die whereas twenty-four remained alive. Of thirty-six patients, all of whom had normal or raised α_2 macroglobulin levels, only eleven died. The difference in survival at the end of one year was 92% versus 72% for the normal versus depressed patients respectively. This advantage was maintained to 5 years when the figures became 84% and 57% respectively. This is an entirely new finding and is particularly useful in that no other factor seemed to affect the level of the α_2 macroglobulin protein.

Elevation of α_2 macroglobulin has been reported in patients with various malignant and non malignant disorders (Sunderman and Sunderman, 1964), and in several of the patients in this group reported presently, high levels were achieved. As α_2 macroglobulin is clearly shown to be a lymphocyte secretion, it may be that a low level of α_2 macroglobulin is indicative of either lymphopenia or dysfunction of lymphocytes. The latter is more likely because the lymphocyte count alone did not correlate with prognosis and tended to drop as treatment ensued in contrast to the α_2 macroglobulin which was constant. As mentioned earlier α_2 macroglobulin has been seen to be associated with cell membranes which is hardly surprising as molecules such as glycoprotein, glycolipids, glycosaminoglycans and acid mucopolysaccharides are a constant finding in the composition of the cell membrane. Previous investigations of glycocalyx components have failed to detect any major difference between cancer cells and normal cells (Nicholson,

1976). If α_2 macroglobulin is an important lymphocyte derived protein its depression may well be surmised to lead to less efficient phagocytosis of tumour cells by tissue macrophages. Clearly further work requires to be done to elucidate the function, if any, of α_2 macroglobulin in this situation. In the meantime, it provides a useful marker for detection of high risk groups of patients.

(e) Hypoalbuminaemia

Although serum protein abnormalities are accepted in the lymphomas (Kaplan, 1972) almost without exception the reports have concentrated on globulin levels. Only Waldmann et al, 1963, noted and investigated reduced serum albumin levels in lymphoma patients and felt that albumin synthesis was possibly decreased leading to low serum levels. Low serum albumin has been discovered in patients with liver disease because of decrease in synthesis, but this cannot be the reason for low albumin levels in the present study. Only nine of thirty-two patients who had hypoalbuminaemia had involvement of the liver with lymphoma. Nor was there any close correlation with involvement of bone marrow or other extranodal structures. Hypoalbuminaemia has been discovered in approximately the same percentage of a large group of patients with various solid tumours in the University Department of Oncology, Glasgow (Soukop et al, 1978). Again there was no clear association with liver disease although the patients who had liver involvement with metastases had a lower mean value of albumin than those who had normal livers. In the context of solid tumours such as carcinomas and sarcomas, hypoalbuminaemia is almost always an accompaniment of poor nutritional status and cachexia. Such patients do notoriously

badly on treatment regardless of whether it is by radiation or drugs and indeed this has led to the evolution of parenteral nutrition as a preamble to cytotoxic therapy. A third of the group of one hundred patients had lost more than 15% of their body weight and might have been expected to do badly according to the Rye classification. Indeed this was so, the 5 year survival of this group being less than the patients who had no weight loss. Eighteen patients had weight loss and low serum albumin levels. Therefore there was a group of fourteen patients who had low albumin levels which were not related to weight loss and therefore were probably not related to malnutrition but possibly to anti-metabolic factors released from the tumour.

Albumin levels were independent of all the other laboratory parameters but related to prognosis as previously described. Patients who had a low albumin before treatment and went into remission were shown without exception to have an increase from their pretreatment level of albumin. Patients who did not survive and who had a high pretreatment albumin again without exception dropped their albumin level throughout the course of treatment. It can be seen therefore that not only is the pretreatment level of albumin useful but follow-up levels give some guide to progress.

It has been suggested that malnutrition is associated with immune depression. In a recent study (Soukop and Nicholson, 1977) PHA transformation of lymphocytes was compared to the nutritional status of a large group of patients with solid tumours. Only patients who had lost greater than 30% of their body weight showed any depression of lymphocyte transformation. In contrast a study from the M.D. Anderson Hospital, Houston, showed that

that anergy as defined by recall of skin antigens was closely correlated with hypoalbuminaemia and weight loss of greater than 15% (Dudrick et al, 1977) and anergy has further been associated with the presence of a peptide in the serum, probably derived from tumour (Glasgow et al, 1977). It may be that the serum albumin will be shown in further studies to be a valuable indicator of immune function as well as of nutritional status in patients with malignant disease.

(f) Tumour-Associated Factors

There is now a wealth of literature on naturally occurring immunosuppressive factors in man such as that of Glasgow's group above. Cooperband et al, 1975, reviewed thirty-nine different factors which have been extracted from various tissues from a variety of animals including man. These factors act on either T cells, B cells, macrophages or on occasion, more than one cell system. It is clear that these factors which include the lymphokines have considerably blurred the classical theory of two clear cut mechanisms of immunity, one humoral, and one cell mediated. It is obvious too that several of the substances, particularly several lymphokines have very powerful feedback properties; for instance, factors generated by lymphocytes may either increase (Rosenthal et al, 1973) or decrease (Tadakuma and Pierce, 1976) the synthesis of antibody by other lymphocytes. Several factors arise from tissues other than those of the reticulo endothelial system. Macrophage inhibition factor for instance, at first thought to be a hall mark of specific antibody antigen reaction has been derived from fibroblasts, lung tissue, brain tissue and cell lines infected by

viruses such as mumps and Newcastle disease (Papageorgiou et al, 1972, Tubergen et al, 1972, and Yoshida et al, 1975). Plasminogen activator can be released by macrophages after stimulation by supernatants from transformed lymphocytes (Klimetzek and Sorg, 1977). The effect of this is clearly not immunological in the least as fibrinolysis may be initiated and both clotting and complement systems triggered.

The two substances reported here have not been classified due to inadequate amount of supernatant remaining after use in test systems. Nevertheless their effect in depressing spontaneous and PHA induced transformation of lymphocytes on one hand and the chemotaxis of monocytes on the other is clearly of relevance to the above discussion of immunodepression associated with lymphoma. It may be too that the inhibition of normal monocyte chemotaxis by the Hodgkin's derived pleural fluid (above) was due to attachment of polypeptides of the lymphokine variety and indeed this may also be the explanation of the sluggish response of lymphoma patients own monocytes to standard chemo-attractants. Fuks et al, 1976, discovered serum factors from patients with Hodgkin's disease which inhibited T lymphocytes and blocked effective binding of sheep erythrocytes (E rosette formation). Long et al, 1977, described soluble immune complexes in the serum of Hodgkin's patients which may have been the same factor as Fuks was describing. The soluble immune complexes interacted with established monolayers of Hodgkin's lymph nodes but not to cultured cells derived from normal spleen tissue.

Direct examination of tumour cells in culture by fluorescent tags failed to show convincing evidence of tumour specific antigens

in the cells; or else the results may be interpreted as failing to show the presence of circulating antibody to the tumour cells. In a similar work with carcinoma of the colon, Nairn et al, 1971, used viable tumour cells to detect membrane staining and although not reported here, this work has not been reproduced in lymphoma cells (Habeshaw, J.A., personal communication). Nairn and Order et al, 1973, used indirect fluorescent methods in contrast to the present study which reports only the direct application of fluorescence labelled gamma globulin. Order and his colleagues found two Hodgkin's associated antigens in specimens of spleen which cross-reacted with tissue from several auto-immune diseases and foetal liver, spleen and thymus. It is possible that in the present study antibody was absorbed out by over zealous absorption with liver powder. On the other hand the cross reactivity of the test reported by Order lessened its specificity and therefore its relevance to further manipulation such as preparation of antibody.

The importance of immune complexes (if antigens exist which form them) as possible blocking factors has been mentioned by Sjogren et al, 1971. If this is relevant to lymphoma it might explain the effective blocking of tumour cell surfaces to host attack and might also account for the lowering of levels of immunoglobulin in the peripheral blood. A clinical study by Amlot et al, 1976, suggested that the presence of circulating immune complexes related to night sweats and fever in Hodgkin's disease thus conferring on the affected patients a poorer prognosis. This was a small study and did not report on the follow-up of patients and therefore is of limited value. Correlation of circulating immune complexes with disease outcome on one hand and other immune

parameters such as macrophage function, albumin, IgM and α_2 macroglobulin levels is an obvious sequel to the present findings.

(g) Association of Infections and Second Malignancies

There have been a distressing number of second malignancies reported after prolonged follow-up of Hodgkin's patients in remission (Canellos et al, 1975). Also Hodgkin's disease has been reported after remission was achieved in acute leukaemia (Woodruff et al, 1977). This is clearly a matter for concern though it is not surprising as all cytotoxic drugs and indeed radiation itself are likely to be carcinogenic. It has been assumed that because studies reported to date show a higher instance of second malignancy in patients undergoing more intense treatment than in those given for instance only one modality of therapy, that second malignancies are a result of treatment. Multiple tumours however, have been known to occur in the same patient before treatment (Haddock et al, 1972) Tashima reported two cases in 1973 of patients who had malignant melanoma and malignant lymphoma. The incidence of second non lymphomatous malignancy not related to treatment in a large group of mycosis fungoides patients is 15% (Mackie, 1977). Langlands, 1976, found that 5% of a group of 350 patients with breast cancer had other malignancies, again before treatment. Eleven of 100 patients studied here had a history of previous malignancy other than lymphoma. None of these patients had had previous cytotoxic drugs, the majority having been treated by surgery alone. One further patient discussed above had a plasmacytoma of stomach and myeloma at the same time. These findings may be explained by chance, on the other hand, there is preliminary evidence that clustering of tumours such as breast, colon and ovarian

carcinoma and lymphoma occur in the same areas epidemiologically (Gillis, 1977). It is possible therefore that there may be a common aetiology in a certain percentage of patients with these malignant diseases. It is too early in the follow-up period of this study to detect any second malignancies which might be due to treatment. It is of interest to note that the group of patients who did have two malignancies has not shown a survival curve which is significantly different from the total group.

On the other hand, the small group of patients who presented with a history of recurrent bacterial or viral infections have done considerably less well than the rest of the cohort group. Hodgkin's disease has classically been associated with concurrent tuberculosis, brucellosis, candida infection and viral infection particularly with herpes. The National Cancer Institute study in 1972, reported 25% incidence overall of herpes infection both zoster and simplex in lymphoma patients of all histologies. Infection was equally common before treatment and on treatment. Bodey, 1975, maintains that more patients die from infection than from cancer. This is clearly an artefact of certification. It could just as easily be said that more patients who suffer cerebrovascular accidents die from infection than from their stroke. This information only tells us about the immediate time before death and gives us no clue as to the relevance of infection to prognosis in the stages leading up to terminal illness. It has clearly been seen from the mention of complications of treatment in the present study that the large majority (75%) of infective complications in particular were found in patients who still had lymphoma and had not achieved remission. From this it must be

inferred that any immunodepression which is predisposing to infection is related to the presence of tumour rather than to a secondary effect of chemotherapy or radiotherapy. Sixty eight per cent of patients survive to the present time and all of these have had potentially immuno-suppressive exposure to cytotoxic therapy. The incidence of infective complications in this group is only of the order of 25%.

Immunoglobulin levels and α_2 macroglobulin levels correlated well with development of infective complications. All episodes of septicaemia were associated with neutropenia but other infections were not so related. Lymphopenia was associated with viral infection and gram negative and gram positive infection and, as mentioned earlier, monocytopenia was not demonstrated.

Depressed lymphocyte transformation and synthesis of interferon have been correlated with repeated or severe herpes virus infections in Hodgkin's patients (Ruckdeschel et al, 1977, Stevens and Merigan, 1972). There is also considerable animal evidence to suggest that the severity of viral infections correlates closely with T cell and macrophage dysfunction (Allison, 1972). Also the congenital immunological deficiency syndromes invariably present with early and repeated infections regardless of whether they are associated with bursal defects such as Bruton-type hypogammaglobulinaemia, thymus deficiency or combined immuno-deficiency (Walton, 1972).

Infections in patients with lymphoma could therefore be seen to be a bad prognostic feature at the time of presentation and associated when they occur throughout treatment with the presence of residual disease. Whether they occur in patients in remission or in relapse however, they would appear to be associated

with considerable defects in immunity which may account for the surprising occurrence of opportunistic infections by viruses, fungi, atypical bacteria such as *Listeria* and *Mycobacteria*, and Protozoa such as *pneumocystis carinii*. It would appear logical not only to treat infections before treatment of the lymphoma commences, but also to make an attempt to restore the immune competence of the patient before or alongside anti-tumour manoeuvres. Such restoration might be achieved by non-specific or specific immunotherapy, with surface active agents, unblocking agents, cytophilic antibody or removal of blocking agents such as antigen antibody complexes by plasmapheresis.

5. Patterns of Disease Spread

Delineation of the extent of disease in an individual patient is important for two reasons. First it may point to the most appropriate therapy for that patient and second, it allows the physician to communicate to others the stage of the disease along with the outcome of therapy. It follows therefore that staging procedures are only of clinical relevance if they establish a pattern of disease which will change the course of treatment planned for the patient. The early exercises in staging by lymphography, staging laparotomy, percutaneous biopsies of marrow, spleen and liver have provided a backcloth of intelligence on the mode of spread of Hodgkin's disease and non Hodgkin's lymphoma. Thus it is likely that the patient who has nodular lymphocytic lymphoma proved on a cervical gland biopsy will also have other glands either easily palpable in the groins, axillae and abdomen or detected on lymphography in the para-aortic region (Goffinet et al, 1977). It is likely that as shown in the above thesis

the majority of non Hodgkin's lymphomas will have spread to the bone marrow or liver before the time of presentation. In contrast, it is now established that Hodgkin's disease presenting in the cervical lymph nodes is likely first to involve the mediastinum (particularly in the nodular sclerosing subdivision of Hodgkin's disease) and then the splenic hilar lymph nodes followed by the spleen, para-aortic nodes and liver (Kaplan, 1972). Marrow involvement is late in Hodgkin's disease in the majority of cases.

Now that this basic information is available, staging procedures which have been taken as routine in the past might well be modified and simplified. Chabner et al, 1977, have clearly shown that most patients with non Hodgkin's lymphoma will be accurately staged by a sequential series of simple tests including lymphangiography, bone marrow biopsy and percutaneous liver biopsy; only the minority of patients will require staging laparotomy to complete investigations prior to treatment. The therapeutic implication is that these tests mentioned above will have converted the majority of patients into a stage of disease which will imply drug treatment because of the evidence of advanced disease. Thus further surgical assault on the patient is unnecessary and will not lead to an alteration of treatment policy. The experience of Chabner and Hermeck et al, 1975, and Spinelli et al, 1975, have supported the findings of this thesis viz. that between 40% and 50% of patients are wrongly staged by clinical means alone. These authors however do not state the percentage of patients in whom treatment alterations ensued. In the present work all patients who changed stage increased their stage and therefore with a few exceptions treatment plans were altered. As mentioned before patients who had stage III disease accompanied by systemic

symptoms were not subjected to a further staging procedure for two reasons, the first was that they were frequently too ill for surgical intervention, and the second reason was that these patients were due to have chemotherapy in any case and it was merely academic to know whether these patients were truly stage IV or stage III.

Investigational studies however require more careful organ sampling particularly if studies are multicentre collaborative projects. Despite the knowledge of disease spread in most of the lymphomas much has yet to be learned of the patterns of response of individual organs to different treatment modalities. It has been mentioned above that different disease sites have responded in different ways to the same therapeutic manoeuvres; for instance, lymph node disease has been relatively successfully eradicated in contrast to lymphoma in liver or skin. Differential response to radiotherapy and to chemotherapy has also been the author's experience in the treatment of solid tumours such as breast cancer, lung cancer and teratoma of testis. In the last example, lymph node disease, particularly when it is bulky, is extremely difficult to sterilise by either radiotherapy or chemotherapy whereas pulmonary metastases are readily ablated by chemotherapy consisting of vinblastine, bleomycin and Cis-platinum. This is exactly the reverse of the lymphoma experience.

The differential uptake of cytotoxic drugs by primary tumours and metastatic lesions in different organs has not been fully investigated in man. There is some animal evidence to suggest that certain malignant masses are relatively avascular but that tumour cells may survive in the centre of such tumours despite relative hypoxia. Different cytotoxic drugs are certainly

transported across cell membranes by different mechanisms and this may account in some way for the difference of results discussed above. Local immunity may differ from organ to organ thus having a bearing on the contribution of host macrophages and lymphocytes to the cytotoxic effort against the tumour. It is therefore important that careful studies are continued which document the site of disease before treatment and include rigorous re-assessment of disease involvement at those sites after treatment has been completed.

For practical purposes patients with Hodgkin's disease who have no systemic complaints and who have lymphocyte predominant or nodular sclerosing histology with a negative lymphangiogram and negative gallium scanning may well be safely spared splenectomy and staging laparotomy. In a small percentage of patients this will be the wrong treatment approach and these patients will relapse in the spleen or coeliac axis nodes. It is likely however that at that time response to either further irradiation or chemotherapy may still lead to favourable survival. An alternative approach is to assume that the spleen is involved and either irradiate it or give cytotoxic therapy in addition to local irradiation to areas of known disease.

Concern about the infective hazards of splenectomy has previously been mentioned and is documented by Donaldson et al, 1972, and Chilcote et al, 1976. Although there were no disturbances of immune function or any unusual intercurrent infections in the present series, splenectomy is still a major consideration for the patient and may have late implications. Alternatives are gray scale ultrasonography which Glees et al, 1977, report as a promising

development, or gallium 67 citrate scanning (Horn et al, 1976) or laparoscopy and needle biopsy of spleen which is advocated by Spinelli et al, 1975. None of these techniques are as reliable as splenectomy and careful dissection and examination of the spleen but each if positive might save a significant percentage of patients from a full laparotomy.

Laparoscopy is particularly useful in non Hodgkin's lymphomas. Chabner et al, 1977, have indicated in a sequential study of 170 patients, two particular areas of value with this technique. One was the highlighting of disease in the sub-diaphragmatic lymphatics on the right side which can easily be seen at the same time as viewing the liver and the other was the ease of liver biopsy under direct vision through the laparoscope. Occasionally mesenteric nodes have been noted at peritonoscopy but they usually require a laparotomy for confirmation of histology (Goffinet et al, 1977).. These represent an important site of relapse in patients who have merely been treated with radiation to the para-aortic and inguinal nodes. Mesenteric nodes and indeed gut lymphoma are quite unusual in Hodgkin's disease but may represent up to 20% of non Hodgkin lymphomas.

The surgical confirmation of lymphangiographic findings in both Hodgkin's disease and non Hodgkin's lymphoma has shown the technique to be of outstanding value in assessment of disease in the lymph nodes of the abdomen inferior to the third lumbar vertebra. Above this region gallium 67 citrate may be of value intravenously or via lymphatics as mentioned earlier. Of much more immediate importance has been the development of computerised axial tomography (CAT) which very accurately estimates the size of

this group of nodes. It will confidently predict and report enlargement of these nodes but gives no insight into the nature of the enlargement unlike the appearances of the lymphogram. As yet, these CAT scanners are few and far between due to their considerable expense and their evaluation in management of disease such as lymphoma has yet to be completed.

The major site of disease which is not detected in the routine manner is the gut. This is nearly always diagnosed primarily at laparotomy. There were nine patients in the present study and they are atypical not only in their histology (predominantly non Hodgkin's lymphoma) but their presentation and natural history as discussed earlier. The results of treatment in this group are important because eight of the nine were treated by chemotherapy. The results of radiotherapy in gut lymphoma have been mixed. In the retrospective study referred to earlier, there were 82 patients who had lymphoma involving the gut and 75% of them were dead less than a year after treatment by radiotherapy. Of the 8 patients who were treated in this study 4 died in the first 7 months, 2 of them before chemotherapy had had an adequate trial (that is, 2 course at 2-3 weekly intervals). The remainder were alive greater than 2 years after treatment with chemotherapy which represents a considerable improvement in management. A further 8 patients with gastro-intestinal lymphoma have since been treated by the author. None of these patients have died and all have achieved complete remission.

One of the problems of comparing results of therapy for gastro-intestinal lymphoma in different centres has been that staging has been inadequate. Patients have frequently presented as emergencies

and been dealt with by junior surgical staff who have little experience in either the macroscopic appearances of lymphoma in the bowel or else the principles of staging at the time of laparotomy. Staging has therefore been inadequate as indeed has primary therapy (surgical removal of the bulk of disease). It would appear from the poor results of radiotherapy that the disease is not localised to the bowel and almost certainly has spread to mesenteric lymph nodes if not by the blood to other sites of the body. Other adverse factors in this group are coincident malabsorption, either due to the lymphoma process itself or due to the presence of pre-existing coeliac disease. These patients tend to have lost a considerable amount of weight and are nutritionally and immunologically deficient. Hypoalbuminaemia in particular is common and as discovered above leads in itself to a poor prognosis.

There are too few patients who have involvement of other extranodal sites to make further comment apart from remarks already made about the differential responses in marrow and liver. It is worth reiterating that of 15 patients, who were not in remission at 2 months but clinically were extremely well, 9 were stage IV poorly differentiated lymphocytic lymphomas. The decision to maintain the original form of treatment which was in all instances chemotherapy was taken on clinical grounds alone and all achieved remission in the next 2 months. It is important to continue to re-biopsy both marrow and liver, particularly if the patient is likely to finish chemotherapy because of good clinical remission. Lastly it should be pointed out again that several patients who had relapse of nodular sclerosing Hodgkin's disease in lung, proved extremely resistant to chemotherapy. This is not reported in the literature

but has been noticed in clinical practice at the Royal Marsden, London by McElwaine (personal communication).

6. Improved Survival

There are two aspects in survival, quantity and quality. The overall actuarial survival figure of 68% at 5 years represents definite evidence of improvement over earlier series (1965-69 figure for Hodgkin's disease was 54%, other lymphomas 32%, Cutler et al, 1975). Reference has already been made to the retrospective study in which patients of a comparable histological spectrum were clinically staged and treated by radiotherapy alone in the decade to 1971. These patients fared less well in every respect than the present group. This is the common experience of numerous other groups (Schein et al, 1974, Hellman et al, 1975, Aisenberg and Qazi 1976, and Donaldson et al, 1976). In each of these studies, similar survival figures are given, the overall 5 year survival for Hodgkin's disease being in the order of 85% compared to 60% for the non Hodgkin's lymphomas; they also report complete remission rates of similar magnitude to the present study - 55% for the total group. Other authors too have noted that complete response status at 2 months from the onset of therapy is the single best prognostic factor in the management of lymphoma (Wollner et al, 1976). Apart from the early achievement of complete response, histology as above had considerable bearing on outcome, in particular, diffuse histiocytic lymphomas have long been notoriously threatening and have now been seen to be responsive in a third to one half of patients. Indeed results from De Vita et al, 1975, indicate not only that a complete remission has been achieved in 40% of the patients, but that this remission if achieved is

maintained for up to 9 years. This is the result of the present study although follow-up time is as yet merely up to 5 years. The remainder of the group of diffuse histiocytic lymphomas die very rapidly (within the first year) irrespective of treatment modality and so progress is only limited to the minority of those patients.

Prolonged survival in the present study has been associated with absence of a history of weight loss or infections at presentation, a normal or high level of serum albumin, α_2 macroglobulin and IgM. The principle of staging has been borne out by the evidence that stage I patients do better than stages II, III or IV and conversely patients with stage IV disease fare considerably less well than the patients with limited disease. There was little difference however between patients who had stage II disease and stage III disease.

Improved survival figures not only reflect the vigour and intensity of investigative procedures themselves but point to the positive gains of collaborative management by radiotherapist and chemotherapist. Radiotherapy remains the distinctive and appropriate treatment for early nodal disease regardless of histology and for limited extranodal disease, particularly in the spinal cord. Chemotherapy has been shown to be not only effective but relatively safe in the treatment of advanced disease outside lymph nodes. The role of surgery in essence has evolved to one of removal of bulk disease particularly in the spleen, gut and occasionally in isolated single metastatic lesions in lung or brain. The use of all these modalities when appropriate and perhaps with the addition of immunotherapy in subsequent protocols would appear the logical direction for progress.

The cost of prolonged survival however has been occasional

intercurrent complications. There have been no treatment related deaths in the present series but many patients have become critically ill as a result of septicaemia, neutropenia and shock. The tables describing complications shown earlier implicated chemotherapy in particular in association with side effects although it was pointed out that the single most relevant feature was the presence or absence of disease. Patients who were responding to chemotherapy infrequently showed severe complications; conversely those patients who went on to die from overwhelming resistant lymphoma represented the group with multiple complications. A treatment plan has evolved which involves the assessment of disease response early after initiation of treatment and the speedy decision to alter treatment in the face of resistant disease or intolerable side effects.

The majority of side effects were due to infection which is not surprising in the light of the immune deficiency in the earlier part of this work. Further, radiotherapy (Ghossein et al, 1975) cancer surgery (Eilber and Merton, 1970) and chemotherapy (McVie, 1977), have each been demonstrated to be immuno-suppressive of their own right. Of further concern in this context is the earlier discussion of second malignancies in lymphoma. It has been known for some time (Penn, 1974) that patients who are immuno-suppressed after renal transplantation show a propensity to develop a subsequent malignant tumour particularly lymphoma. Lately Roberts and Bell, 1976, described three cases of acute myeloid leukaemia following exposure to cyclophosphamide given to patients with renal disease. This is a particularly worrying complication as it represents yet another instance of iatrogenic disease and

represents a real threat to long term improvement in survival from lymphoma.

Patients who were treated for Hodgkin's disease with radiotherapy or chemotherapy had a shorter course of therapy than those treated for non Hodgkin's lymphoma. This is because patients went into remission earlier and treatment could therefore be terminated quickly. Patients with non Hodgkin's lymphoma however tended to have advanced disease and although partial remissions were frequently seen early, complete remissions were occasionally late and indeed a certain group of patients particularly with nodular lymphoma in accordance with the experience of Portlock and Rosenberg, 1977, continued with evidence of disease for considerable periods of time. It was therefore important in this group in particular that drug related immediate toxicity was tolerable.

All chemotherapy and radiotherapy was carried out on an out-patient basis except for the occasional exception which was due to social reasons such as the distance of travel for each visit to hospital. The principal complaints after radiotherapy were nausea, apathy and anorexia and the main problem was myelo-suppression; similarly for chemotherapy depression of blood counts, although of no concern to the patients, was a problem in management for the clinician. Nevertheless, only 20% of courses of therapy required to be delayed for periods of one to three weeks because of failure of bone marrow recovery. The only other reason for alteration of therapy was the development of peripheral neuropathy related to the vinca alkaloids. This occurred in 25% of patients and consisted mainly of tingling in the extremities and occasional sensory loss. Several patients were found to have absent peripheral reflexes

and one patient developed postural hypotension and an autonomic neuropathy involving his gut which allowed the growth of a large bezoar in his stomach. This bezoar consisted mainly of orange pith to which the patient was apparently habituated. The bezoar was confirmed at endoscopy and an attempt made to fragment it, thereafter it passed through the gastrointestinal tract without hold-up. The majority of patients did not have serious side effects which were severe enough to keep them from work for more than 24 or 48 hours. Several patients did not interrupt their work schedules at all though these were all patients receiving cyclophosphamide as opposed to mustine. Mustine invariably caused vomiting for several hours after injection irrespective of concurrent administration of antiemetics. These symptoms apart however, performance status was improved in all patients on chemotherapy who achieved either a partial or complete response. As observed earlier, patients who did not achieve a response either had treatment altered or stopped because inevitably in association with resistant or progressive disease was noted a decrease in performance status and increased intolerance to side effects. In summary, therefore quality of survival was improved in those patients who responded to therapy and survival was lengthened in comparison to the retrospective study and also to others in the literature for the same period summarised by Cutler et al, 1975. The complete remission and survival figures for each histology are in accordance with those other reports in the literature employing similar staging and therapeutic strategies.

Analysis of Failure

The reasons for failure of treatment in lymphoma are generally

speaking either related to properties of resistance by the tumour or else failure to tolerate treatment by the patient. Advanced disease is less readily cured than early disease and several points of discussion were made earlier relating to access of drugs to the centre of bulky tumours and the survival of hypoxic cells despite radiation or chemotherapy. The site of relapse of disease is frequently in areas of bulk and this has led to the adoption of adjuvant radiotherapy to such areas after the completion of chemotherapy for both Hodgkin's disease and non Hodgkin's lymphoma. As many cytotoxic drugs are myelotoxic, patients whose marrow is already compromised by invasion of tumour tend to do less well than the patients who have adequate marrow reserve. Further there is evidence that disease in vital organs of metabolism and excretion such as liver and kidney change the handling of cytotoxic drugs by the patient (McVie, 1976). For instance, cyclophosphamide requires to be activated by liver enzymes before it is of any cytotoxic value. If liver function is inadequate because of the presence of lymphoma, metabolism of cyclophosphamide may not be optimum. Similarly toxicity of adriamycin will be considerably greater in such patients, as the liver is responsible for excreting most of a dose of adriamycin and its metabolites. In a like manner methotrexate toxicity is inevitable in the presence of inadequate renal function. Moreover, it is apparent that several cytotoxic regimes including radiotherapy are still relatively non selective so that damage to normal tissues limits dose and certain cell clones can develop resistance to therapy. Such cells may have altered kinetics and growth fractions or else they may reside in privileged sites mentioned earlier such as testis and the central nervous system; or else they may

develop biochemical defences which block either entrance of drugs into the cell or activation of the drug at its target site within the cytoplasm or nucleus.

Lastly, failure of treatment may depend on the fitness of the patient. Evidence has been produced here to suggest nutritional and immune deficiencies in several individuals manifested by the occurrence of co-existent infections, weight loss, hypoalbuminaemia and hypogammaglobulinaemia. Patients who are elderly and frail have done considerably less well in this and other studies. They tend to have advanced non Hodgkin's lymphoma and particularly severe nutritional problems. In addition elderly people have lower lymphocyte counts and impaired lymphocyte function in any case, (Augener et al, 1974) and may for all those reasons metabolise drugs inadequately (B.M.J. 1975), suffer more toxicity and incur frequent intercurrent and opportunistic infection.

8. Implications for Therapy

It seems logical to continue from the previous section with a discussion of improvements both in therapy and patient support which might be implemented. As far as improvement of tumour response to cytotoxic drugs is concerned, new chemotherapy protocols are emerging involving drugs such as imidazole carboxamide, adriamycin and the nitrosoureas which seem equally effective if not an improvement on existing schedules (Bonadonna et al, 1975, Lenhard et al, 1977, and Lokich et al, 1976). Significantly there are a number of other drugs which spare the marrow while maintaining a cytotoxic effect. These include bleomycin, vincristine, dihydroemetine, streptozocin and razoxane all of which have been shown to have effect in lymphoma (McVie, 1976, Flamery et al, 1976, and Kerr et al, 1976).

Attempts are being made to alter the mode of delivery of established cytotoxic drugs because there is evidence that this alters the relative concentrations in different tissues. Incorporation of drugs into liposomes for instance has been shown to improve the tumour specificity of methotrexate and bleomycin compared to the uptake into normal cells (Gregoriadis, 1976). Liposomes are very adaptable and can be altered not only in shape but in the lipid composition of the outer membrane and in the electric charge on the surface. It is possible to ensure their uptake by reticulo-endothelial cells if that is required or to ensure their access to the central nervous system by increased lipophilicity. Drugs such as methotrexate and 5-fluorouracil do not readily cross into the central nervous system and an attempt has been made to alter certain radicals on their molecules with the result that two important drugs DDMP and Ftorafur have emerged as useful additions to the drug armamentarium. The drawback of both of these drugs is indeed that they are so successful in a sense that their main toxicity is now on central nervous system rather than on marrow (Cox and Farmer, 1977).

Drug interactions are very important in combination chemotherapy and the use of non cytotoxic drugs along with cytotoxic agents is a possible development of note. Examples are fibrinolytic agents which theoretically might reduce the incidence of metastases, theophylline and its derivatives which in animal experiments increase the growth fraction of tumour cells, and enzyme inducing drugs such as barbiturates which improve the metabolism of drugs like cyclophosphamide to their active forms.

Another group of drugs called collectively the radiosensitisers are on trial in association with radiotherapy in lymphoma. The

principal effect of the drugs, the first of which was metronidazole is to improve the oxygen content of otherwise hypoxic cells in the centre of tumour masses to allow the radiation effect to be more complete. There are of course fast neutron sources available which will be of importance in the future because they do not depend on the oxygen effect which limits conventional radiation.

The other logical area for improvement would appear to be the general nutritional and immunological state of the patient. It seems sense to make sure that the patient is in optimal physical state before embarking on therapies which will certainly make his condition deteriorate at least temporarily. Hyperalimentation, restoration of serum albumin and even parenteral nutrition have been advocated as a preliminary to cytotoxic therapy in other solid tumours such as lung cancer (Dudrick et al, 1977). Similarly correction of immune deficiency would appear to be worthwhile in association with restoration of nutritional values. Several studies have indicated that patients who are immune deficient from the outset do less well than patients who are immuno-competent (Pouillart et al, 1976, and Orita et al, 1976) and this is a general inference of the present work.

The best methods of immune restoration however are far from clear. If blocking factors are present in the circulating blood, it would seem obvious to plasmapheresis patients as a first step. This is supported by the clinical results of Israel and Edelstein, 1978. They demonstrated successful removal of acute phase reactants and glycoproteins associated with tumour cell membranes by regular plasmapheresis for 2 weeks prior to any other therapy. They moreover were surprised to note seven partial regressions out of

23 patients with advanced malignant disease as an additional bonus to their study (Israel et al, 1977). These responses were measured before patients went on to receive chemotherapy which in 3 of the patients converted their partial response to complete responses. Bekesi et al, 1976, claim that neuraminidase treatment of leukaemic cells achieves the same function, that is removal of blocking factors from the exterior of tumour cells, while Hogan-Ryan and Fennelly, 1978, have shown in tissue culture that vitamin A alcohol is equivalent to neuraminidase in removal of sialic acid from tumour cell membranes.

The other problems are of hypogammaglobulinaemia which might be treated by giving specific gamma globulin and impaired monocyte function probably resulting in impaired lymphocyte function. A variety of agents have been used in experimental situations to achieve immune restoration. Levamisole, an anthelmintic has been shown to improve survival in stage III breast cancer treated by radiotherapy (Rojas et al, 1976) and Ramot et al, 1976, showed that it improved skin anergy in 10 patients with Hodgkin's disease in association with a rise in the number of E rosette forming cells (T lymphocytes). Levamisole however has side effects and particularly has been associated with agranulocytosis in some patients (Parkinson et al, 1977). This has been a severe problem in the treatment of Sjogren's syndrome as reported by Balint et al, 1977. This is in contrast to the claim that levamisole actually restores bone marrow after chemotherapy in a variety of patients with malignant disease (Lods et al, 1976).

C. parvum, and B.C.G. are non specific immunological stimulants which are mainly effective in improving marrow reserve and by stimulation of monocyte function (Oettingen, 1977). In addition

however, B.C.G. has been claimed to improve K cell numbers (K stands for killer lymphocyte), (Campbell and McLennan, 1976) and *C. parvum* has been associated with increase in circulating IgG levels (James et al, 1975). Monocyte chemotaxis is certainly improved by B.C.G. (Snyderman et al, 1977) and non specific cytotoxicity by macrophages in tissue culture systems is stimulated by either bacterial product (Evans et al, 1972, Germain et al, 1975). Few studies however have shown that immunotherapy alone is of value in treatment of lymphoma though delay in progression has been reported after B.C.G. vaccination by Sokal et al, 1974. The majority of studies indicate that the place of immunotherapy is alongside chemotherapy, both in animal work (Currie and Bagshawe 1970) and clinical studies (Frei and Schlossman, 1974).

Transfusion of macrophages has been suggested by Fakler et al, 1976, and indeed an extension of this might be the use of stem cells harvested from suitable donors by cell separator and transfused to patients after maximum chemotherapy. This treatment might have two effects - restoration of normal bone marrow and an improvement of immune status of the patient. Transfer of serum constituents is another possibility for the future. Transfer factor has been given to Hodgkin's patients (Ng et al, 1975, and Khan et al, 1975) without toxicity and with improvement in cell mediated immune responses and, coupled with a possible availability of interferon in the future, these might be sensible developments for restoring cells which hypothetically might have been impaired by viral damage.

In summary, there would appear to be many avenues of possible development in the management of lymphoma. The accent should be on improving selectivity of cytotoxic treatment, exploitation of

of interactions with non cytotoxic drugs and the restoration of a patient who is nutritionally and immunologically deficient prior to cytotoxic and inevitably immunosuppressive therapy.

CONCLUSIONS

1. Virus-like particles can be found in lymphoma cells on ultrastructural examination.
2. True clustering of lymphoma does occur though epidemiological study has many sources of error. Taken with other evidence of virus association with lymphoma, clustering points to an infective aetiology in lymphoma.
3. The reticulo-endothelial system does attempt a response to the presence of lymphoma cells. Chemotactic factors are present in lymphoma nodes for eosinophils, neutrophils and monocytes. In the intact tissue however, despite close contiguity of numerous host cells to tumour cells, no effective aggressor action is seen. In tissue culture of single cells however time-lapse cine indicates definite cytotoxicity following host-tumour cell contact.
4. Non-specific inhibitors of macrophage and lymphocyte function have been demonstrated in lymphoma culture supernatants and a Hodgkin's-associated pleural fluid.
5. Tumour release of such inhibitors (possibly constituents of normal cells, but present in increased concentrations in malignancy) might also explain defective monocyte chemotaxis, hypoalbuminaemia, low immunoglobulin M and low α_2 macroglobulin levels in peripheral blood from lymphoma patients. Weight loss and concurrent infections might similarly be explained by antimetabolic and immunosuppressive tumour-derived factors.
6. Poor survival in lymphomas is associated with pre-treatment hypoalbuminaemia, low IgM, low α_2 macroglobulin and a history

of weight loss and/or infections. Failure to achieve complete remission quickly after treatment is an independent pointer to poor survival. Stage of disease relates to survival.

7. Prediction of complete response is 75% accurate using a combination of normal IgM level and low age. Also of value are normal E. coli IgM antibody, albumin, and α_2 macroglobulin values.

8. Prediction of complications of treatment is most accurate by lymphocyte and neutrophil counts and levels of albumin, IgM, E. coli antibodies and α_2 macroglobulin. Chemotherapy and presence of disease are accompanied by a tendency to develop complications presumably due to associated immune deficiency.

9. The outcome of treatment of lymphoma with a combination of modalities after vigorous staging has significantly improved survival and quality of life.

10. Improved nutritional and immunological status should be a pre-requisite to cytotoxic therapy in the future.

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APPENDICES

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APPENDIX 1

Preparation of medium 199

From stock medium 10 ml of medium 199 was diluted with 96 ml of distilled water, 4 ml of Hepes buffer and 100 units (0.1 ml) of preservative free heparin. The pH was adjusted if necessary to 7 under aseptic conditions. Medium 199 was made up minus heparin as follows:

10 ml of concentrated medium 199 diluted with 96 ml of distilled water + 4 ml of Hepes buffer. The pH was checked and adjusted to 7.

APPENDIX 2

Composition of lactalbumin for lymph node cultures (Habeshaw, J.A., personal communication).

Dialysable Fraction of Lactalbumin Hydrolysate	4.7 g
Oyster Glycogen (BDH)	1.0 g
Sucrose	0.5 - 1.0 g
Polyvinyl Pyrrolidone (Koch Light)	0.75 g
L. Glutamine	20 mg
Calcium Pantothenate	10 mg
Distilled Water to Sterilise by Filtration	100 ml

The constituents, excluding sucrose were dissolved in warm water and allowed to cool. Sucrose was added until the resulting solution no longer caused sphering of fresh red cells. Polyvinyl Pyrrolidone enhanced the storage qualities of the medium but can either be omitted or replaced with salt poor human

serum albumin 0.65 g/100 ml. This supplement was used at concentrations of 10% in medium 199, and effectively supported cultures for periods in excess of 8 days.

APPENDIX 3

Preparation of Ficoll-Triosil

Solution A. Ficoll 9 g made up to 100 ml with distilled water was placed in a 56° water bath to effect solution.

Solution B. Triosil 56.5 ml was made up to 100 ml of distilled water to give a 56.5% solution of Triosil. The working solution of Ficoll-Triosil consisted of 24 parts of solution A to 10 parts of solution B. This was dispensed in universal containers in volumes of 12.5 ml sterilised by autoclaving at 15 lb for 10 min.

APPENDIX 4

Preparation of casein

Casein, BDH was prepared at a concentration of 10 mg/ml in glass distilled water (200 mg/20ml). In order to dissolve the casein the pH was raised to 11 with normal sodium hydroxide and immersed in a water bath at 56°C. the pH was then reduced to 7 using approximately 20 µl of phosphoric acid. Aliquots of 1 ml were frozen at -40°C and used within 2 months of storage.

To prepare working solution of casein a 1 ml aliquot was thawed and added to 9 ml of medium 199 (without heparin). An aliquot of this working solution was diluted with equal parts of the above medium 199.

Thus two working concentrations of casein were achieved equal to 1 mg/ml and 0.5 mg/ml.

APPENDIX 5

Peroxidase Stain

Solutions

1. Saturated ammonium chloride. 40 g saturated ammonium chloride was made up to 100 ml warm distilled water.
2. Ethyl diamine tetra-acetic acid. (EDTA) 5% of EDTA in distilled water was buffered to pH 6 with 2 normal sodium hydroxide.
3. Benzidine. A saturated aqueous solution of benzidine was obtained by mixing 50 mg of benzidine with 200 ml of distilled water at 80°C. The mixture was cooled and filtered and then stored in the refrigerator.
4. Hydrogen peroxidase. A 3% solution was prepared in distilled water.
5. Scarba red. Scarba red was prepared from 2% aniline water and 2 g of phenol made up to 100 ml of water. 1 g of neutral red was then added, mixed and filtered.

Methods

Staining procedure. Slides or coverslips containing cells were fixed, rinsed in saline and the following were added:

1. 0.5 ml of saturated ammonium chloride
2. 0.5 ml of EDTA
3. 4.5 ml benzidine solution (or distilled water for the control coverslip).

4. One drop of 3% hydrogen peroxidase.

The coverslip was incubated at 37°C for 30 min and then rinsed in saline. The coverslip was then dried and counterstained with scarba red for 5 sec and mounted in DEPX.

APPENDIX 6

Rye Classification of Hodgkin's Disease

Type

Lymphocyte predominance

Nodular sclerosis

Mixed cellularity

Lymphocyte depletion

APPENDIX 7

Histopathological Classification of the Non Hodgkin's Lymphomas
(Rappaport et al, 1956).

	<u>Nodular</u>		<u>Diffuse</u>
NH	Nodular histiocytic	DH	Diffuse histiocytic
NM	Nodular mixed histiocytic lymphocytic	DM	Diffuse mixed histiocytic lymphocytic
NLPD	Nodular lymphocytic poorly differentiated	DLPD	Diffuse lymphocytic poorly differentiated
NLWD	Nodular lymphocytic well differentiated	DLWD	Diffuse lymphocytic well differentiated
		DU	Diffuse undifferentiated

APPENDIX 8

Staging of Lymphoma

Stage I

Involvement of a single lymph node region (1) or of a single organ or site (I_E).

Stage II

Involvement of two or more lymph node regions on the same side of the diaphragm; or localised involvement of an extralymphatic organ or site and of one or more lymph node regions on the same side of the diaphragm (II_E).

Stage III

Involvement of lymph node regions on both sides of the diaphragm (III), which may also be accompanied by localised involvement of the spleen (III_S), and extralymphatic site (III_E) or both (III_{SE}).

Stage IV

Diffuse or disseminated involvement of one or more extralymphatic organs or tissues with or without associated lymph node enlargement.

APPENDIX 9

PUBLICATIONS

Monocyte Function in Cancer Patients

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Abstract—Monocyte function has been studied in patients with lymphoma and lung cancer. Ability of monocytes to phagocytose *Staphylococcus aureus* was not depressed before treatment but was lowered by cytotoxic drugs. Recovery was then seen after a rest period. Bactericidal capacity was significantly lower in lymphoma patients prior to treatment than in normal controls. Chemotherapy lowered the bactericidal indices further and there was little recovery in between courses of treatment. Monocyte chemotaxis was depressed in untreated patients with advanced lung cancer and in half of a group of nineteen lymphoma patients. The implications of these monocyte defects are discussed.

INTRODUCTION

MONOCYTE function is a credible requirement for an intact immune system. New roles in defence for the monocyte are projected almost weekly. Depressed monocyte function might be a predisposing factor in development of cancer. It might lead to inadequate containment of metastases and it might explain intercurrent infections frequent in tumour patients. As more aggressive treatment becomes the vogue, more patients develop serious immunodeficiency. It is no longer uncommon to witness resolution of a tumour such as a lymphoma followed by death due to overwhelming opportunist infection.

Peripheral blood monocytes have therefore been assessed for phagocytic and microbiocidal prowess in a group of lymphoma patients and appropriate controls before and after chemotherapy. A further group of lymphoma patients plus a small number of men with lung cancer have been tested for defects of monocyte chemotaxis.

MATERIAL AND METHODS

(1) *Cell preparation.* 20 mls of venous blood was collected into preservative-free heparin and separated on a Ficoll Hypaque column. The mononuclear cells collected from the gradient interface were washed, counted and adjusted to critical concentrations.

Phagocytosis and bactericidal capacity

Monolayers of 1×10^6 mononuclear cells

were grown in 30% AB serum and HEPES solution for 22 hr. At that time 20×10^6 bacteria (*Staphylococcus aureus*) were added to the culture and the supernatants sampled 20 min later. Duplicate monolayers were then washed and lysed in distilled water at 0, 10, 20, and 40 min. The supernatants and lysates were subcultured on mannitol salt agar. From the bacterial counts at 48 hr the phagocytic capacity of the monolayer is calculated as a percentage of bacteria engulfed in 20 min of culture. The bactericidal index is an expression of the rate of killing of *Staphylococcus aureus* over 40 min.

Chemotaxis

A modification of Boyden's millipore technique was used. The chemo-attractant, either cobra venom factor (2.5%) or casein 5 and 10%, was placed in the bottom of a culture vessel and separated from 0.5 million monocytes in medium 199 by a millipore filter (pore size 5 μ m). After incubation in a moist atmosphere at 37°C for 2 hr, the filters were removed, fixed in ethanol and stained with Giemsa solution. The distance that the "leading front" of monocytes had moved through the filter was measured in cultures containing medium 199 alone or medium 199 plus chemo-attractant. The first reading was subtracted from the second and expressed as the "distance migrated". In each experiment monocytes from a cancer patient were compared to those of an age, sex-matched control.

RESULTS

Phagocytosis

Eighteen patients were studied, each on several occasions. Before treatment patients' monocytes were as proficient as controls in phagocytosing *Staphylococcus aureus*. After one or more courses of combination chemotherapy (COP or MVPP)* phagocytosis was almost invariably inhibited. As shown in Fig. 1, five patients were studied serially: pre-treatment, on day 2 of chemotherapy and after two weeks clear of drug treatment. Depression of phagocytosis was seen while the patients were on treatment and some measure of recovery was seen prior to the next pulse of drugs.

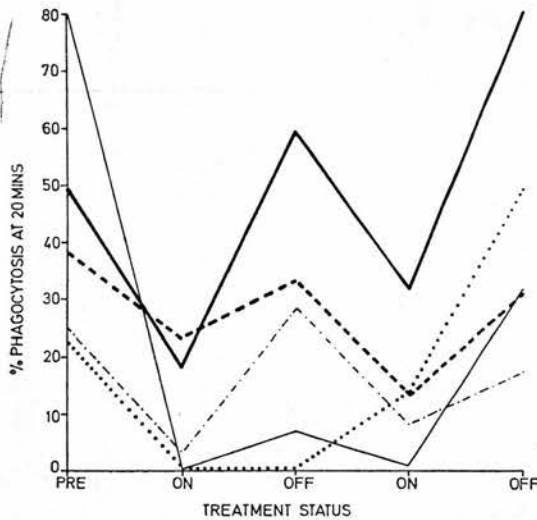


Fig. 1. Effect of cytotoxic drugs on monocyte phagocytosis.

Bactericidal index

Bactericidal capacity was estimated on over fifty occasions on eighteen patients. Their mean bactericidal index before treatment was 23 compared with the control value of 45. The difference was significant ($P < 0.05$). The bactericidal indices of the study group fell gradually below pre-treatment values when drug treatment was initiated and did not recover between cycles. Indeed, in two patients the bactericidal index remained low three months after treatment had been discontinued and the patients' lymphomas had entirely remitted. Although monocyte and total white

counts varied throughout treatment, fluctuations were shown to be independent of alterations in monocyte function.

Chemotaxis

A pilot study unrelated to the investigation of lymphoma patients above showed a marked chemotactic defect in monocytes from ten patients suffering from advanced lung cancer (Fig. 2). A group of nineteen lymphoma patients were therefore studied using the monocyte chemotaxis assay. None of the patients had

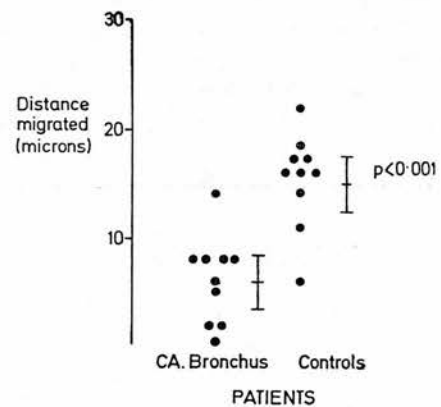


Fig. 2. Monocyte chemotaxis in carcinoma of the bronchus.

received any treatment. The results were not as clear cut as those in the lung cancer group. Ten patients' monocytes were significantly less mobile than age, sex-matched controls; in seven patients the distances migrated by the study monocytes were similar to those of controls and two patients' monocytes were superior to the normals. No obvious correlation was noted between impaired chemotactic ability and histological type or stage of disease. The chemotaxis assays have not yet been repeated after treatment.

DISCUSSION

Impairment of monocyte function has been demonstrated in eight patients, with myelomonocytic leukaemia and two lymphoma patients [1]. Whether the deficiencies reported here represent an intrinsic cell defect or a secondary effect of a circulating factor is not yet elucidated. "Immunosuppressive factors" have been postulated in association with cancer, and in one group of patients [2] a small molecular weight polypeptide has been detected

*COP = cyclophosphamide vincristine prednisolone. MVPP = mustine vinblastine procarbazine prednisolone.

which inhibits phyto-haemagglutinin-induced DNA synthesis in lymphocytes. The latter factor was correlated with anergy, a notable accompaniment of lymphoma, particularly Hodgkin's disease. The drug-induced suppression of monocyte phagocytosis is predictable but would appear to be reversible in contrast to the progressive impairment of bactericidal capacity. It would be more pertinent to measure those functions with a gram negative organism such as *Escherichia coli*, as the infections incurred by the patients studied were usually of this type. The bacterial assays were, however, extremely unpredictable when gram negatives were employed so *Staphylococcus aureus* was retained as a standard.

Depression of monocyte chemotaxis is known to occur during viral illnesses such as influenza [3], again in association with loss of delayed cutaneous hyper-sensitivity and possibly a tendency to super-infection. It may be that tumours are not contained because of a defect in monocyte motility. Reversal of such a phenomenon is one of the aims of non-specific immunotherapy. BCG, *C. parvum* and levamisole may increase the pool of monocytes and conceivably increase their motility. It remains to be seen whether these measures have any anti-tumour effect or whether they may lessen the incidence of secondary immunodeficiency and opportunist infections in cancer patients.

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Eosinophil chemotaxis of supernatants from cultured Hodgkin's lymph node cells

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Eosinophil chemotaxis of supernatants from cultured Hodgkin's lymph node cells

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SYNOPSIS Cultured lymph node cell supernatants from five out of six cases of Hodgkin's disease were relatively more chemotactic for peripheral blood eosinophils than neutrophils. In contrast, supernatants from two cases of lymphocytic lymphoma and four nodes showing reactive hyperplasia were chemotactic for neutrophils but had little eosinophil chemotactic activity. In most instances the degree of eosinophil infiltration observed histologically in the Hodgkin's lymph node correlated with elaboration of eosinophil chemotactic activity from the cultured cells. Following gel-filtration of three of the Hodgkin's lymph node supernatants, four peaks of eosinophil chemotactic activity were demonstrated in each case. One of these corresponded in molecular size to the previously described eosinophil chemotactic factor of anaphylaxis (ECF-A). It is suggested that the eosinophil chemotactic activity of cultured lymph node cell supernatants may be of value in the diagnosis and classification of Hodgkin's disease.

The histological diagnosis of Hodgkin's disease is made by the recognition of the characteristic Reed-Sternberg cell which is a large multinucleate cell containing two or more nuclei with prominent nucleoli (Butler, 1970). Other morphological features of the lymph nodes are variable, and this has led to several attempts at classification with the object of relating the histology to clinical staging and prognosis (Jackson and Parker, 1944a; Jackson and Parker, 1944b; Lukes and Butler, 1966; Smithers, 1967; Smithers, 1970; Dorfman, 1972). One of the characteristics of the Hodgkin's lymph node is the presence of eosinophil leucocytes (Dreschfeld, 1892), and it has been suggested that this may represent part of the inflammatory infiltrate (Stuart, 1970), the eosinophil accumulation in the nodes being a result of chemotaxis (Vianna *et al.*, 1971). In the present study we provide evidence to support this concept by showing that supernatants from cultured Hodgkin's lymph nodes attract a greater proportion of eosinophils from mixed leucocyte populations than do supernatants from cultures of non-Hodgkin's lymph nodes.

Material and Methods

PREPARATION OF LYMPH NODE CELL CULTURE SUPERNATANTS

Lymph nodes obtained at surgery were bisected. One-half was taken for routine histology and the other for cell culture as described (Habeshaw, 1972). Cells for culture were obtained by carefully dividing the tissue into small fragments with scalpel blades, gently homogenizing and passing through a sieve to remove fibrous debris. Cell counts were adjusted to 5×10^6 cells/ml and the viability was assessed by Trypan blue exclusion and phase contrast microscopy. Only those preparations which contained greater than 90% of viable cells were subsequently cultured. Cultures were set up in 'flying cover slips' in glass test tubes, and the cells were contained in 1 ml volumes of medium 199 with 0.5% lactalbumin, 50 units of penicillin, and 5 μ g of streptomycin. The tubes were incubated at 37°C and the supernatants removed at day 3, replaced with culture medium, and removed again at day 5. Supernatants thus obtained were centrifuged to remove particulate material, pooled and dialysed for 24 hours against phosphate buffered saline (PBS), and lyophilized. Before chemotaxis the material

was reconstituted in distilled water and the protein concentrations were measured by the Folin-Ciocalteu method.

MEASUREMENT OF CHEMOTAXIS

Chemotaxis of human peripheral blood eosinophils and neutrophils was assayed by a modification of the Millipore technique of Boyden as previously described (Kay, 1970). Cell counts were performed in duplicate or triplicate. A chemotactic index to show the relative proportion of eosinophils migrating was calculated from the equation:

$$\frac{\text{Eosinophil chemotactic count/}}{\text{Neutrophil chemotactic count}}$$

$$\frac{\% \text{ eosinophils in cell suspension before migration/}}{\% \text{ neutrophils in cell suspension before migration}}$$

GEL FILTRATION

Hodgkin's lymph node cell supernatants were concentrated by rotary evaporation at 37°C under vacuum. Samples of 1 ml containing approximately 11.5 mg/ml of protein were applied to a column of Sephadex G-50 (104 × 2.5 cm) in PBS. From alternate 2 ml fractions 0.5 ml volumes were taken and tested in duplicate for neutrophil and eosinophil chemotaxis.

Results

Chemotactic activity from cultured lymph node cell supernatants was demonstrable in a dose-dependent fashion as shown in fig 1 for cultures from a Hodgkin's and a reactive lymph node cell supernatant.

The chemotactic activity of lymph node cell supernatants from six patients with Hodgkin's disease, two with lymphocytic lymphoma, and four

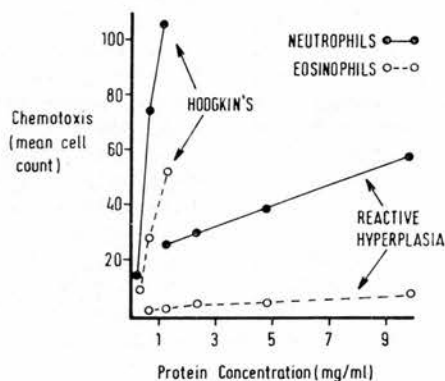


Fig 1 Neutrophil and eosinophil chemotactic activity of a Hodgkin's and reactive hyperplasia cultured lymph node cell supernatant.

nodes showing reactive hyperplasia are shown (fig 2). Neutrophil and eosinophil chemotaxis was demonstrable with all supernatants but five out of the six Hodgkin's supernatants gave greater eosinophil chemotactic indices than the highest index obtained with non-Hodgkin's supernatants.

The degree of eosinophil infiltration in the lymph nodes, arbitrarily assessed 0 to ++, to some extent correlated with the eosinophil chemotactic activity from the cell culture supernatants (fig 2). However, one Hodgkin's node had no eosinophils, and eosinophil infiltration was present in one of the non-Hodgkin's nodes.

When the chemotactic gradient was eliminated by placing supernatants from either Hodgkin's or non-Hodgkin's lymph nodes in both the test and cell compartments of the chemotactic chambers little or no migration was observed for either cell type. This suggests that the leucotaxis was a result of directional, rather than random, migration.

When concentrated cell supernatants from three Hodgkin's lymph nodes were applied to a column of Sephadex G-50 four peaks of eosinophil chemotactic

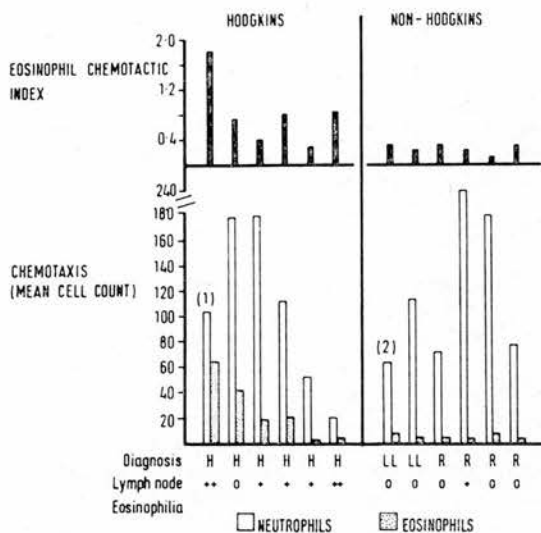


Fig 2 Neutrophil and eosinophil chemotaxis and the eosinophil chemotactic index from Hodgkin's and non-Hodgkin's cultured lymph node cell supernatants. The degree of lymph node eosinophil infiltration is also indicated: H = Hodgkin's disease, LL = lymphocytic lymphoma, R = reactive hyperplasia. The measurements represent the pooled results from three experiments except in (1) and (2) where there was sufficient sample for only one and two estimations respectively. All samples were tested at a protein concentration of 0.6 mg/ml.

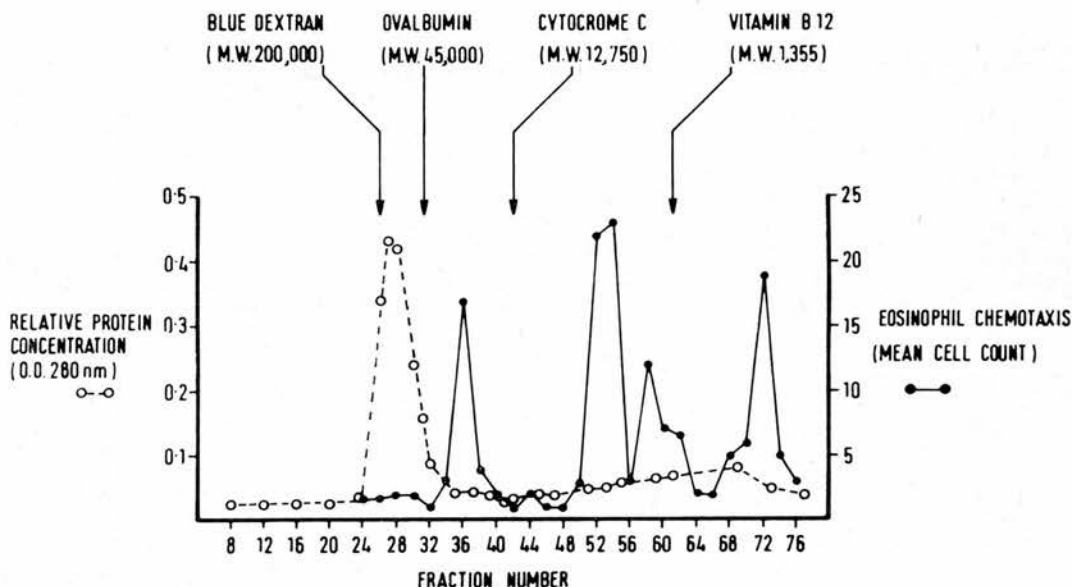


Fig 3 Sephadex G-50 chromatography of a cultured lymph node cell supernatant from Hodgkin's disease. The column was previously calibrated with markers having the molecular sizes indicated.

activity were consistently demonstrable (fig 3). The molecular markers indicated that these four activities were associated with molecules having an approximate molecular size of 30 000, 6000, 2000, and 500 daltons respectively (fig 3).

Discussion

The capacity of human peripheral blood lymphocytes to release chemotactic factors for neutrophils and monocytes following stimulation by mitogens and specific antigen has been reported by a number of workers (Ward *et al*, 1969; Altman *et al*, 1973). In addition, an eosinophil chemotactic factor from lymphocyte supernatants has also been reported, but in this system there was an additional requirement for antigen-antibody complexes (Cohen and Ward, 1971). In the present study we have demonstrated the elaboration of neutrophil and eosinophil chemotactic agents from cultured lymph node cell supernatants but in addition have shown that Hodgkin's lymph node preparations attract relatively more eosinophils than the non-Hodgkin's supernatants (figs 1 and 2).

Care was taken to use the same protein concentrations when making comparisons between the activity of the cell supernatants. A dose-response effect was obtained with each supernatant as well as the ones depicted in figure 1.

Although relatively more eosinophil than neutrophil chemotactic activity was observed in a node in which histologically there were no demonstrable eosinophils, there was in general an association between lymph node eosinophilia and the elaboration of eosinophil chemotactic activity. More direct evidence would be possible by performing these measurements on several nodes from individual patients.

Several peaks of activity were obtained following chromatography on Sephadex G-50, suggesting that there is a heterogeneity of eosinophil chemotactic factors from Hodgkin's lymph node cell supernatants (fig 3). One peak corresponded to a molecular size of the eosinophil chemotactic factor of anaphylaxis (ECF-A) (Kay and Austen, 1971; Kay *et al*, 1971). In a previous report a substance of compatible molecular size was extracted from an undifferentiated carcinoma of lung associated with a peripheral blood eosinophilia (Wasserman *et al*, 1974).

The finding that Hodgkin's lymph node cell supernatants attract relatively more eosinophils than non-Hodgkin's cultures suggests that this may be of value both in diagnosis and in the classification of various forms of the disease.

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Appraisal of Case Clustering Data in Lymphoma

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Summary

An epidemiological study of case clustering in three types of lymphoma (Hodgkin's disease, histiocytic lymphoma and lymphosarcoma) using computer data from the Information Services Division of the Scottish Health Service Common Services Agency, revealed many geographical areas in which the case incidence was markedly raised. Further investigation confirmed the presence of only one of those clusters due to numerous errors in the data source. These errors resulted from inaccurate input of information into the computer and may explain some of the conflicting reports on case clustering in the literature.

Introduction

There has been a flurry of anecdotal reports of case clusters in Hodgkin's disease over the last five years. These have resulted in much speculation amongst physicians and their patients about the possible infectious nature of Hodgkin's disease, other lymphomas and indeed leukaemia. Virologists have found complexes of reverse transcriptase and high molecular weight RNA in human lymphoma tissues but have not proved viral carcinogenesis (Chezzi *et al.*, 1976). Furthermore, studies on unselected populations have failed to confirm the statistical validity of space time clustering in these conditions (Glass *et al.* 1968). There is also a lack of studies in depth of stable populations over a prolonged time interval. The importance of this is that the incubation period of the 'infectious agent' has been claimed to be from two months (Wagener and Hannan, 1975) up to twelve years (Vianna and Polan, 1973). An epidemiological study of Hodgkin's disease of lymphosarcoma and histiocytic lymphoma was instituted in Edinburgh for the period 1961-71. Clusters were sought both in time and according to the area of residence defined by electoral ward.

Methods

Edinburgh before local government reorganisation (1975), was conveniently divided into 23 electoral wards of population between 9,000 and 37,000. The information for the study was obtained from Scottish Health Service case listings for all patients in the Edinburgh area between 1961-71 coded for disease by the International Coding System.

The information obtained which consisted of the patient's identification number, his or her electoral ward (1 to 23) and the year in which the disease was diagnosed was then computed. This information was further screened to delete duplicated cases. The incidence of the disease in each ward each year was calculated from the number of cases

and population statistics obtained from the 1961 and 1971 census. Where the incidence of the disease was high in any area the computer plotted an incidence/time graph, superimposed on a graph of the average values for comparison.

From the graphical output seven suspected clusters of Hodgkin's disease, four of histiocytic lymphoma and three of lymphosarcoma were found. The case notes of all patients in each cluster were scanned to verify the information obtained from the Scottish Home and Health Department statistics. Eleven hospitals provided the case notes.

The address of each patient was plotted on a map of Edinburgh divided into electoral wards. This allowed the recognition of clusters across ward boundaries.

Results

Of the 107 case notes sought 93 were recovered, a retrieval rate of 87%. Of these 93 case notes only 47 people emerged who fulfilled the initial requirements of their selection. This 49% rejection rate was due to inaccuracies in electoral ward, disease type and duplication of hospital registration numbers found in the Scottish Home and Health Department statistics.

At the beginning of 1968 a new medical records system at the Edinburgh Royal Infirmary was introduced whereby the date of birth of the patient became his hospital number. This new system did not succeed entirely as was shown by the duplication of the hospital numbers which still appeared after its introduction.

The major cluster contained 31 patients diagnosed as having lymphosarcoma and reticulum cell sarcoma in the adjacent wards of St Andrews/St Giles/George Square between 1966 and 1968. On obtaining the relevant case notes it was found that eight patients from one hospital had been registered correctly by area but wrongly by disease. The correct diagnosis in each case was tuberculosis and not non-Hodgkin's lymphoma. The international codes (7th Revision of ICD) for these two conditions are 012-019 and 200.2 so confusion is not likely. Some other error must have been reproduced eight times in successive cases.

Such an error could well result from a misunderstanding introduced by the coding doctor. It was also found that a patient had been admitted to one hospital six times, each time with a different patient number giving the impression of multiple identity. Though four of these admissions were in 1968 the patient number never co-incided with

the date of birth. Six other sets of case notes were irrelevant to the study either for the above two reasons or because they were registered in the wrong electoral ward. This left a total of 11 people who had been correctly registered in their case notes. The true incidence of the disease in the area has now fallen to a level that no longer fulfilled the criteria for further investigation as a cluster. Figure 1 shows the case incidence of the disease as shown by Scottish Health Service statistics (A) and after further investigation (B).

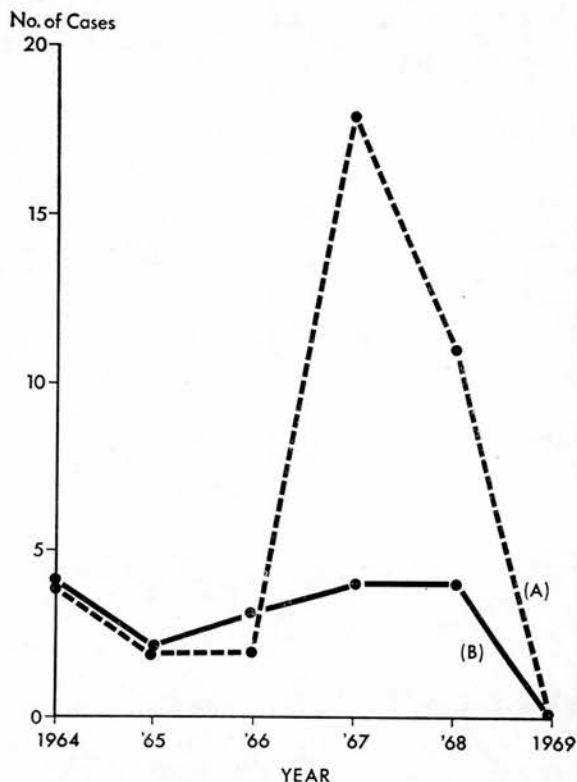


Figure 1. Case incidence of lymphosarcoma and reticulum cell sarcoma in St Andrews/St Giles/George Square 1964-69

- A From Scottish Home and Health Department case listings
- B From patients' case notes

Of the remaining clusters all but one have disappeared in a similar manner to that explained above. The only cluster that has remained after case note study is one that overlaps the Gorgie-Dalry Merchiston wards between 1967-71. Eight cases of Hodgkin's disease were diagnosed within a radius of one-third mile from a central point, the approximate population of this area being 10,000. Further information concerning social contact and possible family links between eight cases was not obtainable from the case

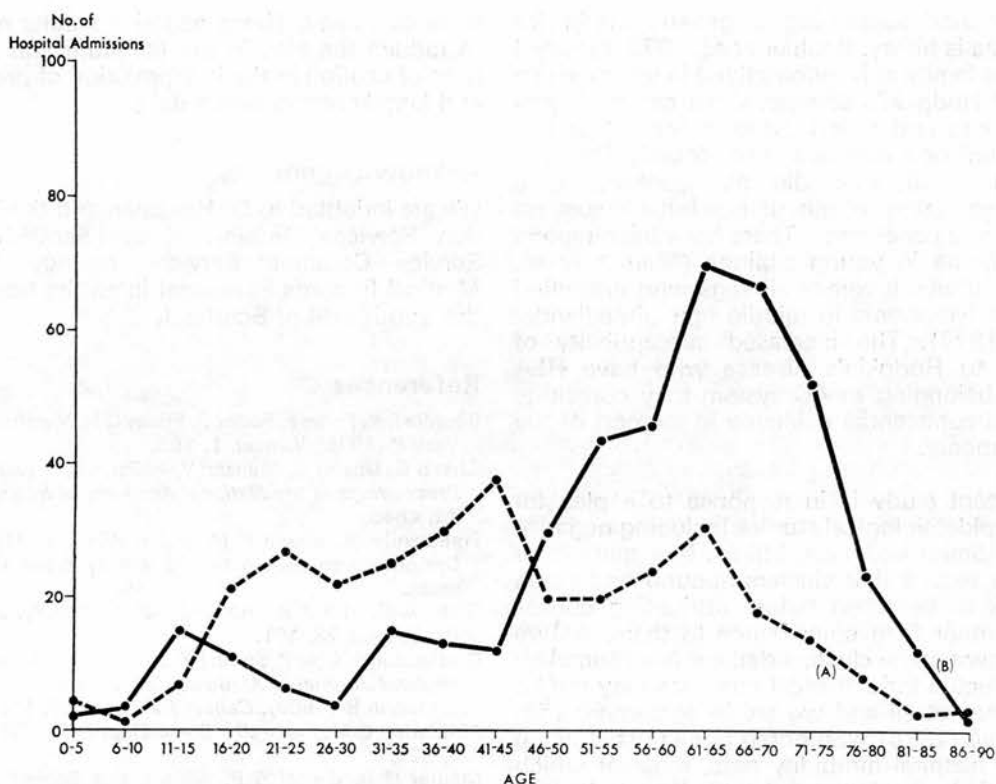


Figure 2. Age specific admission rate in Edinburgh 1961-71 for:

- A Hodgkin's disease
B Non-Hodgkin's lymphoma

notes. The maiden names of married women were not mentioned in the case notes and information concerning family histories did not reveal any occurrence of the disease in other family members. Since most of the eight patients had died personal interviews to obtain this information were not possible.

The age specific incidence of Hodgkin's disease and non-Hodgkin's lymphoma was plotted (Figure 2) and these figures broadly concur with MacMahon, 1966, indicating that the computer is accurate enough for detecting coarse trends. The bimodal distribution of Hodgkin's disease contrasts strikingly with the steep rise with age of onset of non-Hodgkin's lymphoma.

Discussion

At present the state of the debate on whether Hodgkin's disease and other lymphomas are of genetic or environmental origin is inconclusive. The infection hypothesis was mooted from the suggestion that tonsillectomy may predispose to Hodgkin's disease. Vianna (1976) has concluded

from sibling studies that this is not proven. The same author was responsible for the report of an 'outbreak' of Hodgkin's disease in a high school in Albany (Vianna *et al.*, 1971). Amongst the anecdotal reports was one which claimed an increase of mortality from Hodgkin's disease amongst physicians (Vianna *et al.*, 1974). A British survey by Smith *et al.* (1974) supported by another American study (Matanoski *et al.*, 1975) have produced convincing evidence to the contrary. An interesting study of siblings is reported by Grufferman *et al.* (1977) combining their data from a survey of Greater Boston with literature experience. They conclude that there is a two-fold increase in risk of Hodgkin's disease amongst siblings provided the siblings are of the same sex. They explain this by person to person contact which might be more prolonged or more intimate amongst siblings of the same sex who might for instance share a bed or bedroom.

They were discussing 51 sibling pairs which is not a large number and may be an underestimate of the true situation. It is therefore not possible to rule out other environmental factors or indeed, genetic factors.

The evidence supporting a genetic origin for lymphoma is flimsy. Beuhler *et al.*, 1975, reported an inbred family in Newfoundland in which seven cases of Hodgkin's disease, three cases of lymphosarcoma and seven other malignant or immunodeficiency diseases were found. The distribution of diseases did not conform to a Mendelian pattern of inheritance but did suggest limited gene penetrance. There have been reports of lymphoma in young siblings (Maurer *et al.*, 1976) and also a pair of siblings who presented with gut lymphoma in middle age (Freedlander *et al.*, 1977). The increased susceptibility of patients to Hodgkin's disease who have HLA antigen belonging to 4C system may constitute further circumstantial evidence in support of the genetic theory.

The present study is in response to a plea for further epidemiological studies including negative studies (Smith and Pike, 1976). It is quite clear from our results that clusters abound and much care has to be taken before attributing consequence other than coincidence to them. As we have shown some clusters derived from summary data collected for statistical purposes may not be lymphomas at all and we are in agreement with Patel *et al.* (1976) who noted numerous errors in Scottish hospital morbidity data, most of which were entirely the fault of the medical staff. It is important to point out that many of the published reports of clusters in lymphoma have derived their information from tumour registers or from computerised data such as that provided by the Scottish Health Service for this study.

Personal interviews are obviously the best way to go about such a study but this would require to be done prospectively and in a controlled fashion using a large unselected population. Although the present study unearthed one cluster, we fully appreciate that it may be a coincidence and we place more stress on the numerous clusters which

were dissolved. These negative results may help to reduce the bias in the literature and sound a note of caution in the interpretation of preliminary and largely retrospective data.

Acknowledgements

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MONOCYTE CHEMOTAXIS IN BRONCHIAL CARCINOMA AND CIGARETTE SMOKERS

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Summary.—Chemotaxis of blood monocytes was measured in 31 patients with bronchial carcinoma and 19 cigarette smokers. Thirteen patients with metastatic bronchial carcinoma had significantly less ($P < 0.005$) chemotactic response than matched controls. Those with disease confined to the chest, or with recurrent or operable bronchial carcinoma, had no significant depression of monocyte chemotaxis. There was also no significant difference in monocyte chemotaxis between cigarette smokers and matched controls. These results support the concept that in human cancer there is a defect in monocyte chemotaxis, but in bronchial carcinoma significant depression was only apparent in those with advanced disease.

THE role of cells of the mononuclear phagocytic series in immune surveillance has been suggested by a number of workers (Hibbs, Lambert and Remington, 1972; Alexander, 1976). For example, an inhibitor of macrophage chemotaxis produced by various transplanted tumours in mice has been described (Snyderman and Pike, 1976) and in man the capacity of peripheral blood monocytes to respond by chemotaxis *in vitro* was depressed in patients with genito-urinary neoplasms (Hausman *et al.*, 1975), malignant melanoma (Rubin, Cosimi and Goetzl, 1976) and other human cancers (Boetcher and Leonard, 1974). We have studied monocyte chemotaxis from 31 patients with bronchial carcinoma at various clinical stages, and also the monocyte chemotactic response of cigarette smokers who are known to be at risk for developing bronchial neoplasms.

PATIENTS AND CONTROLS

Patients with bronchial carcinoma were classified according to the stage of their

disease. Those with small tumours deemed suitable for surgical resection were termed *operable*. Disease which reappeared locally at the site of a surgical resection was termed *recurrent*. Disease *confined to the chest* had spread locally from the primary site in the bronchus to involve surrounding lung, local lymph nodes and chest wall. The *metastatic* group had deposits of tumour outside the chest, commonly in liver or bone, as demonstrated clinically, or by radionuclide scanning.

Controls for the cancer groups were all convalescent, hospitalized patients who had sustained either myocardial infarctions or respiratory infections and in whom there was no evidence of malignant disease. Controls for cigarette smokers were all healthy, non-smoking volunteers.

MATERIALS AND METHODS

Human peripheral-blood monocytes were separated on a Ficoll-Triosil gradient as previously described (Böyum, 1968). Chemotaxis was quantified either by the "leading front" method using Millipore filters (Millipore Co., Wembley) of 8 μm pore size (Zigmond and Hirsch, 1973) or by the method of Snyderman *et al.*, (1972) employing

Nucleopore filters and polycarbonate "Boyden chambers" Neuroprobe, Bethesda, Maryland, U.S.A.). The only modification was that the suspending medium for monocytes and for dilutions of chemoattractant was Medium 199 containing 30 mM Hepes buffer. The chemoattractant was either human serum in which the complement system had been activated with purified cobra venom factor (CVF) (Ballou and Cochrane, 1969) or solutions of casein (British Drug Houses). Casein was used in the early part of the study, but its chemoattracting properties often deteriorated after a few days, even under a variety of storage conditions. The experiments with casein reported here are with freshly prepared material. In contrast, serum activated with CVF was divided into portions after preparation and stored at -80°C until use.

The chemotactic responses from patients with bronchial carcinoma or cigarette smokers were compared with age- and sex-matched controls and each pair was performed on the same day under the same experimental conditions. A three-point dose-response of chemoattractant was performed for each experiment. Optimal monocyte migration of cells, either from patients or smokers, was achieved with 0.5 mg/ml of fresh casein or 2.5% CVF-activated serum. Each assay was performed in duplicate, and measurements from each filter were the pooled results from 10 random high-power fields. The test and control samples were analysed by the Wilcoxon test of paired differences. The variation between duplicate filters was $\pm 15\%$ as previously described (Turnbull and Kay, 1976; Turnbull, Evans and Kay, 1977).

RESULTS

Bronchial carcinoma

The clinical staging and histology of the 31 patients with bronchial carcinoma are shown in Table I. Apart from one individual, all patients were male, and were matched with controls within 10 years of their age. The monocyte chemotactic response of patients with metastatic disease, and their respective controls, are shown in Table I, together with the histology and the treatment being received either at the time of sampling or before the chemotactic assay. There was a significantly greater depression in the

TABLE I.—*Clinical Staging and Predominant Histology of the 31 Patients Studied with Bronchial Carcinoma*

Clinical stage	
Metastatic	13
Confined to chest	12
Recurrent	2
Operable	4
Histology	
Anaplastic	6
Squamous	10
Oat cell	5
Adenocarcinoma	1
Unknown	9

monocyte chemotactic response in the metastatic group ($P < 0.005$) than in their respective controls. No statistical difference was observed with patients with disease confined to the chest, recurrent cancer or operable disease (Table III, Fig.). It is unlikely that the observed effect with the metastatic group was a result of medication. Three of the 13 were receiving antibiotics and one had treatment with prednisolone, but most of the patients were receiving no treatment at the time of the chemotactic test. The two patients receiving prednisolone in the group with disease confined to the chest had higher chemotactic responses than the control, whereas the one in the metastatic group having corticosteroids had a lower chemotactic response.

In the Fig. the results are expressed as the percent migration of each patient's monocytes as compared to their respective matched control. With the patients as a whole there was a wide scatter; however, the metastatic group responded significantly less in monocyte chemotaxis. The one patient who gave a high response had a pulmonary infection with a white-cell count of $17,000/\mu\text{l}$. There was no significant difference between patients and controls in the other groups, although with operable and recurrent cancer the numbers were very small.

Cigarette smokers

The monocyte chemotactic response of 19 male cigarette smokers, compared with

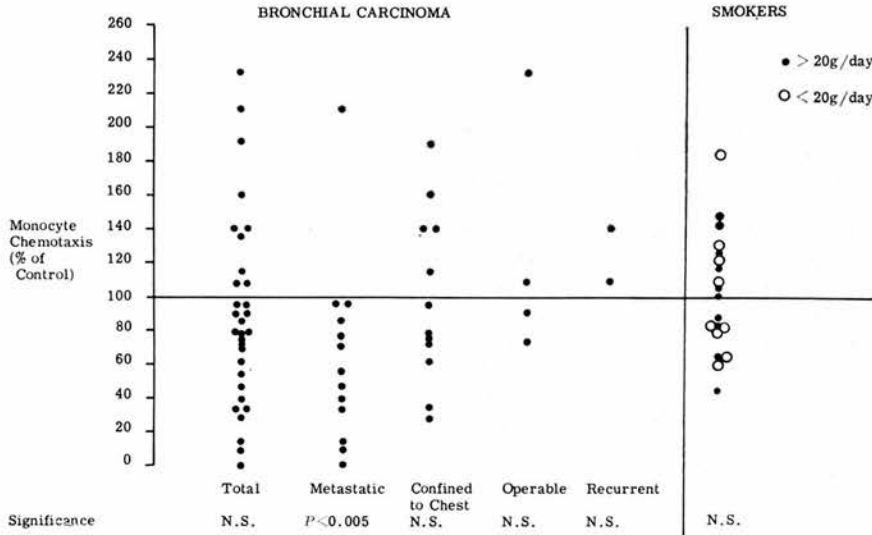


FIG.—The monocyte chemotactic response of patients with various clinical stages of bronchial carcinoma, and cigarette smokers, expressed as the percent migration of each patient's monocytes compared to its matched control. NS = not significant.

TABLE II.—*The Histology, Treatment and Monocyte Chemotactic Response of Patients with Metastatic Bronchial Carcinoma. Controls were Matched for Age and Sex*

Pt. No.	Age (Years)		Histology	Prior treatment (interval between end of treatment and chemotaxis test)	Medication at time of test	Monocyte chemotaxis (distance migrated in μm)	
	Pt.	Control				Pt.	Control
1	67	69	Unknown	—	—	19.7	26.8
2	62	61	Anaplastic	—	—	35.9	17.2
3	55	59	Oat cell	PR (22 months)	Prednisolone	7.6	13.3
4	66	61	Unknown	—	Ampicillin	2.0	16.0
5	54	60	Unknown	—	—	8.0	17.0
6	85	75	Unknown	—	—	2.0	18.0
7	73	73	Unknown	—	Ampicillin	0.5	17.0
8	78	71	Unknown	PR (3 years)	—	8.3	23.7
9	56	55	Oat cell	—	—	26.4	33.5
10	61	55	Oat cell	—	—	13.4	33.5
11	53	53	Anaplastic	PR (1 day)	—	53.6	59.0
12	69	65	Squamous	—	Ampicillin	15.0	17.6
13	65	65	Squamous	—	—	46.4	49.7

PR = palliative radiotherapy. The chemoattractant was complement-activated serum (2.5%) in Patients 1 to 10 and casein (0.5 mg/ml) in Patients 11 to 13.

non-smoking controls, is shown in Table IV and the Fig. There was no significant difference between the two groups as a whole, nor when the smokers were divided into those who smoked more or less than 20 g per day.

DISCUSSION

Our results support previous findings on depressed monocyte chemotactic responses in various human cancers (Hausman *et al.*,

1975; Rubin *et al.*, 1976; Boetcher and Leonard, 1974). In the present study on bronchial carcinoma, only those patients with metastatic disease showed a significant depression (Table II, Fig.). Although this may have been a non-specific effect due to general debilitation it was unlikely to be the result of treatment. Many of the patients were receiving no medication at the time of sampling and had not received prior chemotherapy or radiotherapy

TABLE III.—*The Histology, Treatment and Monocyte Chemotactic Response of Patients with Bronchial Carcinoma that was Confined to the Chest, Recurrent or Operable. Controls were Matched for Age and Sex*

Pt. No.	Age (years)		Histology	Prior treatment (interval since end of treatment until chemotaxis test)	Medication at time of test	Monocyte chemotaxis (distance migrated in μm)	
	Pt.	Control				Pt.	Control
1	61	69	Anaplastic	Confined to chest PR (20 weeks)	Ampicillin, Prednisolone	24.0	12.7
2	68	65	Squamous	—	Oxytetracycline	13.9	18.4
3	62	66	Anaplastic	—	Ampicillin	14.0	50.2
4	61	63	Squamous	—	—	16.1	17.2
5	60	62	Anaplastic	PR (24 weeks)	—	17.1	48.1
6	64	65	Unknown	—	Prednisolone	20.7	12.9
7	51	51	Oat cell	—	—	21.1	15.2
8♀	54	55	Adenocarcinoma	—	—	25.9	33.5
9	67	58	Oat cell	—	—	33.0	23.6
10	80	80	Squamous	PR (23 days)	—	37.8	59.8
11	68	65	Unknown	Chemotherapy (16 weeks)	—	36.4	46.9
12	57	57	Squamous	Chemotherapy (6 weeks) Recurrent	—	58.6	50.2
13	82	81	Unknown	—	Oxytetracycline	42.5	30.6
14	53	55	Squamous	—	—	20.0	18.4
15	65	61	Squamous	Operable	—	18.8	17.2
16	50	48	Squamous	—	—	8.0	11.0
17	57	54	Anaplastic	Post-surgery (3 weeks)	Ampicillin	28.0	31.0
18	68	65	Squamous	—	Ampicillin	28.8	12.3

PR = palliative radiotherapy. The chemoattractant was casein (0.5 mg/ml) in Patients and controls 9 to 12 and 18. In the others it was complement-activated serum (2.5%).

TABLE IV.—*The Monocyte Chemotactic Response of 19 Male Cigarette Smokers Compared with Non-smoking Male Controls*

Age (years)		Tobacco smoked/day (g)	No. of years smoking	Monocyte chemotaxis (mean cell count)	
Smoker	Control			Smoker	Control
56	63	60	41	49	76
62	51	60	45	45	53
49	42	53	31	65	61
42	33	40	17	76	53
40	55	36	19	66	74
52	64	30	38	116	90
49	31	30	35	17	38
58	44	26	40	*46	31
64	60	23	47	57	48
27	22	21	8	82	81
35	34	20	16	43	70
48	54	20	35	76	95
32	28	20	14	37	28
25	27	20	8	84	76
47	34	20	23	84	66
28	23	16	10	134	72
54	55	15	36	65	100
29	29	29	5	75	91
53	49	14	28	50	59

The chemoattractant was 2.5% CVF-activated serum, with the exception of the pair indicated (*) in which the concentration was 1.25%.

(Tables II and III). In a similar study on malignant melanoma (Rubin *et al.*, 1976), only patients with advanced disease had a monocyte defect.

The inhibitor of macrophage chemotaxis produced by various transplanted neoplasms in the peritoneal cavity of mice was partially identified as a protein of mol. wt. 6000-10,000 (Snyderman and Pike, 1976). A similar inhibitor is possibly elaborated from human neoplasms and if it is related to tumour mass this may account for the effect observed in the metastatic group in the present study.

A recent leading article in the *Lancet* (1976), discussing the possible role of macrophages in tumour surveillance, emphasized the present difficulties in relating *in vitro* data from man and experimental animals to the clinical situation. Nevertheless, if tumour-derived material with inhibitory effects on monocyte function can be demonstrated, this may provide some additional evidence to support the concept that tumour products overcome possible tumoricidal effects of mononuclear phagocytes. Experiments currently in progress suggest that extracts of human tumours may inhibit the chemotactic response of normal blood monocytes (Abell, C. and Kay, A. B., unpublished).

Cigarette smokers showed no difference from controls in their monocyte migratory capacity, indicating that monocyte chemotaxis will not be useful in detecting individuals at risk for developing bronchial carcinoma (Table IV). Studies with human alveolar macrophages obtained from smoking and non-smoking volunteers demonstrated both an increase in the number of cells recovered by bronchial lavage and of the chemotactic response of these cells from smokers when compared to controls (Warr and Martin, 1974). This suggests that cigarette smoke probably has an initial non-specific "macrophage-activating effect" analogous to the influx of macrophages into tissues treated with various irritants such as mineral oil and glycogen.

The chemotactic activity of human serum activated with cobra venom factor is due almost entirely to the fragment cleaved from the 5th component of complement (C5a) liberated as a result of activation of the alternate pathway. When CVF-activated serum is placed on either side of the micropore chamber, migration is either minimal or absent, suggesting that this agent evokes chemotaxis, (i.e. directional migration) rather than random migration (Kay, unpublished).

There are difficulties in employing the chemotactic assay for clinical studies. The reasons include possible deterioration of the chemoattractant during storage, variations in an individual's cell response with time, and failure to reproduce this biological assay exactly on each occasion. In the present study these difficulties were largely overcome by matching each patient or smoker with a control individual for age and sex, withdrawing blood from each pair at the same time and performing the test under identical conditions. Comparison of these matched pairs by the Wilcoxon test of paired differences allowed statistical analysis.

Thus the present study suggests that defects in monocyte chemotaxis are only apparent at advanced stages of bronchial carcinoma, and not in those with relatively confined disease or in those individuals who are at risk for developing bronchial neoplasms.

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Extramedullary Plasmacytoma of Stomach

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A single case of gastric plasmacytoma showing unusual clinical and pathological features is described. The patient had gluten sensitive enteropathy, and showed increased circulating IgA levels prior to gastrectomy. Progression of the disease was associated with both K and L light chain proteinuria, hypoalbuminaemia, and vasopressin resistant polyuria. Pathological investigation revealed the coexistence of IgA secreting soft tissue plasmacytoma, with IgG secreting myeloma. The significance of these findings is discussed.

Key words: myelomatosis – plasmacytoma – immunoglobulin A (IgA) – malabsorption syndrome – vasopressin resistant polyuria

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Of all extramedullary plasmacytomas the majority occur in the submucosa of the upper air passages, less than 10 % occurring as primary neoplasms in the gastrointestinal tract (Wiltshaw 1971).

We report here a plasmacytoma of the stomach associated with gluten-sensitive enteropathy and diffuse, systematic myelomatosis of differing clonal derivation.

CASE HISTORY

(Mr. J.M. – Date of birth Oct. 19, 1926)

In 1967 the patient sustained a right hemiplegia related to a left carotid artery stenosis, followed by good recovery of function and he was subsequently given a two-year course of anticoagulants.

In 1969 he was diagnosed as having gluten-sensitive enteropathy with steatorrhea and total villous atrophy on jejunal biopsy; total remission of symptoms followed a gluten-free diet and vitamin supplements.

In Dec. 1971 an osteolytic lesion was demonstrated in the upper end of the left femur following pain in the left thigh. This lesion was presumed to be a metastasis from an undiagnosed primary tumour and was treated in April 1972 by local radiotherapy (to a total of 2,200 rads) followed by a rapid relief of symptoms and marked subsequent radiological improvement.

In July 1972 the patient was admitted for the investigation of persistent iron deficiency anaemia and because the stools were persistently positive for blood. A barium meal and endoscopy showed an infiltrative lesion in the region of the pyloric antrum. At this time there was a marked rise in

TABLE I
Levels of serum immunoglobulin during the course of the disease

	Date		
	May 31, 1972 (prior to removal of gastric primary)	Aug. 25, 1972 (after removal of gastric primary)	Febr. 14, 1973 (recurrence)
IgG	880 mg/100 ml	1,700 mg/100 ml	2,100 mg/100 ml
IgA	2,375 mg/100 ml	550 mg/100 ml	540 mg/100 ml
IgM	22 mg/100 ml	150 mg/100 ml	94 mg/100 ml

Normal values: IgG 800–1500 mg/100 ml, IgA 140–170 mg/100 ml, IgM 50–110 mg/100 ml.

the serum IgA fraction to a level of 2,375 mg/100 ml (see Table I). No protein was detected in the urine. At laparotomy the tumour was found to involve the pyloric region of the stomach, the base of the mesocolon and the origin of the middle colic vessels; there was no obvious glandular enlargement and the liver was macroscopically normal. A Polya gastrectomy was carried out and part of the transverse colon was resected. Histology revealed a plasmacytoma of the stomach (Figure 1). Postoperatively the serum IgA concentration fell to near normal levels (see Table I). At this time the liver function tests were normal except for a raised serum alkaline phosphatase of 26 KA units/100 ml.

He was first seen for assessment at the Royal Infirmary, Edinburgh, in Aug. 1972 when a skeletal survey showed no abnormality and histology of specimens from marrow obtained by aspiration and trephine was normal. No further treatment was given at that time and the patient remained in good health until late Dec. 1972 when he was referred because of marked lymphadenopathy in the right side of the neck and left axilla. Biopsy of a cervical node was consistent with a diagnosis of plasmacytoma. The serum alkaline phosphatase estimation was now 40 KA U/ml being of liver origin on electrophoresis. Repeated bone marrow smears were normal. The patient was started on prednisolone and melphalan in doses of 40 mg/day and 4 mg/day respectively. Therapy was discontinued after five days when signs of small intestinal obstruction developed. Because of copious vomiting, the patient was treated with i.v. fluids and nasogastric suction. Although there was radiographic evidence of hold-up at the site of the gastric anastomosis, the symptoms subsided gradually. On Febr. 15, 1973 a definite mass

was felt in the central epigastrium and the serum IgG was considerably elevated (see Table I). The serum albumin had fallen to 2.1 g% and sacral oedema was noted. For the first time, kappa and lambda chains were found in the urine and a cold haemagglutinin in the serum. The direct Coombs' test was positive to a titre of 1:80. The patient developed remittent fever and the peripheral white blood count rose to 27,000 μ l, 92% of the cells being neutrophils. Repeated blood cultures were sterile and culture of urine and swabs from the nose, throat and axilla yielded no significant growth. The patient was transfused with blood and given kanamycin i.m. 50 mg daily. He then developed hyponatraemia, the serum sodium falling to 114 meq/litre and serum chloride to 88 meq/litre. This was thought to be due partly to loss of sodium into the 'tissue compartment' because of hypoalbuminaemia. Treatment with salt-poor albumin and fluids was instituted and thereafter the serum sodium rose and serum albumin reached 3 g/100 ml. The patient then developed polyuria and within 10 days the diuresis had risen to 11 l. The urinary osmolality varied between 280 and 290 m.osm/l and administration of vasopressin tannate i.m. failed to induce reduction in urine volume or an increase in urine osmolality. Thereafter the patient's condition deteriorated rapidly due to a chest infection and he died on March 8, 1973.

PATHOLOGY

Postmortem findings

Specimens of duodenal and jejunal mucosa were obtained within 2 h of death, and

immediately fixed for microscopic examination. Postmortem was performed 12 h after death showing an emaciated man, with enlarged cervical (1–2 cm), carinal (3–5 cm), mesenteric (3–5 cm) and para-aortic lymph nodes. A tumour measuring $3 \times 5 \times 2$ cm was present in the upper anterior mediastinum, secondary nodules were present in the pancreas, both kidneys, and tumour infiltration was present in both lung bases. The tonsils were involved, but no macroscopic tumour deposits were found in sternal, vertebral, costal or pelvic bone marrow. No osteolytic lesions were found in the femora, or skull. The liver showed fine surface nodularity with dilation of in-

trahepatic bile ducts associated with a degree of obstruction at the porta hepatis by enlarged lymph nodes. The brain showed an old cystic infarct ($2.5 \times 1 \times 5$ cm). Ureters, bladder and prostate were macroscopically normal.

Microscopic examination

The primary tumour was an infiltrating poorly differentiated plasmacytoma replacing gastric mucosa and infiltrating muscle coats (Figure 1).

In all the effected lymph nodes, tonsil, pancreas, and kidney the histological features were those of a poorly differentiated

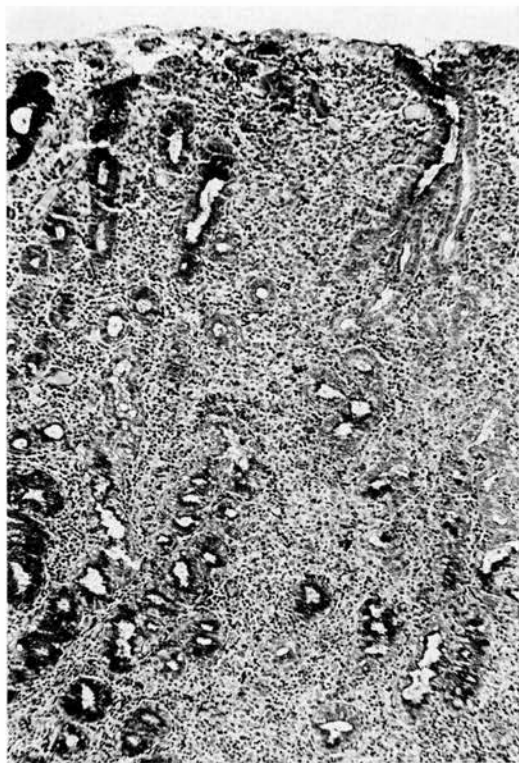


Figure 1. The primary tumour in stomach. The gastric epithelium is darkly staining, and the glands separated by a dense infiltrate of mononuclear cells. – P.A.S. $\times 95$

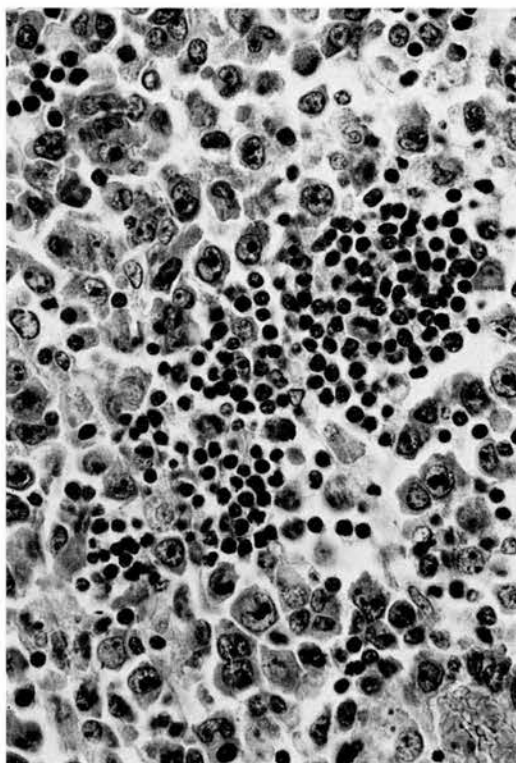


Figure 2. The appearance of tumour in lymph node. A focus of normal lymphocytes contrasts with the large neoplastic cells, some of which show the features of plasma cells. – H. & E. $\times 425$

plasmacytoma composed of large pleomorphic cells with abundant basophilic cytoplasm (Figure 2). Methyl-green pyronine stains showed a number of small islands of tumour cells staining intensely with pyronine scattered in a uniform population of poorly pyroninophilic cells. No stromal component could be identified in reticulin stained preparations. The tumour in the mediastinal mass, tonsil and cervical nodes was less well differentiated than that in other sites. Necrosis was obvious in mesenteric and para-aortic nodes.

The bone marrow from ribs, sternum and vertebral column was excessively cellular, and showed diffuse infiltration with immature, though well differentiated plasma cells,

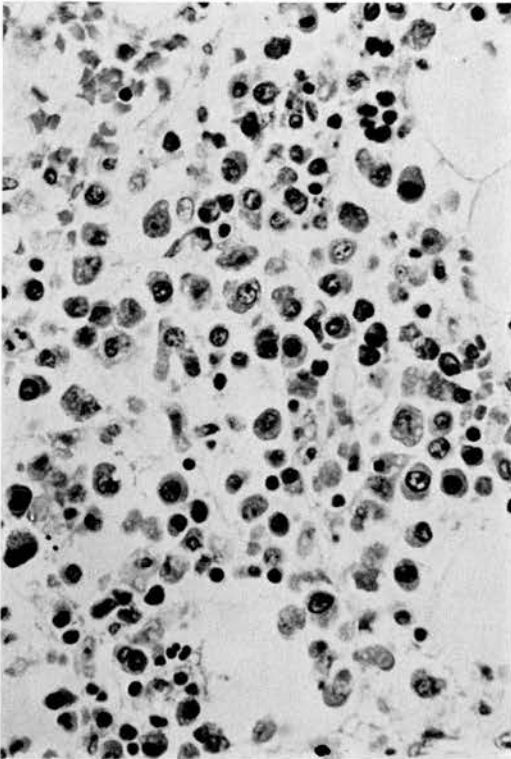


Figure 3. Bone marrow. Numerous immature plasma cells are present as a diffuse infiltrate. — Giemsa stain $\times 425$

quite unlike those found in the lymph nodes (Figure 3).

The portal tracts in the liver were expanded by loose connective tissue containing plasma cells, lymphocytes, and neutrophil polymorphs. The pancreas showed, apart from tumour infiltration, prominent islet tissue and some interlobular fibrosis.

The jejunal mucosa showed the typical light and dissecting microscope appearance of sub-total villous atrophy.

In the kidney the principal features were widespread interstitial oedema of the medulla, with 'myeloma kidney' changes in the proximal cortical tubules, and the presence of cortical tumour nodules. Amyloid was not present.

Immunological studies

Clinical quantitation of immunoglobulin was by the method of radial gel diffusion. In addition, urinary and serum components were identified by immunoelectrophoresis with monospecific antisera.

Immunofluorescent studies

Material was obtained post-mortem from lung, kidney, liver, lymph-nodes and bone marrow. It was immediately snap frozen onto dies. Frozen sections and freeze dried paraffin-impregnated sections were cut and stained for IgM, IgA, IgG and kappa and lambda light chains by both direct (rabbit anti-immunoglobulin — FITC conjugate) and indirect (goat anti-rabbit — FITC conjugate) methods. Appropriate controls were included at each step.

Immunofluorescent studies showed the tumour in lymph nodes to stain exclusively for IgA and lambda chains. Staining was not uniform, occurring in clumps of cells

corresponding to the patchy pyroninophilia already described (Figure 4).

In the portal tracts, IgG and IgA staining cells were both present, IgG cells predominating, and both classes of light chain were found.

In the kidney there was strong tubular staining for both kappa and lambda chains, and tubular IgA was present in the medulla.

The bone marrow showed a very pronounced predominance of well differentiated plasma cells staining for IgG; although both classes of light chain were present there was a marked excess of kappa over lambda chain.

Control sections showed weak non-specific staining in kidney, liver and lung, but lymph nodes showed very little non-specific staining.

Electron microscopy

The plasmacytoid nature of the tumour cells in the lymph nodes was confirmed. Furthermore, several of the tumour cells contained crystalline linear deposits showing a light band periodicity of 11 nm, and a dark band periodicity of 11.8 nm. This material occurred exclusively in dilated endoplasmic sacs, related to the Golgi areas of the tumour cells (Figure 5). In addition nearly all tumour cells contained prominent intranuclear inclusions.

DISCUSSION

This case adds one more report of gastric plasmacytoma to the 21 reported in the



Figure 4. Typical appearance of tumour in lymph nodes by immunofluorescence. Indirect staining for IgA. - $\times 800$



Figure 5. Endoplasmic crystalline inclusion in a tumour cell. - $\times 68,750$

world literature up to 1971. (For reviews see Hampton & Gandy 1957, Annamunthodo & Robertson 1958, Remigo & Klaum 1971). However, the case presented here contributes some further details to the assembling clinico-pathological picture of the entity of gastrointestinal plasmacytoma.

In this case gluten-sensitive enteropathy existed before diagnosis of plasmacytoma. This was proven by biopsy, clinical investigation, and response to therapy. The pathological features of sub-total villous atrophy were also present at autopsy. In the literature, most authors stress gastrointestinal symptoms – often associated with gastric ulceration – and loss of weight as presenting symptoms of gastric plasmacytoma, although documentation of malabsorption syndrome has not been reported to date. Rappaport et al (1972) described the pathological features and demonstrated severe plasma cell infiltration of the intestinal mucosa and submucosa in Mediterranean abdominal lymphoma with malabsorption. In this series 16 out of 20 cases subsequently developed malignant lymphoma. Although no plasma cell tumours were reported, the authors state that in Mediterranean lymphoma with malabsorption – ‘the possibility is suggested that this abnormal, though probably not neoplastic proliferation of plasma cells is a morphological manifestation of an immune deficiency state which predisposes the patient to the development of malignant lymphoreticular neoplasms’.

In contrast to the case of gastric plasmacytoma reported here, the features of Mediterranean abdominal lymphoma are particularly well documented in regard to the abnormal proteins secreted by the tumour cells. In the majority of cases the paraprotein consists of alpha chains only (α -chain disease)

(Seligmann et al 1971). In our case the abnormal protein appeared as a fairly diffuse, myeloma band on electrophoresis, and reacted both with anti- α -chain antisera and with anti-lambda-chain antisera. By immunofluorescence the tumour cells expressed both α -chain and lambda-chain determinants.

In two cases, reported by Hefferman (1947) and Ruland (1954) primary gastric plasmacytoma was followed by the development of skeletal plasma cell tumours. In this respect Line & Lewis's case (1969) is also of interest as a mediastinal tumour was present, with a clavicular deposit of tumour, and light chain of immunoglobulin in the serum. In our case, the patient in the later stages of his illness developed a raised serum IgG, and at this time both kappa and lambda light chains were present in the urine. The direct Coombs' test became positive. The demonstration, post-mortem, of the IgA, lambda chain secreting nature of the plasmacytoma does not account for the presence of the urinary kappa chain, nor the elevated serum IgG. Serum M protein components (light chains) were present in the cases described by Line & Lewis (1969) and Remigo & Klaum (1971). Urinary M proteins, or Bence Jones proteinuria are generally not present in soft tissue plasmacytoma (Snapper 1953, Dolin & Dewar 1956), but may be so when skeletal ‘metastases’ or co-existent multiple myeloma is present. In our case serum IgA was elevated initially but fell when the primary tumour was resected. Light chains were present in urine only when elevated IgG levels were found, and then both classes of light chain were present. This finding supports the view that soft tissue plasmacytomas are not generally associated with Bence Jones proteinuria. This is probably

due to the relatively small mass of plasma cells, but in cases of α -chain disease renal excretion of α -chain is notably poor (Seligmann et al 1971). Lymphomas of the gut, for example Waldenström's disease of the stomach (Froget et al 1972) or Mediterranean abdominal lymphoma (Rappaport et al 1972), are frequently associated with abnormal serum IgM or IgA paraproteins, which are poorly excreted in the urine.

In view of the post-mortem appearance of diffuse myelomatosis of bone marrow, the great preponderance of cells secreting IgG and kappa chain in the bone marrow, the elevated serum IgG and the kappa chain proteinuria, we believe that these features indicate myelomatosis of different clonal origin from the primary plasmacytoma. It is also possible that the previous reports of bone involvement in soft tissue plasmacytoma might, in some instances, have been due to co-existent myelomatosis of different clonal origin. The late onset of light chain proteinuria was related to a period of marked renal dysfunction, ADH resistant polyuria, and low serum albumin. The pathological features found in the kidney were those of myeloma kidney and similar to those described by Remigio & Klaum (1971). Amyloid was not present in our case although para-amyloid deposits were present in cases described by Ruland (1954) and Annamunthodo & Robertson (1958) in lymph nodes. Low serum albumin can exist without the nephrotic syndrome in patients with primary amyloidosis with renal involvement, the serum albumin level being below the threshold value for renal filtration. Although amyloid was not present in this case, both kappa and lambda chains and IgA were present in the renal tubules and interstitium, and may have produced

an equivalent effect to the deposition of amyloid.

Finally, attention is drawn to the crystalline deposits observed in the cells of this tumour which are identical with those described in the cells of acute promyelocytic leukaemia (Cawley & Hayhoe 1973). It is relevant to note that myelomatosis terminating in acute leukaemia has been described in four cases (Videbaek 1971) and monocytic leukaemia in three (Osserman 1971).

The case described conforms to the general features of previously described cases of plasmacytoma of the gastrointestinal tract, notably in respect of widespread lymph node metastasis with haematogenous spread to kidney, lung and pancreas (Wiltshaw 1971). The clinical features illustrate the well documented initial favourable response of a solitary myeloma of bone to radiotherapy, and the relentless progression of a soft tissue plasmacytoma. In retrospect it seems possible that the patient would have benefitted from a course of prednisolone and melphalan following the resection of the gastric tumour, despite the initial good subjective response to surgery and the fall in serum IgA.

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Progression of Polycythaemia Vera to Malignant Myelofibrosis and Reticulum Cell Sarcoma

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A case of polycythaemia vera, known for 4 years and treated with ^{32}P is reported because of the rapidly fatal transition into myelofibrosis and then reticulum cell sarcoma.

Polycythaemia vera progresses slowly to myelofibrosis and death in 20 % of patients (Wasserman 1954). Four months is an uncommonly short time course for such a development. The patient to be described may therefore fit into the category of 'malignant myelosclerosis' (Lewis & Szur 1963), in which there is an acute onset of pancytopenia with occasional blast cells in the peripheral blood; no lymphadenopathy occurs and there is no palpable splenic enlargement; bone marrow aspiration is unproductive and trephine biopsy is indicative of chronic myelofibrosis. The disease is refractory to treatment and survival is short.

CASE REPORT

A retired school janitor, aged 61, was discovered to have polycythaemia in May, 1968, after a routine pre-operative Hb estimation (Table I). Three herniorrhaphies had been carried out over the previous 10 years and full blood counts had been normal on those occasions. A diagnosis of polycythaemia vera was confirmed by high white

cell and platelet counts, a raised leukocyte alkaline phosphatase score and a red cell mass of 57.7 ml/kg (normal range 26-33). Marrow aspiration revealed hyperplasia of all elements. The spleen tip was palpable then but was not found at any time thereafter, follow-up being undertaken monthly.

The patient was treated with a total of 17 mCi of ^{32}P over a period of 17 months, 5 being required in May, 1968, 6 in Dec., 1968, and 6 in Oct., 1969. In addition, 800 ml of blood was removed in Nov., 1970. During that time an episode of cholestatic jaundice occurred following administration of chlorpromazine and a prolonged course of cycloserine was required for a urinary tract infection in conjunction with prostatic hypertrophy. In Dec., 1971, four months prior to his death, the patient's Hb, white cell and platelet counts were normal and he had no symptoms except intermittent claudication.

A month later, he complained of depression and his Hb was noted to have dropped to 9.2 g%. Examination of faeces showed these to be negative for occult blood. Marked symptomatic deterioration had occurred by the following month. Tiredness, listlessness, limb pains and loss of 6.5 kg in weight were the dominant complaints. Although the Hb had not specifically changed, the platelet and white cell counts were low (Table I)

TABLE I
Haematological findings in the course of the patient's illness

	1968	Dec. 1971	Jan. 1972	Febr. 1972	March 1972
Hb (g%)	18.8	12.2	9.2	9.0	7.1
WBC (n/ml)	21,300	4,800	2,700	1,800	1,200
Platelets (n/ μ l)	750,000	250,000	200,000	53,000	<10,000

and the differential WBC had changed from 54 % neutrophils, 36 % lymphocytes and 10 % monocytes in Jan. to 24 % neutrophils, 58 % lymphocytes, 13 % blast cells and 3 % promyelocytes in Febr. No nucleated red blood cells were seen in the peripheral blood film and reticulocyte counts varied between 1 and 2 % during Jan. and Febr. Small numbers of circulating normoblasts were present terminally.

Six bone marrow aspirations were attempted without yield of any marrow. Trephine biopsies were consistent with but not diagnostic of early myelofibrosis though few megakaryocytes were noted alongside the regenerating fibroblasts. A study of chromosomes from bone marrow culture could not be carried out because aspirated material was unsuited. The diagnosis of myelofibrosis which was made at that time, was supported by a $T_{1/2}^{59\text{Fe}}$ in plasma of 98 min (normal range: 70–140) with no significant uptake in the sacrum and maximum uptake in the spleen and liver as determined by surface counting.

A low serum B_{12} was noted with histamine refractory achlorhydria and an abnormal Schilling test was not corrected by intrinsic factor. Nor was any effect noted on treatment with hydroxycobalamin. No other evidence of malabsorption was obtained, notably, serum folate, calcium, phosphate and iron and plasma proteins were normal. A barium meal carried out in mid-Febr. showed a normal stomach and small bowel.

The ESR in the terminal illness was raised to 140 mm/1 h and rouleaux formation was seen in the peripheral blood film. Serum protein electrophoresis showed no abnormal band or M-component and immunoelectrophoresis gave a normal pattern. IgG level was 1475 mg/100 ml (normal range 700–1675), IgA was 312 mg/100 ml (normal range 89–315) and IgM was 178 mg/100 ml (normal range 45–250). The urine contained no Bence-Jones protein and electrophoresis of a concentrate yielded no kappa or lambda chains. Radiological

skeletal survey showed osteoarthritic changes only, these particularly in the lumbar spine. There were no changes in bone texture and no osteolytic or osteosclerotic lesions.

At this time the direct and indirect Coombs tests as well as a test for antinuclear factor were negative. An intensive search for tuberculosis and other infection was made in an attempt to explain the onset of spikes of fever as well as the raised ESR. A Mantoux test and one for serum hepatitis associated antigen were negative. Repeated chest x-rays were normal and cultures of blood, sputum, urine, marrow aspirate and trephine biopsies were consistently negative. Nevertheless, as the patient's clinical state deteriorated a therapeutic trial of ethambutol and isoniazid was instituted – to no effect.

The ultimate demise was heralded by severe thrombocytopenic purpura, unaffected by transfusions of blood and platelets.

AUTOPSY FINDINGS

There was no lymphadenopathy but the spleen weight was 615 g. The organ had undergone extensive autolysis. Vertebral marrow was firm, white and fibrous and marrow in the right femur had been replaced by yellow fatty material. The lungs demonstrated haemorrhage and small areas of bronchopneumonic consolidation in the right middle lobe.

Microscopically, the vertebral marrow was hypercellular due to replacement by a multifocal dense infiltration of monomorphic cells with large oval vesicular nuclei. Silver stainable reticulin was present in increased amounts around groups of such cells, and their appearance was

similar to that seen in reticulum cell sarcoma.

No malignant infiltration was seen in the spleen: histologically the architecture was well preserved and pigment-filled macrophages were prominent within sinusoids. Extramedullary haematopoiesis was present in numerous foci. Microscopic sections of the liver were consistent with previous cholestatic jaundice, namely, considerable numbers of bile thrombi within, and chronic inflammatory cells around, portal tracts; only slight extramedullary haematopoiesis was evident and no infiltration with cells similar to those seen in the marrow. Both kidneys, however, showed foci of primitive reticulum cells resembling those in the marrow, and also numerous primitive anaplastic cells diffusely infiltrating the interstitial tissue. Skeletal muscle and lung parenchyma also showed invasion by abnormal reticulum cells, though to a lesser extent than the kidney.

The histological picture seen in both ante mortem iliac crest trephine biopsies was reviewed and, in summary, showed a diffuse sclerosis of bone trabeculae with hypoplasia of marrow elements much more marked than would be accountable by age alone. There was minimal fibroblastic proliferation and no evidence of replacement of marrow by sheets or foci of primitive reticulum cells as was seen in post mortem vertebral marrow. A few islets of stem cells and occasional megakaryocytes were present.

DISCUSSION

The clinical and laboratory findings de-

scribed above are consistent with a diagnosis of malignant myelofibrosis; particularly the absence of hepatosplenomegaly on palpation, the failure of iron clearance to the sacrum and its uptake by liver and spleen, the rapidly progressive pancytopenia and the appearance of the trephine biopsies are features as described by Lewis & Szur (1963). The autopsy findings of concentrated foci of abnormal reticulum cells in the marrow and other organs suggest a pathological diagnosis of reticulum cell sarcoma; though an increase of reticulum cells is found in myelofibrosis it is usually diffuse in nature, not focal as above. The anaplastic cells infiltrating kidney were not thought to be carcinoma cells and no primary tumour was evident.

It is possible that reticulum cell sarcoma developed de novo, or perhaps it followed from radiophosphorus treatment. Polycythaemia is known to progress slowly into myelofibrosis but hitherto no instance of accelerated deterioration has been noted. Similarly, reticulum cell sarcoma is not a recognised sequel to myelofibrosis. The present case links reticulum cell sarcoma and the myeloproliferative disorders by showing it as an extension or perhaps alternatively as a poorly differentiated form of malignant myelofibrosis.

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Gut lymphoma presenting simultaneously in two siblings

Primary intestinal lymphoma is rare outside the Middle East—there are about 10 cases per million a year. A few cases have been reported in members of the same family but these have presented in childhood or early adult life.^{1 2} We report two cases of gut lymphoma occurring in a middle-aged brother and sister who presented simultaneously.

Case reports

Case 1—A 51-year-old woman presented in September 1976 with weight loss, progressive abdominal distension, vomiting, and anorexia. Examination showed ascites but no enlargement of the abdominal organs. Routine haematological and biochemical investigations and chest radiography gave normal results, and cytology of the ascitic fluid was negative. At laparotomy a large irreducible ileocolic intussusception was found, caused by a tumour, and a right hemicolectomy was performed. Histology showed a malignant lymphocytic lymphoma of diffuse small cell type affecting ileum, colon, and lymph nodes (see figure). A sternal marrow smear also showed infiltration by a well-differentiated lymphoma. A lymphangiogram was attempted but was technically unsatisfactory. Xylose and vitamin B₁₂ absorption were normal, and examination of faeces showed no evidence of steatorrhoea. Serum calcium concentration was low initially—2.02 mmol/l (8.1 mg/100 ml)—and has remained low—2.05 mmol/l (8.2 mg/100 ml). Serum albumin concentration, also low at presentation (16.2 g/l), has returned on chemotherapy towards normal—34.6 g/l. This patient is being treated with intermittent chlorambucil and prednisolone and is doing well.

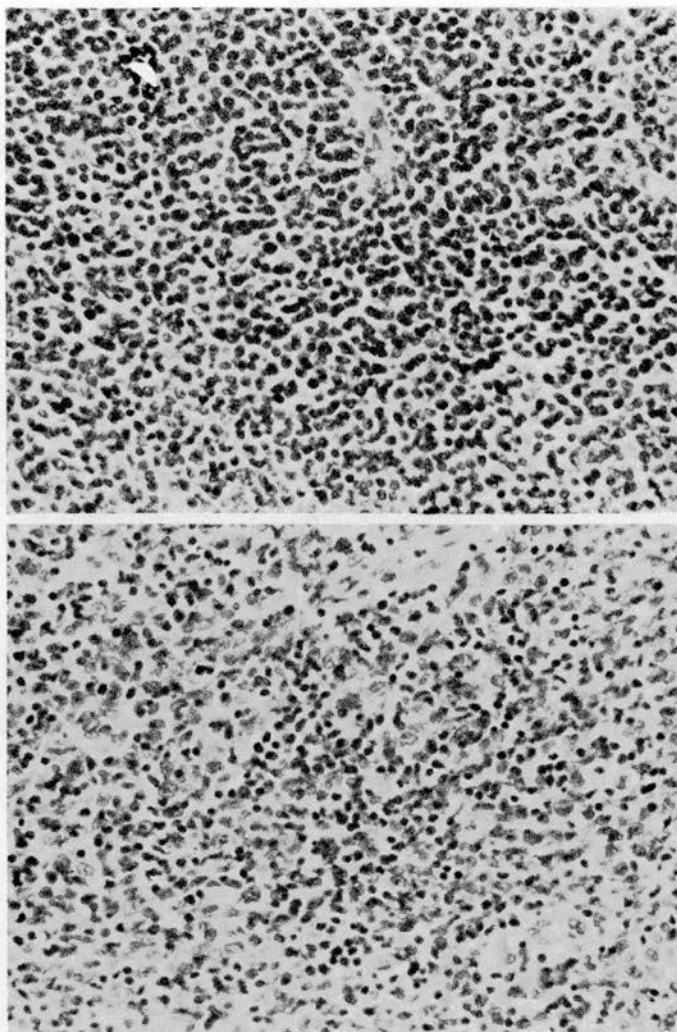
Case 2—A 54-year-old man was admitted to hospital just two weeks after his sister (case 1). He had a three-month history of weight loss, constipation, bleeding per rectum, and tenesmus. On rectal examination a large tumour was easily palpable. Routine haematology, biochemistry, and chest radiography results were normal. At laparotomy the lesion was found to be inoperable and an end colostomy was performed. Biopsies taken at operation showed a diffuse lymphocytic lymphoma of mixed small and large cell type (see figure). Sternal marrow was normal and a lymphangiogram was contraindicated owing to postoperative pulmonary atelectasis. The serum calcium and serum albumin concentrations were low initially—2.04 mmol/l (8.2 mg/100 ml) and 25.5 g/l—and have subsequently returned to normal—2.28 mmol/l (9.1 mg/100 ml) and 42.4 g/l. A xylose absorption test gave a low result, though examination of faeces did not show any appreciable steatorrhoea. This man is being treated with pulses of cyclophosphamide, cytarabine, vincristine, and prednisolone. After an initial deterioration he is now responding to treatment.

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Histological appearances of lymphocytic lymphoma. Above: Case 1, diffuse small cell type. Below: Case 2, mixed small and large cell type. ($\times 300$.)

To establish any common immunological, genetic, or familial factors various investigations were performed. Serum immunoglobulin concentrations were normal in both cases. An immunoperoxidase method failed to detect immunoglobulins in either tumour, and results of routine chromosomal analyses of peripheral white blood cells were normal. Virological screening of the patients, however, disclosed that both had a significant IgG titre to EB virus ($\log_2 5$ and $\log_2 7$), indicating past infection. The brother was said to have had malaria during the war but this could not be confirmed serologically. There was no family history of note and the patients had not lived in the same house for many years.

Comment

Smith and Pike³ warn that anecdotes of space-time clusters are not statistically meaningful as they may occur by chance; their value is commended, however, in the generation of hypotheses. The two cases presented here may point to a genetic link in the development of gut lymphoma in support of previous reports.^{1 2} On the other hand, the EB virus titres were surprisingly high, and one patient had a history of malaria. The association of EB virus, malaria, and Burkitt's lymphoma is well known,⁴ and studies of non-Burkitt's lymphoma have yielded complexes of low molecular weight RNA and reverse transcriptase.⁵ Further study of intestinal lymphoma may yield more clues about the cause of this group of diseases.

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¹ Maurer, H, *et al*, *Cancer*, 1976, **37**, 2224.

² Hambleton, G, *Proceedings of the Royal Society of Medicine*, 1969, **62**, 1093.

³ Smith, P G, and Pike, M C, *Cancer Research*, 1976, **36**, 660.

⁴ Epstein, M A, Achong, B G, and Barr, Y M, *Lancet*, 1964, **1**, 802.

⁵ Spiegelman, S, *et al*, *Cancer Research*, 1973, **33**, 1515.

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CLINICAL AND LABORATORY STUDIES ON HUMAN LYMPHOMA

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