https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

A.C.A. Glen

Thesis for the degree of M.D. 1972

DECLARATION

Some of the work undertaken for this thesis was carried out in collaboration with others and in certain parts the work was facilitated by technical assistance. The author's individual contribution is detailed below.

The studies to validate the methodology, i.e. Section 2 of the thesis, were carried out entirely by the author. In Section 3, the application of the measurements in normal individuals and in pathology, all the determinations of lymphocyte chemistry, lymphocyte enumeration, differential counting and cell diameter measurements were carried out by the author with some minor exceptions. These exceptions were the measurements in the study of neoplastic disease carried out by a technical assistant and in the study of thyrotoxicosis where the chemical measurements but not the leucocyte counts were carried out by the author.

The measurements of lymphocyte chemistry in renal transplantation (Section 4) were carried out by the author initially representing one third of the analyses and later by a technical assistant. The measurements on thoracic duct lymphocytes were shared among three

ProQuest Number: 10647019

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 10647019

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

individuals, the author included. All the serial studies of thoracic duct lymphocyte chemistry were carried out by the author who took a one third share in the investigation of the nature of lymphocyte RNA, and carried out each of the stages in the work personally.

With minor exceptions the clinical data used in diagnosis and in correlation with the lymphocyte chemistry were obtained by others and the author's task was that of abstraction and compilation. Included in the measurements made by others were ESR, rheumatoid factor titre, auto-antibody titres, iodine and radio-iodine studies, LATS assay, tissue typing and routine blood chemistry of the transplant patients. Again, with minor exceptions, the clinical care of the patients studied was not the responsibility of the author.

The thesis was composed by the author and it represents his views although especially in the collaborative projects these views have been developed in discussion with others.

The work has not been presented for a degree previously.

THE MEASUREMENT OF DNA AND RNA IN HUMAN LYMPHOCYTES IN CONDITIONS OF ALTERED IMMUNOLOGICAL ACTIVITY

A thesis submitted to the University of Glasgow

for the degree of M.D.

by

Alastair C. A. Glen

March 1972

CONTENTS OF VOLUME 1

Section 3 Lymphocyte nucleic acid measurements in normal individuals and in pathology

CONTENTS OF VOLUME 2

Section 4 The lymphocyte and renal transplantation

SECTION 1

Introduction

and

Introductory Review of Past Work

1.1 Introduction

'The immunological responses of an animal are put into effect by the activity of the fixed and circulating cells which in aggregate make up its lymphoid tissue.'

This statement which introduces a review of the immunological activities of lymphocytes by Gowans and McGregor (1965) puts forward concisely the reasons for interest in the lymphocytes. Lymphoid tissue in the body is thought to amount to one per cent of the body weight (Yoffey and Courtice, 1970) and although the bulk of this tissue is to be found in lymph nodes, spleen, bone marrow and other organs throughout the body a small fraction is readily accessible to investigation in the peripheral blood. The majority of the lymphocytes in peripheral blood are normally only transiently blood cells; about two thirds of the cells interchange with cells elsewhere in the lymphoid system while others are short lived cells (Everett, Caffrey and Reike, 1964). Lymphocytes of the peripheral blood are therefore more representative of the tissue as a whole than might at first appear.

The blood lymphocyte population is heterogeneous both in morphology and function, and it has become clear that the two principal immune responses, antibody production and infiltration of tissues with sensitised cells, the cellular immune response, are attributable to distinct cell populations (Roitt, Greaves,

Torrigiani, Brostoff and Playfair, 1969). It is now possible to distinguish these cell populations in peripheral blood lymphocytes by recognition of surface immunoglobulins which are found in substantial quantity only on cells of the antibody producing line (Papamichail, Brown and Holborow, 1971). Morphological assessment of the blood lymphocytes has remained unsatisfactory in that classifications of the lymphocytes depend on considerations of cell size and degree of basophilia in stained preparations. Histochemical measurements or electron microscopy might improve the classification, but there are practical difficulties in applying these techniques in quantity.

Lymphocytes in tissue culture will react to specific antigen stimuli in sensitized individuals and more generally to non-specific mitogens such as the kidney bean extract, phytohaemagglutinin (Hirschorn and Ripps, 1965). The reaction known as the transformation response involves cell enlargement nucleic acid synthesis and later cell division. Tests of lymphocyte function based on this transformation response have been applied in human disease and, when there is a known immunological derangement, have frequently shown up an impaired ability of the lymphocytes to respond to mitogens. Although alterations in the proportion of large and small lymphocytes in the blood have been documented in human disease such changes are difficult to define and a simple and objective method of describing the nature of the blood lymphocyte population is not available. It is to this problem that the thesis

is directed.

The work described is the approach of a biochemist to the problem of assessment of the lymphocytes. Measurements of nucleic acids have been carried out on lymphocytes isolated from the peripheral blood using deoxyribose nucleic acid (DNA) to establish cell numbers and ribose nucleic acid (RNA) to determine the metabolic activity of the cells. The mean RNA/DNA ratio of isolated blood lymphocytes has been proposed as a suitable objective measurement of the cells and the ratio has been determined in a series of normal subjects and in diseases of special immunological interest.

Clarity of presentation has demanded that the thesis be written in sections. This introduction is followed by a short general review of past work on the role of the lymphocyte in immune processes. A second separate section deals with investigations of the methodology, and then in the third section a study of normal lymphocytes and the effect of diseases on blood lymphocyte nucleic acids is presented. A fourth section considers the lymphocyte nucleic acids in renal transplantation and includes measurements on human thoracic duct cells with observations on the nature of lymphocyte RNA. A short concluding discussion draws points from the preceding sections in a general review of the work.

1.2 Introductory review of past work

The lymphocytes are directly involved in the production of the immune response, a process so involved that a review of any aspect of these cells must begin with an outline of the events of antibody immunity and cell mediated immunity. Both of these reactions are triggered by the presence of antigen, and depending on the nature of the antigen as well as its presentation, humoral antibody is produced or sensitized lymphocytes appear as effectors of the cellular immune response (Gowans and McGregor, 1965).

An appreciation that there are two mechanisms of immune response developed from the observation that in some instances (antibody immunity) a state of temporary immunity can be transferred from an immune animal to a non-immune animal by means of serum, while in other types of immune response transfer of immunity with serum does not occur. Cell mediated immunity was recognised when it proved possible to transfer cells from sensitized animals and thereby transfer reactions of the delayed hypersensitivity type such as skin contact sensitivity (Landsteiner and Chase, 1942), skin allograft immunity (Billingham, Brent, Medawar and Sparrow, 1954), and tuberculin hypersensitivity (Chase, 1945). Lymphocytes were the important cells in the transfers (Billingham, Silvers and Wilson, 1962) and these cells are regarded as effectors in cell mediated reactions.

It is instructive to look closely at the part played by

lymphocytes in immune processes. In a revealing experiment Gowans (1962) transferred thoracic duct small lymphocytes from an inbred strain of rats into the F1 hybrid between the donor strain and an unrelated strain. He showed that the transferred small lymphocytes reacted against tissue antigens in the hybrid which were foreign to the transferred cells, that is antigens inherited from the unrelated parental strain. A graft versus host reaction ensued. This experiment identified the rat small lymphocyte as a cell capable of recognising antigen and possibly also an effector of tissue damage.

Important evidence of diversity in lymphocyte function has come from studies of mice which have been thymectomized at birth (Miller and Osoba, 1967). The thymectomized animals are partially depleted of small lymphocytes and have a severely limited capacity to produce a cell mediated immune reaction, for example in allograft rejection or in delayed hypersensitivity. Humoral antibody production in response to certain antigens in thymectomized animals is less markedly impaired (Miller and Mitchell, 1969). Developments from this type of experiment led to the study of mice which have been exposed to whole body irradiation and these investigations have clarified our understanding of lymphocyte function. Restoration of radiation depleted animals by means of a thymic graft revealed that thymus derived cells would respond to antigen by mitosis, but they did not make antibody in significant quantity (Davies, Leuchars, Wallis, Marchant and Elliot, 1967).

Nossal and his colleagues (Nossal, Cunningham, Mitchell and Miller, 1968), using chromosomal markers to identify the lymphocytes, were able to show that most antibody producing cells were bone marrow derived and it is now held that some form of co-operation between thymus derived lymphocytes and bone marrow derived lymphocytes is necessary for the processes of recognition of a variety of antigens and production of specific antibody (Claman and Chaperon, 1969).

An earlier observation by Glick introduces additional evidence of diversity of lymphocyte function. It was shown by Glick and his co-workers (Glick, Chang and Jaap, 1956) that a lymphoid organ present in birds, the bursa of Fabricius, was necessary for the proper development of antibody producing cells in chickens. Although a single organ equivalent to the bursa has not been demonstrated in mammals its importance in birds is evidence in favour of the existence of a population of lymphocytes distinct from the thymus dependent cells. The present view, and this has gained wide acceptance, is that mammalian lymphocytes are derived ultimately from bone marrow as undifferentiated stem cells. Some of the stem cells come under the influence of the thymus and proliferate within its substance to become thymus dependent, T-lymphocytes. These are the cells with capacity to recognise antigen and to proliferate outwith the thymus in a cellular immune response. Other lymphocytes, again derived from bone marrow stem cells, proliferate in areas of the lymph nodes, gut and spleen. These B-lymphocytes are of the antibody producing line and are best

considered as bone marrow derived thymus independent cells (Meuwissen, Stutman and Good, 1969; Davies, 1969; Roitt, Greaves, Torrigiani, Brostoff and Playfair, 1969). Anatomical parallel of the functional division of the lymphocytes is observed in the lymph nodes where certain regions known as the paracortical areas are populated almost completely by thymus dependent cells (Parrott, de Sousa and East, 1966), while thymus independent B-lymphocytes proliferate in the germinal centres.

The validity of these observations for human immune reactions has been accepted principally as a result of observations in pathology. Thus, the clearest demonstration of the presence of at least two distinct populations of lymphocytes in man has been the recognition of immunological diseases in which there may be independent failure of either one of the two types of immunity. In 1965 Di George described congenital absence of the thymus in a child and this defect was associated with failure of delayed hypersensitivity reactions, i.e. cell mediated immunity. Such children have a relatively intact capacity to form immunoglobulins (Fulginiti, Hathaway, Pearlman, Blackburn, Reiquam, Githens, Claman and Kempe, 1966). On the other hand a form of sex linked hypogammaglobulinaemia exists, first described by Bruton (1952), in which the thymus gland is normal, circulating small lymphocytes appear normal, but there is a partial lymphocyte depletion from the thymus independent areas of the lymph nodes. Plasma cells are lacking and these infants fail to produce significant quantities of immunoglobulins

(Meuwissen, Stutman and Good, 1969).

Further evidence of distinct populations of lymphocytes has been the detection of surface immunoglobulins on the cells by immunofluorescent technique. Both T-lymphocyte and B-lymphocytes have antigens fixed to their surface by means of specific membrane-bound immunoglobulins, but only in B-lymphocytes is this sufficient to be detected by fluorescein conjugated anti-immunoglobulin. In mice the cells shown to be carrying surface immunoglobulins by this method have been identified as B-lymphocytes (Raff, 1971) and a proportion of the blood lymphocytes in man, of the order of one in three, show detectable surface immunoglobulin typical of B-lymphocytes (Papamichail, Brown and Holborow, 1971). It is now almost certain that in man the presence of surface immunoglobulin detectable by immunofluorescent technique identifies the B-lymphocytes, more especially since in the Bruton type agamma-globulin none are found in peripheral blood (Froland, Natvig and Berdal, 1971).

It is likely that the life span of T-lymphocytes and B-lymphocytes is considerably different. There is undoubtedly a population of lymphocytes in the rat with a life span in excess of two hundred days. This is the interpretation of the persistence of unlabelled cells during a long period of continuous administration of tritiated thymidine to these animals (Robinson, Brecher, Lourie and Haley, 1965). A minority of rat lymphocytes have a life span of less than two weeks (Everett, Caffrey and Rieke, 1964).

It has been possible to study the life span of the lymphocytes in man by investigation of the persistence of small lymphocytes damaged during therapeutic radiation. By this method it is reckoned that the average life span of the human long-lived small lymphocytes is between four and five years (Bucton, Court-Brown and Smith, 1967). The long-lived cells recirculate between blood and lymph nodes either directly or by percolation through the tissues and thence by way of the efferent lymphatics and the thoracic duct reappear in blood (Gowans, 1959). Long-lived and short-lived lymphocytes are to be found in varying proportion in different lymphoid organs and regions of lymphoid organs (Ford and Gowans, 1969), thoracic duct lymph showing a low percentage of the short-lived cells and peripheral blood a greater proportion; about one third of the blood lymphocytes in the rat are these short-lived cells (Everett et al., 1964). This is the proportion of immunoglobulin bearing B-lymphocytes in the peripheral blood in man. Furthermore, it is long-lived lymphocytes which are depleted in thymectomized and therefore T-lymphocyte depleted rats (Rieke and Schwartz, 1966). Definite proof is required however that short-lived lymphocytes are B-lymphocytes and the long-lived variety are T-lymphocytes.

Oort and Turk (1965) have made a detailed study of the changes which occur in regional lymph nodes of the guinea pig during the development of contact sensitivity to the chemical oxazolone, a cell mediated reaction. They observed enlargement of lymphocytes

in the paracortical areas of the local node to form pyroninophilic blast cells. The lymphoblasts reached their maximum concentration four days after contact with oxazolone, the day before the animal became sensitized to this chemical agent. Mitoses were apparent in the lymphoblasts after day four and from auto-radiographic studies it appeared very likely that the resulting daughter cells were small lymphocytes. Similar changes have been described in the days following the application of a skin homograft in the rabbit (Scothorne and McGregor, 1955).

When the antigenic stimulus gives rise to an antibody response the changes in the lymph node are to be found in the medullary region. On the sixth day following intra-dermal injection of a purified pneumococcal antigen there is a marked increase in the number of plasma cells to be found in the areas known as the medullary cords (Oort and Turk, 1965). Paracortical areas of the lymph nodes appear unchanged when this antigen is administered. The expression of the immune response is influenced by the nature of the antigen as recent studies of the response to Salmonella flagellar protein have shown quite clearly (Parish, 1971). Progressive chemical modification of the antigen resulted in a steady reduction of the ability of the antigen to induce antibody production and an enhancement of the cell mediated response. It is however likely that many antigens provoke a combined cell mediated and antibody mediated immune response (Turk, 1967).

The in vivo response to antigen challenge has been examined in a different way by Hall and his colleagues (Hall, 1967; Hall, Morris, Moreno and Bessis, 1967). These authors developed the technique of cannulation of the efferent lymphatics of a lymph node draining the flank region in sheep. They then stimulated the region drained by the node with a suitable antigen and studied the changes in the cellular content of the lymph flowing from the node. It was clear from these experiments that large numbers of basophilic cells appeared in the lymph when the antigen was a skin allograft, and there was a similar response to injected human red blood cells. Although both reactions probably involve a mixed cell mediated and antibody response there were differences in the timing of the greatest outflow of basophilic cells. Unfortunately there are no studies of an uncomplicated cell mediated or antibody immune response using this technique.

An important advance in the study of lymphocyte function was the development of an in vivo model of the immune response. In 1960 Nowell discovered that phytohaemagglutinin, a mucoprotein extracted from the red kidney bean, phaeseolus vulgaris, was able to stimulate cultured white blood cells to divide. It is small lymphocytes which have the capacity to react to non-specific mitogens such as phytohaemagglutinin or to specific antigens where there has been previous exposure of the cell donor to the antigen (Hirschorn and Ripps, 1965). This so called transformation response which includes RNA, DNA and protein synthesis with

enlargement and division in some of the cells, is thought to parallel the *in vivo* response of the lymphocytes (Rubin, 1967). The response to phytohaemagglutinin and other non-specific agents such as Pokeweed mitogen and Streptolysin-S stimulate a high proportion of lymphocytes without prior sensitizing contact. The extent of this *in vitro* reactivity of the lymphocytes relates to the immunological potential of the cells and is diminished when there is deficiency of thymus dependent cells (Davies, 1969). A similar but more specific reaction to bacterial or viral antigens in sensitized individuals is a more close parallel of the natural *in vivo* response but is of lesser degree (Meuwissen, Stutman and Good, 1969). Many reports of alteration in the lymphocyte transformation response in human disease have appeared in the literature, some of which will be detailed later.

This thesis is mainly concerned with the blood lymphocytes. The work of Hall and his colleagues on the cellular content of the lymph implied that changes are to be expected in the blood lymphocytes during an immune response. The large basophilic cells of the efferent lymphatics drain to the blood stream by way of the major lymphatic trunks, furthermore the electron microscopic appearances of these basophilic lymphoid cells in animals (Hall, Morris, Moreno and Bessis, 1967) are very similar to the cells described in the peripheral blood in man in association with an immunological response (Zucker-Franklin, 1969). The striking feature of the cells, and this accounts for their basophilia on

stained preparations are the abundant clustered ribosomes in the cytoplasm which is usually lacking in a developed endoplasmic reticulum.

When a peripheral blood smear stained with Wright's stain is examined by light microscopy it is immediately obvious that the lymphocytes are heterogeneous. Wintrobe (1967) states that the lymphocyte is generally small (ten microns in diameter) but larger forms are common (ten to thirty microns). Attempts to be more precise in a morphological classification of the cells have met with the difficulty of making arbitrary divisions where no natural morphological classes exist. Attention has been drawn to this problem by several reviewers (Cowans and McGregor, 1965; Sell and Asofsky, 1968).

Early classification of blood lymphocytes relied on the microscopic appearance of the cells on stained smears, in particular the size and degree of basophilia (Wiseman, 1931; Reich and Reich, 1933; Pariser, Zucker and Meyer, 1952). With greater understanding of the immune response, interest in this type of measurement revived and because of the difficulty of making objective measurement of basophilia various means of assessing the lymphocytes have been devised. Hernberg made cell diameter measurements on stained smears of peripheral blood (Hernberg, 1954). Lesiewska (1967) adopted histochemical techniques to measure lymphocyte DNA and RNA content, and Crowther and his colleagues (Crowther, Fairley and Sewell, 1969) determined labelled Uridine and Thymidine uptake

of the lymphocytes in an assessment of the rates of RNA and DNA synthesis in the cells. A limited series of conditions have been investigated by these several authors, Hernberg's studies were of thyrotoxicosis, Lesiewska's measurements were in children with viral infections, and Crowther's group examined the effect of immunisation in normal subjects and in patients with Hodgkin's disease. From these studies it is evident that the blood lymphocyte population changes to include a greater proportion of the more basophilic, RNA containing, cells in response to a variety of stimuli.

Each of the methods used in the assessment of the lymphocytes has disadvantages. Cell diameter measurements are laborious and the cell size recorded depends on the techniques used. The histochemical approach requires individual measurements from a large number of cells and although the procedure of Crowther et al. is shorter it is difficult to obtain reproducible results when the uptake of nucleic acid precursors is measured in vitro (Parker and Lukes, 1969). Determination of RNA and DNA content of the lymphocytes may offer some advantages in reproducibility, but this has not been previously investigated.

Chemical measurements of DNA and RNA in blood leucocytes have been undertaken by Davidson, Leslie and White (1951) and by Métais and Mandel (1950) but no chemical measurements of DNA and RNA in blood lymphocytes have been described except in patients with acute and chronic lymphatic leucaemia (Rigas, Duerst, Jump and Osgood,

1956). The opportunity therefore exists to make a study of the nucleic acid content of the blood lymphocytes by chemical analyses in a variety of conditions.

At the time of writing there is no clear evidence which links B-lymphocytes or T-lymphocytes with any particular morphological class of cell; both may be small lymphocytes. There is a suggestion based on measures of cell size and electrophoretic mobility that the B-lymphocytes may be larger than T-lymphocytes (Davies, 1969) and this is supported by independent observations.

Metcalf (1967) compared the size of lymphocytes in suspension by Coulter counter and showed that lymph node cells and these from Peyer's patch were larger and more heterogeneous than thymus small lymphocytes. Heiniger, Riedwyl, Giger, Sordat and Cottier (1967) demonstrated by electron microscopy that small lymphocytes from the thymus of Swiss albino mice had a smaller nucleolar volume than small lymphocytes from the popliteal node in these animals; this was by a considerable margin, 0.11 μm^3 as against 0.46 μm^3. Thymic small lymphocytes also had less cytoplasm. Furthermore, thymic lymphocytes have been shown to be lacking in polyribosome structures (Clawson, Cooper and Good, 1966). From these observations it might be anticipated that T-lymphocytes, at least during the intra-thymic phase of their life span, would have a lower RNA content than B-lymphocytes. Large lymphoid cells which appear in the blood during an immune response may be plasma cells precursors (Birbeck and Hall, 1967) of the B-lymphocyte line, but

it is also possible that the large lymphocytes are T-lymphocytes which have undergone blastoid transformation. Techniques to differentiate these alternatives have hitherto been lacking as have effective methods of monitoring the changes in the circulating lymphocytes.

This review has only introduced topics from the vast literature on the lymphocytes but it has given justification for a study of the blood lymphocytes drawing attention to one of the problems, the difficulty of assessing these cells.

SECTION 2

Methods and their verification

2.1 Introductory review

Isolation of lymphocytes from peripheral blood.

The large number of published methods for the separation of leucocytes and lymphocytes from the peripheral blood is an indication that no technique is entirely satisfactory, for example Skoog and Beck (1956) have compared three methods and given data on yields and purity of the isolated cells, as well as information about the optimum conditions for cell separation. Two general principles have been used in methods for isolating the leucocytes from blood. In one type, advantage is taken of the property of certain high molecular weight materials such as dextran or gelatin to accelerate red cell sedimentation. Separation of polymorphonuclear leucocytes and lymphocytes takes place by differential adhesion of the cells either to the same added material, gelatin is effective (Coulson and Chalmers, 1964), or to additional adsorbants such as cotton wool (Cooper, 1967), or glass beads (Rabinowitz, 1964). In another procedure the cells are separated by centrifugation through layers of different density; solutions of bovine serum albumin (Vallee, Hughes and Gibson, 1947) or silicone oil (Seal, 1959) have been used. In the present investigation methods depending on these two principles were tried and compared.

Measurement of RNA and DNA in human lymphocytes.

Reviewers of the problem of nucleic acid determination in tissues have agreed that the most generally useful method of RNA measurement, and one that is still reasonably convenient, is to apply ultra-violet absorptiometry to the RNA fraction separated by the Schmidt Thannhauser method (Hutchison and Munro, 1961). An important proviso made by these authors is that allowance must be made for the small amount of protein present in the separated RNA fraction and since the amount and nature of the protein is variable depending on the tissue analysed the factors employed in any correction technique must be worked out for the tissue concerned.

Analyses of the DNA fraction following the Schmidt Thannhauser separation engenders similar dangers of contamination with protein as are encountered with the RNA fraction (Hutchison and Munro, 1961). Davidson (1957) recommended the extraction of the DNA with hot perchloric acid, 0.5 N at 80 C for ten minutes, however if ultra-violet absorptiometry is to be employed, correction for the presence of protein will still be necessary (Tsanev and Markov, 1960). Much of this section is concerned therefore with an assessment of the validity of these analytical methods for human lymphocytes.

2.2 The gelatin method for the isolation of blood lymphocytes

The gelatin sedimentation method of Coulson and Chalmers (1964) for the separation of human lymphocytes from peripheral blood was modified in order to increase the purity of the lymphocyte yield and the convenience of sample collection. Ethylene-diamine-tetra acetic acid (EDTA) was used as the anti-coagulant which avoided the need for immediate defibrination of the blood sample, a disadvantage of the original method. Defibrination was retained as a step in the separation however, since the fibrin clot selectively traps and removes polymorpho-nuclear leucocytes; the procedure was used after the bulk of the red cells had been removed and the selection therefore more efficient. In the method, an aqueous solution of gelatin prepared from lime-processed hides was mixed with the blood to accelerate red cell sedimentation. This gelatin has a high content of calcium (8.0 mg per 100 g of the solid), so that an increase in the amount of EDTA anticoagulant was required for the modified method.

Materials

1) 'Repelcote', a 2% solution of dimethyldichlorosilane in carbon tetrachloride (Hopkin and Williams, Ltd.).

2) 0.11 M EDTA, 4 g of the disodium salt per 100 ml water brought to pH 7.4 with 4 M potassium hydroxide to give a solution of

40 mg EDTA per ml.

3) 0.10 M calcium chloride, 1.10 g anhydrous salt per 100 ml in 0.05 sodium chloride (4.0 mg calcium per ml).

4) Gelatin, a 3% solution in 0.15 M sodium chloride. The gelatin was number 277, number 309 or number 66003, obtained from the Gelatin and Glue Research Association, Warwick Street, Birmingham, 12. The solution was freshly prepared for each batch of analyses.

5) 0.15 M sodium chloride.

6) 0.30 M sodium chloride.

Procedure

Venous blood, 20 ml, was collected in a polystyrene tube containing 1 ml of the EDTA solution. After mixing, 6.0 ml of the gelatin solution were added and the solution mixed again. The diluted blood was then transferred to a siliconed tube and stood at a temperature of 37 C for thirty minutes during which time the mixture separated into two visibly distinct layers. The whole of the upper layer, freed from the bulk of the red cells, was removed to a 50 ml conical flask fitted with a stirring rod suitable for defibrination (i.e. a glass rod with a wire paper-clip fixed on its end). Calcium chloride solution, 2 ml, were added and defibrination carried out by swirling. After about fifteen minutes, when defibrination was complete, 3 ml of the EDTA solution

were added and the suspension removed to a centrifuge tube. The cells were then spun down by centrifugation at 350 gav (1400 rev/min in a centrifuge head of 16 cm radius) for ten minutes. The lymphocytes sedimented as a small button tinged with red, there being some red cell contamination at this stage. It was convenient to make a smear of the sediment by spreading a small sample on a glass slide; the fixed and stained preparation was then used to assess the contamination with white cells other than lymphocytes. The red cells were removed by a process of osmotic shock; 2 ml or 3 ml of ice cold distilled water were added to the cell button, the cells suspended and left for exactly fifteen seconds. Normal tonicity was then restored by the addition of an equal volume of ice cold 0.30 M sodium chloride solution and the cells maintained thereafter at 4 C. Centrifugation at 125 gav (900 rev/min in the centrifuge head of radius 16 cm) again gave a cellular button, this time grey-white in colour. The haemoglobin from the lysed red cells remained in solution and was removed by Pasteur pipette together with a thin layer of red cell ghosts overlying the compact button. The lymphocytes were then suspended in 2.5 ml of 0.15 M sodium chloride solution. Some clumping of the cells was occasionally observed and these cell aggregates were removed by allowing them to sediment to the bottom of the tube on standing for five minutes. The suspended cells were then collected by Pasteur pipette and a cell count was carried out.

Usually about 12 million cells were obtained from 20 ml of blood representing a yield of 25 per cent of the lymphocytes. A minimum of three million cells were required for reliable estimation of nucleic acid so that 10 ml of peripheral blood would in most cases give a satisfactory yield of cells. Reduced volumes of blood were used on some occasions with appropriately adjusted reagent volumes but in general a 20 ml sample was used. The few cell suspensions with less than 90 per cent of lymphocytes were regarded as unsuitable for chemical analysis and were discarded.

2.3 The gelatin and cotton wool method for the isolation of blood lymphocytes

At a later stage of the work, following a suggestion by H.L. Cooper (1967), an additional step in the separation was adopted which improved the purity of the final lymphocyte suspension, so that samples with excessive contamination with polymorphonuclear leucocytes were all but eliminated. The new step was to add cotton wool to the defibrinated suspended cells, sufficient to soak up free fluid (the cotton wool was pre-washed in distilled water and dried). After fifteen minutes at 37 C the purified cell suspension was expressed from the cotton wool by pressure and the cells centrifuged ready for osmotic shock as before. This modified method will be referred to as the gelatin

and cotton wool method. The previous method, Section 2.2, is the gelatin method.

2.4 The silicone oil method for the isolation of blood lymphocytes

S.H. Seal (1959) has published a method intended for the isolation of cancer cells from the peripheral blood which allowed separation of lymphocytes from other cells by centrifugation through silicone oil of a particular specific gravity. The method described here has been modified from the published technique in an effort to maintain the viability of the lymphocytes by maintenance of a suitable osmolality and to eliminate possible difficulties with the technique due to fluctuations in room temperature.

Materials

1) Silicone Oil

MS 510 Hopkin and Williams, Ltd. (Relative density at 20 C with respect to water at 20 C 0.9956).

MS 555 Hopkin and Williams, Ltd. (Relative density at 20 C with respect to water at 20 C 1.0715).

The two silicone oils (density measured by density bottle technique) were blended to give an oil of relative density at 20 C with respect to water at 20 C of 1.0695-1.0698;

the preparation being 2.5 ml 510 to 97.5 ml 555; this was a modification from the original technique.

2) Polyvinyl Pyrrolidone (PVP)

35.0 g of PVP (British Drug Houses, Ltd.) was dissolved in 500 ml of 0.85% saline with 0.5 ml of WR 1339 polymer and 2.5 g of EDTA. The pH was adjusted to 7.4 (approximately 5 ml of M KOH) and the volume made up to 1 litre. Water, 100 ml, was then added to give a final osmolality of 290 m osmole/kg.

3) Saponin. A 2% solution of Saponin in water.

Procedure

Ten ml of silicone oil blended to a relative density of 1.0695 to 1.0698, was placed in a 50 ml siliconised centrifuge tube and overlaid with 20 ml of PVP solution and the whole cooled to 4 C. Then 8 ml to 12 ml of defibrinated blood was added to the PVP layer and the tubes spun at 500 gav (1650 rev/min in a head of 16 cm radius) for thirty minutes at 4 C. This was the time required for the red blood cells to penetrate the surface of the oil. Lymphocytes, with a few red cells, then remained at the PVP oil interface in a thin grey and red layer. The supernatant PVP solution was removed down to a level close to the oil surface and the cells washed off with 20 ml of fresh PVP solution using small aliquots and disturbing the oil as

little as possible. Gross contamination with oil droplets was reduced by allowing these to settle out from the washings. Red blood cells were lysed by addition of 1 ml of 2% saponin to 20 ml of PVP cell suspension. The lymphocytes were then centrifuged at 200 gav (1,100 rev/min in a centrifuge head of radius 16 cm) for thirty minutes at 4 C and washed in 10 ml of PVP. It was convenient to suspend the cells in 3 ml of PVP for counting and removal of aliquots.

2.5 Cytology

Lymphocyte counts.

Counting of lymphocytes in suspension was carried out by adding 0.1 ml of the cell suspension to 1.0 ml of 1.5% acetic acid solution, tinted with methyl violet, using accurate pipettes for the dilution. The cells were counted in an improved Neubauer counting chamber.

Whole blood white cell counts.

This was carried out by standard technique using the Coulter counter, model F, (Coulter Electronics Ltd., England).

Differential counts on blood white cells by light microscopy.

One hundred cells were examined on each blood smear stained with Leishman's stain. The battlement technique (MacGregor, Richards and Loh, 1940) was used to ensure representative counting of the cells.

Cell diameter measurements.

Some measurements were carried out of lymphocyte diameters as measured on a fixed and stained blood smear. These measurements were made using the Watson image shearing eyepiece, previously calibrated by a ruled calibration slide. Measurements to 0.1 μm were readily made with this technique.

Cell viability.

Estimation of viability of the isolated cells was carried out by the standard trypan blue method (Parker, 1961) which showed up dead or damaged cells, since only they are stained when the dye is added to a cell suspension. The procedure was as follows:- to 1 ml of isolated lymphocytes suspended in 0.15 M sodium chloride, 0.5 ml of a 0.5% solution of trypan blue in water was added. The cells were examined by light microscopy after ten minutes and the percentage of stained cells recorded. Cells prepared by the gelatin method and the silicone oil procedure were shown to respond to mitogens in tissue culture but no systematic comparison of cell viability by this method was carried out.

2.6 Phosphorus measurement

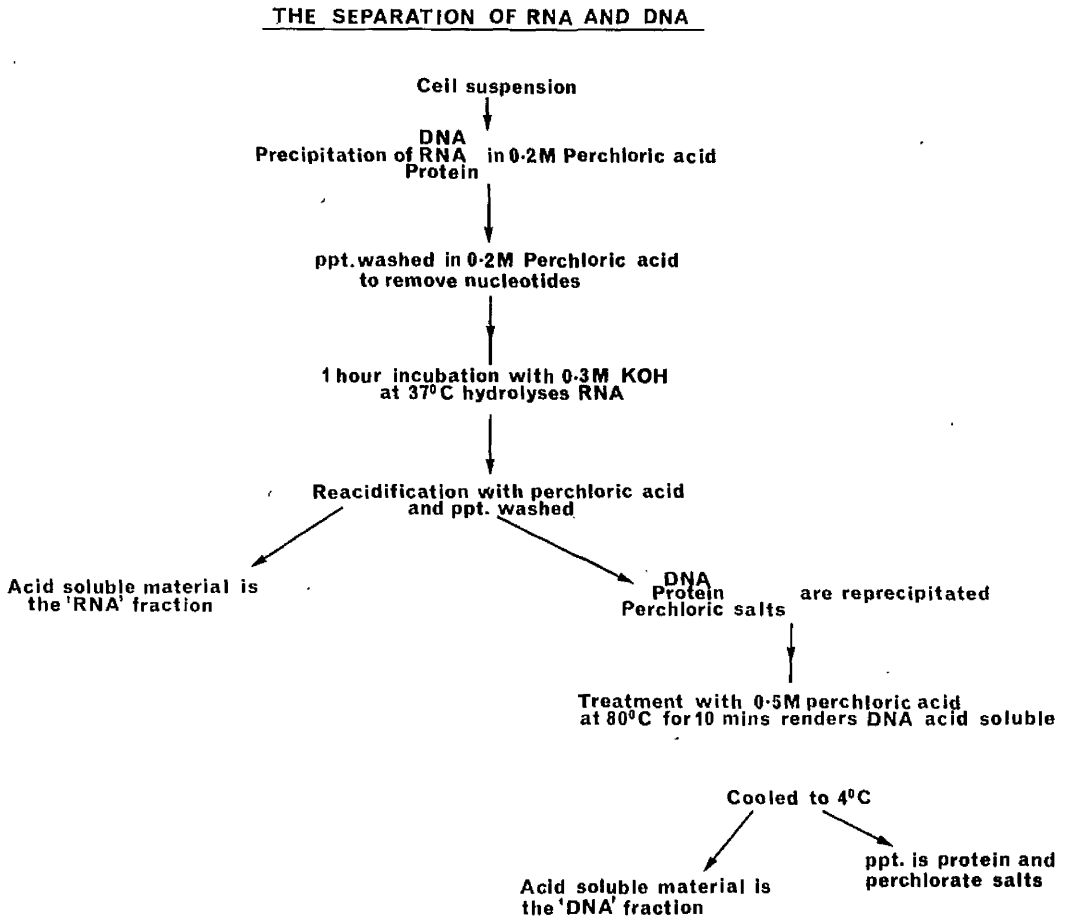
The method of Allen (1940) was used for measurement in the range 10 micrograms to 100 micrograms of phosphorus. Di-potassium hydrogen phosphate, Analar grade, was the standard and was prepared by drying to constant weight at 100 C.

2.7 Protein measurement

Measurements in the range of 10 micrograms to 150 micrograms of protein were carried out by the method of Lowry, Rosebrough, Farr and Randall (1951). Bovine serum albumin fraction V (Armour Pharmaceutical Co., England) was the standard used.

Figure 1

The separation of RNA and DNA by the modified Schmidt Thannhauser technique.



The flow diagram for the method of separation of a cell suspension into RNA and DNA fractions is outlined. Details are in the text (Section 2.8).

2.8 The measurement of RNA and DNA

The method used was that of Schmidt and Thannhauser (1945) as modified by Fleck and Munro (1962), omitting extraction with lipid solvents (Hallinan, Fleck and Munro, 1963), and using Schneider's modification for extraction of DNA (Davidson, 1957). Considerable reduction in scale from the standard procedures was required to give sufficient sensitivity for measurement of the small amounts of nucleic acid present in the samples of lymphocytes. The method makes use of the hydrolysis of RNA in dilute alkali as a means of rendering it acid soluble under conditions in which DNA and protein are stable. Reacidification allows separation of the acid soluble oligonucleotides derived from hydrolysis of the RNA from the insoluble residue of DNA and protein. The DNA protein fraction may then be treated with hot perchloric acid to extract the DNA. The flow diagram for the separation has been drawn up in Figure 1.

Range

The range for the method as described is from 0.4 micrograms to 5.0 micrograms RNA phosphorus and from 1 microgram to 25 micrograms DNA phosphorus.

Procedure

All manipulations were carried out at 4 C unless otherwise stated.

The sample, containing a known quantity of lymphocytes between 2×10^6 and 20×10^6 cells, was pipetted into a 9 cm by 1 cm centrifuge tube usually in 2.0 ml of 0.15 M sodium chloride. To this was added one half volume of 0.6 M PCA making the mixture 0.2 M in PCA. The suspension was left for ten minutes then centrifuged for five minutes at 500 gav (1650 rev/min in a 16 cm radius centrifuge head). The supernatant was discarded and the pellet resuspended in 0.2 M PCA. Centrifugation as before, followed by resuspension and recentrifugation, gave a pellet washed twice in PCA. Excess of acid was carefully drained from the pellet which was then broken up by shaking, treated with 0.5 ml of 0.3 M potassium hydroxide, and incubated at 37 C for exactly one hour.

At the end of the incubation period the solution was cooled to 4 C, and 0.25 ml of 1.2 M PCA added. After standing for ten minutes, centrifugation at 500 gav for five minutes was carried out and the supernatant transferred to a graduated tube. The precipitate was washed twice with 0.75 ml volumes of 0.2 M PCA, the washings being added to the graduated tube. The pooled supernatant and washings were made up to a volume of 2.5 ml or, in some experiments, 3.0 ml with 0.2 M PCA giving the RNA fraction

in 0.2 M PCA. It was found to be necessary to ensure that the graduation on the tubes was accurate.

The washed precipitate which contained the DNA together with cellular protein was treated with 2.0 ml of 0.5 M PCA and incubated at 80 C for ten minutes then cooled to 4 C, allowed to stand for thirty minutes, and centrifuged for five minutes at 500 gav. Supernatant 1.5 ml was removed and mixed with an equal volume of 0.5 M PCA to give the DNA fraction in 0.5 M PCA.

Measurement of RNA.

Measurement of the RNA content of the separated RNA fraction was carried out by ultra-violet absorptiometry.

A small amount of protein may be solubilised by the alkaline digestion procedure and this polypeptide will contribute to the optical density at 260 nm. A correction may be made for this error provided that the specific extinction of the contaminating material is known as well as its concentration. As an alternative to protein assay, a correction for protein contamination may be made by application of a two wavelength correction (Fleck and Munro, 1962). This necessitates the preparation of a sample of the tissue RNA to obtain values for the specific extinction coefficients at two selected wavelengths, together with knowledge of the specific extinction coefficients of the contaminating polypeptide at the selected wavelengths. A sample of human lymphocyte RNA was obtained by the method of

Digirolamo, Henshaw and Hiatt (1964), and the purified polypeptide prepared following the technique of Fleck and Munro (1962).

Correction by protein estimation.

Lymphocytes were isolated in quantity from human blood discarded from the heart lung machine. The polypeptide prepared from them had a maximal absorption at 275 nm, and a minimum at 255 nm. The extinction $E^{1\ cm}_{260\ nm}$ for a solution of the polypeptide in 0.2 M PCA containing 1 µg protein per ml was 0.00075 optical density units. This factor was used to correct the extinction at 260 nm of the RNA fraction for its measured protein contamination.

Correction by two wavelength measurements.

Purified human lymphocyte RNA had, after alkaline digestion and solution in 0.2 M perchloric acid, a molar extinction coefficient with respect to phosphorus of 9200 at 260 nm with a 1 cm light path. This solution had a maximum at 262 nm and a minimum at 235 nm. The ratio of the extinction at 260 nm to the extinction of 280 nm was 1.3. Unhydrolysed RNA had a similar spectrum except that the maximum was at 260 nm. There was no detectable protein contamination of this purified RNA but since some minor protein contamination was found in the fraction obtained by the modified Schmidt Thannhauser procedure described above, correction factors were calculated as follows.

The corrected RNA concentration, cRNAP of a solution, is given by: $cRNAP = A.E_1 - B.E_2$ (Fleck and Munro, 1962), where E_1 and E_2 are the optical density measurements of the solution made at two wavelengths λ_1 and λ_2. A and B are constants depending on the extinction coefficients of the pure RNA and of the contaminating protein. The selected wavelengths were λ_1 = 260 nm and λ_2 = 232 nm. A and B are derived as follows:-

$$A = \frac{1}{r_1 - r_2 . \frac{P_1}{P_2}} \qquad B = \frac{1}{r_1 . \frac{P_2}{P_1} - r_2}$$

r_1 and r_2 are the specific extinction coefficients of the lymphocyte RNA at λ_1 and λ_2; these values are 0.296 at 260 nm and 0.182 at 232 nm for a solution in 0.2 M perchloric acid containing 1 µg of RNA phosphorus per ml. P_1/P_2 is the ratio of the extinction coefficients of the protein contaminant at λ_1 and λ_2. A solution of the pure polypeptide in 0.2 M perchloric acid gave the value for P_1/P_2 of 0.210.

Substitution gives the values of 3.876 for A, and 0.812 for B. Thus $cRNAP = (3.876 . E^{1\ cm}_{260\ nm}) - (0.812 . E^{1\ cm}_{232\ nm})$ µg RNA phosphorus per ml.

A reagent blank obtained by carrying a sample of saline through the procedure of the Schmidt Thannhauser separation gives small but significant extinction values at the two wavelengths used, and a reagent blank was therefore included with each batch of analyses and appropriate correction made.

Measurement of DNA.

The modified Schmidt Thannhauser procedure described above gave the DNA fraction in 0.5 M perchloric acid. Measurement of the optical density at 265 nm was used to estimate the DNA content (Davidson, 1957). The standard for these measurements was highly polymerised calf thymus DNA (Sigma London Chemical Co.). This had a molar extinction coefficient $E^{1\ cm}_{265\ nm}$ of 9500 with respect to its phosphorus content, the measurement being again in 0.5 M PCA, a 1 µg P per ml solution of the DNA in 0.5 M PCA having an extinction at 265 nm of 0.308 after the usual acid and alkali treatments of the separation procedure. The wavelength used represents the maximum for both the standard and the lymphocyte DNA. The separated DNA fraction was found to contain a small amount of solubilized protein so that a protein correction was again applied. This was carried out as described under the measurement of RNA substituting the values applicable to the DNA measurement (Tsanev and Markov, 1960).

Calf thymus standard of concentration 1 µg DNA phosphorus per ml had an extinction $E^{1\ cm}_{265\ nm}$ of 0.308 = r_1 and $E^{1\ cm}_{232\ nm}$ of 0.127 = r_2 in 0.5 M PCA.

Values used for the protein contaminant were P_1/P_2 = 0.224. The corrected DNA concentration was given by:

cDNAP = $(3.571 \cdot E^{1\ cm}_{265\ nm}) - (0.802 \cdot E^{1\ cm}_{232\ nm})$ µg DNA phosphorus per ml. Again corrections to the extinction values for the reagent blank were made.

In some experiments DNA was determined by the Ceriotti (1955) procedure using calf thymus DNA as standard.

In later experiments, when access to the Olivetti programma 101 computer became available, a simple programme was used to calculate the RNAP content per 10^6 cells, DNAP content per 10^6 cells, and the RNAP/DNAP ratio, given the appropriate optical density values, sample volume measurements and the number of cells analysed.

The results have been expressed as DNA phosphorus (DNAP) and RNA phosphorus (RNAP) since values for the standards were arrived at by phosphorus determinations. Conversion from DNAP to DNA values and RNAP to RNA values depends on factors (approximately ten per cent of DNA and RNA is phosphorus) which are not necessarily the same in different tissues. Future determined results are therefore expressed as DNAP and RNAP which are preferable to the derived values for DNA and RNA.

2.9 Statistical methods

The statistical methods used in this and later sections were standard procedures as described by Snelecor and Cochran (1967) and by Armitage (1971). In most instances statistical comparisons of mean values were by Student's t-test but where a preliminary F-test showed significant between-group variance Welsh's test for 'd' was employed (Armitage, 1971), referring to the tables of values of 'd' (Pearson and Hartley, 1954) for values of statistical probability, p.

2.10 Results of comparison of yields, purity and viability of the cells in two methods of isolating lymphocytes from blood

In the initial stages of the work, two methods of lymphocyte separation were compared by measuring the yields of lymphocytes obtained and the purity of the isolated cells by differential counting of a stained preparation. The results of these measurements on samples of blood from normal individuals are listed in Table 1. The silicone oil method was superior to the gelatin separation only on the basis of the yield of lymphocytes obtained from blood. The average yield was 46 per cent of the theoretical possible lymphocyte yield in the silicone oil method, and 25 per cent in the gelatin method. There were only marginal differences in the purity of the isolated cells in the two methods

Table 1

Comparison of yield, purity and cell viability in two techniques of isolating lymphocytes from blood, the silicone oil method and the gelatin method.

		Silicone oil separation	Gelatin separation
Number of specimens studied		8	12
Percentage yield of lymphocytes	mean	46	25
	range	14 to 68	11 to 62
Percentage of lymphocytes in isolated cells	mean	98	94
	range	95 to 100	90 to 98
Percentage of viable cells (3 specimens only)	mean	60	96
	range	30 to 75	90 to 99

Percentage yield is the number of separated lymphocytes expressed as a percentage of the number of lymphocytes present in the whole blood; percentage of lymphocytes in isolated cells expresses the purity of the preparation measured on a stained film; percentage of viable cells is the percentage which do not take up the dye trypan blue in a standard technique described by Parker (1961).

but when cell viability was compared the silicone oil method was strikingly inferior. Sixty per cent of the lymphocytes on average were viable after silicone oil separation while an average of 96 per cent were viable after the gelatin procedure. In normal individuals all the mononuclear cells examined which were isolated by the silicone oil method, the gelatin method or the gelatin and cotton wool method, had the appearance of large or small lymphocytes; contaminating cells, always less than ten per cent and usually less than five per cent, were polymorphonuclear leucocytes.

Experiments to establish the validity of the method of measurement of RNA and DNA.

Determination of the validity of the RNA and DNA analyses requires a consideration of specificity, accuracy, precision and sensitivity of the methods employed. For this assessment, specificity will be defined as the extent to which the method measures either RNA or DNA and not interfering substances; accuracy describes how much individual determinations deviate from the amounts actually present; precision is defined as the repeatability of the analyses, and sensitivity describes the amount of the analysed substance which can be distinguished from zero. In practice, this last parameter corresponds to the standard deviation in replicate measurements (Richterich, 1969).

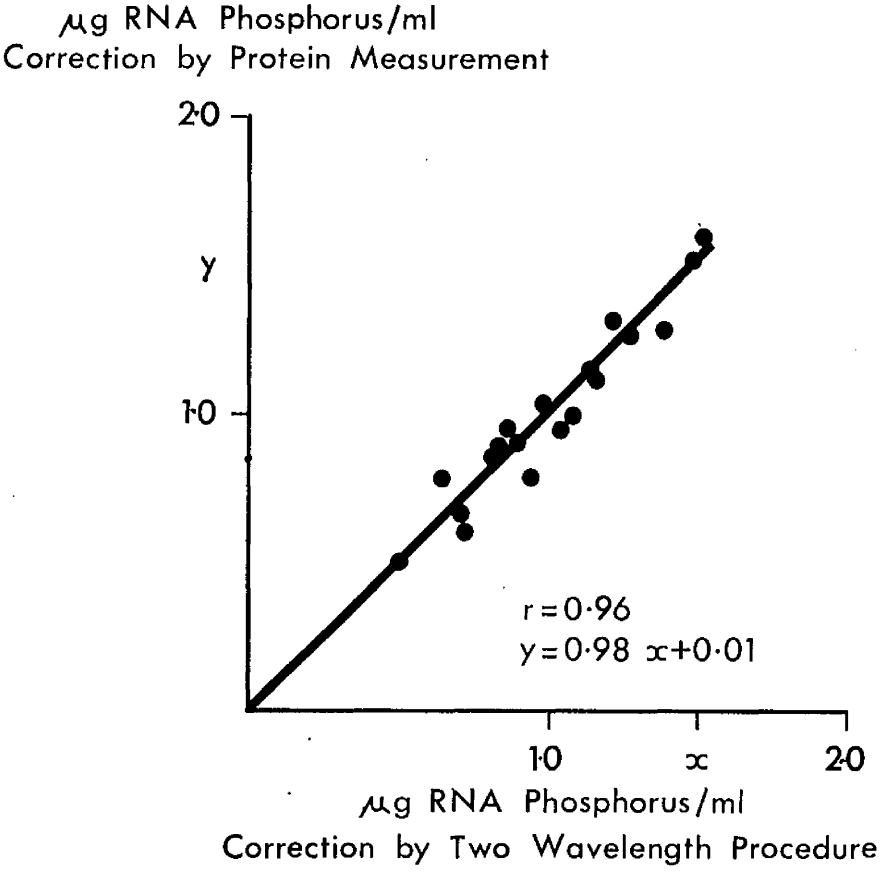
2.11 The specificity of the RNAP measurements

Errors in the determination of RNA by the Schmidt Thannhauser procedure can arise from the ultra-violet absorbancy of protein present in the fraction. Correction for this lack of specificity, as has been indicated earlier in the section, was carried out by measurement at two wavelengths with application of a mathematical correction, as well as by direct measurement of the contaminating polypeptide.

Presented here are the results of a series of measurements to establish that the two wavelength corrections are appropriate. Figure 2 shows the correlation between RNAP determinations by ultra-violet absorptiometry using the two wavelength correction, and RNAP measurements on the same samples with correction by actual measurement of the contaminating protein. The correlation coefficient of 0.96 established that the two wavelength method of correction would be satisfactory. It is also more convenient than the alternative method. More rigorous checking of the specificity has not been undertaken since the method is an established one and the recommended check procedures (Hutchison and Munro, 1961) have been carried out.

Figure 2

Measurement of lymphocyte RNA by ultra-violet absorptiometry; correlation of two methods of correction for protein error.



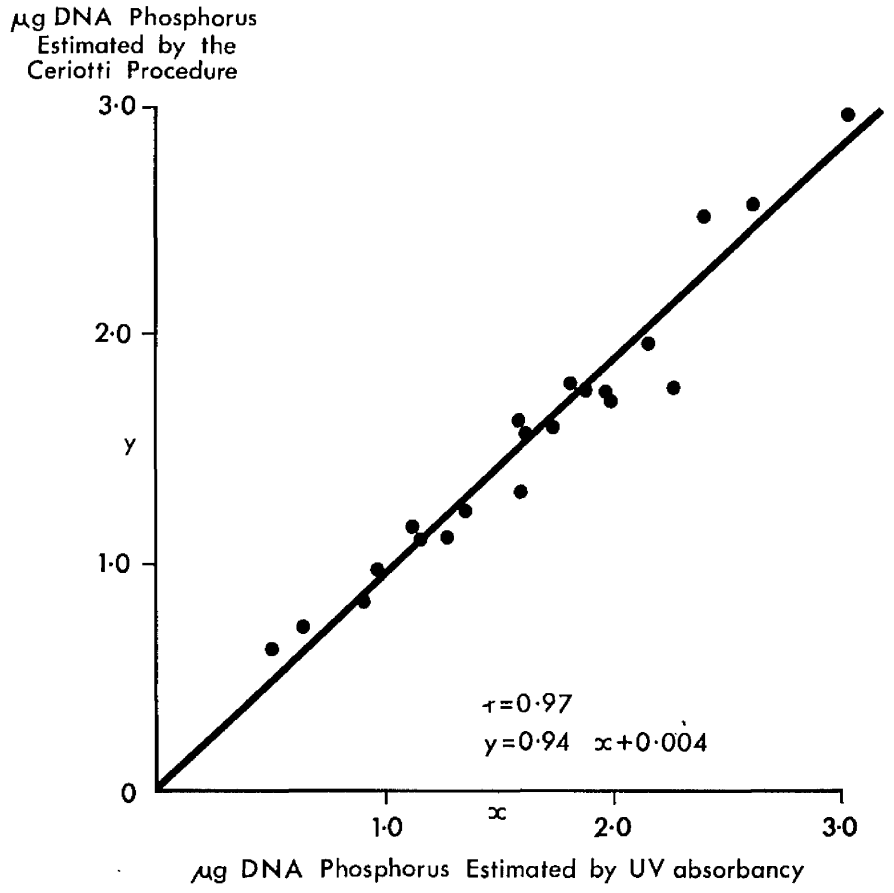
The ordinate (y) gives measurement of RNA by ultra-violet absorption in nineteen samples expressed as µg RNAP phosphorus per ml, corrected by measurement of protein contamination. The abscissa (x) shows the results of the analyses of the same samples using ultra-violet absorptiometry and the two wavelength correction. The regression line of y on x is drawn.

2.12 The specificity of the DNAP measurements

The results obtained in the DNAP analyses of a series of samples of lymphocytes using the two wavelength procedure were compared with the DNAP analyses by an independent method of measurement, i.e. that of Ceriotti (1955). The correlation coefficient of 0.97 was obtained in the comparison (Figure 3). The relationship between the two methods was such that one microgram DNA phosphorus by Ceriotti was equivalent to 0.94 micrograms of DNA phosphorus by the ultra-violet measurement plus 0.004. Thus the Ceriotti reaction gave slightly lower but still well correlated results when compared with ultra-violet absorptiometry but since the measurements by the Ceriotti technique are of lower accuracy than these using ultra-violet absorptiometry (see over) the latter method is considered superior.

Figure 3

Measurement of DNA. A correlation of two methods.



Measurements of DNA by the Ceriotti procedure as the ordinate (y) are compared with measurements of DNA by the two wavelength procedure in the abscissa (x). The regression line of y on x is shown.

2.13 Assessment of accuracy

Recovery experiments for the methods of nucleic acid analyses.

A solution was prepared containing DNA, RNA and protein in amounts similar to those encountered in analyses of lymphocyte preparations, i.e. 4.40 µg DNA phosphorus, 2.70 µg RNA phosphorus, and 3 mg of protein per 1 ml aliquot. The materials used were highly polymerised calf thymus DNA (Sigma London Chemical Co.), yeast RNA (Papst Laboratories, Wisconsin), repurified by the method of Zytko et al.(1958), and bovine serum albumin fraction V (Armour Pharmaceutical Co., England). The phosphorus content of the standard DNA and RNA solutions were determined in triplicate and the results compared with the results of multiple analyses by the modified Schmidt Thannhauser procedure measuring both DNAP and RNAP by ultra-violet absorbancy. DNA was also measured by the Ceriotti procedure. The results of replicate analyses are shown in Table 2. Adequate reproducibility is reflected in the low standard deviations of the recoveries. The Ceriotti method with the mean recovery of 82 per cent was less satisfactory than ultra-violet absorbancy with 100 per cent recovery for measurement of DNA. Ultra-violet absorbancy of RNA gave an 86 per cent recovery.

Table 2

The assessment of accuracy. Recovery experiments with comparison of methods.

Method of analysis	Number of analyses	Percentage recovery		Observed range of recovery (per cent)
DNA by Ceriotti	14	mean	82.0	65 to 98
		SD	9.4	
DNA by UV absorbtiometry	18	mean	100.4	93 to 111
		SD	4.7	
RNA by UV absorbtiometry	19	mean	86.4	75 to 100
		SD	4.5	

The results of multiple analyses of a standard solution of DNA, RNA and protein carried out to assess the recoveries obtainable by methods of nucleic acid measurement when compared with phosphorus estimations of the pure solutions taken as 100 per cent. Each aliquot contained 4.40 μg DNA phosphorus, 2.70 μg RNA phosphorus and 3 mg bovine serum albumin.

2.14 The precision of the lymphocyte analyses

The precision of the entire procedure of cell separation and nucleic acid analyses by ultra-violet absorbancy was examined by comparing the lymphocyte DNAP per 10^6 cells, RNAP per 10^6 cells, and RNAP/DNAP ratio values derived from duplicate samples of blood obtained from five individuals and separately carried through the stages of the analyses. Results for the gelatin method of separation are tabulated and the values of a similar series using silicone oil separation are included in a summarised form for comparison (Table 3). Using gelatin to separate the cells, the mean variation for DNAP measurements was $\pm$ 6.0 per cent and for RNAP measurements was $\pm$ 8.8 per cent. When the ratio $\frac{\text{RNAP}}{\text{DNAP}}$ was calculated from these results, the mean variation fell to $\pm$ 3.8 per cent.

The values for the duplicate blood samples using silicone oil as a method of cell separation show variation of a similar order to the gelatin method. The failure of the RNAP/DNAP ratio to have the lowest variation in this series was brought about by one duplicate result of RNAP/DNAP ratio having a variation of $\pm$ 24.0 per cent. This was an isolated result; in general, the variation was within five per cent on either side of the mean for the two results.

Table 3

Analyses of duplicate blood samples.

3A Gelatin cell separation method.

μg DNAP per 10^6 cells			μg RNAP per 10^6 cells		
Duplicate results	Mean	Variation	Duplicate results	Mean	Variation
0.822,0.763	(0.792)	±3.7%	0.227,0.184	(0.205)	±10.7%
0.713,0.836	(0.774)	±7.9%	0.133,0.167	(0.150)	±11.2%
0.879,0.705	(0.792)	±11.0%	0.162,0.125	(0.143)	±13.3%
0.740,0.911	(0.825)	±10.4%	0.229,0.315	(0.272)	±15.7%
0.898,0.844	(0.871)	± 3.1%	0.233,0.224	(0.228)	± 1.7%
Average Variation = ± 6.0%			Average Variation = ± 8.8%		

RNAP/DNAP ratio

Duplicate results	Mean	Variation
0.276,0.242	(0.259)	±6.8%
0.187,0.199	(0.193)	±3.1%
0.185,0.178	(0.182)	±2.2%
0.310,0.346	(0.328)	±5.5%
0.259,0.265	(0.262)	±3.9%
Average Variation =		±3.8%

Table 3 (cont'd)

3B Silicone oil cell separation method.

µg DNAP per 10^6 cells			µg RNAP per 10^6 cells		
Duplicate results	Mean	Variation	Duplicate results	Mean	Variation
0.694,0.780	(0.737)	5.9%	0.226,0.264	(0.245)	7.8%
0.670,0.710	(0.690)	2.9%	0.180,0.163	(0.171)	5.2%
0.719,0.687	(0.703)	2.3%	0.169,0.208	(0.188)	10.0%
0.746,0.757	(0.751)	0.8%	0.298,0.295	(0.297)	0.5%
0.854,0.926	(0.890)	4.0%	0.242,0.249	(0.245)	1.2%
0.935,1.020	(0.977)	4.3%	0.265,0.295	(0.280)	5.3%
Average Variation =		±3.3%	Average Variation =		±5.0%

RNAP/DNAP ratio

Duplicate results	Mean	Variance
0.326,0.338	(0.332)	1.8%
0.269,0.229	(0.249)	8.0%
0.235,0.305	(0.270)	24.0%
0.400,0.397	(0.398)	0.4%
0.283,0.269	(0.276)	2.5%
0.284,0.290	(0.287)	1.0%
Average Variation =		±6.3%

Duplicate samples of blood from normal individuals were carried through the stages of the analyses for DNA and RNA. The observed values for the nucleic acids are tabulated for each pair of results and shown also as percentage variation either side of the mean.

2.15 The sensitivity of the method of the chemical analyses

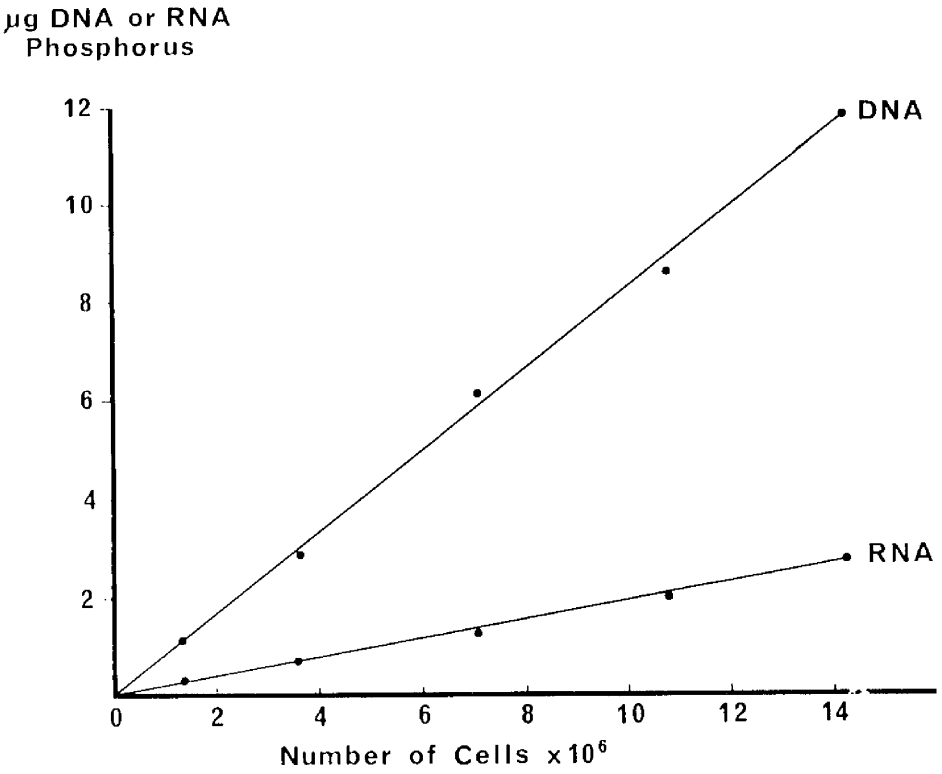
A series of aliquots of a lymphocyte suspension obtained from one normal individual and containing a range of numbers of cells from 1.43 million to 14.3 million lymphocytes were analysed. The individual measurements of DNAP and RNAP gave linear results over this range and the plot of the number of estimated cells against their nucleic acid content passed through the origin (Figure 4). Satisfactory results for both DNAP and RNAP measurements by ultra-violet absorbancy were obtained when from 3×10^6 cells to 14×10^6 cells are analysed.

2.16 Storage of isolated lymphocytes prior to analyses

In certain experiments, after the lymphocytes have been separated from blood, the isolated cells were stored prior to analysis. The presence of active nucleases in lymphocytes (Barnes, 1940) necessitated rigorous storage conditions for the preservation of the cells. Satisfactory conditions were found to be storage in 0.05 M sodium acetate, acetic acid buffer, pH 5.2, at a temperature below -40 C; solid carbon dioxide in a Dewar flask is a convenient method of maintaining the buffered cells below this temperature. A correction table for the

Figure 4

Assessment of the sensitivity of the method of DNA and RNA analysis.



Aliquots of a sample of lymphocytes containing different numbers of cells were analysed. The results plotted show the measured amounts of DNA and RNA phosphorus in relation to the number of cells analysed.

Table 4

The effect of storage of a lymphocyte suspension at -40C in 0.05 M acetate buffer pH 5.2, on the results of nucleic acid analysis.

Measurement	No storage	1 day storage	5 days storage	27 days storage
ug DNA phosphorus per sample	9.12	10.00	9.75	8.04
	9.49	10.05	9.28	9.10
	9.50	9.80	9.00	8.06
	9.50	9.40	8.61	-
Mean DNAP	9.40	9.81	9.16	8.40
Percentage change	0	+4.4	-2.6	-11.7
ug RNA phosphorus per sample	3.57	3.13	3.10	3.10
	3.42	3.44	3.13	3.10
	3.34	3.45	3.41	2.93
	3.35	3.08	3.73	2.94
Mean RNAP	3.42	3.28	3.34	3.02
Percentage change	0	-4.9	-2.3	-11.8
RNAP/DNAP ratio in sample	0.39	0.31	0.32	0.39
	0.36	0.34	0.34	0.32
	0.35	0.35	0.38	0.36
	0.35	0.33	0.43	-
Mean ratio	0.365	0.334	0.366	0.357
Percentage change	0	-8.5	+0.2	-2.2

Multiple samples of human thoracic duct lymphocytes containing 13×10^6 cells were analysed in batches of four by the standard procedure either immediately or after the stated period of storage at -40C.

possible small losses of nucleic acid which might occur in storage at -40 C was prepared from the results of serial quadruplicate analyses of replicate samples of human thoracic duct lymphocytes analysed fresh, and after storage in the buffer for one day, five days and twenty-seven days at -40 C. Details of the method of obtaining the cells appear in Section 4. The results of the serial analyses of the cells appear in Table 4, from which it can be seen that the losses in storage by this method are negligible if a five day storage is to be used. If the storage is longer a correction for losses of both DNA and RNA amounting to about ten per cent at twenty-seven days would be required.

Whole blood may be stored at room temperature for up to one and a half hours before starting the separation without alteration to the value obtained for the lymphocyte RNAP/DNAP ratio.

2.17 Discussion

The gelatin sedimentation method of separating lymphocytes from the peripheral blood was preferred for several reasons. The most important of these was the impaired viability of the cells in the alternative method. Viability is a requirement since RNA is a labile polymer readily attacked by cell nucleases which

are likely to be released in damaged cells. The yield of cells by the gelatin method was adequate for chemical analyses provided 20 ml of blood was taken and the need to discard samples because of impaired cell purity in the gelatin preparation was overcome by the addition of an incubation with washed cotton wool as has been described. The silicone oil method of cell separation was used for an experimental period only, and in view of its cumbersomeness as well as the impaired viability cells, it is not recommended. In the alternative method the choice of gelatin to be used is of interest. The critical properties of the material are not known though it is believed that the long gelatin molecule accelerates rouleaux formation of the erythrocytes, and therefore assist sedimentation. Chalmers (1967) and Johns (1970) have some further comments on the nature of the process. The gelatin was of recent manufacture and similar to that used by Coulson and Chalmers in their published method (1964). An important detail is the period of time allowed for the fibrin strands, and later the cotton wool, to trap polymorphonuclear leucocytes; fifteen minutes is the time recommended for each of these stages since prolongation of this results in poor cell yields. The rigorous conditions for cell lysis were satisfactory for chemical work but could require modification if cell culture was contemplated and here the work of Thomson, Bull and Robinson (1966) on the resistance of lymphocytes to osmotic shock was valuable in confirming the fifteen second exposure to distilled

water as optimum for lysing red cells without destroying leucocytes.

The specificity of the chemical method is not in doubt since satisfactory answers have been given to the question of protein contamination of the separated DNA and RNA fractions in the validation of the chemical methodology. Accuracy has been determined by measuring solutions of known nucleic acid content. The mean recovery for the DNA analyses by ultra-violet absorptiometry of 100.4 per cent needs no comment. Minor losses during acid precipitation steps or suboptimal hydrolysis of RNA by alkali are the most likely reasons for the incomplete (86 per cent) recovery of RNA but avoiding these possible losses is difficult. Alternative methods of analyses which rely on hot acid extraction of the tissue without precipitation stages (Schneider, 1945) are less satisfactory for the DNA analysis (Hutchison and Munro, 1961) and prolongation of alkali digestion in the Schmidt Thannhauser procedure results in release of protein into the RNA fraction which makes ultra-violet absorptiometry even with protein corrections less accurate (Fleck and Munro, 1962). Direct phosphorus determination of the RNA fraction has been used (Davidson, Leslie and White, 1951) but there is evidence that this fraction contains organic phosphorus components other than ribonucleotides. This has not been examined in leucocytes, but in rat liver (Davidson and Smellie, 1951) and rabbit spleen (Davidson, Fraser and Hutchison, 1951)

the non-nucleotide phosphorus represents 20 per cent and 25 per cent respectively of the phosphorus present in the fraction. The recovery of 86 per cent for the RNA measurements in the present method is therefore acceptable accuracy since this recovery is consistent.

In the experiments to measure precision, comparisons of determinations of DNAP content, RNAP content and RNAP/DNAP ratio in duplicate blood samples showed clearly that the ratio RNAP/DNAP has the smallest between duplicate variation and showed adequate precision. This finding illustrates the technical advantage of using the measurement which does not depend on cell counting, the lymphocyte RNAP/DNAP ratio. It is clear from Table 3 that an individual estimate of lymphocyte DNAP or RNAP expressed per million cells lacks precision. Cell counting error is the principal source of variation and since this error is dependent on the square root of the number of cells counted it could be reduced by repeated counting. In the present study, time for cell counts was limited since it was considered important that the isolated cells should be treated with cold perchloric acid as soon as possible after their removal from the blood circulation, thereby limiting degradation of nucleic acid particularly the more labile RNA. Any nucleic acid degradation would give consistently low values for the mean cell content while the error of cell counting tends to be a random one.

The lower limit of sensitivity of the method has not been

exactly determined but the method gives accurate results for the more critical RNA measurements using 1.4×10^6 cells. This limit might be variable depending on the protein content of the specimen, so in practice the limit on the number of cells required for reliable determination of nucleic acids has been 3.0×10^6 cells. This limit has been observed in all the results and is well above the theoretical limit set by the standard deviation obtained in the determination of accuracy, i.e. 4.5 per cent of 2.7 µg RNAP corresponding to 0.5×10^6 cells.

2.18 Summary

The investigations of the methodology may be summarised as follows:

1) The gelatin method as well as the gelatin and cotton wool method are satisfactory techniques for the preparation of lymphocyte suspensions from whole blood.

2) The modified Schmidt Thannhauser technique for the separation of RNA and DNA and subsequent ultra-violet absorptiometry of the separated fractions using two wavelength corrections for the presence of protein is the method of choice for human lymphocyte nucleic acid analyses. The method had adequate specificity, accuracy, precision and sensitivity.

3) In the combined technique, applying nucleic acid measurements to lymphocytes isolated from blood, determination of cell DNA and RNA content lacked precision due to cell counting errors. Precision for the measurement of the lymphocyte RNA/DNA ratio was good, and satisfactory analyses may be carried out provided at least 3×10^6 cells are analysed.

SECTION 3

Lymphocyte Nucleic Acid

Measurements in Normal Individuals and in the Presence of Immunological Abnormality

3.1 Introduction and review of past work

There are no normal values for the blood lymphocyte DNA, RNA or RNA/DNA ratio in the literature so the first step in the recognition of changes in the lymphocyte nucleic acid content in pathology is to define these normal values. Three methods of isolating the lymphocytes have been described earlier and initial studies compare results by these methods. When the normal range has been established possible differences in the lymphocyte chemistry related to age or sex will be considered, and the variation in lymphocyte DNA, RNA and RNA/DNA ratio on repeated analyses in an individual over a period of time investigated.

The blood lymphocyte count is a variable of possible importance in the study of the lymphocyte nucleic acid content. Changes in the blood lymphocyte count dependent on age, sex and pathology are well known (Wintrobe, 1967; Cruikshank and Alexander, 1970), and a valuable compilation of these has appeared recently (Eastham, 1970). In infancy the lymphocyte count is high relative to young adult values and gradually declines with increasing age. The blood lymphocyte count is on average marginally higher in females than in males. The differences between individuals are however much greater than these age or sex differences and this is recognised in the normal range for the blood lymphocyte count which is usually

reported as 1500 to 3500 cells per mm^3. The relationship between the blood lymphocyte count and the lymphocyte nucleic acid content is not known and will be included in the investigation. Blood lymphocyte counts were not determined in some studies, carried out before the importance of this measurement was appreciated.

Three conditions of known immunological abnormality have been examined in detail, namely rheumatoid arthritis, Sjøgren's syndrome and thyrotoxicosis. Blood analyses from patients with systemic and discoid lupus erythematosus, infection and with neoplastic disease are in sufficient number to justify listing as three separate groups. In addition, small numbers of patients with a variety of conditions have had measurements of blood lymphocyte nucleic acids and these form a miscellaneous group. The lymphocyte chemistry will be examined in each condition, starting with the DNA measurement since if the DNA content of the cell is constant, the lymphocyte RNA/DNA ratio is validated as an index of the cell RNA. The relationship between severity of the disease and the lymphocyte results will be considered as well as the influence of drug therapy where this is appropriate. In some conditions the relationship between the lymphocyte chemistry and the appearance of the cells on a peripheral blood film has been investigated.

The lymphocytes in rheumatoid arthritis.

Rheumatoid arthritis is a common disease and immunological abnormalities are such a striking feature of the pathology that this condition was the first choice for the study of lymphocyte nucleic acids in disease. Abnormalities of the antibody and the cell mediated immunity systems are well documented in rheumatoid arthritis, for example rheumatoid factor is present in the sera of about 80 per cent of patients with the disease (Glynn, 1965). It has the characteristics of an auto-antibody and can react with a patient's own IgG (Hannestad, 1968). Other antibodies are found with abnormal frequency in the sera of patients with rheumatoid arthritis, including anti-nuclear factor (Alexander, Bremner and Duthie, 1960) and thyroid auto-antibodies (Buchanan, Crooks, Alexander, Koutras, Wayne and Gray, 1961). The presence of rheumatoid factor in the serum is not restricted to patients with rheumatoid arthritis (Anderson, Buchanan and Goudie, 1967) and the factor is unlikely to be the cause of the lesions, Harris and Vaughan (1961) having demonstrated that infusion of serum positive for rheumatoid factor into healthy volunteers did not produce disease in the recipients, although autologous rheumatoid factor injected locally into a joint can sustain joint inflammation (Restifo, Lussier, Rawson, Rockey and Hollander, 1965). Attention has therefore been directed to cell mediated immunity in the disease process.

Cell mediated effects have proved more difficult to

investigate. Skin hypersensitivity to the chemical agents 2,4-dinitrochlorobenzene and para-nitro-sodimethyl analine is known to be cell mediated and this response has been tested in patients with rheumatoid arthritis. The ability to become sensitized to these materials is depressed compared with normal individuals (Epstein and Jessar, 1959), however, these authors noticed no correlation of abnormal tests with a particular stage of the disease. The in vitro response of the blood lymphocytes from patients with rheumatoid arthritis to the non-specific mitogen phytohaemagglutinin (PHA) and to streptolysin O has been examined by Leventhal, Waldorf and Talal (1967). Their results clearly demonstrated impaired lymphocyte responses to both these agents, confirming that depressed lymphocyte function is a feature of the disease. The skin hypersensitivity reaction and the phyto-haemagglutinin response are thought to be mediated by thymus dependent lymphocytes (Davies, 1969) so T-lymphocyte function is impaired in rheumatoid arthritis. Recently it has been shown that a greater than normal proportion of B-lymphocytes are present in the blood of patients with the disease (Papamichail, Brown and Holborow, 1971) so impaired T-lymphocyte function may be linked with a relative or absolute deficiency of these cells.

A relative but not necessarily an absolute lymphocytosis is a well-recognised feature of rheumatoid arthritis and there is unmistakable evidence of ectopic lymphocyte activity, recognisable by the classical histology of the lesions in the rheumatoid nodule,

and by lymphocytic infiltration of the synovia in affected joints. Only two references to the morphology of the blood lymphocyte in rheumatoid arthritis have been found in the literature, thus Zucker-Franklin (1969) recorded the presence of atypical lymphocytes in the blood of patients with the disease, and in an earlier less sophisticated study Kristenson (1949) found increased numbers of large lymphocytes in the peripheral blood of a patient with arthritis. It may be possible to measure the extent of these changes by examining the lymphocyte chemistry.

The American Rheumatism Association have graded rheumatoid arthritis as probable or definite depending on the number of easily recognisable features of the disease present in a particular patient (Ropes, 1959). In an initial analysis results from patients with probable and definite rheumatoid arthritis are combined but later the results of the lymphocyte analyses are listed according to the two American Rheumatism Association categories. Any relation between severity of the disease and the lymphocyte abnormality should then be apparent. It may also be possible to relate measurable features of the disease, e.g. the E.S.R. or the rheumatoid factor titre to the lymphocyte RNAP/DNAP ratio in a series of patients. Certain features of rheumatoid arthritis are associated with a poor prognosis. Duthie, Brown, Truelove, Barger and Lawrie (1964) have found a tendancy to progressive disease in patients with a high rheumatoid factor titre, and in those with subcutaneous nodules. A study of these points

will be made to establish the relationship between disease activity and the lymphocyte chemistry.

The steroid therapy commonly used in rheumatoid arthritis could be a factor in influencing the cell chemistry since corticosteroids are known to have a lympholytic action (Dougherty and Frank, 1953). This will require to be tested.

The demonstration of abnormality of the lymphocytes both by chemistry and by morphology would be more convincing than either alone. Studies will be included which make this comparison of lymphocyte nucleic acid content and measurements of the cells as they appear on a stained blood film.

The lymphocytes in sicca and Sjøgren's syndrome.

The study of Sjøgren's syndrome contributes to knowledge of lymphocyte nucleic acid content in immunologic disease since the syndrome includes two conditions with similar frequencies of abnormal circulating antibodies and yet differing degrees of tissue involvement.

Sjøgren's syndrome consists of kerato-conjunctivitis sicca (dry eye), xerostomia (dry mouth) and rheumatoid arthritis or other connective tissue diseases. The first two, when present by themselves, constitutes the sicca syndrome (Block, Buchanan, Wohl and Bunim, 1965). About half of the patients with Sjøgren's syndrome

have rheumatoid arthritis as the associated disorder. These individuals frequently have multiple antibodies in their serum and there are widespread chronic inflammatory changes with lymphocyte infiltration in a variety of tissues. In sicca syndrome multiple antibodies are present, perhaps in greater frequency than in the complete Sjögren's syndrome, and although chronic inflammatory changes may be present in a variety of tissues, notably the lacrimal and salivary glands, there is no connective tissue disease.

In parallel with their studies on rheumatoid arthritis, Leventhal, Waldorf and Talal (1967) have produced evidence of functional abnormalities of lymphocytes in Sjogren's syndrome. They found in a significant number of patients with Sjögren's syndrome that the lymphocytes failed to produce a normal in vitro transformation response to the mitogens, phytohaemagglutinin and streptolysin O. There was a similar frequency of abnormalities in the response of patients to the contact allergen 2,4-dinitro-chlorobenzene, failure to become sensitised to the chemical is evidence of an impaired delayed hypersensitivity response. The patients with the combination kerato-conjunctivitis, xerostomia and rheumatoid arthritis, i.e. the full Sjögren's syndrome, showed a greater proportion of abnormal responses than the group with sicca syndrome alone. In Sjogren's syndrome a leucopenia with a relative increase in a proportion of lymphocytes has been reported (Bloch et al., 1965) but otherwise information on the nature of the

blood lymphocytes in the disease is lacking. In the nucleic acid measurements on the blood lymphocytes which follow, answers are sought for two questions, firstly, is there biochemical evidence that the lymphocytes are abnormal in the sicca syndrome or in Sjögren's syndrome, and secondly, is there a difference in degree of abnormality between sicca syndrome and the complete Sjogren's syndrome.

The lymphocytes in thyrotoxicosis.

A general discussion of the aetiology of thyrotoxicosis would be out of place in this introduction, but it is clear from a recent review (Havard, 1969) that immunological factors or, more precisely, the circulating immunoglobulin 'long acting thyroid stimulator' (LATS) is involved in the over-stimulation of the thyroid gland in most patients with thyrotoxicosis. Excepted from this are the rare occasions when an autonomous toxic adenoma is present. The existence of LATS became apparent when Adams and Purves (1956) observed that material in the blood of thyrotoxic patients which was thyroid stimulating was not pituitary thyroid stimulating hormone. This was a protein material later identified as an immunoglobulin (Kriss, Pleshakov and Chien, 1964; Dorrington and Munro, 1965), and is of the class IgG. Depending on the assay technique used and whether sera are concentrated

before analysis, LATS can be demonstrated in up to 80 per cent of patients with thyrotoxicosis (Carneiro, Dorrington and Munro, 1966). When sera are analysed without prior concentration the LATS assay is positive in about 30 per cent of patients (Sellers, Awad and Schonbaum, 1970). The most convincing evidence that LATS can produce thyrotoxicosis is derived from the study of thyrotoxicosis in pregnancy. Mothers who have a high titre for LATS pass the immunoglobulin via the placenta to the foetus and thyrotoxicosis is produced in the infant, a condition which resolves as the LATS activity disappears (McKenzie, 1964). Attempts to produce LATS experimentally by injecting thyroid antigens into animals have been only partially successful (Burke, 1968) but natural auto-antibodies do occur frequently in the sera of patients with thyrotoxicosis; as many as 85 per cent of patients showing abnormalities (Anderson, Buchanan and Goudie, 1967). There is sufficient evidence therefore to regard thyrotoxicosis as an auto-immune disease although an unusual one since the affected organ is stimulated rather than depressed.

The presence of an abnormality in cell mediated immunity in thyrotoxicosis is less well established. A variable degree of thyroiditis with infiltration of lymphocytes into the gland tissue is a feature of the pathology of thyrotoxicosis (Buchanan, Alexander, Crooks, Koutras, Wayne, Anderson and Goudie, 1961), and lymphocytes have been observed invading the thyroid vesicles in the disease (Irvine and Muir, 1963). Patients with thyrotoxicosis

may show a relative lymphocytosis in the peripheral blood (Williams, 1965). A single author, Hernberg (1954), has published data on the nature of the lymphocytes in thyrotoxicosis. Hernberg measured the size of the lymphocytes in fixed and stained blood films, and found that the mean lymphocyte diameter was at the upper limit of normal or above normal in thyrotoxicosis, and returned to normal when the patients were euthyroid after partial thyroidectomy. Despite this finding, De Groot and Jaksina failed to show a significant functional abnormality in thyrotoxic lymphocytes in the courses of studies of the phytohaemagglutinin response of these cells. The near normality of the phytohaemagglutinin response and the absence of massive infiltration of lymphocytes in the tissues suggests that the immunological abnormality of thyrotoxicosis may be slight and related to activity of B-lymphocytes, cells of the antibody producing line. Measurements of lymphocyte RNAP/DNAP ratio would not of course specifically identify this abnormality but the chemical determinations may confirm Hernberg's data. Furthermore, it is now possible to compare lymphocyte from blood positive in the LATS bio-assay with blood negative in the LATS assay. Carneiro et al.(1966) have shown a correlation between the iodine turnover per unit weight of the gland and the LATS activity, and although the severity of thyrotoxicosis is difficult to measure it is possible to test for a correlation between the I^{131} uptake of the gland and chemical abnormalities of the lymphocytes.

The lymphocytes in systemic lupus erythematosus.

Changes in the lymphocytes are not a striking feature of the pathology of systemic lupus erythematosus although there is often a leucopenia and lymphopenia. The important histological features are widespread fibrinoid degeneration and necrosis in the walls of small blood vessels associated with areas of collagenous fibrosis and hyalinization. A perivascular cellular infiltrate, mainly of lymphocytes, is also seen. The tissues which may be involved include skin, myocardium, pericardium and endocardium, kidney and spleen. When there is clinical evidence of skin involvement alone the lesion is referred to as discoid lupus erythematosus.

A variety of antibodies are found in the sera of patients with the disease; the list includes antibodies to nucleohistone complex, DNA, histone and other nuclear and nucleolar proteins. Antibodies to cytoplasmic constituents may also be present (Anderson, 1963). Some understanding of the immunopathology of the vascular lesions in systemic lupus erythematosus has come from study of the analogous spontaneous disease in hybrid mice, the offspring of the cross between NZB and NZW strains. It has been shown in these animals (Lambert and Dixon, 1969) that there is a close relationship in time between the appearance of antinuclear antibodies and the development of kidney damage. It is probable that the kidney

lesions result from glomerular localisation of antigen-antibody complexes (Seegel, Accinni, Andres, Christian, Erlanger and Hsu, 1969). Antigen-antibody complexes are thought to be important in the human disease and cell mediated reactions less so. However, the present views on the importance of thymic dependent lymphocytes in control of the antibody as well as cell mediated response make investigation of the blood lymphocytes in lupus erythematosus of interest. The number of patients in the present study is small and includes patients with discoid as well as systemic lupus erythematosus (Haserick, 1965). Within this limitation, measurement of the DNA and RNA content of the blood lymphocytes should give an indication of abnormality in these cells.

The lymphocytes in infection.

Hyperbasophilic medium sized lymphocytes, plasma cells and primative lymphoid cells appear in the blood after immunisation and infection and, from the evidence outlined in the introduction, it is likely that these are derived from lymphoid tissue in response to immune stimuli. Detailed investigation of the response to immunisation in man has been studied by two groups, Pariser, Zucker and Meyer (1952) and Crowther, Fairley and Sewell, (1969). The earlier study was of the secondary response to diptheria toxoid while both primary and secondary responses were examined by Crowther and his group. From these studies it is

clear that following both primary and secondary immunisation large lymphoid cells appear in the peripheral blood with the maximum response on the fourth to sixth day following immunisation. Although there is considerable individual variation in the response, in no case were there more than six per cent of atypical lymphoid cells present in the peripheral blood.

Lesiewska (1967) has investigated the blood lymphocytes following bacterial and viral infection and shown that only minor changes in the proportion of large basophilic lymphocytes take place following bacterial infection in children. Much more marked changes occurred following virus infection. In a study of chicken-pox and measles the absolute number and percentage of large lymphoid cells increased more than two-fold. Lesiewska confirmed this data with histochemical determinations of lymphocyte RNA content. The virus-induced changes in the lymphocytes may well be produced by blastoid transformation as has been demonstrated in leucocytes from patients with a previous history of infectious mononucleosis, exposed in culture to EB virus (Diehl, Henle, Henle and Kohn, 1968).

A group of patients with bacterial and viral infections are included in the present study to assess the degree of change which might occur in the lymphocyte RNA and DNA content during an infection, allowing that only a proportion of the samples were taken at the optimum time of four to six days after exposure to

the viral or bacterial antigens.

The lymphocytes in neoplastic disease.

In a review of immunological factors affecting tumour growth, Klein (1970) maintains that the immune response against tumours resembles a homograft reaction and that cellular immunity plays the major part in this response. Failure of the mechanisms of immunological surveillance have been proposed as important in the proliferation of neoplastic cells (Keast, 1970). There is undoubtedly an association between immunological disorders and cancers of the reticulo-endothelial system, but this need not be cause and effect, and the concept of impaired immunological surveillance and the development of cancer in man remains as a hypothesis at present (Doll and Kinlin, 1970).

There are functional abnormalities of cellular immunity in the presence of established neoplasm. The tuberculin response shows a marked depression in cancer patients (Hughes and Mackay, 1965). The in vitro phytohaemagglutinin response of blood lymphocytes from patients with carcinoma is impaired (Iwao, 1968) and this impairment is attributable to abnormalities of the cells, not the plasma (Golob, Israsena, Quatrale and Becker, 1969).

A study of the morphology of the blood lymphocytes in a series of patients with breast cancinoma was made by Smetana, Janele and

Malinsky (1966). They recorded the average number of nucleoli per lymphocyte nucleus and made the interesting observation that the number of cells with multiple nucleoli is increased in malignancy. Since the nucleoli are concerned with RNA bio-synthesis, chemical measurements of DNA and particularly RNA content may show changes from normal in the presence of neoplasm. Lymphopenia is a feature of established malignancy and is recorded in the two papers cited (Iwao, 1968, and Smetana et al, 1966).

The lymphocyte in a miscellany of conditions.

A detailed study of the lymphocyte chemistry in a wide range of conditions is beyond the scope of this thesis but inclusion of results from small numbers of patients with a variety of conditions does broaden the knowledge of the lymphocyte in disease processes. The conditions represented in the miscellaneous group show a spectrum of involvement of the immunity system in the disease process.

The conditions with a recognisable abnormality of immunity will be considered first. Macroglobulinaemia is a disease characterised by a proliferation of lymphoid cells usually recognisable by lymphadenopathy, splenomegaly and infiltration of the bone marrow by atypical lymphoid cells. The serum shows an excessive quantity of an immunoglobulin of the class IgM.

Atypical lymphocytes are recognised in the peripheral blood and it appears that these plasmacytoid lymphocytes are the source of the excessive immunoglobulin (Zucker-Franklin, Franklin and Cooper, 1962).

Neoplastic overproliferation of plasma cells may occur in the bone marrow and elsewhere in the distinct condition of myelomatosis. A patient with light-chain myeloma is included in this study and here the plasma cells are producing excessively the shorter so called light amino-acid chains of an immunoglobulin. Abnormal cells in the peripheral blood are not usually a feature of this disease. The presence of non caseating granulomatae in a variety of tissues is the principal feature of sarcoidosis. It is considered to be a disease of hypersensitivity (Epstein, 1967) and it is recognised that there is a deficiency of some forms of delayed hypersensitivity reactions (Good, Kelly, Rotstein and Varco, 1962) while the lymphocyte response to phytohaemagglutinin is also impaired (Topilsky, Siltzbach, Williams and Glade, 1972). Functional abnormality of the blood lymphocyte in sarcoidosis is therefore established.

In other conditions represented in the miscellaneous section the presence of a cellular immune abnormality is less certain. Crohn's disease may affect any level of the gastro-intestinal tract from stomach to colon, but most commonly involves the terminal ileum. The diseased areas show ulceration and destruction of the mucosa, marked submucosal fibrosis with chronic

inflammation and infiltration with neutrophils, lymphocytes, histiocytes and plasma cells. Aggregation of lymphocytes into follicles may occur in subserosal and submucosal zones and these areas may show sarcoid-like granulomas (Robbins, 1967). The presence of aggregates of lymphocytes has suggested that an immunological reaction is a feature of the disease, however there is no consistent change in the levels of serum immunoglobulins, and tests for organ specific cellular hypersensitivity to intestinal mucosal components by the leucocyte migration test proved negative (Weeke and Bendixen, 1969).

Broberger and Perlman (1959) have pointed out features of the immunopathology of ulcerative colitis. Anti-colonic antibodies are found in the sera of a proportion of patients with established disease, and more recently it has been shown that some patients with the disease have elevated levels of serum IgG, furthermore leococytes from the majority of patients show sensitization to pooled foetal colonic or jejunoileal mucosal antigens in the leucocyte migration test (Weeke and Bendixen, 1969).

These abnormalities of both cellular and antibody immunity may be reflected in changes in the blood lymphocytes although they may just as readily be secondary features of the disease as of primary importance in the pathogenesis.

Although pemphigus and pemphigoid are regarded as auto-immune diseases it is the presence of circulating antibodies to intercellular material in pemphigus and to the basement zone of

skin in pemphigoid which are important. The primary lesions can occur without local infiltration with chronic inflammatory cells so the typical changes associated with a cell bound immune reaction are absent (Beutner, Jordon and Chorzelski, 1968).

There has been considerable speculation on the cause of Whipple's disease since the original description in 1907 (Whipple, 1907). Recently Maxwell, Ferguson, McKay, Irvine and Watson drew attention to the sparcity of the lymphocyte and plasma cell response in the gut lesions and showed a functional impairment of the lymphocyte responses to phytohaemagglutinin in patients with this disease. Colonic histiocytosis, although affecting a more distal part of the gut, is histologically similar to Whipple's disease and both these conditions have been included in the study of patients from the miscellaneous group as conditions with possible abnormality of immunity.

The conditions which are unlikely to be associated with an abnormality of immunity include patients with salicylate over-dosage, acne, osteoporosis and nutritional osteomalacia.

Three patients with leucaemia have been studied and their results included in the miscellaneous group. Measurements of the lymphocyte DNA and RNA content in leucaemia have been reported by Rigas et al., (1956), and the opportunity is taken to compare the findings in a limited series here with nucleic acid measurements reported in the literature.

MATERIALS AND METHODS

3.2 Clinical material

Normal individuals.

Three groups of normal individuals were studied in initial measurements to investigate possible effects of the cell isolation technique on the normal range.

Normal group 1.

There were seven individuals in this group, all hospital staff, five were males. Their mean age was twenty-seven years with a range of from twenty-two years to thirty-one years. Fifteen analyses using the silicone oil method of cell separation were carried out on this group.

Normal group 2.

The gelatin method of cell separation was used for the separation of lymphocytes from blood in a study of the thirty-nine normal individuals in group 2. All but three were hospital staff. Their mean age was thirty-five years with a range of from eighteen years to seventy-seven years; nineteen of the group were males. Some details of these individuals are listed in Appendix 1. Six

individuals were members of both group 1 and group 2.

Normal group 3.

Eleven males and nine females, all but one hospital staff, made up this group. Their mean age was thirty years with a range of from eighteen years to sixty-three years. Only one individual was common to another of the normal groups. Details of the individuals in group 3 appear in Appendix 2.

Rheumatoid arthritis.

Thirty-five patients with rheumatoid arthritis were studied. Twenty-four had definite rheumatoid arthritis by the American Rheumatism Association criteria (Ropes, 1959); twenty-one of this group were female, and their mean age was fifty-five years with a range of from twenty-five years to seventy-eight years. The remaining eleven patients, eleven of whom were female, had probable rheumatoid arthritis. Their mean age was forty-seven years with a range of from twenty-five years to seventy years. Details of the patients are listed in Appendix 3.

Sicca syndrome and Sjøgren's syndrome.

Two groups of patients were studied, patients with the sicca syndrome and a second group with Sjøgren's syndrome.

The diagnostic criteria for the sicca syndrome were the presence of kerato-conjunctivitis sicca and xerostomia as recognised by dry mouth and an abnormal sialogram. All eight of the patients were females and the average age was sixty-four years with the range from fifty-five years to seventy-six years. Eight patients with Sjøgren's syndrome had the features of the sicca syndrome with, in addition, definite rheumatoid arthritis by the American Rheumatism Association criteria (Ropes, 1959). One of the eight patients was male and the age range for the group was fifty-one years to seventy-eight years with an average age of sixty-six years.

Details of the patients with sicca syndrome and Sjøgren's syndrome are listed in Appendix 4.

Thyrotoxicosis.

Thirty-four patients with thyrotoxicosis, initially untreated, were the subjects of a study. There were thirty women and four men with an age range of from twenty-two years to seventy years and a mean age of forty-nine years. The clinical thyrotoxicosis was confirmed by radio-iodine uptake studies, serum protein bound

iodine determinations and serum tri-iodothyronine resin sponge tests. The results of a selection of these measurements are included in Appendix 5.

The initial studies of untreated thyrotoxicosis were followed by lymphocyte nucleic acid determination on patients euthyroid as a result of treatment. Ten patients received Carbimazole and the lymphocyte RNA and DNA measurements were carried out after control of the thyrotoxicosis and while a maintenance dose of 15 mg or 20 mg of Carbimazole was continued. This was an average of four months (range two to six months) after starting drugs. Twenty-one patients were treated with radioactive iodine and the nucleic acid measurements were carried out on the lymphocytes after control of the thyrotoxicosis, again four months (range two to six months) from the date of the dose. Of the remaining five patients, three patients had a partial thyroidectomy and two patients did not return.

Lupus erythematosus.

Eight patients with lupus erythematosus comprised the first of the minor studies. All but one were females. The age range was eighteen years to sixty-five years. Details of these patients, including the degree of systemic involvement according to Haserick's classification (1964), have been recorded. A single patient had

skin manifestations only (group 1). Four patients (Haserick group 2) showed evidence of LE cells in the blood as well as skin lesions and three patients had unequivocal systemic disease (group 3). Details of these patients are listed in Appendix 6.

Infection.

Eight patients with bacterial infection and two patients with viral infection were the subjects of a study. Six of the group were males. The age range of the group was seventeen years to sixty-three years with a mean age of thirty-one years. Details of the individual patients and the nature and duration of their infections are drawn up in Appendix 7.

Neoplasm.

Six patients with histologically confirmed malignant neoplasms were studied, all were females. Their age range was fifty-eight years to seventy-five years. Details of these patients are listed in Appendix 8. Four had invasive spread with metastases and two had local neoplasm only.

Miscellaneous.

This group comprised twenty-two patients and fifteen different conditions are represented. No advantage is to be gained from analysing these patients as a group and their individual data appear in Appendix 9.

3.3 Methods

Three different methods of isolation of lymphocytes from blood, the gelatin method (Section 2.2), the gelatin and cotton wool method (Section 2.3), and the silicone oil method (Section 2.4), have been used in lymphocytes from normal individuals. In the patients with rheumatoid arthritis, Sjøgren's syndrome, lupus erythematosus and infection, the gelatin method was used. For all the remaining patient groups the gelatin and cotton wool technique was used.

Chemical analysis of the lymphocytes in all the studies of nucleic acid content was by the method described in Section 2.8

Special techniques.

Certain specialised methods have been referred to and results

recorded in succeeding sections. These techniques were not all part of the work of the thesis but contribute information to it. References for these methods are included here - Erythrocyte sedimentation rate was measured by the method of Westergren; rheumatoid factor measurements followed the sensitised sheep red cell agglutination technique of Ziff, Brown, Lospalluto, Baden and McEwan (1956); bioassay for long acting thyroid stimulator (LATS), the technique used was that of Kriss, Pleshakov and Chien (1964). The LATS activity was measured using 0.5 ml of test serum injected intraperitoneally into white male Swiss mice whose thyroid iodine was labelled with I^{131}. The thyroid stimulation was monitored by measuring the increase in blood radio-activity at twenty-four hours; the net increase in blood radio-activity is expressed as counts per minute per 0.1 ml of blood and the results are recorded as percentage increase. A blood radio-activity of 150 per cent of that before administration of serum was taken as a LATS positive response. These measurements were carried out by Dr. J.F.B. Smith.

RESULTS

3.4 The normal range for lymphocyte DNA phosphorus and RNA phosphorus content and the ratio RNAP/DNAP, and the effect of differing cell separation techniques

The results for the analyses of the lymphocyte nucleic acid content in normal individuals by three methods of cell separation are set out in Table 5 which shows DNAP per 10^6 cells, RNAP per 10^6 cells, and the RNAP/DNAP ratio for each of the methods, giving the mean result, the standard deviation and the observed range in each case. The method for chemical measurement of the isolated cells was the same throughout. There were differences in variance between the methods as shown in Table 5A. The most important of these for future work is the observation that the gelatin and cotton wool method has a significantly lower variance than either of the other two methods. Where a significant difference in variance was found the d-test was used in the comparison of mean values (see Section 2.9). Analyses of the results by Student's t-test and where appropriate, the d-test, appear in Table 5B. The only significant difference found in the mean value for normal individuals between methods was in the comparison of the RNAP/DNAP ratio for the gelatin method and the gelatin plus cotton wool method. The mean for the latter method was significantly lower

Table 5

Lymphocyte nucleic acid analyses in normal individuals; three cell separation techniques compared.

Method of lymphocyte separation	μg DNA phosphorus per 10^6 cells	μg RNA phosphorus per 10^6 cells	RNAP/DNAP ratio
Silicone oil	Range 0.66 to 0.92	Range 0.16 to 0.30	Range 0.18 to 0.40
	Mean 0.752	Mean 0.223	Mean 0.299
	SD 0.085	SD 0.048	SD 0.064
Gelatin	Range 0.55 to 1.15	Range 0.12 to 0.36	Range 0.16 to 0.37
	Mean 0.744	Mean 0.203	Mean 0.274
	SD 0.142	SD 0.061	SD 0.055
Gelatin plus cotton wool	Range 0.59 to 1.01	Range 0.16 to 0.29	Range 0.21 to 0.35
	Mean 0.732	Mean 0.215	Mean 0.295
	SD 0.114	SD 0.041	SD 0.036
All methods combined	Range 0.55 to 1.15	Range 0.12 to 0.36	Range 0.16 to 0.40
	Mean 0.742	Mean 0.210	Mean 0.284
	SD 0.124	SD 0.054	SD 0.055

The nucleic acid analyses were obtained from fifteen estimations from seven individuals (silicone oil method), thirty-nine estimations from thirty-nine individuals (gelatin method), twenty estimations from twenty individuals (gelatin plus cotton wool) and the combined results seventy-four estimations from sixty individuals.

Table 5A

Analysis of the variance of the results in Table 5 by the F-test.

Comparison	Degrees of freedom	F	Probability
Silicone oil v gelatin method	14 + 38		
DNAP		2.79	<0.01
RNAP		1.62	N.S.
RNAP/DNAP ratio		1.35	N.S.
Silicone oil v gelatin and cotton wool method	14 + 19		
DNAP		1.80	N.S.
RNAP		1.37	N.S.
RNAP/DNAP ratio		3.17	<0.05
Gelatin v gelatin and cotton wool method	38 + 19		
DNAP		1.55	N.S.
RNAP		2.21	<0.05
RNAP/DNAP ratio		2.34	<0.05

Mean values are compared in Table 5B overleaf.

Table 5B

Statistical analysis of mean values in Table 5.

Where variance between the groups is significant, Welsh's test for 'd' has been used to calculate the probability.

Comparison	*t	*d	Probability
Silicone oil v gelatin method			
DNAP	0.19	0.25,0.5	N.S.
RNAP	1.15	-	<0.3>0.2
RNAP/DNAP ratio	1.47	-	<0.2>0.1
Silicone oil v gelatin and cotton wool method			
DNAP	0.97	-	<0.4>0.3
RNAP	0.57	-	<0.6>0.5
RNAP/DNAP ratio	0.25	0.22,0.8	N.S.
Gelatin v gelatin and cotton wool method			
DNAP	0.33	-	<0.8>0.7
RNAP	0.78	0.54,0.5	N.S.
RNAP/DNAP ratio	1.55	1.84,0.5	<0.05

*Degrees of freedom as for Table 5A.

at the five per cent level.

Since the cell separation technique showed only a minor effect on the results of the lymphocyte analyses the results for the three methods of cell separation in normal individuals were combined. The range of normal for an analysis, usually calculated as two standard deviations either side of the mean value, applies when the distribution of results about the mean is of the gaussian type. The distribution of the present results was therefore examined. The values obtained in seventy-four determinations from sixty normal individuals are shown in frequency histogram form in Figure 5.

DNA phosphorus per 10^6 cells.

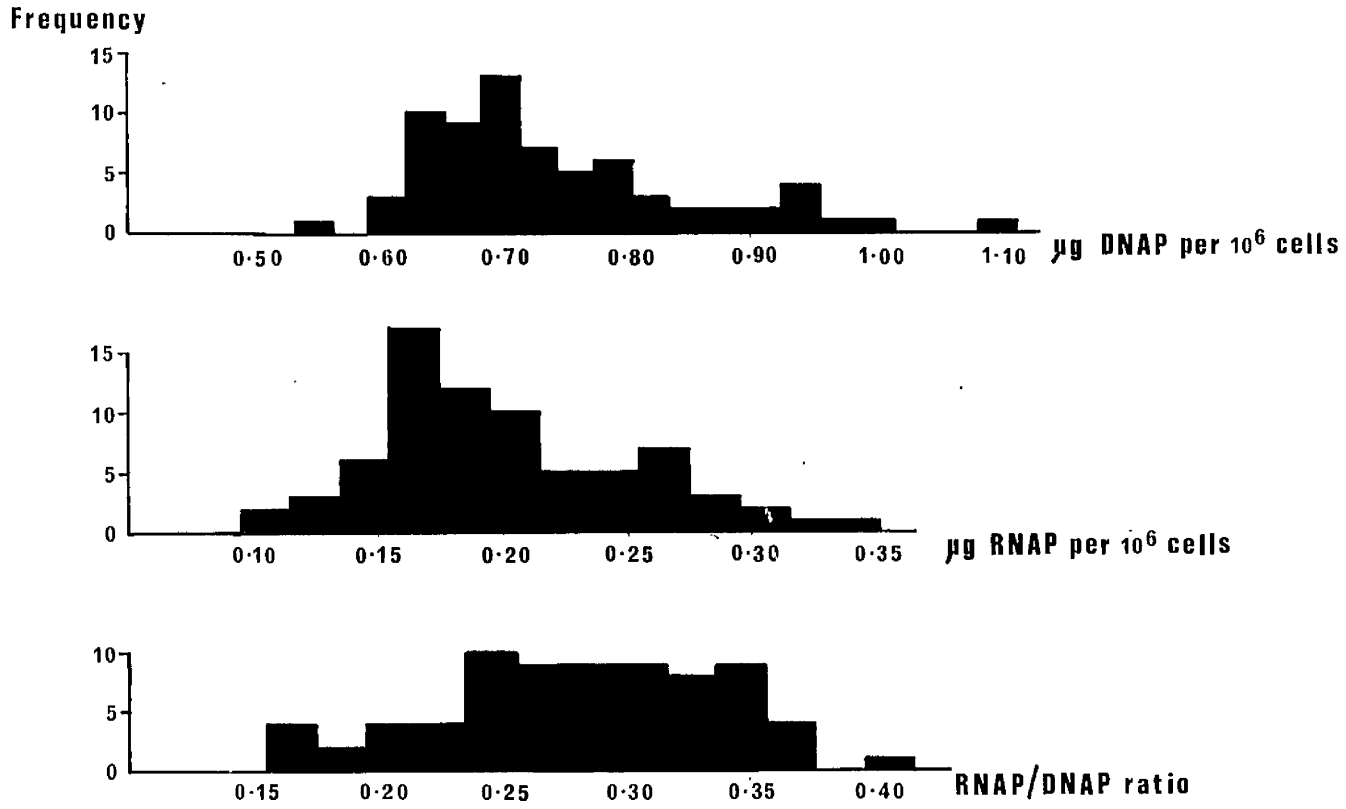
The class interval for the histogram was 0.03 µg DNAP per 10^6 cells. The distribution is skewed with a tail on the side of high levels of DNA phosphorus. The mode is at 0.69 µg to 0.71 µg DNAP per 10^6 cells. The coefficient of skewness (Snedecor and Cochrane, 1967) was +1.29 indicating positive skew with a 'p' value of less than 0.01.

RNA phosphorus per 10^6 cells.

The class interval here was 0.02 µg RNAP per 10^6 cells. The

Figure 5

The distribution of the results of nucleic acid analyses from normal lymphocytes.



The three histograms show the frequency with which the various levels of DNA phosphorus, RNA phosphorus and the calculated RNAP/DNAP ratio were found in seventy estimations of the lymphocyte nucleic acids in sixty normal individuals.

distribution shows a skew similar to that observed for the DNA analyses with a bias towards the higher levels of RNAP content. The coefficient of skewness was +1.00 showing positive skew with a 'p' value of less than 0.01.

The ratio RNAP/DNAP.

The class interval for the ratio is 0.02. The distribution of results does not show skew (the coefficient of skew was zero) and there is no significant kurtosis, i.e. flatness or peaked deviation from the normal distribution (Snedecor and Cochrane, 1967). The mode is not well defined around 0.24 to 0.25 for these results but by the available tests these results are normally distributed.

Since the analyses of both DNA phosphorus and RNA phosphorus content of the lymphocytes in the sixty normal individuals show a significant skew towards positive values the normal range for these measurements is best defined as the observed range. For DNA phosphorus the range was 0.55 μg to 1.15 μg DNAP per 10^6 cells with a mean value of 0.742 μg DNAP per 10^6 cells. The normal range for RNA content was 0.12 μg to 0.36 μg RNAP per 10^6 cells with a mean value of 0.210 μg RNAP per 10^6 cells. The normal distribution of the values for the ratio RNAP/DNAP permits the use of a normal range calculated as two standard deviations on either side of the mean.

The mean value was 0.284 and the normal range therefore 0.174 to 0.394.

The majority of the measurements which follow were carried out by the more satisfactory gelatin and cotton wool method. This method has a lower coefficient of variation for the RNAP/DNAP ratio than the other two methods, i.e. 12 per cent as against 21 per cent and 20 per cent. The normal range for the gelatin and cotton wool method is re-stated with values rounded off for convenience.

DNAP	0.60 - 1.00 μg DNAP per 10^6 cells
RNAP	0.16 - 0.30 μg RNAP per 10^6 cells
RNAP/DNAP ratio	0.22 - 0.37

3.5 The demonstration that sex of the donor has no effect on the results of lymphocyte nucleic acid analyses

The results for the comparison of the lymphocyte analyses from thirty-one normal male and twenty-nine normal female individuals are shown in Table 6. These were again the results of the combined normal group first referred to in Section 3.4.. There were no significant differences between males and females in the observed values for DNA phosphorus content, RNA phosphorus content or the RNAP/DNAP ratio of the lymphocytes when analysed by the t-test.

Table 6

Comparison of lymphocyte analyses in males and females.

	μg DNAP per 10^6 lymphocytes		μg RNAP per 10^6 lymphocytes		RNAP/DNAP	
Male	Mean	0.737	Mean	0.204	Mean	0.280
	SD	0.095	SD	0.050	SD	0.065
Female	Mean	0.738	Mean	0.209	Mean	0.286
	SD	0.125	SD	0.058	SD	0.051

Comparison of the results of lymphocyte analyses from thirty-one normal males and twenty-nine normal females. There were a total of seventy-four analyses, forty from the males.

Statistical analysis by t-test

Comparison	Degrees of freedom	t	Probability
DNAP Males v Females	72	0.04	>0.9
RNAP Males v Females	72	0.38	$<0.9 >0.8$
RNAP/DNAP Males v Females	72	0.46	$<0.8 >0.7$

There are no significant sex differences in the nucleic acid analyses, either by mean value or variance. The values of F for the comparisons of variance in DNAP, RNAP and RNAP/DNAP are 1.73, 1.34 and 1.62 respectively.

3.6 The effect of circadian rhythm on the results of the analyses

The possible effect of circadian rhythm on the results has not been studied but it can be discounted in the study of blood lymphocytes which follow, since all the blood samples were taken in the period 0830 hours to 1130 hours. The study of human thoracic duct lymphocytes reported in Section 4 was the only study in which samples were taken at other times throughout the day.

3.7 The effect of the age of the individual on the lymphocyte nucleic acid analyses

When the results from the sixty normal individuals were analysed by age in decades, no age effect on the DNA analyses could be detected. The advantages in precision of using RNAP/DNAP ratio as a measure of the RNAP content of the cell has been established so the RNAP/DNAP ratios of the lymphocytes in normal individuals have been tabulated according to the age of the donor. These RNA/DNA results appear in Table 7. The data is insufficient to make a complete assessment of the effect of age on the results. Although a significant difference has been shown between the mean results for age group thirty to thirty-nine, and the mean result for age group twenty to twenty-nine ($p = < 0.05$ by Student's t-

Table 7

Age group and the lymphocyte RNAP/DNAP ratio.

Age of group in years	Less than 20	20 to 29	30 to 39	40 to 49	50 to 59	60 or over
Number in group	4	41	10	4	8	3
Mean RNAP/DNAP of lymphocytes	0.298	0.290	0.323	0.283	0.259	0.270
Standard deviation of the RNAP/DNAP	0.028	0.054	0.044	0.010	0.058	0.034

The results of 70 estimations of the lymphocyte RNAP/DNAP in 60 normal individuals are grouped by age in decades.

Analyses by t-test shows differences at the 5% level of significance between the mean values for age group 30 to 39 and both the 20 to 29 group and the 50 to 59 age group. The t values were 2.13 and 2.71 respectively. However, since 15 intercomparisons have been made it is not established that a real difference exists between the age groups.

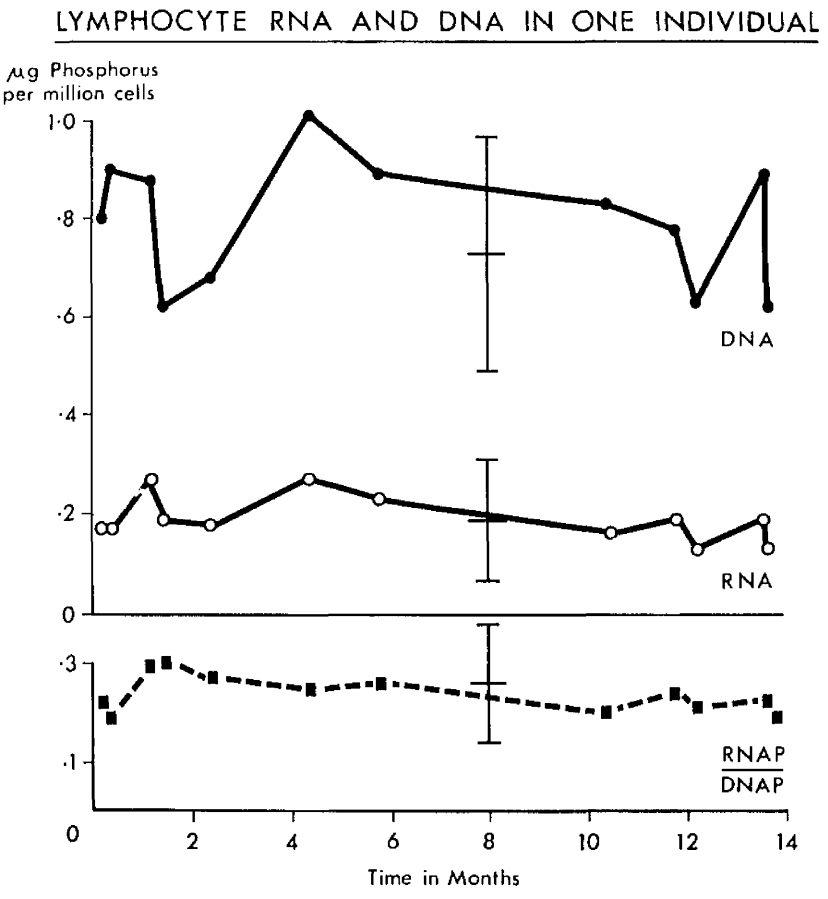
test) and a similar statistically significant difference is present between age group thirty to thirty-nine and age group fifty to fifty-nine, i.e. the mean RNAP/DNAP ratio of the lymphocytes appears to be slightly higher for individuals in their fourth decade than at other periods, these findings must be interpreted with caution. There is unlikely to have been a methodological influence on the finding since six of the results for the age group thirty to thirty-nine were obtained by the standard gelatin method, and four by the cotton wool modification. A more important point is that fifteen intercomparisons have been made, two of which showed a significant difference with a probability of one in twenty. The data are therefore insufficient evidence of a real difference in lymphocyte RNAP/DNAP ratio between age groups.

3.8 The variation in the lymphocyte nucleic acid content in two individuals studied serially

The peripheral blood lymphocytes were analysed over a period of one year in one individual, a male of twenty-nine years, using the gelatin cell separation method. The serial analyses are presented in Figure 6. During this period, measurement of the DNAP content of the lymphocytes varied from 1.05 µg to 0.62 µg per

Figure 6

Serial determinations of lymphocyte nucleic acids in one individual.



Serial analyses of DNA phosphorus per 10^6 cells, RNA phosphorus per 10^6 cells and theRNAP/DNAP ratio of the blood lymphocytes are plotted for one individual, and measured at intervals over one year. Average normal values and the normal range are indicated.

10^6 cells, and the RNAP content per 10^6 cells from 0.27 to 0.13 μg. The variation in the DNAP and RNAP tended to be coincidental while the calculated ratio RNAP/DNAP of the lymphocytes showed less variation (0.20 to 0.30) than the individual RNAP and DNAP values, particularly when the estimations were repeated after an interval of a few days. In a second individual, a female aged twenty-three years, the lymphocytes were analysed five times over a period of nine months. Again the RNAP/DNAP ratio of the blood lymphocytes showed a lower variability than either the DNA phosphorus or the RNA phosphorus results. The observed range of the RNAP/DNAP ratio was from 0.21 to 0.33 in the nine month period.

3.9 The blood lymphocyte nucleic acid content in rheumatoid arthritis

DNA analyses.

The results of the blood lymphocyte DNA analyses are summarised in Table 8 which shows normal values for comparison. The mean DNAP content of the lymphocytes in rheumatoid arthritis is 0.736 μg DNAP per 10^6 cells. There is a high probability that the small difference between this result and that found in the normal group, 0.744 μg DNAP per 10^6 cells, is a chance finding ($p = > 0.7$). There is no significant difference in variance between the two groups.

Table 8

The nucleic acid content of the lymphocytes in rheumatoid arthritis.

Group	Number of analyses	DNAP μg per 10^6 cells	RNAP μg per 10^6 cells	RNAP/DNAP ratio
Rheumatoid Arthritis	35	Mean 0.736 SD 0.133	Mean 0.254 SD 0.069	Mean 0.346 SD 0.062
Normal	39	Mean 0.744 SD 0.142	Mean 0.203 SD 0.061	Mean 0.274 SD 0.055

The comparison of the lymphocyte nucleic acid determinations in patients with rheumatoid arthritis and the results in normal individuals by the same method.

Statistical analysis by t-test

Comparison		Degrees of freedom	t	Probability
Rheumatoid Arthritis	DNAP	72	0.26	$< 0.8 > 0.7$
v	RNAP	72	3.45	$*< 0.01 > 0.001$
Normal	RNAP/DNAP	72	5.41	$*< 0.001$

*These differences are significant.

There are no significant differences in variance; the F values for the comparisons of DNAP, RNAP and RNAP/DNAP ratio are 1.19, 1.27 and 1.27 respectively.

RNA analyses.

The results for these analyses are listed in Table 8. The mean value for the RNA content of the lymphocytes from patients with rheumatoid arthritis is 0.254 μg RNAP per 10^6 cells and the corresponding value in the normal group is 0.203 μg RNAP per 10^6 cells. There is no significant difference in variance but the difference between mean values is significant ($p = <0.01$).

The RNAP/DNAP ratio.

The values for the RNAP/DNAP ratio calculated from the measurements of RNA and DNA content in the cells are listed in Table 8 which shows mean values and standard deviations in the patient and normal groups. In rheumatoid arthritis patients the mean value is 0.346 and in the normal group 0.254. Differences in variance are not significant but the mean RNAP/DNAP ratio is significantly greater than normal in rheumatoid arthritis ($p = <0.001$).

It was possible to match for age and sex, ten patients with definite rheumatoid arthritis and ten controls. Pairings were carried out by random selection where more than one match was available. The lymphocyte RNAP/DNAP was again different in the matched groups; the mean ratio for the rheumatoid arthritis patients was 0.37, standard deviation 0.04, and for the normal

subjects 0.26, standard deviation 0.03 ($p = < 0.001$).

3.10 The relationship between the severity of rheumatoid arthritis and the lymphocyte chemistry

The American Rheumatism Association classification of rheumatoid arthritis was applied to the patients studied and the results of lymphocyte chemistry re-analysed in two groups, definite rheumatoid arthritis and probable rheumatoid arthritis.

DNA analyses.

There is no significant difference in blood lymphocyte DNA content between definite rheumatoid arthritis, probable rheumatoid arthritis and the normal group. The mean values were 0.73 µg DNAP per 10^6 cells, 0.76 µg per 10^6 cells and 0.74 µg per 10^6 cells respectively. These results are recorded in Table 9.

Table 9

Lymphocyte nucleic acid content in probable and definite rheumatoid arthritis and the comparison with normal.

Group	Number of individuals	DNAP μg per 10^6 cells	RNAP μg per 10^6 cells	RNAP/DNAP ratio
Definite rheumatoid arthritis	24	Mean 0.726 SD 0.121	Mean 0.267 SD 0.064	Mean 0.367 SD 0.054
Probable rheumatoid arthritis	11	Mean 0.756 SD 0.159	Mean 0. 225 SD 0.074	Mean 0.299 SD 0.053
Normal	39	Mean 0.744 SD 0.142	Mean 0.203 SD 0.061	Mean 0.274 SD 0.055

Mean values and standard deviations are shown for blood lymphocyte DNA phosphorus and RNA phosphorus content and the RNAP/DNAP ratio. There are no significant differences in variance (F-test) in the comparisons listed for analysis by t-test.

Table 9 (cont'd)

Statistical analysis by t-test

	Comparison	Degrees of freedom	t	Probability	F
DNAP	No significant differences	-	-	-	N.S.
RNAP	Definite RA v normal	61	3.97	*<0.001	1.10
	Probable RA v normal	48	1.04	<0.40>0.30	1.47
	Definite RA v probable RA	33	1.73	<0.10>0.05	1.34
RNAP/DNAP	Definite RA v normal	61	6.70	*<0.001	1.04
	Probable RA v normal	48	1.09	<0.30>0.20	1.08
	Definite RA v probable RA	33	3.51	*<0.01>0.001	1.04

*These differences are significant.

RNA analyses.

The RNA content of the blood lymphocytes in definite rheumatoid arthritis was 0.27 μg RNAP per 10^6 cells. The mean RNA content of the lymphocytes from the patients with probable rheumatoid arthritis was 0.23 μg RNAP per 10^6 cells. Intercomparisons of the groups, definite rheumatoid arthritis, probable rheumatoid arthritis and normal, revealed a significant difference in the comparison of the group with definite rheumatoid arthritis and the normal results ($p = \leq 0.001$).

RNAP/DNAP ratio results.

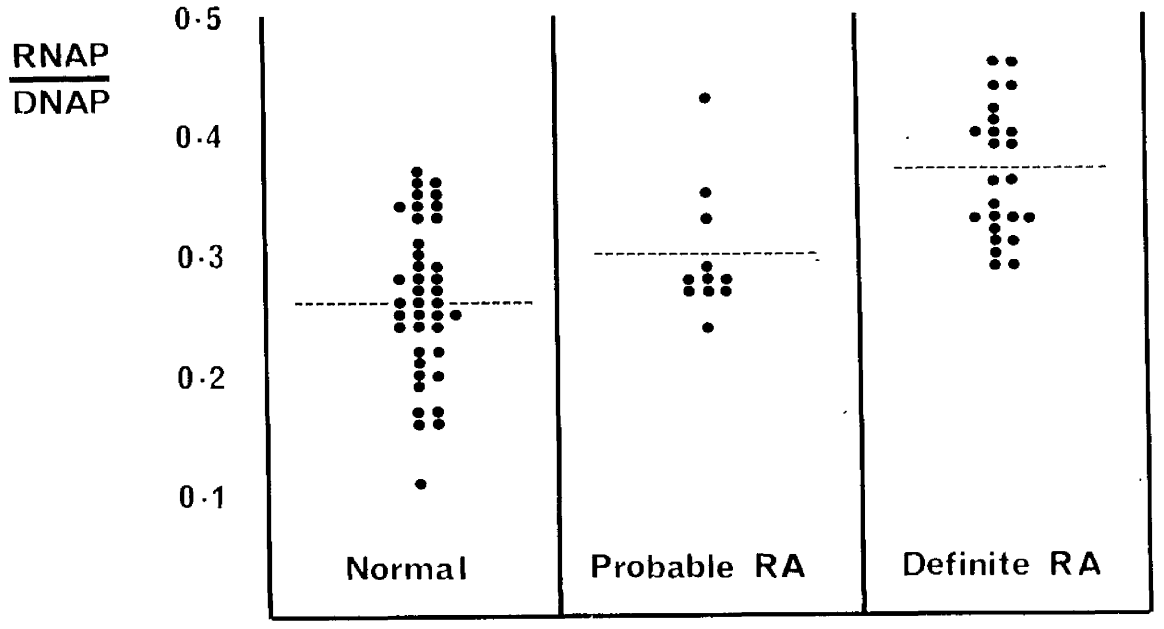
The results of the lymphocyte RNAP/DNAP ratio for the three groups, definite rheumatoid arthritis, probable rheumatoid arthritis and normal individuals, are summarised in Table 9 and the individual results displayed in Figure 7.

Statistical analysis of the results reveals a significant difference between the mean RNAP/DNAP ratio result in definite rheumatoid arthritis, 0.37 and the mean ratio in the normal group 0.27 ($p = \leq 0.001$). A further significant difference was demonstrable between the RNAP/DNAP ratio in probable rheumatoid arthritis 0.30 and the ratio in definite rheumatoid arthritis ($p = \leq 0.01$).

Results presented in Section 3.5 and Section 3.7 support the

Figure 7

The RNAP/DNAP of the blood lymphocytes in patients with rheumatoid arthritis.



The patients with rheumatoid arthritis were grouped according to the American Rheumatism Association criteria for probable and definite rheumatoid arthritis and the blood lymphocyte RNAP/DNAP ratio results for these groups separately plotted. A series of normal values are also shown.

belief that age and sex differences between control and patient groups would not themselves produce the results shown.

3.11 The relationship between the lymphocyte RNAP/DNAP ratic and measurable features of the disease (probable and definite rheumatoid arthritis)

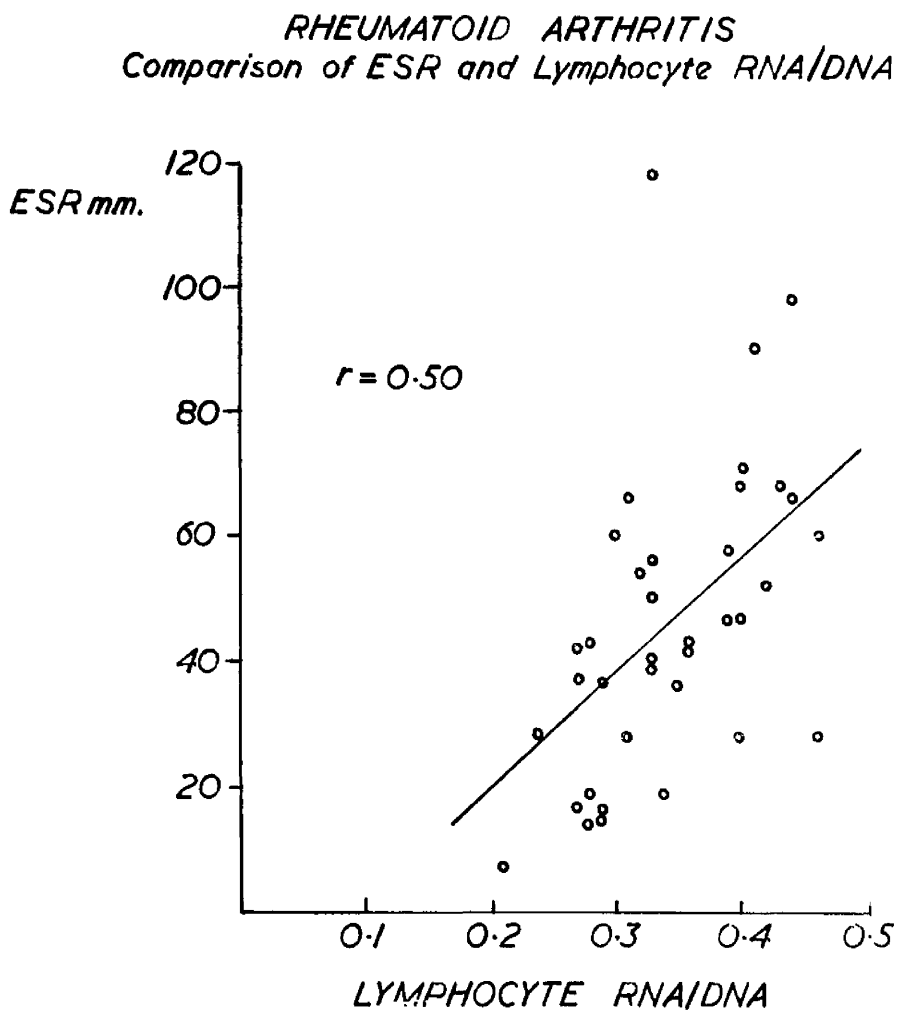
Figure 8 shows the relationship between the blood lymphocyte RNAP/DNAP ratio and the ESR (Westergren) in mm per hour. The correlation coefficient is 0.50 ($t = 3.34$, $n = 37$, $p = < 0.01$) indicating that some correlation is present but the correlation of ESR and ratio is not a close one.

A similar degree of correlation exists between the plasma level of rheumatoid factor, as measured by the sheep red cell agglutination titre, and the lymphocyte RNAP/DNAP ratio. The results of the sheep cell agglutination were expressed as the log of the reciprocal of the titre and an arbitrary value of 0.5 on the log scale was given to negative titres, i.e. those less than $^1/16$. The linear correlation coefficient for the comparison calculated at 0.56 ($t = 3.76$, $n = 33$, $p = < 0.001$).

A comparison was made in patients with rheumatoid arthritis, either probable or definite, between individuals with a high rheumatoid factor titre, i.e. $^1/256$ or greater and those with a

Figure 8

Studies on probable and definite rheumatoid arthritis; the correlation of the blood lymphocyte RNAP/DNAP ratio and the erythrocyte sedimentation rate.



Individual values of the erythrocyte sedimentation rate by the Westergren method are correlated with the results of the lymphocyte RNAP/DNAP ratio in patients with rheumatoid arthritis. The coefficient of linear correlation was 0.50.

lower or negative titre. The mean value of the RNAP/DNAP ratio of the blood lymphocytes in the high titre group was 0.39 and in the low titre group 0.29; the means were significantly different ($p = < 0.001$). When this study was extended in a comparison of sero-positive rheumatoid arthritis, i.e. all patients with a titre greater then $^{1}/16$ and sero-negative rheumatoid arthritis, titre less than $^{1}/16$, the mean values for the lymphocyte RNAP/DNAP ratio were: sero-positive 0.36; sero-negative 0.29. These mean values were still significantly different ($p = < 0.01$).

The comparison of eight patients with subcutaneous nodules and twenty-seven patients not showing this sign, again revealed a significant difference when the lymphocyte RNAP/DNAP ratio was compared. The means for the groups were: with nodules 0.39; no nodules 0.33, ($p = < 0.05$).

3.12 The influence of drug treatment of the RNAP/DNAP ratio of the lymphocyte in rheumatoid arthritis

The possibility that treatment could have affected the lymphocyte RNAP/DNAP in the present study was tested by grouping the results of thirty-five patients with probable or definite rheumatoid arthritis according to the drug therapy they were receiving at the time of the analysis. The four groups were: patients on salicylates and steroids, salicylates alone, steroids

Table 10

Drug treatment and the lymphocyte RNAP/DNAP ratio in rheumatoid arthritis.

Group and therapy	Number	Lymphocyte RNAP/DNAP Mean	SD	Comparison with untreate normal results p value
RA, salicylates and steroids	11	0.344	0.065	$p = < 0.001$
RA, salicylates only	15	0.364	0.066	$p = < 0.001$
RA, steroids only	3	0.293	0.032	Not significant
RA, no therapy	6	0.330	0.042	$p = \leq 0.05$
Normal, no therapy	39	0.269	0.058	-

The results of lymphocyte RNAP/DNAP ratio in thirty-five patients with rheumatoid arthritis classified by drug treatment regime and the comparison by t-test with normal individuals. There was a significant difference in variance between the group on steroids only and the normal group ($F = 3.28$, $p = <0.05$), otherwise there were no differences in variances and the remaining comparisons by t-test are valid.

alone, and no therapy. The mean values of the lymphocyte RNAP/DNAP ratios for the patients in each group appear in Table 10. The lymphocyte RNA content as measured by the RNAP/DNAP ratio was still elevated in patients on no therapy (mean value = 0.33) compared with 0.27 in normal individuals ($p = < 0.05$). However more striking differences from normal were found in the treated groups except in the case of steroid treatment alone where the group was too small for satisfactory statistical analysis.

Two patients with probable rheumatoid arthritis were studied at the time steroid therapy was started. The blood lymphocyte RNAP/DNAP ratios for the patients before treatment were 0.19 and 0.26. After twenty-four hours on prednisone 5 mg q.i.d., the lymphocyte RNAP/DNAP ratios were 0.20 and 0.22 respectively, and in the second patient, after one week of treatment, the ratio had risen marginally from the initial 0.26 to 0.29.

3.13 The nature of the blood lymphocyte in rheumatoid arthritis

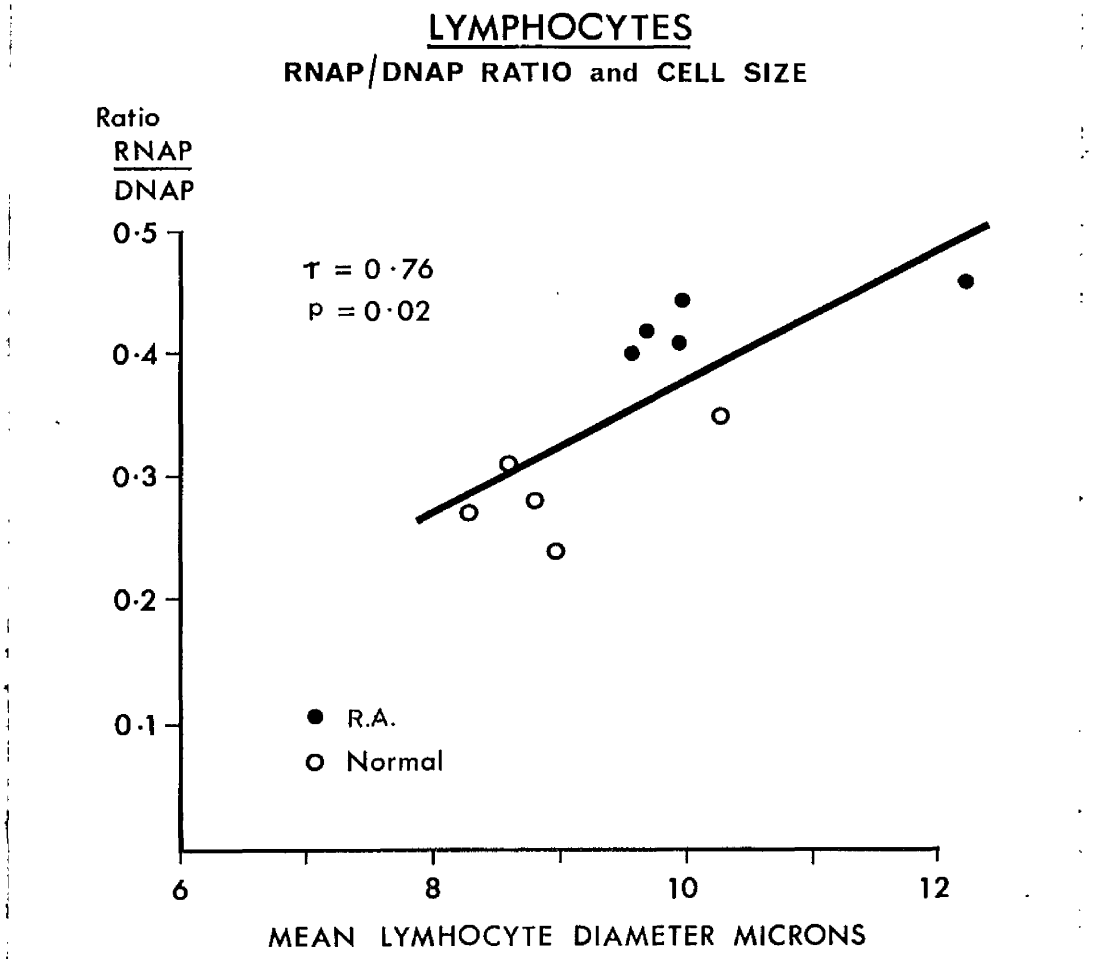
The measurements on the lymphocytes in rheumatoid arthritis and in some of the studies which follow were carried out at an early stage of the work when the importance of the blood lymphocyte count was not yet appreciated. Differential white cell counts on a stained blood film were not carried out in all patients with rheumatoid arthritis, but films from five patients with definite

rheumatoid arthritis were closely examined. Strikingly abnormal cells were rare, less then one per cent of the lymphocytes, although an increased number of deeply basophilic medium sized and large lymphocytes were seen.

A comparison was made between the lymphocyte diameter measured on a blood film stained with a standard technique, and the chemical analysis of the lymphocytes separated from blood drawn at the same time. One hundred cell diameters from each blood film were measured to obtain a value of the mean lymphocyte diameter in microns (μm). Blood films from five normal individuals and from five patients with definite rheumatoid arthritis were examined in this way. To avoid observer bias, the slides were coded and all the cell diameter measurements were made without knowledge of the diagnosis or the chemical analysis. The relationship between the mean lymphocyte diameter and the RNAP/DNAP ratio of the cells, illustrated in Figure 9, shows that an increase in the average lymphocyte diameter occurs as their RNAP/DNAP increases. The coefficient of linear correlation of the ten results was 0.76 ($t = 3.23$, $p = < 0.02$). The possibility that the square of the lymphocyte diameter might give a better correlation with the chemical analysis was not found in practice. The mean lymphocyte diameter averaged for five normal individuals was 8.98 microns and the mean of the measurements in five patients with definite rheumatoid arthritis was 10.27 microns. The normal group and the patients did not differ significantly by cell diameter measurements ($p = > 0.05$

Figure 9

Correlation of the lymphocyte RNAP/DNAP ratio and the mean lymphocyte diameter.



Values for the lymphocyte RNAP/DNAP ratio in five normal individuals and five patients with rheumatoid arthritis are correlated with the mean lymphocyte diameter measured on a fixed and stained blood smear. The lymphocyte diameters are mean values of one hundred cells for each patient. The coefficient of linear correlation was 0.76.

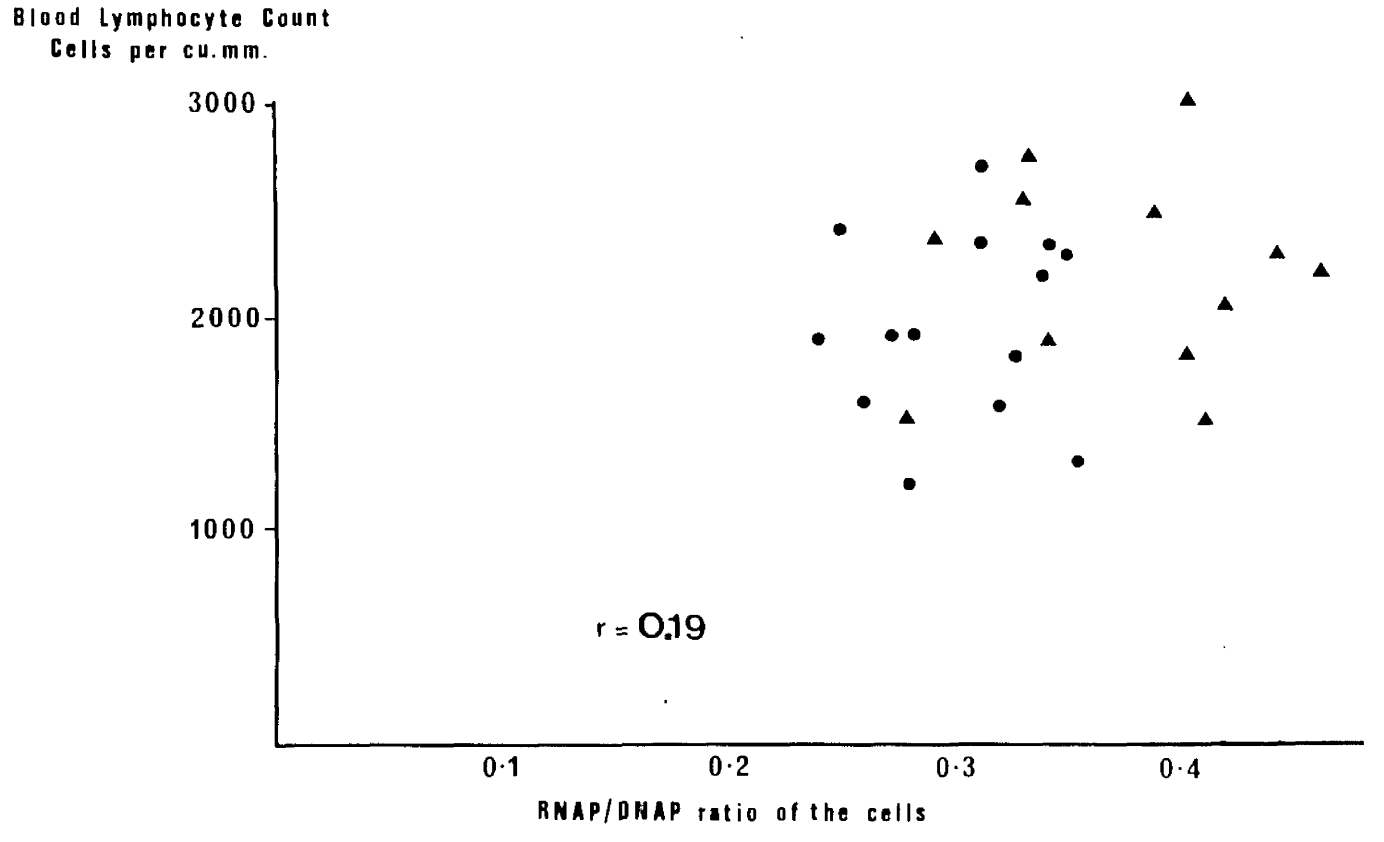
< 0.10) although by chemical analysis the difference was highly significant ($p = < 0.001$).

3.14 The relationship between the blood lymphocyte count and the lymphocyte RNA/DNA ratio

The blood lymphocyte count (cells per mm^3) and the lymphocyte RNAP/DNAP ratio were simultaneously determined in fourteen normal individuals and twelve patients with rheumatoid arthritis. The results, plotted in Figure 10, show different symbols for the normal and patients' results. The blood lymphocyte count and the RNAP/DNAP ratio are essentially independent variables. The coefficient of linear correlation calculated at 0.11 ($t = 0.38$, $n = 14$, $p = > 0.6$) for the normal results and 0.37 ($t = 1.38$, $n = 12$, $p = > 0.1$) for the results in rheumatoid arthritis. The correlation coefficient for the combined results was 0.19 ($t = 1.85$, $n = 26$, $p = > 0.05$). In no case was there a significant correlation between the lymphocyte count and the RNAP/DNAP ratio.

Figure 10

The relationship between lymphocyte RNAP/DNAP ratio and the blood lymphocyte count.



The blood lymphocyte count in cells per mm^3 derived from a leucocyte count and a differential count on one hundred cells is compared with the RNAP/DNAP ratio of the lymphocytes measured in the same individuals. Results from normal subjects (solid circles) and patients with rheumatoid arthritis (solid triangles) are shown. The coefficient of linear correlation was 0.19.

3.15 The blood lymphocyte nucleic acid content in sicca syndrome and in Sjøgren's syndrome

DNA analyses.

The mean lymphocyte DNA content is very similar in the two groups of normal individuals, in patients with sicca syndrome and in Sjøgren's syndrome, as may be seen from the results recorded in Table 11. The mean lymphocyte DNA content in sicca syndrome and in Sjøgren's syndrome was 0.74 µg and 0.77 µg DNAP per 10^6 cells respectively. The corresponding results for the groups of normal individuals were 0.74 µg and 0.73 µg DNAP per 10^6 cells. No statistically significant differences either of variance or of mean values were found between normal and patient groups.

RNA analyses.

The results of the RNA analyses summarised in Table 11 were used to calculate the lymphocyte RNAP/DNAP ratio results for the groups listed above.

Lymphocyte RNAP/DNAP ratio results.

Evidence that the average lymphocyte DNA content is not a

Table 11

Lymphocyte nucleic acid content in sicca syndrome and Sjøgren's syndrome with comparison with two groups of normal individuals.

Group	Number	DNAP μg per 10^6 cells	RNAP μg per 10^6 cells	RNAP/DNAP ratio
Sicca syndrome	8	Mean 0.735 SD 0.122	Mean 0.239 SD 0.057	Mean 0.330 SD 0.098
Sjøgren's syndrome	8	Mean 0.755 SD 0.106	Mean 0.276 SD 0.053	Mean 0.366 SD 0.060
Normal (gelatin and cotton wool method)	20	Mean 0.732 SD 0.114	Mean 0.215 SD 0.041	Mean 0.295 SD 0.036
Normal (gelatin method)	39	Mean 0.744 SD 0.142	Mean 0.203 SD 0.061	Mean 0.274 SD 0.055

In this comparison, two normal groups are included since the cell separation technique used in the study of sicca syndrome was the gelatin and cotton wool method and that used in the Sjøgren's syndrome study was the gelatin method.

Table 11 (cont'd.)

Analysis of the results of Table 11 by t-test.

	Comparison			Degrees of freedom	t	Probability	F
DNAP	Sicca syndrome	v	normal	26	0.06	>0.9	1.14
	Sjögren's syndrome	v	normal	45	0.18	$<0.9>0.8$	1.79
RNAP	Sicca syndrome	v	normal	26	1.27	$<0.3>0.2$	1.93
	Sjögren's syndrome	v	normal	45	2.93	$^{*}<0.01>0.001$	1.33
RNAP/ DNAP ratio	Sicca syndrome	v	normal	26	1.38	$<0.2>0.1$	7.44
	Sjögren's syndrome	v	normal	45	4.27	$^{*}<0.001$	1.19

*These differences are significant.

Consideration of the values of F, the ratio of the variances in these comparisons validated the use of the t-test to compare mean values. The exception was the comparison of the RNAP/DNAP ratio in sicca syndrome and the normal group. Here F calculated at 7.44, significant with $p = <0.01$. The d-test, d = 0.99, 0.9 showed no significant difference in mean values for this particular comparison.

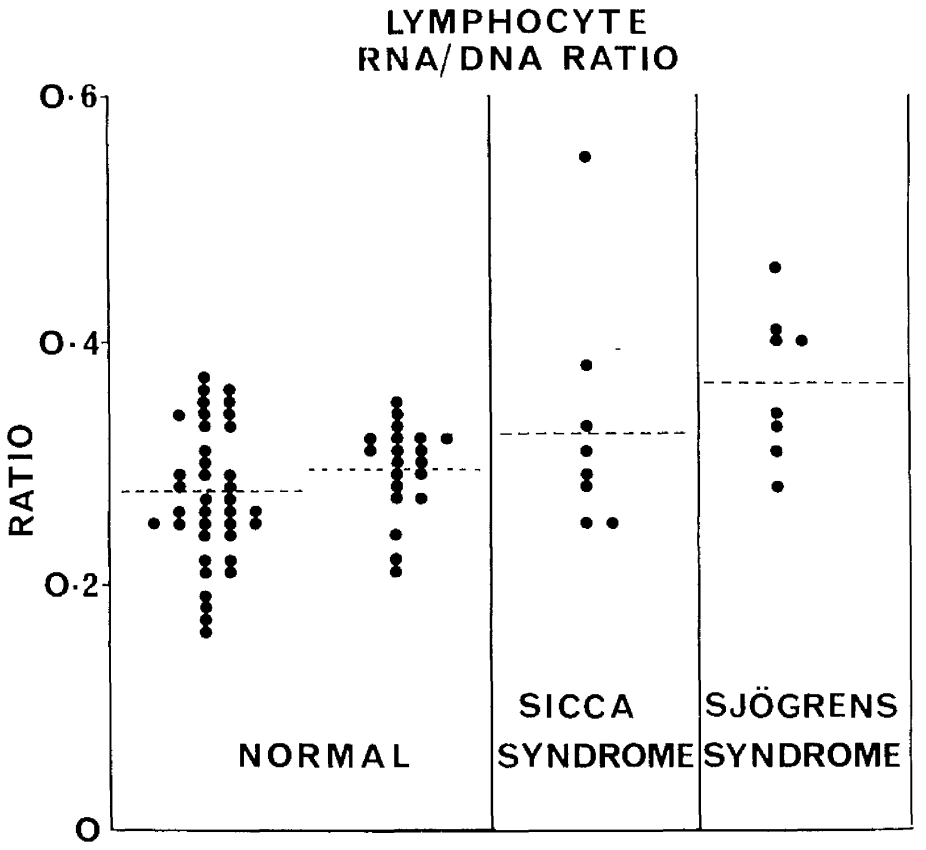
significant variable in patients with sicca syndrome and Sjøgren's syndrome allows legitimate use of the RNAP/DNAP ratio in considering changes in the average cell RNA.

The results of the analyses of lymphocyte RNAP/DNAP in sicca syndrome and Sjøgren's syndrome as well as the two normal groups are summarised in Table 11, and the distribution of the results is illustrated in Figure 11. When comparisons are made between the patients' results and their appropriate control group, two observations may be made. Firstly, the lymphocytes in the eight patients with sicca syndrome have a mean RNAP/DNAP ratio of 0.33, not statistically different from the normal control subjects who have a mean ratio of 0.30. Secondly, the blood lymphocytes in Sjøgren's syndrome were significantly abnormal; their mean RNAP/DNAP ratio was 0.37. The corresponding control value was 0.27 ($p = <0.001$). Consideration of differences in variance did not alter these conclusions.

On the evidence presented in Section 3.5 and Section 3.7, the age and sex differences between patients and control would not influence the findings.

Figure 11

Lymphocyte RNAP/DNAP ratio in sicca syndrome and Sjøgren's syndrome.



Individual results of the lymphocyte RNAP/DNAP ratio are plotted for patients with sicca syndrome and Sjøgren's syndrome. Normal results, using two methods of isolating the lymphocytes from blood, the gelatin method and the gelatin plus cotton wool method, are included for comparison. The broken lines indicate mean values.

3.16 The relationship of the severity of the disease and the lymphocyte RNAP/DNAP ratio. A comparison of sicca syndrome and Sjøgren's syndrome

Although it has been demonstrated that the blood lymphocytes in Sjøgren's syndrome, a disease with widespread tissue manifestations, are significantly different from normal while the lymphocytes in the less severe sicca syndrome do not show this degree of abnormality, direct comparison of the lymphocyte RNAP/DNAP ratios, 0.37 in Sjogren's syndrome and 0.33 in sicca syndrome, fails to show a significant difference ($p = >0.3$). Inspection of Figure 11 shows that there was only one result in the sicca syndrome which could be considered abnormal, while in Sjøgren's syndrome there were four lymphocyte RNAP/DNAP ratio results at the abnormal level of 0.40 or greater.

3.17 The effect of drug treatment on the RNAP/DNAP ratio of the lymphocytes in sicca syndrome and Sjøgren's syndrome

Sicca syndrome.

Six of the patients studied were receiving no therapy, one was taking indomethacin and one was having mefenamic acid both in a

Table 12

The results of the blood lymphocyte RNAP/DNAP ratio determination in Sjögren's syndrome listed by treatment regimes.

Therapy	Lymphocyte RNAP/DNAP ratio
Not on treatment	0.46, 0.34 Mean = 0.40
Salicylate and steroid	0.40
Salicylate alone	0.41, 0.40, 0.31 Mean = 0.37
Steroid alone	0.33, 0.28 Mean = 0.31

Blood lymphocyte analyses from eight patients with Sjögren's syndrome were grouped according to therapy at the time of the analysis. The group mean values for the RNAP/DNAP ratio are recorded where this is appropriate.

standard therapeutic dose. The lymphocyte RNAP/DNAP ratio of these last two patients were within the normal range while the one patient with abnormal lymphocyte chemistry was having no drug treatment at the time of the measurement.

Sjøgren's syndrome.

The majority of patients with Sjøgren's syndrome were on drug treatment and this is most simply shown by a table (Table 12). Although the numbers of patients are small there is no evidence that drug treatment effects are an explanation of the finding of an abnormal lymphocyte RNAP/DNAP ratio in Sjøgren's syndrome.

3.18 The blood lymphocyte nucleic acid content in thyrotoxicosis

DNA results.

Table 13 shows the summarised results of the DNA analyses of the lymphocytes from the patients with untreated thyrotoxicosis and the results in normal individuals for comparison. The mean DNAP content of the lymphocytes was almost identical in patients and the normal group.

Table 13

The nucleic acid analyses of the lymphocytes from patients with untreated thyrotoxicosis, with results in normal individuals analysed by the same method.

Group	Number	DNAP μg per 10^6 cells	RNAP μg per 10^6 cells	RNAP/DNAP ratio
Thyrotoxicosis	20	Mean 0.737 SD 0.119	Mean 0.229 SD 0.039	Mean 0.316 SD 0.060
Normal	34	Mean 0.732 SD 0.114	Mean 0.215 SD 0.041	Mean 0.295 SD 0.036

Mean values and standard deviations are shown for measurements of lymphocyte DNA phosphorus, RNA phosphorus and RNAP/DNAP ratio.

Analysis by t-test

Comparison		Degrees of freedom	t	Probability	F
Thyrotoxicosis v normal	DNAP	52	0.21	$<0.9 >0.8$	1.09
	RNAP	52	1.26	$<0.2 >0.1$	1.11
	RNAP/DNAP ratio	52	1.41	$<0.2 >0.1$	2.79

Comparison of variance between test and control group showed a significant difference for the RNAP/DNAP ratio only ($F = 2.79$, $p = <0.01$). Analysis of the means by d-test (d = 1.45, 0.86 N.S.) confirmed the t-test result.

RNA results.

These results also appear in Table 13. The mean RNA content of the lymphocytes in patients with untreated thyrotoxicosis was 0.23 µg RNAP per 10^6 cells, slightly greater than the mean value in the normal group, but the difference is not statistically significant.

RNAP/DNAP ratio results.

The mean of the result for the lymphocyte RNAP/DNAP ratio in patients with untreated thyrotoxicosis was found to be 0.32 although greater than that found in the normal control group 0.30; the difference was not statistically significant ($p = > 0.10$). A difference in variance between test and control group did not alter this finding.

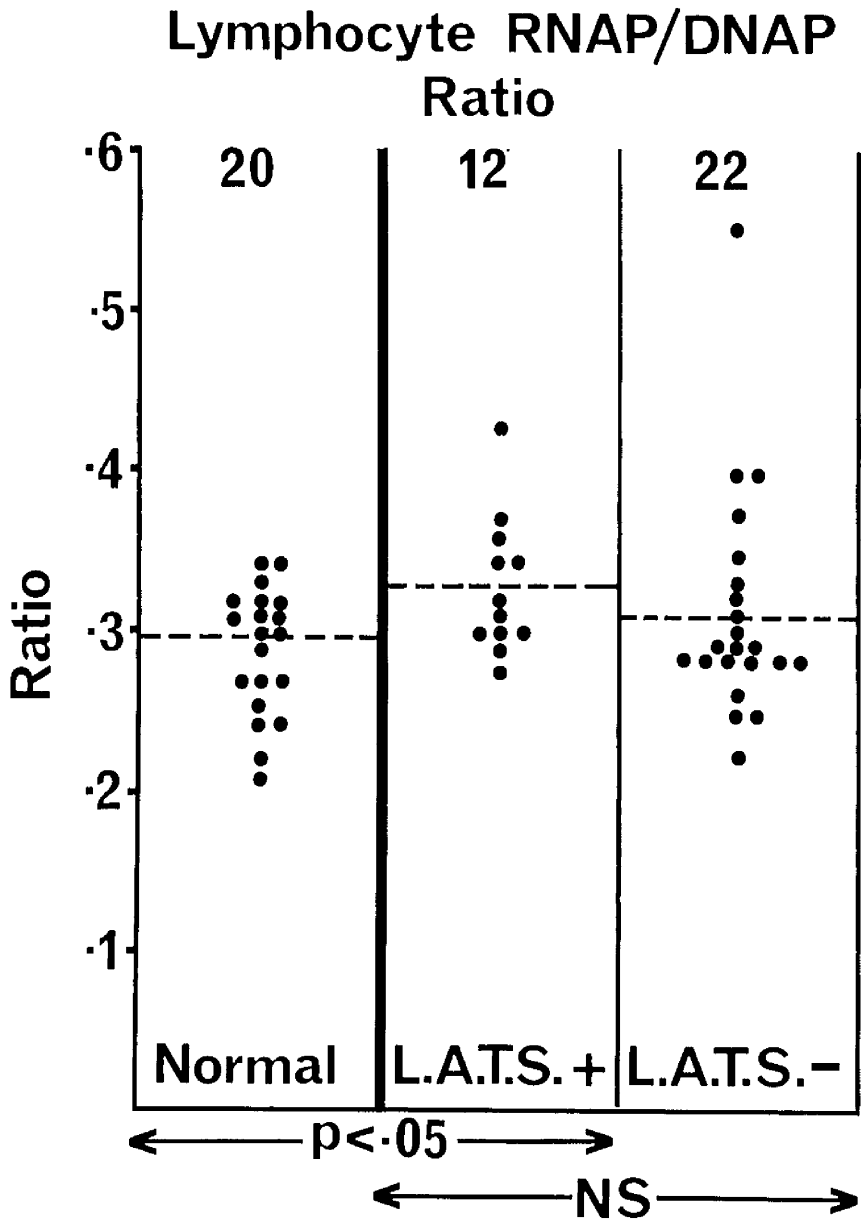
3.19 The blood RNAP/DNAP ratio in relation to measurable features of thyrotoxicosis

The lymphocyte RNAP/DNAP ratio in LATS positive and LATS negative patients.

Figure 12 shows the results of the blood lymphocyte RNAP/DNAP

Figure 12

Lymphocyte RNAP/DNAP ratio in thyrotoxicosis. A comparison of results from patients with sera positive and negative for LATS.



Individual results for the lymphocyte RNAP/DNAP ratio in patients with thyrotoxicosis are grouped according to the results of the LATS bio-assay. Normal results are shown and the result of the t-test comparison, patient and normal results indicated. Broken lines denote mean values.

ratio measurements from twelve thyrotoxic patients whose sera were positive in the LATS bio-assay and compares the lymphocyte results with those from twenty-two patients negative in the LATS assay. The lymphocyte chemistry and the LATS assays were determined prior to treatment of the hyperthyroidism. Lymphocyte RNAP/DNAP ratio data from twenty normal individuals are included for comparison. The mean RNAP/DNAP ratio of the lymphocytes from thyrotoxic patients with LATS positive sera was 0.326 (S.D. 0.044), and from those with LATS negative sera it was 0.302 (S.D. 0.046). Intercomparisons were made and it was found that the mean lymphocyte RNAP/DNAP ratio was significantly higher in the LATS positive thyrotoxic group than in normal individuals ($p = < 0.05$). The difference in the direct comparison of LATS positive and LATS negative individuals did not reach statistical significance ($p = > 0.10$).

The lymphocyte RNAP/DNAP ratio and the 24-hour I^{131} uptake of the gland.

No attempt was made to correlate directly lymphocyte chemistry and thyroid radio-iodine uptake. By the technique used, 15 to 40 per cent of an administered test dose of I^{131} is normally retained by the gland. A high uptake of I^{131} was arbitrarily defined as 60 per cent or greater of the test dose, and the

lymphocyte RNAP/DNAP ratio from patients with a high uptake compared with the ratio in patients with a 24-hour I^{131} uptake less than 60 per cent. There was no significant difference in the lymphocyte chemistry between the high and low uptake groups, indeed the mean ratio was marginally greater in patients with a lower I^{131} uptake, 0.32 as against 0.31.

3.20 The effect of treatment of thyrotoxicosis on the lymphocyte nucleic acid content

Treatment of the thyrotoxicosis did not produce a consistent change in the DNA values. In patients rendered euthyroid by the various forms of treatment the mean DNA content of the lymphocyte was 0.666 µg DNA phosphorus per 10^6 cells with a standard deviation of 0.159. Although lower than the levels found in normal individuals the difference was not significant by 't'-test ($p = > 0.10$).

When the post treatment results for the DNA values were separated by treatment regimes the following results were obtained: in Carbimazole treated patients the mean lymphocyte DNA content was 0.675 µg DNA phosphorus per 10^6 cells, in radio-iodine treated patients the mean DNA content of the lymphocytes was 0.645 µg DNA phosphorus per 10^6 cells, and in patients treated by surgery the

mean post-treatment lymphocyte DNA content was 0.778 μg DNA phosphorus per 10^6 cells. Only in the case of radio-iodine treated patients was the difference significantly different from normal ($p = <0.02$).

Table 14 shows the RNAP/DNAP ratios of the lymphocytes from thyrotoxic patients before and after treatment with Carbimazole, radio-active iodine or partial thyroidectomy, and the data are compared to results from twenty normal individuals. The mean lymphocyte RNAP/DNAP ratio following therapy with Carbimazole in ten patients was 0.365, higher than in the normal control group with a mean ratio of 0.295 ($p = <0.02$). A statistically significant difference was again found when the mean ratio, after treatment, was compared to pre-treatment results for these Carbimazole treated patients. When the lymphocyte RNAP/DNAP ratio data from patients treated with radio-iodine therapy was examined there was a difference between the mean ratio after therapy and the mean ratio found in normal people. The details are tabulated and it may be noted that the mean post-radio-iodine treatment RNAP/DNAP ratio was 0.352, significantly greater than the value in normal individuals. There was not however a significant difference between the mean lymphocyte RNAP/DNAP before and after therapy in the radio-iodine treated patients.

The results in the three patients after treatment by partial thyroidectomy are also tabulated. The mean post-treatment RNAP/DNAP ratio of the lymphocytes 0.432 is substantially higher than

Table 14

The effect of treatment of thyrotoxicosis on the blood lymphocyte RNAP/DNAP ratio.

Treatment	Numbers in groups	Pre-treatment (thyrotoxic)	Post-treatment (euthyroid)	Comparison of pre and post treatment mean value t	Probability
Carbimazole	10,10	Mean 0.308 SD 0.053	*Mean 0.365 SD 0.050	2.55	$<0.02 >0.01$
I^{131}	21,19	Mean 0.323 SD 0.068	*Mean 0.352 SD 0.048	1.60	$<0.20 >0.10$
Partial thyroidectomy	3,3	Mean 0.336 SD 0.054	Mean 0.432 SD 0.057	2.14	$<0.10 >0.05$
Normal untreated individuals	20	Mean 0.295 SD 0.036			

*Mean values denoted by asterisk are significantly different from the results in the normal untreated control subjects.

The results of comparison of the lymphocyte RNAP/DNAP ratio in pre-treatment thyrotoxic and post-treatment euthyroid patients are tabulated, showing the mean values and the standard deviation with the number of individuals in each group. The results of the t-test of this comparison are listed. There were no significant differences in variance between the groups compared, and t-test results are therefore valid.

either the pre-treatment value or the results in normal individuals, but the small sample prevents adequate statistical analysis.

3.21 Blood lymphocyte count results in thyrotoxicosis

The mean leucocyte count in the peripheral blood of twenty-three patients with thyrotoxicosis prior to treatment was 7140 cells per mm^3, and the mean lymphocyte count for the nineteen of these patients in which it was determined was 2350 cells per mm^3 with a standard deviation of 960 cells per mm^3. The observed range of the lymphocyte count was from 1060 cells to 4600 cells per mm^3. Two patients had a leucopenia with a total white cell count of less than 4000 cells per mm^3 and in these patients there was a relative lymphocytosis. The correlation between the blood lymphocyte count and the RNAP/DNAP ratio is not significant, $r = 0.30$ ($t = 0.99$, $n = 12$, $p = > 0.3$).

Data following treatment is incomplete, but for five Carbimazole treated patients the mean blood lymphocyte count was 2670 before therapy, and after treatment in three the mean was 2170 cells per mm^3. In patients treated with radio-iodine the mean lymphocyte count before treatment was 2100 cells per mm^3, and in seven patients after therapy with radio-iodine the mean blood lymphocyte count was 1900 cells per mm^3. Statistical significance

was not established for these results but the trend is for the blood lymphocyte count to fall following Carbimazole therapy and to be unchanged when radio-iodine is the treatment.

3.22 The blood lymphocyte nucleic acid content in lupus erythematosus

The results of the blood lymphocyte nucleic acid analyses in lupus erythematosus appear in Table 15.

DNA results.

The mean lymphocyte DNAP content in the eight patients with lupus erythematosus was 0.744 μg DNAP per 10^6 cells, identical with the average value found in normal individuals.

RNA results.

The mean lymphocyte RNA content in the patients with lupus erythematosus was 0.276 μg RNAP per 10^6 cells, significantly greater than the normal value ($p = < 0.01$).

Table 15

Lymphocyte nucleic acid analyses in lupus erythematosus.

Group	Number	DNAP μg per 10^6 cells	RNAP μg per 10^6 cells	RNAP/DNAP ratio
Lupus erythematosus	8	Mean 0.744 SD 0.113	Mean 0.276 SD 0.080	Mean 0.376 SD 0.102
Normal	39	Mean 0.744 SD 0.142	Mean 0.203 SD 0.061	Mean 0.274 SD 0.102

The nucleic acid analyses of the lymphocytes from patients with lupus erythematosus with results of normal individuals, analysed by the same methods, for comparison. Mean values and standard deviations for lymphocyte DNA phosphorus, RNA phosphorus, RNAP/DNAP ratio in patient and control groups are shown.

Analysis by t-test

	Comparison	Degrees of freedom	t	Probability	F
DNAP	Normal v lupus erythematosus	45	0.01	>0.09	1.58
RNAP	Normal v lupus erythematosus	45	2.92	*<0.01 >0.001	1.72
RNAP/DNAP	Normal v lupus erythematosus	45	4.09	*<0.001	1.00

*Differences are significant.

There were no significant differences in variance in these comparisons and the t-test results are valid.

RNAP/DNAP ratio results.

The mean lymphocyte RNAP/DNAP ratio is significantly increased in the group of patients with lupus erythematosus when compared with the normal control group. The mean ratio of the eight patients was 0.376 compared with the control value. This is a significant difference ($p = < 0.001$).

Data on the blood lymphocyte count was not obtained in these patients.

The relationship of the lymphocyte nucleic acid analyses to the disease process.

There were eight patients in this study of lupus erythematosus, one had the skin manifestations only, four had skin lesions with LE cells present in the blood, and three had the systemic form of the disease. The lymphocyte RNAP/DNAP ratio in four patients was above the upper limit of normal of 0.40. Of these, three had general systemic manifestations of the disease and one had skin lesions with LE cells in the blood.

Treatment of lupus erythematosus and the lymphocyte RNAP/DNAP results.

Steroid therapy was being administered to two of the four patients with an elevated lymphocyte RNAP/DNAP ratio. One patient on no therapy had a lymphocyte RNAP/DNAP ratio of 0.41, one patient with a lymphocyte RNAP/DNAP ratio of 0.43 was receiving salicylate therapy only. The patient with the highest lymphocyte RNAP/DNAP ratio, a value of 0.57, was on steroid therapy, but also had undergone thymectomy two years prior to the measurement.

3.23 The blood lymphocyte nucleic acid content in infection

The results of the analyses in patients with infection are summarised in Table 16.

DNA results.

The mean lymphocyte DNA content for nine patients with infection was 0.728 μg DNA phosphorus per 10^6 cells, not significantly different from normal.

Table 16

Lymphocyte nucleic acid analyses in viral or bacterial infection.

Group	Number	DNAP μg per 10^6 cells	RNAP μg per 10^6 cells	RNAP/DNAP ratio
Infection	9	Mean 0.728 SD 0.102	Mean 0.226 SD 0.050	Mean 0.313 SD 0.047
Normal	39	Mean 0.744 SD 0.142	Mean 0.203 SD 0.061	Mean 0.274 SD 0.055

Mean values and standard deviations of the results of lymphocyte nucleic acid analyses from patients with infection, either viral or bacterial, are listed.

Analysis by t-test

Comparison				Degrees of freedom	t	Probability
DNAP	infection	v	normal	46	0.32	$<0.8>0.7$
RNAP	Infection	v	normal	46	1.02	$<0.4>0.3$
RNAP/ DNAP ratio	infection	v	normal	46	2.07	$*<0.05>0.01$

*This difference is significant.

There were no significant differences in variance in these comparisons, validating the t-test.

RNA results.

The mean lymphocyte RNA content in these patients was 0.266 μg RNA phosphorus per 10^6 cells, slightly greater than the average normal value by this method of 0.203 μg RNAP per 10^6 cells but not significantly different.

RNAP/DNAP ratio results.

The mean RNAP/DNAP ratio of the patients with infection was 0.313, significantly greater than the normal mean for the gelatin method of 0.274 ($p = < 0.05$), but not significantly different from normal when compared with the normal group by all methods.

A comparison of the lymphocyte chemistry between the seven patients with bacterial infection and three patients with viral infection is hardly justified and mean values for the lymphocyte RNAP/DNAP, 0.31 and 0.32 respectively, were similar.

3.24 The blood lymphocyte nucleic acid content and the lymphocyte count in neoplasm

The results of the analyses in patients with neoplastic disease are summarised in Table 17 which shows values and standard

Table 17

The nucleic acid analyses of blood lymphocytes from patients with neoplasm histologically confirmed, with results from normal individuals analysed by the same methods for comparison.

Group	Number	DNAP μg per 10^6 cells	RNAP μg per 10^6 cells	RNAP/DNAP ratio
Neoplasm	6	Mean 0.710	Mean 0.298	Mean 0.417
		SD 0.113	SD 0.090	SD 0.105
Normal	20	Mean 0.732	Mean 0.215	Mean 0.295
		SD 0.114	SD 0.041	SD 0.036

Mean values and standard deviations for the results of lymphocyte DNA phosphorus, RNA phosphorus and RNAP/DNAP ratio are shown.

Analysis by F-test

Group	Degrees of freedom	F	Probability
Neoplasm v normal			
DNAP	5, 19	1.02	N.S.
RNAP	5, 19	4.82	<0.01
RNAP/DNAP ratio	5, 19	8.54	<0.001

There are significant differences in variance in some comparisons, so the d-test and t-test are required in analysis of the mean values.

Table 17A

Statistical analysis of results from Table 17.

Group	Degrees of freedom	t	d	Probability
Neoplasm v normal				
DNAP	5, 19	0.41	-	$<0.7 >0.6$
RNAP	5, 19	3.27	2.19,0.9	$^{*}<0.05$
RNAP/DNAP ratio	5, 19	4.49	2.80,1.0	$^{*}<0.05$

*Significant differences by d-test. The probabilities are greater when the less valid t-test is used.

deviations of the patients and normal results.

DNA results.

The mean DNA content of the lymphocytes in the patients with neoplastic disease 0.710 μg DNAP per 10^6 cells was slightly lower than the value found in normal lymphocytes, but the mean values were not significantly different by 't'-test.

RNA results.

The mean RNA content of the blood lymphocytes in these patients was 0.298 μg RNAP per 10^6 cells. There was greater variance in the patients' results than the control results and comparison of the mean values by the d-test showed the value of 0.298 μg RNAP per 10^6 cells to be significantly elevated ($p = < 0.01$).

RNAP/DNAP results.

The mean value for the lymphocyte RNAP/DNAP ratio in the patients with neoplastic disease was 0.417 compared with 0.295 in the normal group, a significant difference ($p = < 0.05$). This value was obtained by the d-test which allows for the difference

in variance between control and test groups.

The lymphocyte chemistry and the nature of the disease.

In a small group of patients it is not justifiable to classify the results by tissue of origin although this information is noted for each patient in Appendix 8. It is striking however that all four patients with metastatic spread of carcinoma had blood lymphocyte RNAP/DNAP ratios above 0.45 and the ratios in the two patients with local neoplasm were normal, i.e. 0.26 and 0.31.

The blood lymphocyte count in patients with neoplastic disease.

All but two of the blood lymphocyte counts in the patients with neoplastic disease were in the normal range 1500 to 3500 cells per mm^3. One abnormal count was 1034 cells per mm^3 in a patient with extensive secondary neoplasm from breast carcinoma. The blood lymphocyte RNAP/DNAP ratio in this patient was 0.47. The other was 1452 cells per mm^3, just at the borderline of normal, in a patient with liver secondary neoplasm from carcinoma of colon, and associated with a lymphocyte RNAP/DNAP ratio of 0.46. The mean blood lymphocyte of the group was 2060 cells per mm^3.

3.25 The blood lymphocyte nucleic acid content in miscellaneous conditions

The summary of the results of the lymphocyte analyses in twenty-two patients divided into fifteen different disease categories is listed in Table 18. The individual results are drawn up in Appendix 9. The mean DNA content of the lymphocytes for the entire group was 0.700 µg DNAP per 10^6 cells, not significantly different from the value in normal individuals. Analysis of this heterogeneous group would be unprofitable, but there are several points of interest in the results listed in Table 18. The highest lymphocyte RNAP/DNAP ratio of the group was a value of 0.40 in the only patient with acute leucaemia. This was almost certainly due to the presence of blast cells since a very high yield of cells was obtained from this patient's blood. Allowing that the analyses were made on groups of one, two, or sometimes three patients, it is of interest that high normal values for the lymphocyte RNAP/DNAP ratio were found in ulcerative colitis and macroglobulinaemia. Normal or low normal ratios were recorded in pemphigus, colonic histiocytosis, following typhoid-paratyphoid immunisation, acne, osteoporosis, osteomalacia, light chain myeloma, and chronic lymphatic leucaemia. Intermediate between these, that is with RNAP/DNAP ratios within the normal range but slightly above the mean for normal individuals, were the

Table 18

The blood lymphocyte nucleic acid content in a miscellaneous series of conditions.

Group	Number in group	Mean DNAP per 10^6 cells (μg)	Mean RNAP per 10^6 cells (μg)	Mean RNAP/DNAP ratio per 10^6 cells
Acute leucaemia	1	0.66	0.26	0.40
Ulcerative colitis	2	0.64	0.23	0.36
Macroglobulinaemia	1	0.65	0.23	0.36
Crohn's disease	2	0.53	0.19	0.34
Sarcoidosis	1	0.98	0.32	0.33
Salicylate poisoning	1	0.98	0.28	0.33
Whipple's disease	2	0.58	0.17	0.31
Pemphigoid	1	0.74	0.22	0.30
Colonic histiocytosis	2	0.57	0.17	0.29
Acne	1	0.80	0.23	0.29
Post-TAB immunisation	3	0.83	0.23	0.28
Osteoporosis	1	0.71	0.19	0.27
Osteomalacia	1	0.43	0.10	0.24
Light chain myeloma	1	0.57	0.13	0.22
Chronic lymphatic leucaemia	3	0.95	0.22	0.22

Mean values for the lymphocyte DNA phosphorus, RNA phosphorus and RNAP/DNAP ratio are shown where there is more than one patient with the same condition.

lymphocytes from patients with Crohn's disease, sarcoidosis, salicylate poisoning and Whipple's disease.

3.26 Discussion

Results obtained in normal individuals.

DNA results.

The value of 0.74 µg DNA phosphorus per million lymphocytes found in normal individuals, the mean value obtained by combining the results of three cell separation techniques (Table 5), is close to that found by Davidson, Leslie and White (1951) 0.70 µg DNA phosphorus per million cells, and Metais and Mandell (1950) 0.69 µg DNA phosphorus per million cells, in their measurements on preparations of mixed leucocytes. Although the measurements of Davidson et al. (1951) and those described here were carried out by different methods it appears that the lymphocyte and the polymorphonuclear leucocyte of peripheral blood contain similar amounts of DNA. This conclusion is supported by Hale and Wilson (1961) who used microdensitometry analyses of blood cells and found that the amount of Feulgen stained material (DNA) in the mature polymorph is the same as in the small lymphocyte and that the leucocyte of the peripheral blood is practically exclusively diploid (Hale and Wilson, 1959). In a later paper Hale (1963) found that certain polymorphonuclear leucocytes apparently contained less DNA than other human cells, a finding of interest in view of the small discrepancy between the results in lymphocytes

reported here and previous work on mixed leucocyte preparations. The important point is that provided the DNA content of the lymphocyte is constant, variation in the RNA content of the cell may be assessed by measuring the RNAP/DNAP ratio of the cell preparation thus eliminating cell counting error.

Recently Sakai (1971) determined the mean DNA content of normal human lymphocytes by the chemical method described in this thesis and published elsewhere (Glen, 1967). Although his cell separation technique was slightly different, he obtained a value of 0.71 μg per 10^6 cells for the mean DNAP content of the lymphocytes.

The DNA content observed in the lymphocytes is also in accord with the values in the literature for the mean DNA content in other human cells (Leslie, 1955). For cells regarded as predominately diploid, e.g. brain and kidney cells, the values are 0.68 μg and 0.83 μg DNAP per 10^6 cells respectively. A proportion of liver cells are polyploid and in this tissue the mean cell DNA content is slightly higher, 1.00 μg DNAP per 10^6 cells. In the light of these values and the mean DNAP content found in the lymphocytes, 0.74 μg DNAP per 10^6 cells, it is reasonable to accept that the majority of normal blood lymphocytes are diploid cells.

When lymphocytes are separated from blood by methods differing in principle, gelatin sedimentation methods and the silicone oil technique, the mean values for the DNA content and RNA content and

the calculated RNAP/DNAP ratio of the lymphocytes obtained from normal blood are similar in each case (Table 5). Ideally, the same blood sample should have been analysed by all three methods, but practical difficulties of running the methods simultaneously prevented this. The similarity of the chemical analyses of lymphocytes by the three methods is evidence that the cells isolated from blood are representative of the circulating lymphocytes. This point has to be considered since the yields of cells obtained in the separation are low (Table 1). In the gelatin and cotton wool method, the method adopted for most of the work presented, losses of lymphocytes occur when red cells are removed by sedimentation and some lymphocytes are trapped in the cotton wool. It is possible that differential losses of either large or small lymphocytes could occur during lymphocyte isolation in pathological conditions, but the results reported in Section 3.13 would counter this belief since there is an acceptable correlation between the RNAP/DNAP ratio of the isolated cells measured chemically and the mean diameter of the lymphocytes measured on a peripheral blood smear. There is evidence that thymus independent lymphocytes may be relatively more adherent to glass, and presumably cotton wool, than thymus dependent cells (Davies, 1969). Somewhat surprisingly, the mean lymphocyte RNAP/DNAP ratio of normal lymphocytes is marginally but significantly greater when the cells are isolated in the presence rather than the absence of cotton wool. A probable explanation for this is the removal of

residual polymorphonuclear leucocytes (which have a low RNA content) by the exposure to cotton wool.

Although no important differences between methods for the lymphocyte nucleic acid measurements have been shown, the ranges observed in normal individuals differ slightly and it has been the practice to compare results in pathology with a normal series appropriate to the method of cell separation.

RNA results and the RNAP/DNAP ratio results in normal individuals.

The mean value for the RNA phosphorus per 10^6 cells in the series of normal results was 0.210 µg with a standard deviation of 0.054 for sixty individuals studied. These were the combined results obtained by the three methods of cell separation reported individually and in combined form in Table 5.

The calculated mean RNAP/DNAP ratio for the sixty normal individuals was 0.284 with a standard deviation of 0.055. These values are lower than these obtained by Davidson et al. (1951) who found a mean RNA phosphorus of 0.24 µg per million cells which gave a value for the RNAP/DNAP ratio of 0.35 ± 0.16. Although the measurements of Davidson et al. were carried out on cell preparations containing both lymphocytes and polymorphs and could be expected to give different results, the analytical methods used are also important. Davidson et al. (1951) estimated RNA by

measuring the total phosphorus content of the Schmidt Thannhauser RNA fraction. There is evidence that this fraction contains significant amounts of organic phosphorus compounds other than ribonucleotides. Davidson and Smellie (1952) investigated this point, although not specifically in leucocytes, and found that 20 to 25 per cent of the phosphorus in the fraction from rat liver was other than ribonucleotide phosphorus while in rabbit spleen (Davidson, Fraser and Hutchison, 1951) the figure was 10 to 15 per cent. In order to arrive at absolute values and to compare the results in lymphocytes reported here with the leucocyte measurements of Davidson, Leslie and White (1951), it should be noted that the method described here for the analysis of RNA gives an 86 per cent recovery. If an allowance is made for the less than one hundred per cent recovery in the present ultra-violet measurements (add 14 per cent), and the error due to the likely presence of non-ribonucleotide phosphorus in the measurements of Davidson et al. (subtract 12 per cent), the content of RNA phosphorus is similar in normal lymphocytes and leucocytes, being close to 0.24 µg per million cells for the present measurements and 0.21 for these of Davidson et al. When the corrections are applied to the RNA values and the RNAP/DNAP ratio calculated from them, the results are 0.32 and 0.31 for the present observation and those of Davidson et al. respectively. These are low values for the RNAP/DNAP ratio when compared with other tissues, e.g. liver with an RNAP/DNAP of 2.48 (Leslie, 1955); similarly low

values have been reported from leucocytes, thymus and lymphoid tissues in animals (Leslie, 1955).

In the determinations of lymphocyte nucleic acid, no advantage is to be had from making the small corrections arising from the less than one hundred per cent recovery and no such corrections were made.

Individual differences in the lymphocyte RNAP/DNAP ratio observed in the normal subjects are unexplained although each was questioned about recent illnesses, allergies and immunisations. Some of this data is included in Appendices 1 and 2.

The inconclusive findings in relation to age effect in the result of the RNAP/DNAP determinations in lymphocytes from normal individuals requires little further comment. Although the data pointed to the possibility that the lymphocyte RNAP/DNAP in age group thirty years to thirty-nine years was higher than in the preceding decade or in the decade fifty years to fifty-nine years, for the reasons stated in Section 3.7 this is uncertain. Functional differences in the lymphocytes which appear to be age related have been reported. Pisciotta, Westring, Deprey and Walsh (1967) observed that the in vitro response to phytohaem-agglutinin in lymphocytes decreased as the age of donor of the cells increased. Curiously, their data show an impoverished response of the blood lymphocytes to the mitogen when the donor of the cells was in the age group thirty years to forty years.

Serial measurements of the lymphocytes in two individuals

(Section 3.8) illustrate the advantage to be had from measuring the lymphocyte RNAP/DNAP ratio rather than relating the nucleic acid measurement to a cell count. The coefficient of variation of twelve results from one individual over a period of fourteen months were as follows: for DNAP per 10^6 cells, 18 per cent; for RNAP per 10^6 cells, 24 per cent; for the RNAP/DNAP ratio, 16 per cent. The coefficient of variation of the RNAP/DNAP ratio results of this individual (16 per cent) was less than coefficient of variation of single analyses from forty normal individuals by the same method (20 per cent).

The frequency distributions of the measurements, DNA phosphorus per 10^6 cells and RNAP per 10^6 cells, are demonstrably skewed with an excess of individuals in whom the calculated DNAP content and RNAP content of the lymphocytes is higher than the mode (Figure 5). These findings are thought to be evidence that the error of cell counting has not been a completely random one probably because cell clumping has occurred during the isolation procedure resulting in an underestimate of the number of lymphocytes in the suspension. The contrasting normal distribution of the RNAP/DNAP ratios, calculated from the same results, has confirmed that the likely cause of the skewed distribution is cell counting error. Other authors (Rigas, Duerst, Jump and Osgood, 1956) have found a normal distribution of the results of DNA phosphorus and RNA phosphorus per 10^6 cells in analyses of cells from patients with chronic lymphatic leucaemia.

From the evidence presented in Section 3.8 it is apparent that the lymphocyte RNAP/DNAP ratio changes only gradually over periods of some days, at least in normal individuals. Considerable day to day variation in apparent DNAP and RNAP content of the lymphocytes can therefore be attributed to the cell counting errors and would confirm the value of the RNAP/DNAP ratio in assessing the circulating lymphocytes.

Similar results for the lymphocyte nucleic acid content have been recorded in males and females so the theoretical sex difference in the DNA content of the lymphocytes resulting from the different sex chromosome has not been observed.

Results in Pathology.

DNA results.

Table 19 provides a list of the values found for the mean lymphocyte DNA content in the pathological conditions studied in this section as well as the values found in normal individuals. With the single exception of I^{131} treated patients none of the mean values in the pathological conditions is significantly different from the normal, indeed the mean values are remarkably similar. This observation is a notable one since even in conditions where there is increased immunological activity there is no significant change in the average DNA content of the blood

Table 19

The mean DNA content of the blood lymphocytes summarised from a series of studies on patient and normal groups.

Group	Number in group	Mean lymphocyte DNAP μg per 10^6 cells
Sjogren's syndrome	8	0.76
Rheumatoid arthritis	39	0.74
Lupus erythematosus	8	0.74
Normal, combined results	60	0.74
Thyrotoxicosis	34	0.74
Sicca syndrome	8	0.74
Infection	9	0.73
Neoplasm	6	0.71

The mean values of the lymphocyte DNA phosphorus are corrected to two significant figures. None is significantly different from normal.

lymphocytes. It is possible that a more sensitive procedure might show such a change since a small proportion, less than one per cent, of large lymphocytes from human peripheral blood will incorporate tritiated thymidine into their nuclei (Bond, Cronkite, Fliedner and Schork, 1958). Small lymphocytes do not incorporate labelled DNA precursors, and these cells appear to become labelled only as a result of division of already tagged cells. Cooper and Firkin (1965) were able to demonstrate DNA synthesis in peripheral blood mononuclear cells of three patients with rheumatoid arthritis, but once again the percentage of mononuclear cells actively synthesising DNA was low, less than 0.25 per cent had measurable tritiated thymidine uptake by auto-radiography.

Present understanding of the DNA synthesis cycle in mammalian cells indicates that dividing cells are in a hyperdiploid state during the period of DNA synthesis and for the period thereafter known as the G_2 phase. Measurements for example by Lajtha, Oliver and Ellis (1954) in mouse L cells revealed that these two periods can make up half of the generation time. Where the proportion of lymphocytes in the blood that are actively dividing, is sufficiently great it may be possible to detect an increase in the mean cell DNA content. Lesiewska (1967) was able to show a small increase in the DNA content of the blood lymphocytes in some children with virus infections and the evidence was that this was due to the presence of a proportion of hyperdiploid cells. The present restricted study of virus and bacterial infection did not

however reveal an alteration in the blood lymphocyte DNA content.

In the next section it will be shown that the lymphocytes from patients with a renal allograft on therapy with azathioprine and prednisolone have a mean lymphocyte DNA phosphorus content slightly but significantly greater than normal. These patients are of course subject to considerable immunological stimuli and it is possible that active DNA synthesis has contributed to the increase in mean lymphocyte DNA content. The only other group which shows a significant increase in the lymphocyte DNAP content is the group of patients rendered euthyroid by radio-iodine therapy. No definite explanation can be given for this last finding although a tentative one might be the direct effect of radiation damage on the lymphocytes. Tissue culture experiments with mouse L cells (Whitmore, Stanners, Till and Gulyas, 1961) have shown that substantial irradiation (2000 r) allows continued DNA synthesis without division so that the average DNA content per cell reaches three or four times the diploid value. This effect might be operative at a much lower radiation level in radio-sensitive lymphocytes.

The possibility that failure of the system of immunological surveillance occurred in advanced neoplastic disease has been introduced. In this context the observation that in patients with neoplastic disease the mean lymphocyte DNA content is low although not significantly less than normal, may be relevant and could point to a reduced DNA turnover in these cells. This is

of course the converse of the argument which suggests that an elevated DNA content points to an increase in the proportion of cells which are synthesising DNA. If the two other conditions in which there may be immunologic deficiency are considered together, i.e. Whipple's disease and colonic histiocytosis, the mean lymphocyte DNA content for the four patients is 0.572 μg DNAP per 10^6 cells. This value is significantly lower than control values at the five per cent level. Because of this low mean value and repeated low values found in one patient with Whipple's disease who attended regularly for routine clinical surveillance, chromosome analysis of the lymphocytes was carried out. Normal male karyotype was found with no evidence of a hypodiploid line; however, one member of the D-group chromosomes had a deleted short arm. Expert opinion (Ferguson-Smith, 1970) was that this was a possible rare normal variant. To date it has not been possible to test for this abnormality in other patients with Whipple's disease.

RNA and RNA/DNA ratio results.

Rheumatoid arthritis.

The data on the RNAP content lymphocytes, supported by the calculated ratio RNAP/DNAP of the cells, is regarded as

Table 20

The mean RNAP/DNAP ratio of the blood lymphocytes summarised from a series of studies in patient and normal groups.

Condition	Number in group	Mean RNAP/DNAP
Neoplasm	6	*0.42
Lupus erythematosus	8	*0.38
Sjogren's syndrome	8	*0.37
Rheumatoid arthritis	39	*0.35
Sicca syndrome	8	0.33
Thyrotoxicosis	34	0.32
Infection	9	*0.31
Normal	60	0.27

The mean values for the ratio of lymphocyte RNA phosphorus to DNA phosphorus are corrected to two significant figures. The values marked with an asterisk are mean values significantly greater than the normal.

unequivocal evidence that the average RNA content of the blood lymphocytes in patients with rheumatoid arthritis is increased. Although twenty-three out of thirty-five patients had lymphocyte RNAP/DNAP levels within the two standard deviation normal range, in only one patient (a patient with sero-negative probable rheumatoid arthritis) was the mean lymphocyte RNAP/DNAP ratio below 0.27, the mean for normal individuals. Backing for the reality of these changes comes from the correlation of the RNAP/DNAP ratio and cell diameter measurements in Section 3.13. This is evidence that there are alterations to the peripheral blood and that an artefact of the cell separation procedure has not produced the abnormal ratios.

The work of Leventhal, Waldorf and Talal (1967) has suggested that depressed lymphocyte function is a feature of rheumatoid arthritis. More specifically, these authors, in their study of classical rheumatoid arthritis, found that the lymphocytes from eight out of seventeen patients (47 per cent) gave an abnormal in vitro transformation response to either phytohaemagglutinin or streptolysin O. In the present results, eleven out of twenty-four patients with definite rheumatoid arthritis (46 per cent) had lymphocytes with an abnormal mean RNAP/DNAP ratio. It is not of course legitimate to link the functional and chemical abnormalities in two independent series of patients but their frequencies are remarkably in accord.

It is difficult to be certain that drug treatment has not

influenced the result of the analyses. Patients on no therapy are the group with the lowest average lymphocyte RNAP/DNAP in Table 10 where different therapeutic regimes are compared. However, the untreated group tend to have less active disease than those on salicylates or steroids, but even so the patients not on treatment have a mean RNAP/DNAP ratio significantly greater than normal. Also convincing is the high level of the lymphocyte RNAP/DNAP in patients on salicylate therapy alone. Salicylates are not known to have any direct action on the lymphocyte nucleic acids although the site of action may be interference with the supply of ATP to the cell. The anti-inflammatory action of the drug is not understood (Smith, 1959). An immunosuppressive action is only apparent at very high dosages (Jager and Nickerson, 1947) and it is worth recording that nucleic acid measurements carried out on a patient with salicylate overdosage were within normal limits. Steroids might be expected to raise the average RNAP/DNAP of the lymphocytes initially by virtue of their cytolytic action on the smaller cells and data to be presented in the study of renal transplantation confirms this, at least for the high dose levels of prednisone which are necessary for initiation of immunosuppression. Evidence that lymphocytolysis continues on the more usual maintenance dosages is lacking. In the small study reported in Section 3.12 no changes occurred in the blood lymphocyte RNAP/DNAP ratio as therapy with 20 mg per day of prednisone was started.

The action of steroids on human lymphocyte nucleic acid metabolism is a dose related inhibition of DNA and RNA synthesis (Tormey et al. 1967), and since active DNA synthesis is very limited in blood lymphocytes, a fall in the RNAP/DNAP ratio could therefore result in long-term therapy. For three reasons then, the abnormalities in untreated patients, the absence of an effect on the lymphocytes of high salicylate dosage, and the observed as well as the theoretical effects of moderate levels of steroid dosage, it is considered unlikely that the abnormalities demonstrated in the lymphocyte RNA/DNA ratio are the result of drugs alone.

It is concluded that the average RNAP/DNAP ratio of the lymphocytes is increased in rheumatoid arthritis as part of the disease process or the reaction to it, and this implies that there is an increase, either absolute or relative, in the number of the larger lymphocytes circulating in the blood in these patients. The observations of Papamichail, Brown and Holborow (1971) referred to earlier, that the percentage of IgG bearing lymphocytes in the peripheral blood, regarded as B-lymphocytes, is twice the normal in patients with definite active rheumatoid arthritis, are relevant. B-lymphocytes may carry more RNA than T-lymphocytes; an increase in the proportion of the former could account for the impaired phytohaemagglutinin response of the blood lymphocytes and the altered RNAP/DNAP ratio. Atypical mononuclear cells of the type described by Crowther et al. (1969) may be contributing to the

elevated RNAP/DNAP ratio, and could be of the B-lymphocyte line. No correlation has been found between the lymphocyte count and the RNAP/DNAP ratio of the cells and it has not been possible to distinguish between an absolute or relative increase in the number of larger cells.

Sicca syndrome and Sjøgren's syndrome.

Knowledge of the mean lymphocyte DNA content in sicca syndrome and Sjøgren's syndrome allows use of the lymphocyte RNAP/DNAP ratio of the cells as a measure of their RNA content.

A positive answer has been obtained to the first of the two questions posed in the introduction; abnormalities of the lymphocytes have been demonstrated in Sjøgren's syndrome but are only rarely present in patients with sicca syndrome. It appears unlikely that this difference has resulted from drug therapy.

Information on the second question, that of a difference of degree of abnormality between sicca syndrome and Sjøgren's syndrome, is contained in the statistical comparison of each condition with control values (Table 11). Sjögren's syndrome shows a significantly elevated lymphocyte RNAP/DNAP ratio while sicca syndrome does not. Comparison between two conditions, one showing marked abnormality in circulating antibodies and the other combining abnormal circulating antibodies with the presence of a

collagen disease, is of theoretical interest and a table has been prepared (Table 21) which compares data of the present study with that from a similar group of patients in the U.S.A.

Three disease categories are included in the table, sicca syndrome, sicca syndrome with rheumatoid arthritis (Sjögren's syndrome), and rheumatoid arthritis without sicca syndrome. The present results on the mean blood lymphocyte RNAP/DNAP ratio are compared with the published data of Leventhal, Waldorf and Talal (1967) on the in vitro response of the blood lymphocytes to phytohaemagglutinin as well as streptolysin O measured by per cent transformation. Also tabulated are the results by these authors of the skin hypersensitivity response to dinitrochlorobenzene (DNCB), failure of the patient to become sensitized to the chemical after skin application is recorded as an abnormal response. The disease categories in the study by Leventhal and her group were defined in the same way as has been followed in the study of lymphocyte nucleic acids, except that classical rheumatoid arthritis is required for inclusion in the rheumatoid arthritis group in the American study. Patients with probable and definite rheumatoid arthritis were included in the group studied by lymphocyte nucleic acid determination. Patients in this last group numbered twenty-three and were drawn from the previous study on rheumatoid arthritis.

All of the tests show uneven distribution of abnormality between the three patient groups. Abnormalities are most frequent

Table 21

The frequency of abnormal findings in sicca syndrome, Sjogren's syndrome and uncomplicated rheumatoid arthritis, comparing lymphocyte nucleic acid analyses with published results for *in vitro* transformation studies and skin sensitivity tests (Leventhal et al, 1967).

Test		Sicca syndrome	Sjogren's syndrome	Rheumatoid arthritis	Normal individual
Lymphocyte RNAP/DNAP	Number of tests	8	8	*23	59
	Number abnormal	2	4	6	0
	per cent abnormal	25	50	26	0
PHA response	Number of tests	13	12	17	20
	Number abnormal	4	9	4	0
	Per cent abnormal	31	75	24	0
Streptolysin O response	Number of tests	13	12	17	20
	Number abnormal	4	8	8	2
	Per cent abnormal	31	67	47	10
DNCB skin sensitivity	Number of tests	9	9	15	17
	Number abnormal	4	8	5	1
	Per cent abnormal	45	89	33	6

The frequency of abnormal tests in the present lymphocyte analyses is compared with the frequency with which the in vitro response to the two mitogens phytohaemagglutinin and Streptolysin O are abnormal and the frequency of failure to become sensitised to skin application of dinitrochlorobenzene.

*This group is limited to patients without any of the features which suggest sicca syndrome or Sjogren's syndrome.

in patients with Sjögren's syndrome and are at a lower frequency in both sicca syndrome and uncomplicated rheumatoid arthritis. This observation held for the results obtained in the present series of patients, so in addition to the functional abnormality of the lymphocyte pointed out by the American study, there would appear to be a greater proportion of lymphocytes with a high RNA content in Sjögren's syndrome than in uncomplicated rheumatoid arthritis or sicca syndrome. This was not statistically significant however by the χ^2 test nor were the mean values significantly different on intercomparisons by t-test. Some tests show more abnormalities than others, thus the failure of DNCB to sensitise skin was the most frequent of the abnormalities, although it should be noted that some control individuals responded abnormally to the test. The mean lymphocyte RNAP/DNAP ratio of the blood lymphocytes was the test with the lowest frequency of abnormal results, but a direct comparison cannot be made with the other tests since different patients were studied. None the less, when account is taken of the percentage of abnormalities in the control group, the frequencies of abnormal responses to streptolysin O and to PHA are of the same order as the percentage abnormalities of lymphocyte chemistry.

Some authors consider that the <u>in vitro</u> transformation response measures the potential effectiveness of both cellular and antibody response (Benezra, Grey and Davies, 1969) and the conventional view now is that the ability of lymphocytes to respond to the non-

specific stimuli of PHA and perhaps to streptolysin O relates to the functional capacity of the T-lymphocytes. Leventhal's group found a poor correlation between a failure of DNCB to sensitize the skin and an abnormal *in vitro* response of the patients' lymphocytes to the mitogens. Their suggestion that the two tests might be measuring different parameters of lymphocyte function is not in line with the conventional view. However, other investigators have found a good correlation between failure of skin sensitization and an impaired PHA transformation response in other diseases, for example primary biliary cirrhosis (Fox, James, Scheur, Sharma and Sherlock, 1969). On the present information it is reasonable to accept normal skin sensitization specifically and a normal lymphocyte phytohaemagglutinin response less specifically, as measures of the integrity of the delayed hypersensitivity response.

To the acknowledged presence of impaired lymphocyte function in Sjogren's syndrome, the present contribution has been to demonstrate that there is an abnormal lymphocyte constitution in this disease. Once again it is not possible to decide whether an excess of large lymphocytes, possible B-lymphocyte or a relative or absolute deficiency of T-lymphocytes, are directly related to the abnormal mean lymphocyte RNAP/DNAP ratio in the disease.

Thyrotoxicosis.

The finding that thyrotoxic patients whose sera were positive in the LATS bio-assay had statistically significant chemical abnormality in their blood lymphocytes, is confirmation of Hernberg's observation (1954) of morphological abnormality of the lymphocytes in thyrotoxicosis. That the difference was not significant when all the untreated thyrotoxic patients were included in the comparison with normal control subjects is an indication that the changes in the lymphocytes are small. In the present measurements, including all the thyrotoxic patients, the mean lymphocyte RNAP/DNAP ratio was increased by an average of 7.5 per cent over normal control values. The results recorded by Hernberg for the mean cell diameter of normal lymphocytes (11.70 microns) and for the mean cell diameter of thyrotoxic lymphocytes (12.42 microns) differ by 6.2 per cent. Hernberg's data was presented graphically and a statistical analysis was not carried out. Geometric consideration might suggest that the cube or square of the cell diameter would be the important value, but the similarity of the magnitude of abnormality between the cell diameter measurements and the chemical analysis is in accord with the correlation between lymphocyte diameter measurements and the mean cell RNAP/DNAP ratio established in the section on rheumatoid arthritis (Section 3.13). That the absolute values for blood lymphocyte diameters recorded in Section 3.13 are lower than

Hernberg's observations is of little consequence since the actual diameter depends on the method of preparation and staining of the blood film.

Close examination of the data from De Groot and Jaksina (1969), that is their comparison of the phytohaemagglutinin response of lymphocytes from thyrotoxic patients and normal lymphocytes, shows a trend towards an increased radioactive thymidine uptake by lymphocytes from patients with thyrotoxicosis on exposure of the cells to mitogen. Once again the differences between patients' values and control values were small; the average percentage uptake of label was 1.38 for control lymphocytes and 2.07 for lymphocytes from thyrotoxic patients, and this difference of the means did not reach statistical significance.

The possibility of an abnormality in the lymphocytes in some patients with thyrotoxicosis is an expected finding since, as was outlined in the introduction, there is evidence of immunological abnormality in the disease. The total lymphocyte counts observed in the untreated thyrotoxic patients in the present study are in the middle of the accepted range for blood lymphocyte numbers, i.e. 1500 to 3500 cells per cubic mm, and once again the likely explanation of the elevated RNAP/DNAP ratio found in the lymphocytes from thyrotoxic patients is an increased proportion of the larger cells in the blood. Perhaps here there is only a small increased proportion of circulating B-lymphocytes, a possibility which is supported by the finding of De Groot and Jaksina and by the present

observations on patients with sera positive in the bio-assay for the immunoglobulin LATS.

The data presented in Table 14 and in Section 3.20 suggests that the abnormality of the lymphocytes is intensified following radio-iodine therapy; the mean lymphocyte RNAP/DNAP ratio tends to increase although not significantly over the pre-treatment value, while the lymphocyte count is unchanged. Release of antigen by the radiation damaged thyroid gland could be the explanation of an intensified immunological reaction following radio-iodine therapy. Thus, Einhorn, Fagraeus and Johnsson (1965) showed that antibodies to cytoplasmic antigens but not to thyroglobulin were more frequently found in patients after treatment for hyperthyroidism with I^{131} than before treatment. This rise in the number of patients with antibody was temporary and most marked in the first year following treatment. It is established that the likely cause of post-irradiation hypothyroidism following I^{131} therapy in thyrotoxicosis is damage to thyroid cell nuclei which renders the cell incapable of division so that cell replacement is halted and the number of active cells decreases (Greig, 1966). It also remains possible in view of the antibody studies and the present results, that the immunological disease may continue after the hormonal disorder has been corrected.

The data following surgical treatment is limited in the present study, but once again there appears to be evidence of greater abnormality of the lymphocytes after therapy than before

it. Of interest here are the observations that the tendency for patients thus treated to become myxoedematous is correlated with the degree of lymphocytic infiltration of the gland (Whitesell and Black, 1949) and with the presence of thyroid antibodies in the serum (Buchanan, Koutras, Crooks, Alexander, Brass, Anderson, Goudie and Gray, 1962).

The possible effect of the antithyroid drug Carbimazole requires consideration in view of the results in Table 14. Once again the lymphocytes in drug treated patients show an elevated RNAP/DNAP ratio. In this case there may be an associated reduction in lymphocyte count (Section 3.21) although the point is uncertain in view of the very limited data. An extra-thyroidal action of thiouracil drugs has been postulated previously. Borell and Holmgren (1948) demonstrated a decrease of oxygen uptake of thyroidectomised rat tissues on exposure to methylthiouracil, but this was not confirmed in rat kidney slices (Braverman and Ingbar, 1962). It is of considerable interest that a study of the thymus in thyroid disease carried out in 1967 (Michie, Beck, Mahaffy, Honein and Fowler) revealed that thymic atrophy occurred following treatment of patients with thyrotoxicosis with antithyroid drugs. The implied repression of thymic dependent lymphocytes offers an explanation for the significant elevation of the lymphocyte RNAP/DNAP in patients on this form of therapy.

Lupus erythematosus.

It is clear from the introductory review of the pathogenesis of lupus erythematosus that circulating antibody and resultant immune complex formation are of paramount importance in the disease process. The significant elevation of the blood lymphocyte RNAP/DNAP ratio recorded in Section 3.22 indicates that the circulating lymphocytes may be distinctly abnormal, four of the eight patients had a ratio above the normal range. Since the principal immunological abnormality is of antibody formation, once again the possibility of an increased proportion of circulating B-lymphocytes has to be considered. It is possible that these are present as atypical mononuclear cells or, less likely, plasma cells in the peripheral blood. The possibility has not been tested in the present study, but descriptions of the disease do not include reference to this type of abnormal cell in the blood.

A single report of the response of the lymphocytes to phytohaemagglutinin has appeared in the literature, a study of fourteen patients with systemic lupus erythematosus (Patrucco, Rothfield and Hirschhorn, 1967). The measurements of the blast cell response to the mitogen were not significantly different from normal although the mean response was slightly impaired and there was a blast response to native DNA. This evidence might suggest that the thymic dependent cell function is normal in systemic lupus erythematosus. The method of assessment of lymphocyte

transformation used by these authors was measurement of percentage transformed cells and their data less conclusive since their measurements on lymphocytes from patients with rheumatoid arthritis were also within the range of normal.

Infection.

The mean RNAP/DNAP ratio of the blood lymphocytes recorded for the patients with infection is less markedly elevated than that observed in conditions such as neoplasm, systemic lupus erythematosus, Sjögren's syndrome and rheumatoid arthritis (Table 20) although the ratio is just significantly greater in patients with infection than in normal individuals. The data support observations of altered morphology of the lymphocytes in infection which have been outlined in the introduction. The group of patients with infection show a relatively minor abnormality of their lymphocytes, of interest since in uncomplicated and acute infection the response of the host is mainly directed to antibody formation. Germinal centre formation and the appearance of plasma cells at cortico-medullary junction and medullary cords of the lymph nodes are the specific features of humoral antibody formation (Turk, 1967) the development of which (although not the initiation) is a function of the thymus independent B-lymphocytes. The demonstration of atypical mono-

nuclear cells in the blood detailed earlier is a well recognised feature of this response. The present results have shown that the measurement of lymphocyte RNAP/DNAP ratio is sufficiently sensitive to show up alterations to the blood lymphocytes in patients with infection although the timing of the blood sampling has not been optimum.

Neoplasm.

The group of patients with neoplasm heads the list of patient groups drawn up in Table 20, as the condition with the most marked chemical abnormality of the lymphocytes. Inspection of the individual results in Appendix 8 shows that the RNAP/DNAP ratio results recorded in patients with carcinoma associated with secondary spread, up to 0.51, are the highest values recorded in the entire study. The only comparable values were found during rejection of renal allografts and will be reported in Section 4. In their review of tumour immunology, Meuwissen, Stutman and Good (1969) are strongly of the belief that tumour destruction is a cell mediated immune reaction. Their evidence is extensive, based mainly on experimental tumour transplantation work although backed by the circumstantial evidence of the association of immunological deficiency, either natural or iatrogenic, with an increased incidence of malignancy in man.

Meuwissen et al.(1969) have drawn attention to cases in which a tumour was accidently transplanted in man during allograft transplantation to become widely disseminated during immunosuppressive therapy, only to regress again when immunosuppressive therapy was withdrawn (Wilson, Hagar, Hampers, Corson, Merril and Murray, 1968). The observation that the only two patients with localised carcinoma in the study had near normal lymphocyte chemistry raises the question whether the abnormality of the lymphocytes arises as a result of the tumour or whether a deficiency of immunocompetent T-lymphocytes is the prelude to metastatic tumour spread. The small study of the lymphocytes in malignancy presented here was not intended to answer this question, however, within the limits set by the measurement of average cell nuclei acid content, the lymphocytes are near normal in the presence of neoplasm without metastatic spread and are grossly abnormal when metastatic spread has occurred. Further, the presence of metastatic spread may be associated with a low blood lymphocyte count.

The evidence that the phytohaemagglutinin response is impaired in malignancy has been documented in the introduction, an indication of functional impairment of thymus dependent cells. The present results show unequivocal elevation of the RNAP/DNAP ratio of the circulating lymphocytes in the presence of metastatic neoplasm. If it is the case that T-lymphocyte depletion accounts for these results this would have important implications for the effectiveness of immunosurveillance mechanisms.

Miscellaneous group.

Diseases in the miscellaneous group were not analysed individually because of the limited numbers of patients with any one disease. However, the results recorded in full in Appendix 9 do provide an essential background for interpretation of other studies. The results will be taken in descending order for the ratio RNAP/DNAP, the order listed in Table 18, allowing brief comment on each condition.

The lymphocyte nucleic acid content in acute leucaemia has not been extensively measured either previously or here, but the single value of 0.40 for the RNAP/DNAP ratio in the table is in keeping with the findings of Rigas et al. (1956). The range of RNAP/DNAP ratio found by Rigas and colleagues was 0.46 to 0.40 in four analyses in patients with acute leucaemia, values well above those anticipated in normal lymphocytes and appropriate to the morphologic appearance of the blast cells of the blood in acute leucaemia.

The average RNAP/DNAP ratio of the lymphocytes from the two patients with ulcerative colitis, 0.36, is above the average value for normal individuals. Whether this is an indication of abnormality in the lymphocytes is uncertain, and further study of this condition might reveal a significant abnormality. Similar comments apply to the results found in the very limited studies of macroglobulinaemia, sarcoidosis and Crohn's disease. The only RNAP/DNAP results slightly above the mean for normal individuals

and lacking a known or suspected abnormality of immunity, is the single result from a patient with salicylate overdosage. Change in the blood lymphocytes induced by stress could account for this result since the overdosage was self-administered, and an increase in the size of the circulating lymphocytes may be associated with stress in man (Frank and Dougherty, 1953).

Interest in the results in Whipple's disease centre on the DNA measurements and these have been discussed. The values for the RNAP/DNAP ratio in patients with pemphigoid, colonic histiocytosis, acne, osteoporosis, osteomalacia, and one week after typhoid and paratyphoid immunisation, are close to the results in normals. This last observation is of interest since minor changes in the morphology of the blood lymphocytes have been reported following such immunisations (Crowther, Hamilton, Fairley and Sewell, 1969). The changes appear to affect only a small proportion of the circulating lymphocytes, atypical cells constituting at most five per cent of the lymphoid cells in the study of Crowther et al.(1969).

Reprinted from CLINICAL CHEMISTRY
Vol. 13, No. 4, April 1967
Copyright © by American Association of Clinical Chemists
HOEBER MEDICAL DIVISION of Harper & Row, *Publishers*
Printed in U.S.A.

Measurement of DNA and RNA in Human Peripheral Blood Lymphocytes

*A. C. A. Glen**

A method is described, modified from a published technic, for obtaining lymphocytes from human peripheral blood. The cell preparations containing greater than 90% lymphocytes were analyzed for their DNA and RNA content, using a modified Schmidt-Thannhauser procedure. The peripheral blood lymphocytes of 40 normal adults were examined, and normal values established for the 2 compounds. The mean lymphocyte DNA was 0.73 μg DNA phosphorus per million cells, and the mean lymphocyte RNA was 0.19 μg RNA phosphorus per million cells. These values are consistent with results published for human leukocytes. The ratio RNA phosphorus:DNA phosphorus is proposed as a reliable index of changes in the RNA content of the blood lymphocytes.

THE LYMPHOCYTE of the peripheral blood is a cell of considerable interest. Its exact function is unknown, but it is closely involved in the mechanism of homograft rejection (*1*), and in cellular immunity of the delayed hypersensitivity type, and hence it appears implicated in the processes of autoimmune disease (*2*). Study of the lymphocyte is complicated by the considerable variation which occurs in the morphology of the cells. Wintrobe states (*3*) that the lymphocyte is generally small (10 μ), but larger forms (10–20 μ) are common. Attempts to be more precise in classification of the cells have met with difficulties. Wiseman pointed out the considerable difference in size between fixed cells and those vitally stained (*4*), and decided on the degree of cytoplasmic basophilia as a more satisfactory means of classification. Other classifications have been used by Reich and Reich (*5*), Kristenson (*6*), Hernberg (*7, 8*), and Torelli *et al.* (*9*), but in each case the criteria have been subjective, and sometimes laborious, where multiple cell measurements had to be taken. The present work describes the use of the measurement

From the Department of Biochemistry, the Western Infirmary, Glasgow, Scotland.
I wish to express my gratitude to Dr. E. B. Hendry for permission and facilities to undertake this work, to my colleagues who gave advice and donated blood, and to Professor H. N. Munro for guidance at various stages in the work.
Received for publication Oct. 7, 1966; accepted for publication Nov. 28, 1966.
*Address until May 1, 1967: Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass. 02139.

of DNA and RNA in peripheral blood lymphocytes as an objective description of the circulating lymphocytes. An additional reason for undertaking these studies was that, although there have been measurements carried out by microdensitometry of the amount of DNA in human blood cells relative to cells of other organs (*10*), and the absolute DNA and RNA content of human leukocytes has been determined by Davidson *et al.* (*11*), no values exist in the literature for the absolute levels of DNA and RNA in human lymphocytes carried out by chemical means. The establishment of a range of normal values for these compounds was a necessary preliminary in the investigation of variations which might occur in disease, particularly those involving immune or autoimmune states.

Materials and Methods

The Isolation of Lymphocytes from Peripheral Blood

The method of Coulson and Chalmers (*12*) for the separation of human lymphocytes from peripheral blood was modified in order to increase the purity of the lymphocyte yield and the convenience of sample collection. The use of ethylenediaminetetraacetic acid (EDTA) as the anticoagulant avoided the need for immediate defibrination of the blood sample, a disadvantage of the original method. Defibrination is retained, however, as a step in the separation, since the fibrin clot selectively traps and removes polymorphonuclear leukocytes. The procedure was used after the bulk of the red cells had been removed, and the selection was therefore more efficient. In the method, an aqueous solution of gelatin prepared from lime-processed hides was mixed with the blood to accelerate red-cell sedimentation. This gelatin has a high content of calcium ion (8.0 mg./100 gm. of the solid), so that an increase in the amount of EDTA anticoagulant was required for the modified method.

Reagents

1. *"Repelcote"* 2% solution (w/v) of dimethyldichlorosilane in carbon tetrachloride.

2. *0.11 M ethylenediaminetetraacetic acid* 4 gm. of the disodium salt per 100 ml. water, brought to pH 7.4 with 4N potassium hydroxide, to give a solution of 40 mg. EDTA per milliliter.

3. *0.10 M calcium chloride* 1.10 gm. anhydrous salt per 100 ml. in 0.05 M sodium chloride (4.0 mg. calcium per milliliter).

4. *Gelatin* A 3% solution (w/v) in 0.15 M sodium chloride. The gelatin was No. 277 and No. 66003.* The solution must be freshly prepared.

*Gelatin and Glue Research Association, Warwick St., Birmingham 12, England.

5. *0.15 M sodium chloride*
6. *0.30 M sodium chloride*

Method

A total of 20 ml. of blood was collected from a vein and run into a polystyrene tube containing 1 ml. of the EDTA solution. After mixing, 6.0 ml. of the gelatin solution was added, and the solution mixed again. The diluted blood was then transferred to a siliconized tube and stood at a temperature of 37° for 30 min., during which time the mixture separated into 2 visibly distinct layers. The whole of the upper layer, freed from the bulk of the red cells, was removed to a 50-ml. conical flask fitted with a stirring rod suitable for defibrination (i.e., with a wire paper clip fixed on its end). Calcium chloride solution (2 ml.) was added and defibrination carried out by swirling. After approximately 15 min., when defibrination was complete, 3 ml. of the EDTA solution was added and the suspension removed to a centrifuge tube. The cells were then spun down by centrifugation at 350 g (1400 rpm in a head of 16-cm. radius) for 10 min. The lymphocytes sedimented as a small button tinged with red, there being some red-cell contamination at this stage.

It was convenient to make a smear of the sediment by spreading a small sample on a glass slide; the fixed and stained preparation was then used to assess the contamination with white cells other than lymphocytes. The red cells were removed by a process of osmotic shock, lymphocytes being relatively resistant to hypotonic conditions. Ice-cold distilled water (2 ml.) was added to the cell button, the cells being suspended and left for exactly 15 sec. Normal tonicity was then restored by the addition of 2 ml. of ice-cold 0.30 M sodium chloride solution, and the cells maintained thereafter at 4°. Centrifugation at 125 g (900 rpm in the 16-cm. head) again gave a cellular button, this time gray-white in color. The hemoglobin from the lysed red cells remained in solution and was removed by Pasteur pipet together with a thin layer of red-cell ghosts overlying the compact button.

The lymphocytes were then suspended in 2.5 ml. of 0.15 M sodium chloride solution. Some clumping of the cells was occasionally observed. This was reduced by the use of 0.15 M saline containing 5 ml. of the EDTA solution per 100 ml. to suspend the cells. Clumped cells were removed by allowing them to sediment to the bottom of the tube on standing for 5 min. The suspended cells were then collected by Pasteur pipet, and a cell count was carried out by adding 0.1 ml. of the cell suspension to 1.0 ml. of a 1.5% acetic-acid solution tinted with methyl violet, using accurate pipets for the dilution. The cells were counted in

an improved Neubauer counting chamber. Usually about 12 million cells were obtained from 20 ml. of blood, representing a yield of 20% of the lymphocytes. A minimum of 2 million cells was required for reliable estimation of nucleic acid in the sample so that a 10-ml. sample of peripheral blood would in most cases give a satisfactory yield of cells. Reduced volumes of blood were used on some occasions, with appropriately adjusted reagent volumes, but in general a 20-ml. sample was used. Most cell preparations contained at least 95% lymphocytes, the contaminating cells being mainly polymorphonuclear leukocytes, with occasional eosinophils. The few cell suspensions with less than 90% purity were regarded as unsuitable for chemical analysis and were discarded.

The Separation of RNA and DNA

The method employed was essentially that of Schmidt and Thannhauser (*13*) as modified by Fleck and Munro (*14*), omitting extraction with lipid solvents (*15*), and using Schneider's modification for extraction of DNA (*16*). The procedure makes use of the hydrolysis of RNA in dilute alkali, as a means of rendering it acid soluble, under conditions in which DNA and protein are stable. Reacidification allows separation of the acid-soluble polynucleotide derived from hydrolysis of the RNA, from the acid-insoluble residue DNA and protein. The DNA protein fraction may then be treated with hot perchloric acid to extract the DNA. Considerable reduction in scale from the standard procedures was required to give sufficient sensitivity for measurement of the small amounts of nucleic acid present in the samples of lymphocytes. Magnesium chloride (0.05 M) was included in the reagents used for nucleic acid precipitation, as Mg^{++} facilitates the precipitation of ribonucleic acid (*17*).

Reagents

1. *1.2N, and 0.6N, and 0.2N perchloric acid (PCA)* Each made up in 0.05 M magnesium chloride.
2. *0.5N perchloric acid (PCA) in distilled water*
3. *0.3N potassium hydroxide*

Procedure

All manipulations were carried out at 4° unless otherwise stated. The sample, containing a known quantity of lymphocytes between 2×10^6 and 20×10^6 cells, was pipetted into a 4- × 0.5-in. centrifuge tube, usually in 2.0 ml. of 0.15 M sodium chloride. To this was added one-half volume of 0.6N PCA, making the mixture 0.2N in PCA. The suspension was left for 10 min., then centrifuged for 5 min. at 500 g. The superna-

tant was discarded and the pellet resuspended in 0.2N PCA. Centrifugation as before, followed by resuspension and recentrifugation, gave a pellet washed twice in PCA. Excess of acid was carefully drained from the pellet, which was then treated with 0.5 ml. of 0.3N potassium hydroxide, and incubated at 37° for exactly 1 hr.

At the end of the incubation period the solution was cooled to 4°, and 0.25 ml. of 1.2N PCA added. After the solution had stood for 10 min., centrifugation at 500 g for 5 min. was carried out, the supernatant being transferred to a graduated tube. The precipitate was washed twice with 0.75-ml. volumes of 0.2N PCA, the washings being added to the graduated tube. The pooled supernatant and washings were made up to a volume of 2.5 ml. with 0.2N PCA, giving the RNA fraction in 0.2N PCA. It is advisable to ascertain that the 2.5-ml. graduation on the tubes is accurate.

The washed precipitate which contained the DNA together with cellular protein was treated with 2.0 ml. of 0.5N PCA and incubated at 80° for 10 min., then cooled to 4°, allowed to stand for 10 min., and centrifuged for 5 min. at 500 g. A 1.5-ml. portion of the supernatant was removed and mixed with an equal volume of 0.5N PCA to give the DNA fraction in 0.5N PCA.

Measurement of RNA

Measurement of the RNA content of the separated RNA fraction was conveniently carried out by determining the absorbance at 260 $m\mu$ of the acid-soluble material obtained by alkaline digestion of the tissue. A small amount of protein may be made soluble by the alkaline-digestion procedure, and this polypeptide will contribute to the absorbance at 260 $m\mu$. A correction may be made for this error, provided that the specific extinction of the contaminating material is known, as well as its concentration. As an alternative to protein assay, a correction for protein contamination may be made by application of a 2 wavelength correction (*14*). This necessitates the preparation of a sample of the tissue RNA to obtain values for the specific extinction coefficients at 2 selected wave lengths, together with knowledge of the specific extinction coefficients of the contaminating polypeptide. A sample of human lymphocyte RNA was obtained by the method of Digirolamo *et al.* (*18*), and the purified polypeptide prepared following the technic of Fleck and Munro (*14*).

Correction by Protein Estimation

Lymphocytes were isolated in quantity from human blood discarded from the heart-lung machine. The polypeptide prepared from them had

a maximal absorption at 275 mμ, and a minimum at 255 mμ. The specific extinction coefficient $E^{1\text{ cm.}}_{260\text{ m}\mu}$ for a solution of the polypeptide in 0.2N PCA containing 1 μg. protein per milliliter was 0.00075 absorbance units. Protein was measured by the method of Lowry *et al.* (*19*), and this factor was used to correct the extinction at 260 mμ of the RNA fraction for its measured protein contamination. Bovine serum albumin was the reference standard (bovine serum albumin Fraction V from Armour Pharmaceutical Company, England).

Correction by 2 Wave Lengths

Purified human lymphocyte RNA had, after alkaline digestion and solution in 0.2N perchloric acid, a molar extinction coefficient with respect to phosphorus (*20*) of 9200 at 260 mμ with a 1-cm. light path. This solution had a maximum at 262 mμ and a minimum at 235 mμ. The ratio of the extinction at 260 mμ to the extinction at 280 mμ was 1.3. Unhydrolyzed RNA had a similar spectrum except that the maximum was at 260 mμ. There was no detectable protein contamination of this purified RNA, but since some minor protein contamination was to be expected in the fraction obtained by the modified Schmidt-Thannhauser procedure described above, correction factors were calculated as follows.

The corrected RNA concentration, cRNA (*14*), of a solution is given by:

$$\text{cRNA} = A \cdot E_1 - B \cdot E_2$$

where E_1 and E_2 are the absorbance measurements of the solution made at 2 wave lengths λ_1 and λ_2. A and B are constants, depending on the extinction coefficients of the pure RNA and of the contaminating protein. The selected wave lengths were $\lambda_1 = 260$ mμ and $\lambda_2 = 232$ mμ. A and B are derived as follows:

$$A = \frac{1}{r_1 - r_2 \cdot \frac{P_1}{P_2}} \qquad B = \frac{1}{r_1 \cdot \frac{P_2}{P_1} - r_2}$$

r_1 and r_2 are the specific extinction coefficients of the lymphocyte RNA at λ_1 and λ_2; these values are 0.296 at 260 mμ and 0.182 at 232 mμ for a solution in 0.2N perchloric acid containing 1 μg. of RNA phosphorus per milliliter. P_1/P_2 is the ratio of the extinction coefficients of the protein contaminant at λ_1 and λ_2; a solution of the pure polypeptide in 0.2N perchloric acid gave the value for P_1/P_2 of 0.471/2.241—i.e., 0.210.

Substitution gives the values of 3.876 for A and 0.812 for B. Thus

$$\text{cRNA} = (3.876 \cdot E^{1\text{ cm.}}_{260\text{ m}\mu}) - (0.812 \cdot E^{1\text{ cm.}}_{232\text{ m}\mu})\ \mu\text{g. RNA phosphorus/ml.}$$

A reagent blank obtained by carrying a sample of saline through the Schmidt-Thannhauser separation gives small but significant extinction

values at the 2 wave lengths used. Thus a reagent blank is used with each batch of analyses and appropriate correction made.

Measurement of DNA

The modified Schmidt-Thannhauser procedure described above was used to obtain the DNA fraction in 0.5N perchloric acid. Measurement of the absorbance at 265 mμ was used to estimate the DNA content (*16*). The standard for these measurements was highly polymerized calf thymus DNA (Sigma London Chemical Co.). The molar extinction coefficient $E^{1\text{ cm.}}_{265\text{ m}\mu}$ was 9500 with respect to its phosphorus content, the measurement being again in 0.5N PCA, a 1-μg. p per milliliter of solution of the DNA in 0.5N PCA having an extinction at 265 mμ of 0.308 after the usual acid and alkali treatments of the separation procedure. The wave length used represents the maximum for both the standard and the lymphocyte DNA. The separated DNA fraction was found to contain a small amount of dissolved protein so that a protein correction was again applied. This was carried out as described under the measurement of RNA, substituting the values applicable to the DNA measurement (*21*).

Calf thymus standard, of concentration 1 μg. DNA phosphorus per milliliter, had an extinction $E^{1\text{ cm.}}_{265\text{ m}\mu}$ of $0.308 = r_1$ and $E^{1\text{ cm.}}_{232\text{ m}\mu}$ of $0.127 = r_2$ in 0.5N PCA. Values used for the protein contaminant were $P_1/P_2 = 0.224$.

The corrected DNA concentration, cDNA, was given by:

$$\text{cDNA} = (3.571 \cdot E^{1\text{ cm.}}_{265\text{ m}\mu}) - (0.802 \cdot E^{1\text{ cm.}}_{232\text{ m}\mu})\ \mu\text{g. DNA phosphorus/ml.}$$

Again corrections to the extinction values for reagent blank were made. The method of Ceriotti (*22*) was also used for DNA analysis in some experiments.

Results

Experiments were carried out to assess the reliability of the methods employed for the analysis of the nucleic-acid content of lymphocytes.

A solution was prepared containing DNA, RNA, and protein in amounts similar to those encountered in the analysis of lymphocyte preparations. The materials used were highly polymerized calf thymus DNA, yeast RNA (from Pabst Laboratories, and purified by the method of Zytko *et al.* (*23*)) and bovine serum albumin. The phosphorus content of the standard DNA and RNA solutions was determined in triplicate (*20*) and the results compared with those obtained by the modified Schmidt-Thannhauser procedure, measuring both DNA and RNA by UV absorbance. Two wave length protein corrections were applied using values appropriate for these materials. DNA was also measured

by the Ceriotti (*22*) procedure. The results obtained are shown in Table 1. Adequate reproducibility is reflected in the low standard deviations of the recoveries. The Ceriotti method, with a mean recovery of 82%, was less satisfactory than UV absorbance, with 100% recovery, for the measurement of DNA. UV absorbance measurements of RNA gave an 86% recovery.

When the Ceriotti and UV methods for DNA were compared using lymphocyte preparations, a correlation coefficient of 0.97 was obtained (Fig. 1). The relationship between the 2 methods was such that measurement of DNA phosphorus in micrograms by the Ceriotti method equaled 0.94 μg. DNA phosphorus by UV + 0.004. Thus, the Ceriotti reaction gave consistently lower, but still well-correlated results when compared with measurement by UV absorbance.

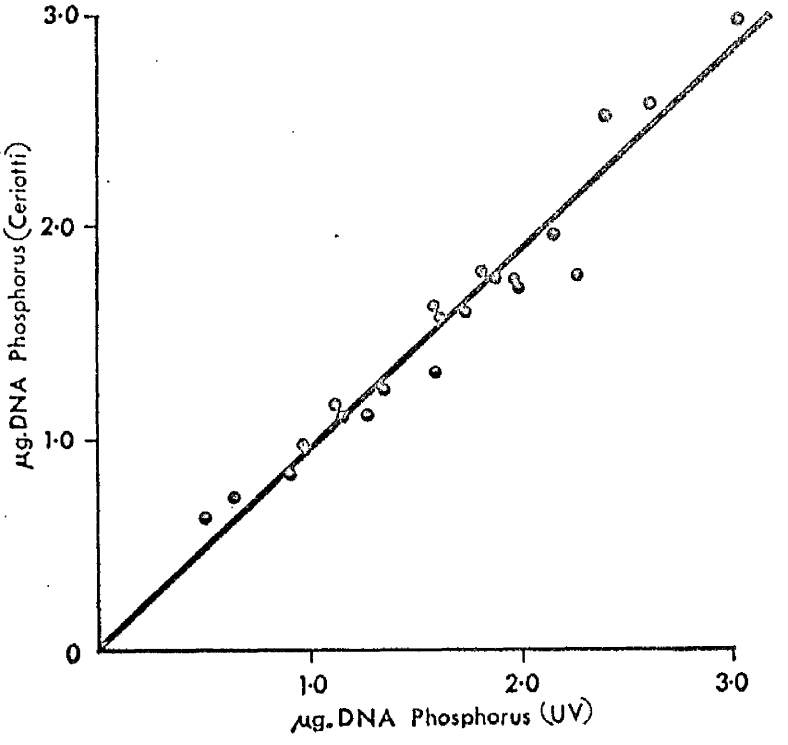


Fig. 1. Ceriotti method (y axis) and that of ultraviolet absorbance at 265 mμ (x axis) for estimating DNA in 22 samples from lymphocytes ($r = 0.97$; $y = 0.94\ x + 0.004$).

Table 1. Recovery Experiments with Comparison of Methods

Method of analysis	*No. of analyses*	*Mean % recovery and S.D.*	*Observed range of recovery (%)*
DNA by Ceriotti	14	82.0 ± 9.4	65–98
DNA by UV absorbance	18	100.4 ± 4.7	93–111
RNA by UV absorbance	19	86.4 ± 4.5	75–100

Analyses were made on standard solutions of DNA, RNA and protein, carried out to assess the recoveries obtainable by the methods of nucleic-acid measurement when compared with phosphorus estimation of the pure solutions taken as 100%.

The extinction at 260 mμ as a measure of RNA may be subject to error owing to the presence of protein (*14*). Two methods of correction for the error may be used: either direct protein estimation with subtraction of an appropriate amount (see above) from the UV absorbance, or correction by measurement at 2 wave lengths in the ultraviolet spectrum. There is close agreement between the 2 methods (Fig. 2), with a correlation coefficient of 0.96.

Measurement of DNA and RNA by UV absorbance, using the 2 wavelength corrections for protein in each case, were found to be the most convenient and reproducible methods, and were selected for all subsequent experiments.

The reproducibility of the entire procedure was examined by comparing lymphocyte DNA and RNA values derived from duplicate samples of blood obtained from 5 individuals. The mean variation for the DNA measurements was ± 6.0% and for RNA measurements ± 8.8%. When the ratio RNA phosphorus/DNA phosphorus was calculated from these results, the mean variation fell to ± 3.8%. These findings express in part the error of cell counting. The RNAP/DNAP ratio of the lymphocytes does not include this error, so the ratio is the most reproducible of the estimates.

Samples from 1 individual, containing a range of cell numbers from 1.43 million to 14.3 million lymphocytes, were analyzed. The individual measurements of DNA and RNA gave linear results over this range, and the graph of estimated cells against their nucleic acid content passed through the origin.

The nucleic-acid content of the peripheral blood lymphocytes of 40 healthy individuals, 20 male and 20 female, was examined. The age range was 18–66, with a mean of 29, for the males, and 18–77, with a mean of 40, for the females. The results of the DNA and RNA analyses are summarized in Table 2. There were no significant differences between males and females in DNA, RNA, or in RNA/DNA ratio. The mean value of the lymphocyte DNA in the male subjects was 0.74 μg. DNA phosphorus per million cells, and in the females 0.72 μg. DNA phosphorus per million cells. The RNA results were 0.19 μg. and 0.20 μg. RNA phosphorus per million cells for the males and females, respectively.

The peripheral blood lymphocytes were analyzed at intervals over a period of a year in 1 man (Fig. 3). During this period the DNAP/10^6 cells result varied from 1.05 μg. to 0.62 μg., and the RNAP/10^6 cells from 0.27 μg. to 0.13 μg. The variation in these 2 measurements tended to be coincident, while the calculated ratio RNAP/DNAP of the lymphocytes showed less variation (0.20–0.30) than the individual RNA

and DNA values, particularly when the estimations were repeated after an interval of a few days. These 2 findings supported the view that much of the variability of the estimation was the result of errors in cell counting.

Discussion

The modified cell-separation procedure has been fully described, and only details will be discussed. The first is the choice of gelatin that is used to accelerate cell sedimentation The critical properties for this step are not known, and it is only possible to describe the characteristics of a gelatin (No. 66003) which has proved satisfactory in practice. The stated properties of the gelatin were: source, limed hide; bloom jelly strength at 6.67%, 225 g; viscosity at 40° and 6.67%, 6.6 centistokes; moisture content, approximately 14%. The gelatin was of recent manufacture, and similar to that used by Coulson and Chalmers (*12*). An important detail of the modified procedure is that sufficient time must be allowed after defibrination to allow the fibrin strands to trap polymorphonuclear leukocytes. The recommended period is 15 min., but this

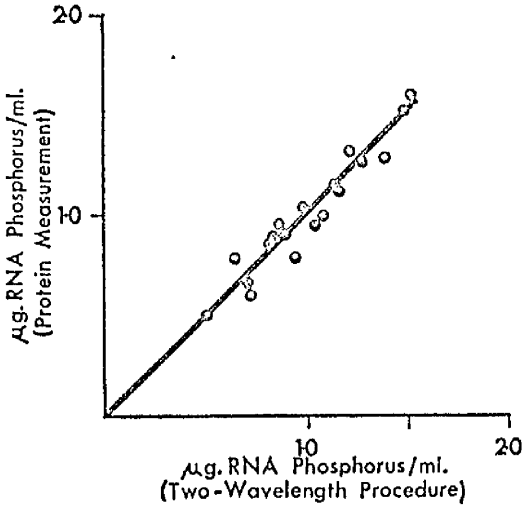


Fig. 2. Measurement of lymphocyte RNA by ultraviolet absorption showing comparison of 2 methods of correction for protein error. RNA phosphorus results corrected by measurement of protein in 19 samples are shown on y axis; and on x axis, same samples corrected by 2 wave-lengths procedure ($r = 0.96$; $y = 0.98\ x + 0.01$).

Table 2. The DNA and RNA Content of Normal Human Lymphocytes

	DNAP (*μg./10⁶ cells*)	*RNAP* (*μg./10⁶ cells*)	*Ratio RNAP/DNAP*
Observed range	0.55–1.05	0.10–0.35	0.11–0.36
Mean	0.73	0.19	0.26
Standard deviation	0.12	0.06	0.06
Coefficient of variation	16%	32%	24%

Analyses were made of the peripheral blood lymphocytes of 40 normal individuals (20 adult males and 20 adult females). RNA and DNA are expressed as micrograms of DNA or RNA phosphorus per million cells.

should be increased if defibrination is slow, to maintain the purity of the lymphocytes The viability of the cells as tested by trypan blue is close to 100%. The rigorous conditions for red-cell lysis were satisfactory for chemical work, but may require modification if cell culture is contemplated.

It is clear, particularly from Fig. 3, that an individual estimate of DNA or RNA expressed per million cells is not a reliable measure. Cell counting is the principal source of variation, and since this error is dependent on the square root of the number of cells counted, it could be reduced by repeated counting. The analytical methods show reliability adequate to give results within ± 5% (Table 1), and provided that accurate cell counting is available, reliable measurements of DNA

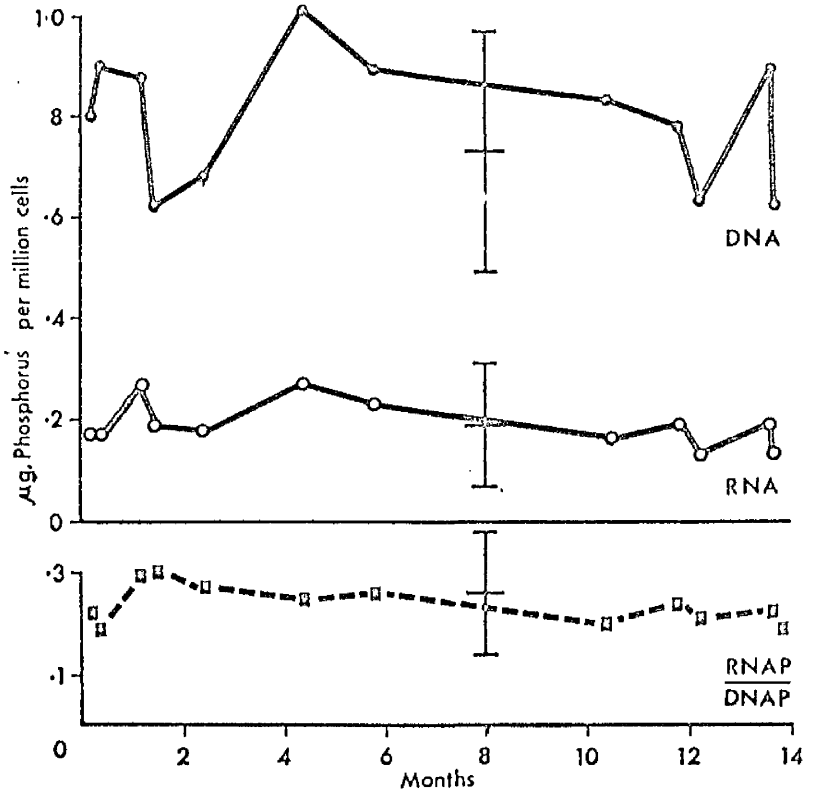


Fig. 3. DNA phosphorus per 10^6 cells, RNA phosphorus per 10^6 cells, and ratio RNAP/DNAP of blood lymphocytes measured in 1 individual (male) at intervals over 1 year. Average normal values are indicated, showing 2 standard deviation limits.

phosphorus or RNA phosphorus per 10^6 cells may be carried out in 1 sample. However, in the present study it was considered important that the isolated cells should be treated with cold perchloric acid as soon as possible after their removal from the peripheral blood, thus limiting degradation of nucleic acid, particularly of the more labile RNA. Any nucleic-acid degradation would give consistently low values for the mean cell content, while the error of cell counting is a random one. The actual estimates of DNA phosphorus per million cells in this study were distributed normally about the mean.

The value of 0.73 μg. DNA phosphorus per million lymphocytes reported here (0.73 pg. DNA phosphorus per average cell) is close to that found by Davidson *et al.* (0.70 μg. DNA phosphorus per million cells) in their measurements on human leukocyte preparations (*11*), and again slightly greater than the value of 0.69 μg. DNA phosphorus per million cells obtained by Metais and Mandel (*24*) for human leukocytes. Although the measurements of Davidson *et al.* (*11*) and those described here were carried out by different methods, it appears that the lymphocyte and the polymorphonuclear leukocyte of peripheral blood contain similar amounts of DNA. This conclusion is supported by Hale and Wilson (*25*), who found, using microdensitometry, that the amount of Feulgen-stained material (DNA) in the mature polymorph is the same as in the small lymphocyte, and that the leukocyte of the peripheral blood is practically exclusively diploid (*10*). Provided the DNA content of the lymphocyte is constant, variation in the RNA content of the cell may be assessed by measuring the RNAP/DNAP ratio of cell preparations, thus eliminating cell-counting error.

The criticism may be leveled that the gelatin-sedimentation method of lymphocyte purification may be selective, and produce cell suspensions of a particular DNA value. This is unlikely since an independent separation method based on cell flotation on silicone oil (*26*) gives values for the measurement of DNA phosphorus and RNA phosphorus of the lymphocyte in 15 normal individuals of 0.75 μg. ± 0.09 and 0.22 μg. ± 0.05 per million cells. In a series of 40 lymphocyte suspensions prepared by gelatin sedimentation from pathologic blood specimens, and containing an increased proportion of large lymphocytes, the mean DNA phosphorus content was 0.74 μg. ± 0.12 per million cells (*26*). The case for the constancy of blood lymphocyte DNA is a strong one, and hence the ratio RNAP/DNAP may be considered an accurate reflection of the mean cell RNA. The question of whether the mature blood leukocyte contains less DNA than other diploid somatic (*27*) cells does not alter this conclusion.

The observed range of RNA phosphorus, 0.10–0.35 (mean 0.19) μg. per million lymphocytes, when calculated as RNAP/DNAP ratio gave a mean value of 0.26 ± 0.06 for the 40 normal individuals tested. These mean values are lower than those obtained in the leukocyte by Davidson *et al.* (*11*), who found a range for the RNA phosphorus of 0.11–0.31 μg., with a mean of 0.24 μg. per million cells, which gave a value for the RNAP/DNAP ratio of 0.35 ± 0.16. Although the measurements of Davidson *et al.* were carried out on cell preparations containing both lymphocytes and polymorphs, and could be expected to give different results, the analytical methods used are also important. Davidson *et al.*

(*11*) estimated RNA by measuring the total phosphorus content of the Schmidt-Thannhauser RNA fraction. There is evidence that this fraction contains significant amounts of organic phosphorus compounds other than ribonucleotides, with differences of degree among tissues. Davidson and Smellie (*28*) investigated this point, although not specifically in leukocytes, and found that 20–25% of the phosphorus in the fraction from rat liver was other than ribonucleotide phosphorus, while with rabbit spleen (*29*) the figure was 10–15%. In comparing our results with those of Davidson *et al.* (*11*), it should also be noted that the method described here for the analysis of RNA gives 86% recovery. If an allowance is made for the less than 100% recovery in the present ultraviolet measurements (add 14%) and the error due to the presence of nonribonucleotide phosphorus in the measurements of Davidson *et al.* (subtract 12%), the content of RNA phosphorus is similar in leukocytes and normal lymphocytes, being close to 0.21 μg. per million cells in each case. This is in keeping with the observation that cell samples rejected from this series because of greater than 10% contamination with polymorphonuclear leukocytes, when analyzed, gave results which fell within the normal range for lymphocytes. When the corrections are applied to the RNA values and the RNAP/DNAP ratio calculated from them, the results are 0.29 and 0.30 for the present observations and those of Davidson *et al.*, respectively. These are low values for the RNA/DNA ratio when compared with other cells; similar values have been reported only from leukocytes and thymus and lymphoid tissue in animals (*30*).

Repeated measurement of the RNAP/DNAP ratio in an individual (Fig. 3) shows that this measure is subject to variation. That these variations are real and not analytical errors is confirmed by the reproducibility of the results when close in time. Variation in the ratio would be brought about by a change in the proportion of cell types circulating, or by synthesis of RNA by the cells of the blood. Both these possibilities may occur, since Gowans (*31*) has demonstrated that lymphocytes recirculate between the blood and lymphatic tissues, and evidence of synthesis of RNA in blood lymphocytes has been reported by Torelli *et al.* (*9*) and by Cooper and Rubin (*32*) and by others (*33*). Blood lymphocytes respond in vitro to a variety of stimuli by increasing in size (*34, 35*) and basophilia. Synthesis of RNA is established as part of this transformation (*36, 37*), which appears to parallel the in-vivo changes in the blood of individuals exposed to diphtheria toxoid antigen reported by Pariser *et al.* (*38*).

How far the RNAP/DNAP ratio of blood lymphocytes represents a measure of their biologic activity remains uncertain, nonetheless the

ratio does represent a quantitative assessment of the nature of the lymphocytes circulating in the blood.

References

1. Gowans, J. L., McGregor, D. D., Cowen, D. M., and Ford, C. E., Initiation of immune responses by small lymphocytes. *Nature* **196**, 651 (1962).
2. Doniach, D., and Roitt, I. M., Auto-antibodies in disease. *Ann. Rev. Med.* **13**, 213 (1963).
3. Wintrobe, M. M., *Clinical Haematology*. Kimpton, London, 1961.
4. Wiseman, B. K., Criteria of the age of lymphocytes in the peripheral blood. *J. Exp. Med.* **54**, 271 (1931).
5. Reich, C., and Reich, E., A study of a lymphocytic hemogram. *Am. J. Med. Sci.* **186**, 278 (1933).
6. Kristenson, A., The variation in size (the lymphocyte profile) of the lymphocytes circulating in the blood in some normal and pathological conditions. *Acta Med. Scand.* **133**, 157 (1949).
7. Hernberg, C. A., Observations on the size of lymphocytes in the blood in Addison's disease; panhypopituitarism and Cushing's syndrome during treatment. *Acta Med. Scand.* **144**, 380 (1953).
8. Hernberg, C. A., Thyrotoxicosis and the size of lymphocytes. *Acta Med. Scand.* **149**, 37 (1954).
9. Torelli, U., Grossi, G., Artusi, T., and Emilia, G., RNA and protein synthesis in normal peripheral mononuclear leukocytes. *Acta Haematol.* **30**, 129 (1963).
10. Hale, A. J., and Wilson, S. J., The DNA content of leukocytes in normal and in leukaemic human blood. *J. Pathol. Bacteriol.* **77**, 605 (1959).
11. Davidson, J. N., Leslie, I., and White, J. C., Quantitative studies on the content of nucleic acids in normal and leukaemic cells from blood and bone marrow. *J. Pathol. Bacteriol.* **63**, 471 (1951).
12. Coulson, A. S., and Chalmers, D. G., Separation of viable lymphocytes from human blood. *Lancet* **1**, 468 (1964).
13. Schmidt, G., and Thannhauser, S. J., A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoprotein in animal tissues. *J. Biol. Chem.* **161**, 83 (1945).
14. Fleck, A., and Munro, H. N., The precision of ultraviolet absorption measurements in the Schmidt-Thannhauser procedure for nucleic acid estimation. *Biochim. Biophys. Acta* **55**, 571 (1962).
15. Hallinan, T., Fleck, A., and Munro, H. N., Loss of ribonucleic acid into lipid solvents after acid precipitation. *Biochim. Biophys. Acta* **68**, 131 (1963).
16. Davidson, J. N., Some critical comments on the chemical estimation of nucleic acids in tissues. *Exp. Cell Res.* Supp. **4**, 260 (1957).
17. Razzell, W. E., The precipitation of polyribonucleotides with magnesium salts and ethanol. *J. Biol. Chem.* **238**, 3053 (1963).
18. Digirolamo, A., Henshaw, E. C., and Hiatt, H. H., Messenger ribonucleic acid in rat liver nuclei and cytoplasm. *J. Mol. Biol.* **8**, 479 (1964).
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265 (1951).
20. Allen, R. J. L., The estimation of phosphorus. *Biochem. J.* **34**, 858 (1940).
21. Tsanev, R., and Markov, G. G., Substances interfering with spectrophotometric estimations of nucleic acids and their elimination by the two-wavelength method. *Biochim. Biophys. Acta* **42**, 442 (1960).
22. Ceriotti, G., Determination of nucleic acid in animal tissues. *J. Biol. Chem.* **214**, 59 (1955).
23. Zytko, J., de Lamirande, G., Allard, G., and Cantaro, A., Ribonucleases of rat liver. *Biochim. Biophys. Acta* **27**, 495 (1958).
24. Metais, P., and Mandel, P., Teneur en acide désoxypentose-nucléique des leucocytes chez l'homme normal et à l'état pathologique. *Compt. Rend. Soc. Biol.* **144**, 277 (1950).
25. Hale, A. J., and Wilson, S. J., The deoxyribonucleic acid content of the leucocytes in human blood, bone marrow and lymph glands. *J. Pathol. Bacteriol.* **82**, 483 (1961).
26. Glen, A. C. A., Unpublished data.

27. Hale, A. J., The leucocyte as a possible exception to the theory of deoxyribonucleic acid constancy. *J. Pathol. Bacteriol.* **85**, 311 (1963).
28. Davidson, J. N., and Smellie, R. M. S., Phosphorus compounds in the cell, 3. *Biochem. J.* **52**, 599 (1952).
29. Davidson, J. N., Fraser, S. C., and Hutchison, W. C., Phosphorus compounds in the cell, 1. *Biochem. J.* **49**, 311 (1951).
30. Leslie, I., "The Nucleic Acid Content of Tissues and Cells." In *The Nucleic Acids* (Vol. II), Chargaff, E., and Davidson, J. N., Eds. Acad. Press, New York, 1955.
31. Gowans, J. L., The role of lymphocytes in the destruction of homografts. *Brit. Med. Bull.* **21**, 106 (1965).
32. Cooper, H. L., and Rubin, A. D., RNA metabolism in lymphocytes stimulated by phytohemagglutinin:Initial responses to phytohemagglutinin. *Blood* **25**, 1014 (1965).
33. Levin, W. C., and Ritzmann, S. E., Relation of abnormal proteins to formed elements of blood:Cellular sources. *Ann. Rev. Med.* **16**, 187 (1965).
34. Elves, M. W., Roath, S., and Israels, M. C. G., The responses of lymphocytes to antigen challenge in vitro. *Lancet* **1**, 806 (1963).
35. Yoffey, J. M., Winter, G. C. B., Osmond, D. G., and Meek, E. S., Morphological studies in the culture of human leucocytes with phytohaemagglutinin. *Brit. J. Haematol.* **11**, 488 (1965).
36. Cooper, H. L., and Rubin, A. D., Lymphocyte RNA metabolism, comparison between antigen and phytohaemagglutinin stimulation. *Lancet* **2**, 723 (1965).
37. Epstein, L. B., and Stohlman, F., Jr., RNA synthesis in culture of normal human peripheral blood. *Blood* **24**, 69 (1964).
38. Pariser, S., Zucker, R. A., and Meyer, L. M., Hematologic changes associated with the immune response in man. *Acta Med. Scand.* **144**, 201 (1952).

LYMPHOCYTE DNA AND RNA CONTENT IN RHEUMATOID ARTHRITIS

BY

A. C. A. GLEN AND M. K. JASANI

Reprinted from

ANNALS OF THE RHEUMATIC DISEASES

Vol. 27 No. 2 March 1968

COPYRIGHT © 1968 by the

Annals of the Rheumatic Diseases

LONDON

BRITISH MEDICAL ASSOCIATION

TAVISTOCK SQUARE, W.C.1.

Ann. rheum. Dis. (1968), 27, 170

LYMPHOCYTE DNA AND RNA CONTENT IN RHEUMATOID ARTHRITIS

BY

A. C. A. GLEN*

Department of Biochemistry, Western Infirmary, Glasgow,

AND

M. K. JASANI†

Centre for Rheumatic Diseases, Glasgow

Rheumatoid factor and antinuclear factors present in the sera of a high proportion of patients with rheumatoid arthritis have many of the characteristics of autoantibodies (Glynn and Holborow, 1965). These serum autoantibodies do not appear to be primary in the pathogenesis of the disease (Harris and Vaughan, 1961) although rheumatoid factor may sustain joint inflammation (Restifo, Lussier, Rawson, Rockey, and Hollander, 1965). It is possible that cell-bound immunity or delayed hypersensitivity may be the important mechanism in the disease process (Doniach and Roitt, 1962) which would implicate sensitized lymphocytes as the effectors of tissue damage (Holborow, 1967). We have therefore studied the lymphocytes of peripheral blood in rheumatoid patients. The DNA and RNA content of suspensions of isolated cells was measured; the DNA to establish cell numbers, and RNA content as an index of their protein synthetic activity.

Material and Methods

Patients

A total of 35 patients with rheumatoid arthritis was studied. Of these 24 had definite rheumatoid arthritis by the American Rheumatism Association criteria (Ropes, 1959); 21 of this group were female, and their mean age was 55 years (range 25 to 78). The remaining eleven patients had probable rheumatoid arthritis; ten were female, and their mean age was 47 years (range 25 to 70). Forty healthy individuals, mostly hospital staff, served as controls; twenty controls were female, and their mean age was 35 years (range 18 to 77).

Estimation of DNA and RNA

The method for analysis of the DNA and RNA content of peripheral blood lymphocytes has been described elsewhere (Glen, 1967). It was carried out in two stages.

(1) To separate the lymphocytes from the other blood cells, 20 ml. venous blood were withdrawn from each patient and the lymphocytes separated by a modification of the method of Coulson and Chalmers (1964) which utilizes a gelatin solution to accelerate cell sedimentation. Red cells were removed by osmotic shock, and the lymphocytes were obtained with less than 10 per cent. contamination with other cells, usually polymorphonuclear leucocytes; a smear from each sample being checked for purity before analysis.

(2) Measured numbers of cells, always greater than 3×10^6 were then submitted to a modified Schmidt Thannhauser separation (Fleck and Munro, 1962), and the separated DNA and RNA fractions of the lymphocytes analysed by ultraviolet absorbtiometry. The results obtained gave the DNA and RNA content of the peripheral blood lymphocytes in each patient expressed as micrograms (μg.) DNA phosphorus (DNAP) or RNA phosphorus (RNAP) per million cells.

A previous report (Glen, 1967) showed that the above analytical method was reproducible within $\pm$ 5 per cent. When the error introduced by cell counting was eliminated by expressing the results as the ratio of RNAP/DNAP, the mean variation in duplicate was reduced to $\pm$ 3.8 per cent.

Results

DNA

The results are summarized in Fig. 1 and Tables I and II (opposite).

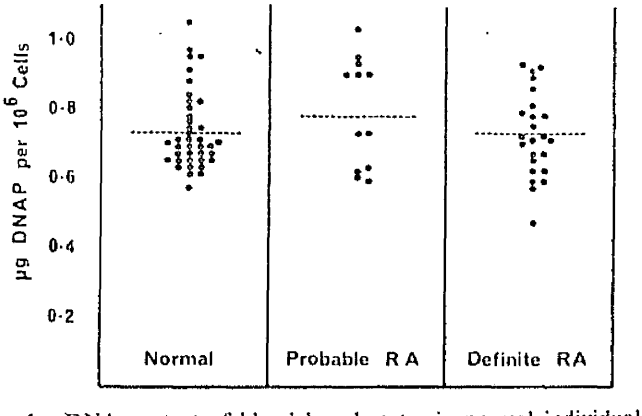


Fig. 1.—DNA content of blood lymphocytes in normal individuals and patients with probable and definite rheumatoid arthritis.

Present Appointments:
*Senior Registrar, Department of Biochemistry, Victoria Infirmary, Glasgow.
†C.I.B.A., Clinical Research Fellow.

TABLE I
MEAN DNAP AND RNAP

Group	Number of Individuals	Mean DNAP (μg. per 10^6 Cells) ± S.D.	Mean RNAP (μg. per 10^6 Cells) ± S.D.	Ratio RNAP/DNAP ± S.D.
Normal	40	0·73 ± 0·12	0·19 ± 0·06	0·26 ± 0·06
Probable Rheumatoid Arthritis	11	0·76 ± 0·16	0·23 ± 0·07	0·30 ± 0·05
Definite Rheumatoid Arthritis	24	0·73 ± 0·12	0·27 ± 0·07	0·37 ± 0·05

TABLE II

Measure	Analysis	Degrees of Freedom ν_1	Degrees of Freedom ν_2	F	P
RNAP	Normal v. Definite + Probable RA	1	73	17·5	<0·001
	Normal v. Definite RA	1	62	24·7	<0·001
	Normal v. Probable RA	1	49	2·5	>0·05
	Probable v. Definite RA	1	33	2·9	>0·05
RNAP/DNAP Ratio	Normal v. Definite + Probable RA	1	73	31·2	<0·001
	Normal v. Definite RA	1	62	43·0	<0·001
	Normal v. Probable RA	1	49	2·8	>0·05
	Probable v. Definite RA	1	33	11·95	<0·01

No significant difference was found between the DNA phosphorus content of the circulating lymphocytes from patients with rheumatoid arthritis and normal individuals. The mean for the definite rheumatoid arthritis group was identical with that for the normal series, 0·73 μg. DNAP per 10^6 cells; the lymphocytes from the probable rheumatoid group had a higher mean DNA phosphorus content, 0·76 μg. DNAP per 10^6 cells, but this difference was not significant. No significant difference in the DNA phosphorus content of the lymphocytes was noted between males and females in either the rheumatoid patients or the control subjects.

RNA

The results are summarized in Fig. 2 and Tables I and II. The mean RNA phosphorus content of the circulating lymphocytes in definite rheumatoid arthritis, 0·27 μg. RNAP per 10^6 cells, was significantly different from the control group, 0·19 μg. RNAP per 10^6 cells ($P < 0·001$). The mean RNA phosphorus content of the lymphocytes of patients with probable rheumatoid arthritis was 0·23 μg. per 10^6 cells, and analysis of variance of the results showed no significant difference from the control values.

RNAP/DNAP Ratio

This was calculated for each patient and control from the results of the RNAP and DNAP analyses. The RNAP/DNAP ratios of the lymphocytes in patients and controls are shown in Fig. 3 and summarized in Tables I and II. The means for the ratios in the three groups were: 0·30 for patients with probable rheumatoid arthritis, 0·37 for patients with definite rheumatoid arthritis, and 0·26 for normal subjects. The definite rheumatoid group

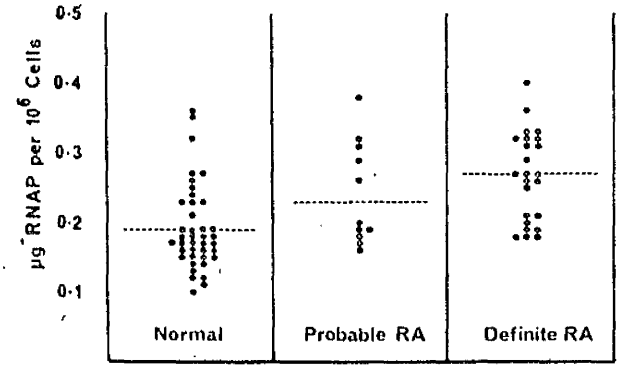


Fig. 2.—RNA content of blood lymphocytes in normal individuals and patients with probable and definite rheumatoid arthritis.

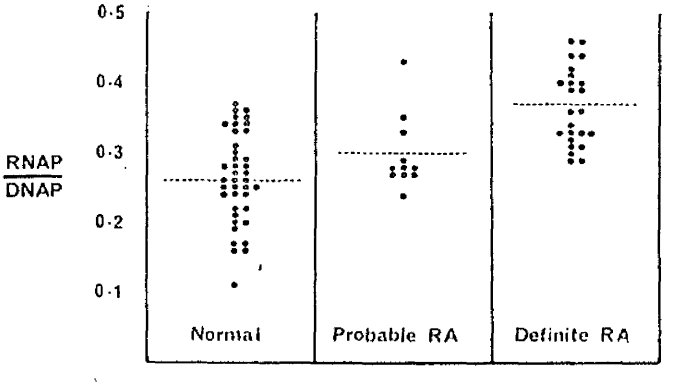


Fig. 3.—RNA/DNA ratio of blood lymphocytes in normal individuals and patients with probable and definite rheumatoid arthritis.

was significantly different from the normal ($P < 0{\cdot}001$). The probable rheumatoid group was not significantly different from the normal, but differed significantly from the definite rheumatoid group ($P < 0{\cdot}01$).

It was possible to match for age and sex, ten patients with definite rheumatoid arthritis and ten controls. Pairings were carried out by random selection when more than one match was available. The lymphocyte RNAP/DNAP ratio was again different in the matched groups; the mean ratio for patients with definite rheumatoid arthritis was $0{\cdot}37 \pm 0{\cdot}04$ and for normal subjects $0{\cdot}26 \pm 0.03$ $P < 0{\cdot}001$).

No close correlation was found between the RNAP/DNAP ratio of the lymphocytes and the clinical or laboratory features of rheumatoid arthritis, although, as shown above, clear differences were noted between the patients with probable and definite rheumatoid arthritis when considered as groups.

Discussion

The results of analyses of the DNA phosphorus (DNAP) content per 10^6 cells of the blood lymphocytes in rheumatoid arthritis and in normal individuals are close to the expected diploid amount for man (Leslie, 1960). DNA phosphorus is thus a good index of cellularity, and hence the mean RNA of the lymphocytes may be expressed as the RNA phosphorus/DNA phosphorus (RNAP/DNAP) ratio of the cells.

The interpretation of the rise in the average RNA content of the blood lymphocytes in rheumatoid arthritis is complicated by the diversity of the blood lymphocyte both in origin and in morphology.

Blood lymphocytes are in part newly-formed cells derived from bone marrow or spleen (large and medium-sized cells) and part recirculating lymphocytes (small lymphocytes) which enter the tissues from the blood stream, percolate through to the lymphatics passing through lymph nodes, and re-enter the blood stream by way of the major lymphatic trunks (Gowans, 1959). Some of these small lymphocytes are known to have a prolonged life span, recirculating from blood to lymph repeatedly over a number of years (Buckton, Jacobs, Court Brown, and Doll, 1962). The lymphocytes of the thoracic duct which comprise the bulk of the recirculating cells were studied in animals by Everett, Caffrey, and Rieke (1964), who observed that exposure of the animal to antigen produced an increase in the size and the protein synthetic rate of the small lymphocytes entering the circulation. These alterations must also imply an increase in the RNAP/DNAP ratio of the lymphocytes. Thus a rise in the mean RNA content of the peripheral blood lymphocytes can occur in several ways. Firstly, by an increase in the numbers of circulating large lymphocytes (either absolute or relative), or secondly, by an increase in the RNA content of the individual cells, either large lymphocytes, or the smaller lymphocytes, or both.

It is likely that increased immunological activity will result in an increased production of newly-formed and hence large lymphocytes. Wardle and Attan (1967) observed hyperplasia of lymphoid elements in the bone marrow of patients with rheumatoid arthritis. Everett and others (1964), in their studies of the effect of antigens on the lymphocytes, observed an increase in the size of the long-lived lymphocytes and called the enlarged small lymphocytes "activated cells". It is possible that, in rheumatoid arthritis, in the presence of increased immunological reaction, a proportion of the lymphocytes carry increased amounts of RNA and are akin to Everett's activated cells.

It is well established that small lymphocytes respond *in vitro* to a series of agents, either specific, such as tuberculin purified protein derivative and pertussis vaccine, or non-specific, such as phytohaemagglutinin (Hirschhorn, Bach, Kolodny, Firschein, and Hashem, 1963). This transformation response is characterized by an increase in several features of the cell, including size, rates of nucleic acid synthesis, and RNA content. A parallel has been drawn between the behaviour of the cells *in vivo* and their response *in vitro*, and this is of special interest in rheumatoid arthritis. Lymphocytes will transform *in vitro* when exposed to antisera to γ-globulin (Sell and Gell, 1965). A similar response *in vivo* to rheumatoid factor, circulating antibody to γ-globulin, could be an explanation for increased amounts of RNA in the circulating lymphocytes in rheumatoid arthritis.

The possibility that drug treatment of patients with rheumatoid arthritis has influenced the RNAP/DNAP ratio of the lymphocytes in this series has been considered. All of the patients investigated were taking salicylates or steroids or both. High lymphocyte RNAP/DNAP ratios occurred in the patients studied irrespective of these categories. It is known that the immediate effect of steroids is to reduce the number of small lymphocytes, however the effect of prolonged therapy tends towards recovery of the pre-treatment state (Glen, Cooper, Jacob, Boak, Murray, and Munro, 1967). In the present series there was no significant difference in the RNAP/DNAP ratios of those receiving steroids and the rest.

Although the precise cause of the change in the lymphocyte RNAP/DNAP ratio has not been

defined, the data show that the ratio is frequently increased in the blood lymphocytes of patients with rheumatoid arthritis. How this observation may be linked to the pathogenesis of the disease remains an open question; however the demonstration by Birbeck and Hall (1967) of the transformation *in vivo* of large basophilic lymphocytes (released by a lymph node on antigenic stimulation) into plasma cells makes it very likely that lymphocytes are indeed the effectors of cell-bound immunity.

Summary

The average cell DNA and RNA was measured in lymphocytes obtained from the peripheral blood of 24 patients with definite and eleven with probable rheumatoid arthritis, according to the American Rheumatism Association diagnostic criteria. Similar measurements were made in forty healthy individuals. A significant increase in the RNA content of the lymphocytes in patients with definite rheumatoid arthritis when compared with the controls was demonstrated. Possible reasons for this difference are discussed in relation to the known reactions of the lymphocyte to immunological stimuli and to drug treatment. It is concluded that the increased lymphocyte RNA content found in patients with rheumatoid arthritis is evidence of the participation of the lymphocyte in the immunological derangements of the disease, and that the RNA/DNA ratio of the lymphocyte may be a useful means of measuring immunological activity.

REFERENCES

Birbeck, M. S. C., and Hall, J. G. (1967). *Nature* (*Lond.*), **214**, 183 (Transformation, *in vivo*, of basophilic lymph cells into plasma cells).

Buckton, K. E., Jacobs, P. A., Court Brown, W. M., and Doll, R. (1962). *Lancet*, **2**, 676 (A study of the chromosome damage persisting after *x*-ray therapy for ankylosing spondylitis).

Coulson, A. S., and Chalmers, D. G. (1964). *Ibid.*, **1**, 468 (Separation of viable lymphocytes from human blood).

Doniach, D., and Roitt, I. M. (1962). *Ann. Rev. Med.*, **13**, 213 (Auto-antibodies in disease).

Everett, N. B., Caffrey, R. W., and Rieke, W. O. (1964). *Ann. N.Y. Acad. Sci.*, **113**, 887 (Recirculation of lymphocytes).

Fleck, A., and Munro, H. N. (1962). *Biochim. biophys. Acta*, **55**, 571 (The precision of ultraviolet absorption measurements in the Schmidt-Thannhauser procedure for nucleic acid estimation).

Glen, A. C. A. (1967). *Clin. Chem.*, **13**, 299 (Measurement of DNA and RNA in human peripheral blood lymphocytes).

——, Cooper, W. C., Jacob, S. T., Boak, J. L., Murray, J. E., and Munro, H. N. (1967). In preparation.

Glynn, L. E., and Holborow, E. J. (1965). "Autoimmunity and Disease". Blackwell Scientific Publications, Oxford.

Gowans, J. L. (1959). *J. Physiol.* (*Lond.*), **146**, 54 (The recirculation of lymphocytes from blood to lymph in the rat).

Harris, J., and Vaughan, J. H. (1961). *Arthr. and Rheum.*, **4**, 47 (Transfusion studies in rheumatoid arthritis).

Hirschhorn, K., Bach, F., Kolodny, R. L., Firschein, I. L., and Hashem, N. (1963). *Science*, **142**, 1185 (Immune response and mitosis of human peripheral blood lymphocytes *in vitro*).

Holborow, E. J. (1967). *Lancet*, **1**, 1208 (An ABC of modern immunology).

Leslie, I. (1960). "The Nucleic Acids". Academic Press, New York.

Restifo, R. A., Lussier, A. J., Rawson, A. J., Rockey, J. H., and Hollander, J. L. (1965). *Ann. intern. Med.*, **62**, 285 (Experimental production of arthritis by the intra-articular injection of purified 7s gamma globulin).

Ropes, M. W. (1959). *Ann. rheum. Dis.*, **18**, 49 (Diagnostic criteria for rheumatoid arthritis. 1958 revision).

Sell, S., and Gell, P. G. H. (1965). *J. exp. Med.*, **122**, 423 (Stimulation of blast transformation with an antiallotype serum).

Wardle, E. N., and Attan, J. (1967). *Brit. J. Haematol.*, **13**, 194 (An electron microscope study of bone marrow in rheumatoid disease).

Le teneur des lymphocytes en ADN et en ARN dans la polyarthrite rhumatoïde

RÉSUMÉ

On détermina le taux moyen d'ADN et d'ARN dans les lymphocytes provenant du sang périphérique de 35 malades atteints de polyarthrite rhumatoïde; chez 24 d'entre eux la maladie était "definie" et chez 11 "probable", selon les critères diagnostiques de l'*American Rheumatism Association*. On fit la même chose chez 40 sujets sains. On mit en évidence une augmentation appréciable du taux lymphocytaire d'ARN chez les malades atteints de polyarthrite rhumatoïde "definie" par rapport aux témoins. On discute les raisons probables de cette différence à propos des réactions bien connues du lymphocyte à l'égard des stimulants immunologiques et des produits médicamenteux. On conclut que la teneur en ARN augmentée des lymphocytes, trouvée chez des malades atteints de polyarthrite rhumatoïde, constitue une preuve de la participation du lymphocyte dans le dérangement morbide et que la raison ARN/ADN du lymphocyte peut être utile pour mesurer l'activité immunologique.

Las cifras linfocitarias de ADN y de ARN en la poliartritis reumatoide

SUMARIO

Se determinaron las cifras medias de ADN y de ARN en linfocitos de las sangre periférica de 35 enfermos con poliartritis reumatoide, que fué "definida" en 24 casos y "probable" en 11 casos, según los criterios diagnósticos de la *American Rheumatism Association*. Se hizo lo mismo en 40 sujetos sanos. Se evidenció un aumento apreciable de las cifras linfocitarias de ARN en enfermos con poliartritis reumatoide "definida" en comparación con los testigos. Se discuten las causas probables de esta diferencia en relación con las reacciones conocidas del linfocito hacia estímulos inmunológicos y productos medicamentosos. Se concluye que las cifras aumentadas de ARN en leucocitos, encontradas en enfermos con poliartritis reumatoide, ofrecen una prueba de la participación del linfocito en el desarreglo morboso y que la razón ARN/ADN del linfocito puede utilizarse para medir la actividad inmunológica.

Sonderdruck aus · Reprint from · Tiré à part
Modern Gastroenterology
Verhandlungsbericht — Proceedings — Communications
VIIIth International Congress of Gastroenterology, July 7th-13th, 1968, Prague
Edited by Prof. Dr. O. Gregor and Doz. Dr. O. Riedl, Prague
F. K. SCHATTAUER VERLAG · STUTTGART - NEW YORK 1969, p.

University Dept. of Medicine, Royal Infirmary, Dept. of Biochemistry, Glasgow University, Victoria Infirmary, Glasgow, Scotland, Great Britain

23. Immunological Defect as the Cause of Whipple's Disease and Colonic Histiocytosis

A. Ferguson, J. D. Maxwell, R. C. Imrie and A. C. A. Glen

In Whipple's disease, abnormal macrophages and many bacilli are present in the intestinal mucosa. A variety of organisms have been cultured, suggesting that this disease is due to a host abnormality rather than to a specific infection. In colonic histiocytosis many macrophages are present, but do not contain bacilli. Both disease states could result from an abnormal cellular response to foreign material. To test this hypothesis the immunological functions of 4 patients have been studied.

All 4 patients were in complete remission and on no treatment. Case 1 (Whipples) had apparently been cured by a course of chloramphenicol 1 year earlier. Case 2 (Whipples) had been well since a course of tetracycline 3 years before. In cases 3 and 4 the diagnosis of colonic histiocytosis had been made on the basis of a history of chronic intermittent diarrhoea, and rectal biopsy containing many PAS-positive macrophages.

Methods

DNA and RNA were assayed in peripheral blood lymphocytes using a modification of the Schmidt-Thannhauser procedure and ultra-violet absorbtimetry (1). The uptake of ^{14}C labelled thymidine was used as a measure of lymphocyte transformation in response to varying concentrations of phytohaemagglutinin (PHA) (2). Mueller, Tormay and Imrie have reported preliminary studies on a human serum fraction which will further stimulate the DNA synthesis produced by PHA. The effect of this material on PHA-stimulated lymphocytes was studied in 3 cases.

Results

Screening tests revealed normal number and morphology of the circulating white blood cells. Several normal antibodies were detected. There were no autoantibodies. Serum immunoglobulin-M was low in case 2.

The results of studies on lymphocytes are summarised in Tables 1, 2 and 3.

Table 1. Nucleic acid content of peripheral blood lymphocytes.

	DNAP ($\mu g/10^6$ lymphocytes)	RNAP ($\mu g/10^6$ lymphocytes)
Normal human lymphocytes		
Mean	0.73	0.19
Standard deviation	0.12	0.06
Case 2	0.54	0.11
Case 3	0.45	0.10
Case 4	0.68	0.21

Table 2. Maximum lymphocyte response to PHA (percentage of control).

Control	100%
Case 1	52%
Case 2	54%
Case 3	47%
Case 4	102%

Table 3. Effect of serum fraction on PHA stimulated lymphocytes (percentage of response to PHA alone).

	PHA	PHA + extract
Control	100	⟶ 135%
Case 2	100	⟶ 94%
Case 3	100	⟶ 310%
Case 4	100	⟶ 251%

Discussion

The RNA content of cells reflects their protein synthetic activity. In auto-immune diseases there is an increase in the RNA content of circulating lymphocytes; with immunosuppressive therapy the lymphocyte RNA content falls. The low RNA in cases 2 and 3 suggests that the immunological activity of these cells is low. The low mean lymphocyte DNA may indicate that, in these patients, a significantly reduced proportion of lymphocytes are actively synthesising DNA, hence containing more than the diploid amount of DNA.

Lymphocyte transformation in response to PHA probably reflects the immunological capacity of the cells; a low response was found in 3 patients. Normally the serum fraction will increase DNA synthesis by up to 100%. In case 2 there was no increase in response. In cases 3 and 4 the response was more than doubled. This suggests either that

PHA had exerted a submaximal effect, or that the amount of the factor in the patients' cells was low.

These results could be due to generalised, mild lymphocyte abnormality. Alternatively, as the circulating lymphocytes are of heterogeneous origin, they may indicate a smaller number of lymphocytes with grossly impaired function — perhaps those associated with the gastrointestinal tract. Whipple's disease and colonic histiocytosis may form part of the spectrum of disease states which result from impaired cell-mediated immune responses.

References

(1) Glen, A. C. A.: Clin. Chem. *13:* 299 (1967).
(2) Tormey, D. C., G. C. Mueller: Blood *26:* 569 (1965).

THE NUCLEIC ACID CONTENT OF LYMPHOCYTES IN THYROTOXICOSIS

J. F. B. SMITH, A. C. A. GLEN AND W. R. GREIG

Reprinted from
Clinical and Experimental Immunology
Vol. 8, No. 6, June 1971

BLACKWELL SCIENTIFIC PUBLICATIONS
OXFORD AND EDINBURGH

Clin. exp. Immunol. (1971) 8, 911–916.

THE NUCLEIC ACID CONTENT OF LYMPHOCYTES IN THYROTOXICOSIS

J. F. B. SMITH, A. C. A. GLEN AND W. R. GREIG

University Department of Medicine, Glasgow Royal Infirmary, and Biochemistry Department, Victoria Infirmary, Glasgow

(*Received* 2 *November* 1970)

SUMMARY

In LATS positive thyrotoxic patients the blood lymphocyte RNA content was significantly greater than normal, but not significantly different from normal in LATS negative thyrotoxicosis patients. Changes occurred in the RNA content of the lymphocytes following treatment whether by radioactive iodine or carbimazole.

INTRODUCTION

The blood of a proportion of patients with thyrotoxicosis (Graves' disease) contains a circulating immunoglobulin (IgG) the long acting thyroid stimulator, LATS (McKenzie, 1967). Blood lymphocytes have been identified as one site of IgG production (Turner & Forbes, 1966), and McKenzie (1967) showed that lymphocytes from thyrotoxic patients produced LATS when stimulated *in vitro* by phytohaemagglutinin. It is therefore relevant to reconsider the nature of the lymphocytes in thyrotoxicosis. Hernberg (1954) has reported that the size of the blood lymphocytes was increased in thyrotoxicosis and returned to within normal limits when the patients were euthyroid after partial thyroidectomy. The measurement of the DNA and RNA content of the peripheral blood lymphocyte (Glen, 1967) provides an objective method of assessing the nature of the circulating lymphocytes; in some conditions, for example rheumatoid arthritis, there is a relationship between the RNA content of blood lymphocytes and the severity of the disease (Glen & Jasani, 1968).

On the assumption that thyrotoxicosis is in part an immunological disease, we thought it of interest to measure the RNA content of the blood lymphocyte and to compare measurements before and after carbimazole and radioactive iodine treatment. In addition, since the presence of the immunoglobulin LATS might be an index of the degree of the immuno-

Correspondence: Dr J. F. B. Smith, University Department of Medicine, Glasgow Royal Infirmary, Glasgow, C.4.

logical disturbance, we compared lymphocytes from blood positive for LATS with cells from blood negative for LATS.

PATIENTS AND METHODS

Methods. Blood samples from thirty-six patients, twenty-eight women and eight men (age range 22–70 years, mean age 49 years) were studied. The clinical thyrotoxicosis was confirmed with radioiodine studies, serum protein bound iodine estimations and serum triiodothyronine resin sponge tests. Blood from healthy control subjects was taken from twenty adults, eleven men and nine women (age range 18–63 years, mean age 30 years).

The LATS activity of sera from the thyroid patients was measured using the bioassay of Kriss in which test and control sera (0·5 ml) are injected intraperitoneally into white male Swiss mice whose thyroid iodine is labelled and the thyroid stimulation monitored by measuring the increase in blood radioactivity at 24 hr (Kriss, Pleshakov & Chien, 1964). The net increase in blood radioactivity which represents the discharge of iodine from the gland is expressed as counts/min per 0·1 ml of blood and the results are recorded as a percentage increase for each sera. A blood radioactivity of 150% of that before administration of sera was taken as a LATS positive response.

The method for analysis of the DNA and RNA content of the blood lymphocyte has been described previously (Glen, 1967); there are two stages, separation of lymphocytes from blood, and chemical analysis of DNA and RNA.

20 ml of venous blood were withdrawn from each patient with EDTA as anticoagulant and the lymphocytes separated using a modification of the gelatin sedimentation procedure of Coulson & Chalmers (1964). The technique used was as described by Glen except that a suggestion by Cooper (1967) has been adopted in these experiments which has improved the reliability of the lymphocyte separation by eliminating polymorphonuclear leucocyte contamination of the isolated cells. In this modification, the cell suspension was allowed to soak into cotton wool at 37°C for 15 min and is thereafter expressed by gentle pressure. Red cells were removed by osmotic shock and a final cell suspension obtained which contained at least 97% lymphocytes. Measured numbers of cells always greater than 4×10^6 were submitted to a modified Schmidt-Thannhauser separation, and the separated DNA and RNA fractions analysed by UV absorptiometry making appropriate correction for the presence of protein (Fleck & Munro, 1962). The results were expressed as μg DNA phosphorus (DNAP) per 10^6 cells and RNA phosphorus (RNAP) per 10^6 cells. It is an advantage to use the RNAP/DNAP ratio of the cells rather than RNAP content expressed per million cells since this eliminates cell counting error. The RNAP/DNAP ratio is a valid index of cell RNA provided the DNA content of the lymphocyte is constant. It has previously been shown that the lymphocyte RNAP/DNAP can be measured with an error of within $\pm 5\%$ when duplicate blood samples are analysed.

Patients studied. Ten patients were treated with carbimazole and the nucleic acid measurements were carried out before and after control of the thyrotoxicosis but during maintenance treatment with carbimazole 15–20 mg daily, an average of 4 months (range 2–6 months) after starting drugs. Twenty-one patients were treated with radioactive iodine and nucleic acid measurements were carried out on the lymphocytes before and after control of thyrotoxicosis, 4 months (range 2–6 months) from the time of the dose. Of the remaining five patients, three patients underwent partial thyroidectomy and two patients did not return.

RESULTS

The mean of the DNA values of the blood lymphocytes in the twenty normal individuals was 0·732 μg DNAP per 10^6 cells (SD = 0·114) and from the thirty-six thyrotoxic patients before treatment it was 0·740 μg (SD = 0·117). These data are shown in Fig. 1. Treatment of thyrotoxicosis did not affect the DNA values so that the RNAP/DNAP ratio was considered a valid index of mean RNA content of the lymphocytes and this applied irrespective of the treatment regimes.

Table 1 shows the RNAP/DNAP ratios of lymphocytes from thyrotoxic patients before and after treatment with either carbimazole or radioactive iodine and these data are

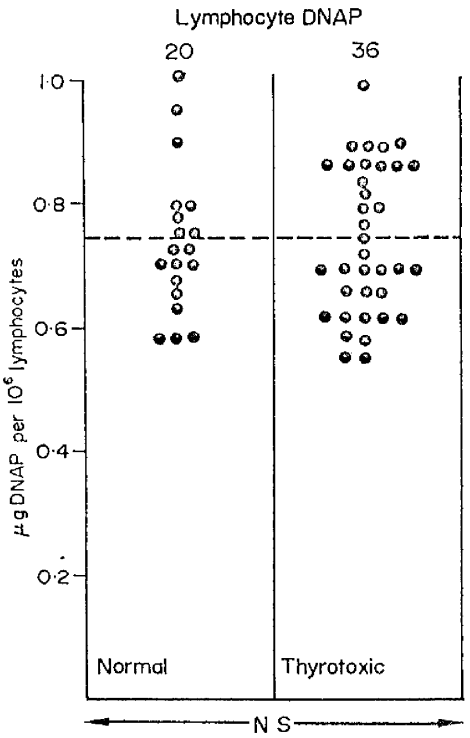


FIG. 1. The blood lymphocyte DNA content in normal and thyrotoxic patients. The mean values were not significantly different (NS) by *t*-test.

compared to those from normal individuals which are also shown in the Table. The results from the group of ten patients treated with carbimazole are higher than normals but only after drug treatment are the differences significant ($P<0·02$). This applies when the mean, after treatment, is compared to that before treatment or with normal people. When the RNAP/DNAP ratio data from patients treated with radio-iodine therapy is similarly examined there is a difference between means after therapy compared to that of normal people. In contrast, however, there is not a significant difference between the mean lymphocyte RNAP/DNAP before and after therapy, but the pre-treatment values are relatively high (mean = 0·323).

Fig. 2 shows the lymphocyte RNAP/DNAP ratios from the patients whose sera was negative in the LATS bioassay (twenty-two) and these from patients positive in the LATS

TABLE 1. Blood lymphocyte RNAP/DNAP ratios in untreated (thyrotoxic) and treated (euthyroid) patients with the results in normal individuals for comparison

	Before treatment (thyrotoxic)	After treatment (euthyroid)	Comparison of pre- and post-treatment means by *t*-test
Treated by carbimazole	Mean = 0·308 (10) SD = 0·053	*Mean = 0·365 (10) SD = 0·050	$t = 2·55$ $P < 0·02$ Significant
Radioactive iodine	Mean = 0·323 (21) SD = 0·068	*Mean = 0·352 (19) SD = 0·048	$t = 1·60$ $P < 0·20$ Not significant
Normal individual untreated	Mean = 0·296 (20) SD = 0·036		

* Values denoted by the asterisk are significantly different from the results in the normal untreated control subjects.

The results of comparison of the lymphocyte RNAP/DNAP ratio in pre-treatment thyrotoxic and post-treatment euthyroid patients are tabulated, showing the mean values and the standard deviation with the number of individuals in parentheses. The results of a comparison of mean values by Student's *t*-test are listed.

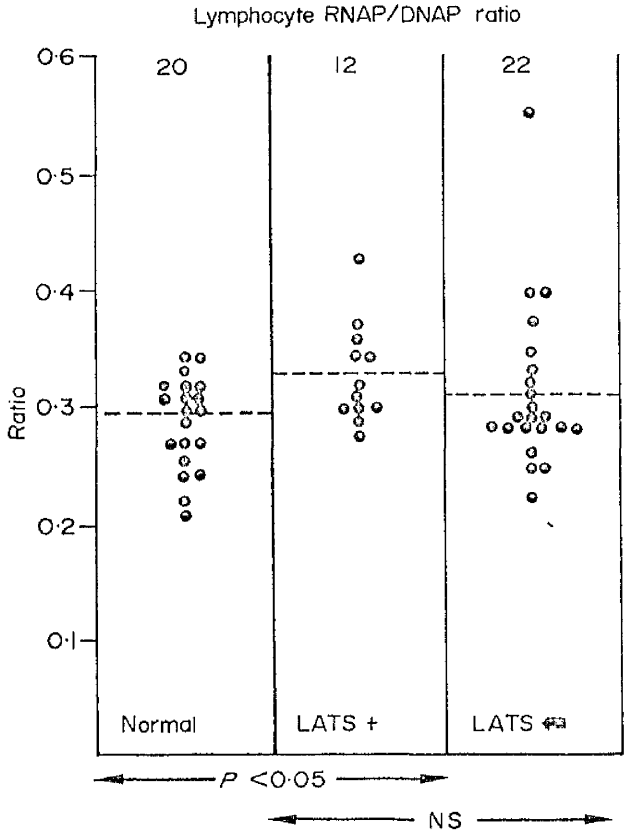


FIG. 2. The RNAP/DNAP ratios of the blood lymphocytes from LATS positive and LATS negative patients with normals for comparison. The *t*-test was used to compare mean values.

bioassay (twelve); the normal data are shown for comparison. The mean ratio values from those with LATS negative sera was 0·302 and from those with LATS positive it was 0·326. Intercomparisons were made and it was found that the mean lymphocyte ratio RNAP/DNAP from LATS positive blood was higher (mean 0·326) than that from normal blood (mean 0·295), with a P value of $<0·05$.

The mean lymphocyte count in the peripheral blood of nineteen of the patients with untreated thyrotoxicosis was 2250 cells/mm^3 with a range of from 1060 to 4600 lymphocytes/mm^3. Two patients had a total white cell count of 4000 or less/mm^3 with a relative lymphocytosis. Peripheral blood lymphocyte counts following treatment are incomplete but it appears from limited data that the lymphocyte count increases following radioiodine therapy and decreases in patients treated with carbimazole. This aspect of the study is being further pursued.

DISCUSSION

The results of the lymphocyte RNAP/DNAP ratio determinations do not show a large difference when thyrotoxic blood lymphocytes are compared with normal lymphocytes; however, the lymphocytes from blood positive for LATS had a higher RNA content than that present in the normal lymphocytes. An elevated RNAP/DNAP ratio of the lymphocytes is found when there is an absolute increase in the number of the larger cells or when the increase is relative due to a reduction in the number of small lymphocytes. The former is the case when there is increased immunological activity as for example in response to viral infection (Lesiewska, 1967; Pariser, Zucker & Meyer, 1952; Zucker-Franklin, 1969; Crowther, Fairley & Sewell, 1969).

In untreated thyrotoxicosis the lymphocyte RNAP/DNAP ratio is significantly higher than normal only in patients with a positive LATS bioassay and this, in the presence of normal blood lymphocyte counts, would be consistent with an increase of immunological activity. The data also suggest that immunological activity may be intensified by radioactive iodine therapy, possibly as a result of antigen release by radiation damage to the thyroid gland.

The interpretation of data from drug-treated patients is difficult. Whether the significant increase in the mean lymphocyte RNA content, apparently produced by carbimazole treatment, indicates a special extra thyroidal effect of the drug in lymphocytes, requires further study.

It is of interest that thymic lymphoid follicle formation is present in adults with thyrotoxicosis (Gunn, Michie & Irvine, 1964) and that there is a reduction in thymic size in patients on anti-thyroid drugs (Michie *et al.*, 1967). Our data would suggest that the anti-thyroid drugs also have a measurable effect on blood lymphocytes. We would interpret the data as indicating a reduction in thymic dependent lymphocytes resulting in an increase in the proportion of large lymphocytes in the blood. This has the implication that immunosuppression may be one of the actions of carbimazole.

ACKNOWLEDGMENT

W. R. Greig is a Senior Wellcome Clinical Research Fellow.

REFERENCES

COOPER, H.L. (1967) Personal communication.

COULSON, A.S. & CHALMERS, D.G. (1964) Separation of viable lymphocytes from human blood. *Lancet*, **i**, 468.

CROWTHER, D., FAIRLEY, G.H. & SEWELL, R.L. (1969) Lymphoid cellular responses in the blood after immunisation in man. *J. exp. Med.* **129**, 849.

FLECK, A. & MUNRO, H.N. (1962) The precision of ultra violet adsorption measurements in the Schmidt-Thannhauser procedure for nucleic acid estimation. *Biochim. biophys. Acta*, **55**, 571.

GLEN, A.C.A. (1967) Measurement of DNA and RNA in human peripheral blood lymphocytes. *Clin. Chem.* **13**, 299.

GLEN, A.C.A. & JASANI, M.K. (1968) Lymphocyte DNA and RNA content in rheumatoid arthritis. *Ann. rheum. Dis.* **27**, 170.

GUNN, A., MICHIE, W. & IRVINE, W.J. (1964) The thymus in thyroid disease. *Lancet*, **ii**, 776.

HERNBERG, C.A. (1954) Thyrotoxicosis and the size of lymphocytes. *Acta med. scand.* **149**, 37.

KRISS, J.P., PLESHAKOV, V. & CHIEN, J.R. (1964) Isolation and identification of the long acting thyroid stimulator and its relation to hyperthyroidism and circumscribed pretibial myxoedema. *J. clin. Endocr.* **24**, 1005.

LESIEWSKA, J. (1967) Nucleic acids in the lymphocytes of the peripheral blood in the course of some infectious diseases in children. *Folia histochem. cytochem.* **5**, 297.

MCKENZIE, J.M. (1967) The long acting thyroid stimulator: its role in Graves' disease. *Recent Prog. Horm. Res.* **23**, 1.

MICHIE, W., BECK, J.S., MAHAFFY, R.G., HONEIN, E.F. & FOWLER, G.B. (1967) Quantitative radiological and histological studies of the thymus in thyroid disease. *Lancet*, **i**, 691.

PARISER, S., ZUCKER, R.A. & MEYER, L.M. (1952) Haematologic changes associated with the immune response in man. *Acta med. scand.* **144**, 201.

TURNER, K.J. & FORBES, I.J. (1966) Synthesis of proteins by human leucocytes *in vitro*. II. Chemical characterisation. *J. Immunol.* **96**, 926.

ZUCKER-FRANKLIN, D. (1969) The ultrastructure of lymphocytes. *Semin. Hemat.* **6**, 4.

IMMUNOLOGICAL RESPONSES IN SJØGREN'S SYNDROME AND RHEUMATOID ARTHRITIS

K. WHALEY, A. C. A. GLEN, R. N. M. MACSWEEN, S. DEODHAR, W. C. DICK, G. NUKI, J. WILLIAMSON AND W. W. BUCHANAN

Reprinted from
Clinical and Experimental Immunology
Vol. 9, No. 6, December 1971

BLACKWELL SCIENTIFIC PUBLICATIONS
OXFORD AND EDINBURGH

Clin. exp. Immunol. (1971) **9**, 721–732.

IMMUNOLOGICAL RESPONSES IN SJØGREN'S SYNDROME AND RHEUMATOID ARTHRITIS

K. WHALEY, A. C. A. GLEN, R. N. M. MACSWEEN, S. DEODHAR, W. C. DICK, G. NUKI, J. WILLIAMSON AND W. W. BUCHANAN

The Centre for Rheumatic Diseases and the University Department of Medicine, Royal Infirmary, The University Department of Pathology, Western Infirmary, the Department of Biochemistry, Victoria Infirmary, and the Department of Ophthalmology, Southern General Hospital, Glasgow

(*Received* 24 *May* 1971)

SUMMARY

Cellular and humoral immune responses have been measured in patients with Sjøgren's syndrome with, and without, rheumatoid arthritis, patients with sero-positive and sero-negative rheumatoid arthritis, and a control group of patients. Several patients with sero-positive rheumatoid arthritis and patients with Sjøgren's syndrome complicated by rheumatoid arthritis failed to respond to 2–4 Dinitrochlorobenzene, and exhibited poor secondary responses to tetanus toxoid. Mantoux responses, however, were diminished only in patients with the sicca syndrome. Estimation of RNA/DNA ratios of peripheral blood lymphocytes showed increased ratios in those groups of patients responding poorly to DNCB and tetanus toxoid. As the lymphocyte RNA/DNA ratio is correlated to mean lymphocyte diameter, this means that patients with elevated ratios have an absolute, or relative, increase in the number of large lymphocytes circulating in the peripheral blood. It is suggested that failure to respond to DNCB may be due to a deficiency of uncommitted small lymphocytes.

INTRODUCTION

Both rheumatoid arthritis and Sjøgren's syndrome, which is frequently associated with rheumatoid arthritis (Bloch, Buchanan, Wohl and Bunim, 1965), are characterized by lymphoid infiltrates in affected tissues, dysproteinaemia, and a multiplicity of serum autoantibodies (Bloch *et al.*, 1965; Anderson, Buchanan & Goudie, 1967; Whaley & Buchanan, 1969). In addition, generalized lymphadenophy and splenomegaly may occur in both diseases (Bloch *et al.*, 1965; Robertson *et al.*, 1968). In Sjøgren's syndrome without rheumatoid arthritis it is now well established that extra-salivary lymphoid abnormalities including 'pseudolymphoma', lymphoma and Waldenström's macroglobulinaemia may occur (Talal & Bunim, 1964; Talal, Sokoloff & Barth, 1967). In rheumatoid arthritis there is statistical evidence to suggest an increased prevalence of lymphosarcoma (Lea, 1964).

Correspondence: Dr K. Whaley, The University Department of Pathology, Western Infirmary, Glasgow, W.1, Scotland.

Abnormalities of the thymus including germinal centre formation have been reported in patients with rheumatoid arthritis (MacSween, Anderson & Milne, 1967), but to date, to our knowledge, no such changes have been found in Sjøgren's syndrome, although only a few cases have been examined (Bloch *et al.*, 1965).

Results of immunization studies in rheumatoid arthritis have yielded conflicting results. Normal antibody responses have been found to heterologous blood groups in patients with rheumatoid arthritis (Creger *et al.*, 1951). Meiselas *et al.* (1961) demonstrated hyper-responsiveness to *Brucella abortus* whereas normal responses were found by another group of workers (Shearn, Epstein & Engleman, 1963). Results of immunization with tetanus toxoid have produced even more conflicting results; Greenwood & Barr (1960) showed higher antitoxin titres than controls, Vaughan & Butler (1962) detected no differences in antibody response, while Barr *et al.*, (1964) noted a significant number of rheumatoid patients having subnormal responses to tetanus toxoid. Normal antibody responses to pneumococcal polysaccharide have been observed (Larson & Tomlinson, 1953).

In view of the obvious anatomical and functional abnormalities of the immune system in Sjøgren's syndrome and rheumatoid arthritis, it was decided to investigate both cellular and humoral immune responses in these conditions.

MATERIALS AND METHODS

Patients studied

Five clinical groups of patients were studied; control subjects who suffered from osteo-arthrosis, disc degeneration or non-articular rheumatism, patients with sero-positive rheumatoid arthritis, patients with sero-negative rheumatoid arthritis, patients with rheumatoid arthritis and Sjøgren's syndrome, and patients with the sicca syndrome. All the patients studied were females, and none were taking corticosteroid or immunosuppressive therapy. Sjøgren's syndrome was diagnosed using the criteria of Bloch *et al.* (1965). Patients having Sjøgren's syndrome without an associated connective tissue disease were said to have the sicca syndrome. Keratoconjunctivitis sicca and xerostomia were diagnosed as previously described (Williamson *et al.*, 1967; Whaley *et al.*, 1969). Rheumatoid arthritis was diagnosed using the diagnostic criteria of the American Rheumatism Association (Ropes *et al.*, 1958). The patients included fell into the 'probable', 'definite', or 'classical' categories.

Clinical details included the age of the patient, disease duration, functional grade and X-ray stage (Steinbroker, Traeger & Batterman, 1949), articular index (Ritchie *et al.*, 1968), and the presence or absence of granulomatous nodules, lymphadenopathy and splenomegaly Laboratory details included haemoglobin concentration (g/100 ml), white cell and platelet counts (per cu mm), erythrocyte sedimentation rate (ESR mm in lst hour-Westergren) and serum albumin and globulin concentrations (g/100 ml).

Examination for rheumatoid factor was performed by the latex agglutination technique (Hyland Laboratories), at a 1/20 serum dilution. Positive sera were then tested by the sensitized sheep cell agglutination test at a titre of 1/32 and then in doubling dilutions until an end point was reached (Ziff *et al.*, 1956). Antinuclear factor was detected by the technique of Beck (1961). Sera was screened at a dilution of 1/16 and positive sera were titrated in quadrupling dilutions until an end point of nuclear staining was reached.

Cellular immune responses

Chemical contact sensitization with DNCB was performed by applying 2000 μg of DNCB in 0·1 ml of acetone to an area of approximately 3 cm diameter on the volar aspect of the upper forearm. The area was covered for 7 days and challenge doses of 25, 50 and 100 μg of DNCB in 0·1 ml of acetone were applied to the opposite forearm after 14 days to see whether sensitization had occurred. The results were read—2–4 days later when signs of delayed hypersensitivity, viz. erythema, induration and vesiculation, were recorded.

Old tuberculin, 0·1 ml of 1/10,000 dilution, was given intradermally, and the response read after 48 hr. An area of induration of 5 mm or more was regarded as positive. Patients not responding to this dose were subsequently challenged with 0·1 ml of 1/1000 dilution, and if still negative 0·1 ml of 1/100 dilution. Patients not responding to 0·1 ml of 1/100 dilution were said to be Mantoux negative.

Humoral immune responses

Patients received two subcutaneous injections of 1 ml (about nine L.F.) of the same batch of tetanus toxoid with an interval of 6 weeks between injections. The tetanus toxoid (Burroughs Wellcome) was stored in a refrigerator at 2°C until used. Blood samples were taken before the first injection, and 14 days following the second injection. Tetanus antitoxin concentrations were determined in mice by the method of Glenny & Stevens (1938), the result being expressed as units of tetanus antitoxin per ml of blood. None of the patients had received tetanus toxoid prior to the study.

Isohaemagglutinin titres were estimated using standard 2% suspensions of human group A_1, A_2 and B erythrocytes and doubling dilutions of serum. The degree of agglutination titre was read after 60 min at room temperature. Control sera of known isohaemagglutinin titre were always included (Dacie & Lewis, 1968). The number of pregnancies in patients was similar in all groups. Four patients with sero-positive rheumatoid arthritis had had blood transfusions, none of which had been associated with transfusion reactions.

Lymphocyte studies

Lymphocyte separation technique. Pure suspensions of peripheral blood lymphocytes were obtained by two different methods: (1) a modification of the method of Coulson & Chalmers (1964) using EDTA as anticoagulant and separating the red cells from the leucocyte-rich fraction by adding 6 ml of 3% gelatin in 0·15 M saline to 20 ml of blood and allowing the tube to stand for 30 min at 37°C. The leucocyte-rich plasma was then defibrinated by stirring for approximately 15 min, thus also removing the majority of polymorphonuclear leucocytes; (2) a second method, identical to the first, except that cotton wool was added to the defibrinated suspension to reduce still further polymorphonuclear leucocyte contamination of the lymphocyte preparations. Only the lymphocyte suspensions from patients with the sicca syndrome were purified by this method.

Residual red cells were then removed from the lymphocyte preparations by osmotic shock, and following centrifugation at 125 *g* the supernatant containing free haemoglobin and red cell ghosts was pipetted off, leaving a button of lymphocytes over 95% pure. The cells were resuspended in 2·5 ml of 0·15 M saline and a cell count performed. Approximately 12 million lymphocytes were obtained from each 20 ml blood sample.

Estimation of the size of peripheral blood lymphocytes. The mean size of 100 lymphocytes on peripheral blood smears stained with Leishman's stain was obtained using a Watson

image-shearing eyepiece. Smears were examined without knowledge of lymphocyte RNA/DNA ratios.

Estimation of RNA/DNA ratio of lymphocytes. At least 2 million cells were necessary to estimate the cellular RNA and DNA content. RNA/DNA ratios were measured using the technique of Glen (1967). The RNA content of the separated RNA fraction was determined by measuring the absorbance at 260 mμ and then correcting for protein contamination (Fleck & Munro, 1962). DNA concentrations were measured by estimating the absorbance at 265 mμ (Davidson, 1957), using highly polymerized calf thymus DNA (Sigma London Chemical Co.) as a standard.

Statistical methods

Results of DNCB and Mantoux tests and tetanus immunization were analysed using the chi square test, with Yates' correction for small numbers where necessary. Isohaemagglutinin titres were analysed using the Student's *t*-test.

RESULTS

Sensitization by DNCB

Three challenge doses of DNCB 25, 50 and 100 μg were used to attempt to increase the sensitivity of the test by revealing greater differences between the test groups and the controls at the lower doses. However, this was not observed, and so all patients responding to 100 μg DNCB were said to have been sensitized.

Table 1 shows the clinical features of the patients tested and the results of exposure to DNCB. Twenty-two of twenty-four (91·7%) control subjects were sensitized to DNCB. This is approximately the number one would expect to become sensitized in a normal population (Waldorf *et al.*, 1966). Normal responses to DNCB were observed in patients with the sicca syndrome (nineteen of twenty-four; 79·7%) and sero-negative rheumatoid arthritis (eleven of twelve 91·7%). Reduced numbers of patients sensitized to DNCB were found in sero-positive rheumatoid arthritics (ten of seventeen; 58·8%; $P<0·05$) and rheumatoid arthritics with Sjøgren's syndrome (eight of sixteen; 50%; $P<0·005$). The results noted in sero-positive and sero-negative rheumatoid arthritics did not differ significantly from each other, probably because too few patients with sero-negative rheumatoid arthritis have been examined.

TABLE 1. Results of DNCB sensitization

Clinical group	Number	Age (yrs)	No. DNCB positive		
			25 μg	50 μg	100 μg
Controls	24	59·3± 8·1	16 (66·7%)	17 (70·8%)	22 (91·7%)
Sero-positive RA	17	55·3± 9·1	5 (29·4%)	6 (35·3%)	10 (58·8%)
Sero-negative RA	12	46·4±13·2	7 (58·3%)	8 (66·7%)	11 (91·7%)
RA+Sjøgren's syndrome	16	57·1±10·7	3 (18·8%)	5 (31·3%)	8 (50·0%)
Sicca syndrome	24	63·8±10·8	10 (41·7%)	15 (62·5%)	19 (79·2%)

TABLE 2. Results of Mantoux tests

Clinical group	Number	Age (yrs)	Mantoux positive		
			1/10,000	1/1,000	1/100
Controls	26	59·9± 8·5	4 (15·4%)	11 (42·3%)	21 (80·8%)
Sero-positive RA	32	54·8± 8·7	4 (12·5%)	11 (34·4%)	22 (68·8%)
Sero-negative RA	21	51·0±13·7	3 (14·3%)	5 (23·8%)	13 (61·9%)
RA+Sjøgren's syndrome	19	55·7±10·4	1 (5·3%)	2 (10·5%) $P<0·05$	11 (57·9%)
Sicca syndrome	24	63·8±10·8	2 (8·3%)	6 (25%)	11 (45·0%) $P<0·05$

An attempt to correlate DNCB unresponsiveness with clinical and laboratory indices revealed that the sero-positive rheumatoid arthritic patients had a significantly lower serum albumin level ($P<0·01$) and a higher mean articular index ($P<0·025$) than sensitized patients No correlations were seen in the other clinical groups.

Mantoux tests

The mean age and sex distribution of patients Mantoux tested are shown in Table 2. Although all four test groups contained fewer Mantoux positive individuals than the control group at all three dilutions, only the groups of patients with rheumatoid arthritis and Sjøgren's syndrome, when tested with the 1/1,000 dilution ($P<0·05$), and patients with the sicca syndrome, when tested with the 1/100 dilution ($P<0·05$), had significantly reduced Mantoux responses. Patients with sero-positive rheumatoid arthritis who were Mantoux negative had a significantly higher mean erythrocyte sedimentation rate ($P<0·025$) than those who were Mantoux positive, and patients with sero-negative rheumatoid arthritis who were Mantoux positive had a significantly lower mean serum albumin ($P<0·01$) and a significantly higher mean erythrocyte sedimentation rate ($P<0·005$).

No relationship between the responses to DNCB and Mantoux testing were observed in any of the clinical groups (Table 3).

TABLE 3. Comparison of results of DNCB skin sensitization and Mantoux tests

Clinical group	DNCB positive		DNCB negative	
	Mantoux positive	Mantoux negative	Mantoux positive	Mantoux negative
Controls 24	17 (77·3%)	5 (22·7%)	2 (50·0%)	0 (50·0%)
Sicca Syndrome 24	10 (52·6%)	9 (47·4%)	1 (20·0%)	4 (80·0%)
RA+Sjøgren's syndrome 16	6 (75·0%)	2 (25·0%)	5 (62·5%)	3 (37·5%)
Sero-positive RA 17	8 (80·0%)	2 (20·0%)	5 (71·4%)	2 (28·6%)
Sero-negative RA 12	5 (45·4%)	6 (54·5%)	1 (100%)	0 (0%)

Lymphocyte RNA/DNA ratios

The results of the nucleic acid estimations in normal subjects, using the two techniques for obtaining pure lymphocyte suspensions, showed a small but insignificant variation (Fig. 1). However, it was decided to compare the mean lymphocyte RNA/DNA ratio in each group of patients to the values noted in control patients using the same method of purifying suspensions of lymphocytes.

The distribution of RNA/DNA ratios is shown in Fig. 1. The mean ratios for patients with the sicca syndrome (0·330±S.D. 0·098) and sero-negative rheumatoid arthritis (0·293 ±S.D. 0·033) do not differ significantly from those found in normal subjects. Significantly

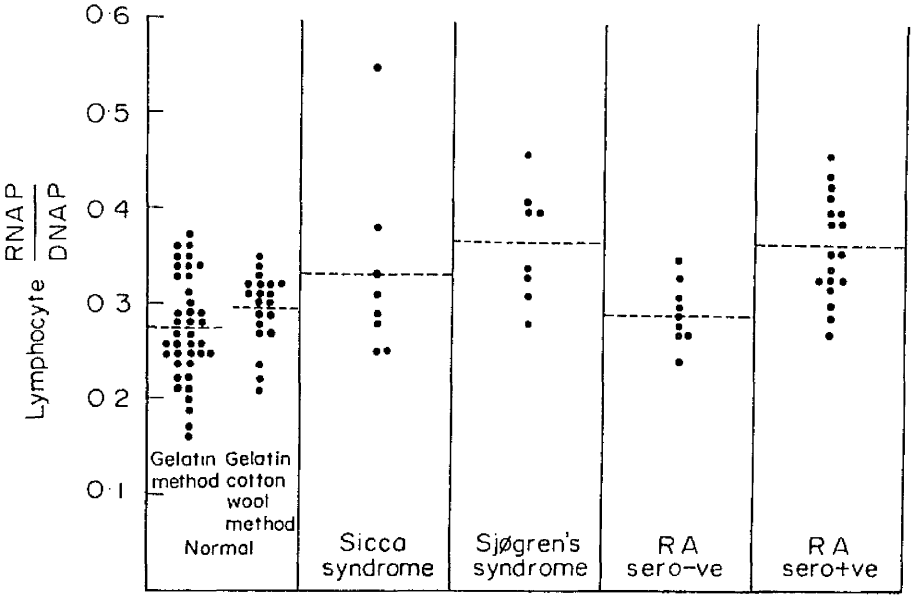


FIG. 1. Distribution of lymphocyte RNA/DNA ratios in the clinical groups studied

raised values were however noted in sero-positive rheumatoid arthritics (0·364±S.D. 0·054; $P<0·001$), and rheumatoid arthritics with Sjøgren's syndrome (0·366±S.D. 0·060; $P<0·001$). The difference between the RNA/DNA ratios in patients with sero-positive rheumatoid arthritis is higher than that found in sero-negative individuals ($P<0·01$).

Relationship between RNA/DNA ratio of peripheral blood lymphocytes and lymphocyte size

A correlation was found ($r = 0·76$) between the mean diameter of lymphocytes in the peripheral blood, and the RNA/DNA ratio of the patient's peripheral blood lymphocytes (Fig. 2). This suggests that patients with sero-positive rheumatoid arthritis, or rheumatoid arthritics with Sjøgren's syndrome, who have elevated lymphocyte RNA/DNA ratios, either have increased numbers of large lymphocytes in their peripheral blood, or a reduction in the number of circulating small lymphocytes.

Response to tetanus immunization

The results of tetanus immunization are shown in Table 4. There is a wide range of values in normal subjects and also in the various disease groups. The anti-toxin responses to secondary immunization were reduced in six cases of rheumatoid arthritis complicated by

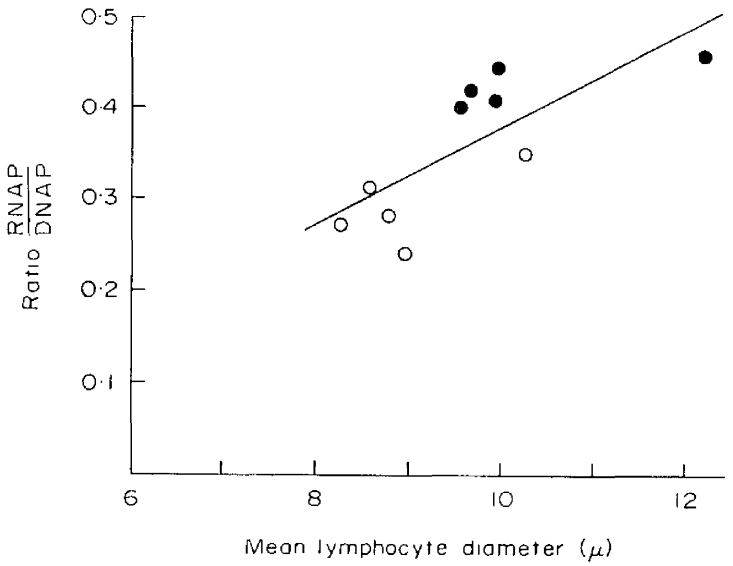


FIG. 2. Relationship between lymphocyte RNA/DNA ratio, cell size and mean lymphocyte diameter. ●, RA; ○, Normal. $P = 0{\cdot}02$, $r = 0{\cdot}76$.

Sjøgren's syndrome, and eight cases of sero-positive rheumatoid arthritis. The lowest secondary response in the controls was between 0·02 units/ml and 0·05 units/ml and responses less than this we have arbitrarily considered subnormal. In those groups with sero-positive rheumatoid arthritis and those with Sjøgren's syndrome associated with rheumatoid arthritis, the number of poor responders was significantly greater than in the control group ($P<0{\cdot}001$ for sero-positive rheumatoid arthritis, $P<0{\cdot}001$ for patients with rheumatoid arthritis associated with Sjøgren's syndrome).

Isohaemagglutinin titres

Table 5 shows the distribution of isohaemagglutinin titres in the clinical groups studied Compared to the control groups, patients with the sicca syndrome ($P<0{\cdot}05$), and sero-positive rheumatoid arthritis ($P<0{\cdot}01$) had significantly reduced levels of anti-A_1. Reduced levels of anti-A_2 were found in patients with Sjøgren's syndrome ($P<0{\cdot}05$) and sero-positive rheumatoid arthritis ($P<0{\cdot}02$). Normal levels of anti-B were found in all clinical groups.

DISCUSSION

In this study we have measured the cellular and humoral immune responses in patients with sicca syndrome, rheumatoid arthritis complicated by Sjøgren's syndrome, and rheumatoid arthritis alone. The delayed hypersensitivity response to the chemical contact sensitizing agent DNCB was found to be normal in patients with the sicca syndrome, and those with sero-negative rheumatoid arthritis. Patients with rheumatoid arthritis complicated by Sjøgren's syndrome, and sero-positive rheumatoid arthritics exhibited impaired responses. Skin sensitization with DNCB has been attempted previously in patients with Sjøgren's syndrome (Leventhal, Waldorf & Talal, 1967) and rheumatoid arthritis (Epstein & Jessar, 1959; Leventhal *et al.*, 1967). Our results are similar to those of Talal's group, but they also

TABLE 4. Secondary response to tetanus toxoid

Clinical group	Tetanus antitoxin titre (units/ml)											
	<0·01	>0·01 <0·02	>0·02 <0·05	>0·05 <0·1	>0·1 <0·2	>0·2 <0·5	>0·5 <1·0	>1·0 <2·0	>2·0 <5·0	> 5·0 <10·0	⩾10·0	Total
Controls	—	—	2	6	8	8	5	4	4	—	—	37
Sicca Syndrome	—	—	1	—	1	4	1	2	2	—	—	11
RA+Sjøgren's	3	3	1	—	—	2	—	—	4	—	—	13
Sero-positive RA	5	3	—	1	1	3	5	1	1	—	1	21
Sero-negative RA	—	—	—	—	—	—	—	2	2	1	2	7

TABLE 5. Isohaemagglutinin titres in patients with Sjøgren's syndrome and rheumatoid arthritis

Isohaem-agglutinin titre (log_2)	Controls			Sicca syndrome			RA+Sjøgren's syndrome			RA sero-positive			RA sero-negative		
	Anti-A_1	Anti-A_2	Anti-B	Anti-A_1	Anti-A_2	Anti-B	Anti-A_1	Anti-A_2	Anti-B	Anti-A_1	Anti-A_2	Anti-B	Anti-A_1	Anti-A_2	Anti-B
0	11	11	10	13	13	3	13	13	8	20	20	4	4	4	1
2	—	—	—	—	2	1	—	1	—	—	2	1	—	—	—
4	—	4	4	1	2	2	1	3	2	2	4	3	—	—	2
8	2	8	9	3	3	11	1	4	8	4	3	14	—	4	2
16	6	10	18	2	7	5	4	12	14	3	8	18	1	2	—
32	10	11	8	7	4	6	13	7	8	8	13	6	4	—	5
64	13	6	4	2	—	4	9	7	5	13	2	4	2	1	2
128	7	3	2	3	—	—	4	1	2	2	—	2	—	1	—
256	6	3	2	—	1	—	2	—	—	—	1	—	1	—	—
512	2	1	—	1	—	—	1	—	1	1	—	1	—	—	—
Mean titre	82·8	46·3	29·1	40·9	16·6	19·6	54·3	21·6	34·2	36·7	18·3	30·8	44·0	21·3	26·0
±S.E.	14·9	11·5	6·8	16·6	8·0	3·5	12·8	3·8	11·0	10·2	5·1	10·0	20·4	10·9	5·8
No. patients		57			32			48			53			12	

observed a markedly diminished ability for patients with the sicca syndrome to become sensitized. This may reflect differences in patient selection and disease severity, as his patients have a relatively high incidence of lymphoid neoplasms (Talal & Bunim, 1964; Talal *et al.*, 1967), and patients with the sicca syndrome having this complication have been shown frequently to have neuropathy, arteritis, lymphadenopathy, splenomegaly and hyperglobulinaemia (Talal *et al.*, 1967), all of which are indications of severe disease. The observation that patients with the sicca syndrome complicated by 'pseudo-lymphoma' usually could not be sensitised to DNCB (Leventhal *et al.*, 1967) supports this suggestion. Epstein & Jessar (1959), when assessing contact-type delayed hypersensitivity in patients with rheumatoid arthritis, used two antigens, DNCB and paranitrosodimethyl aniline (NDMA). Their results demonstrate a significant reduction in the number of rheumatoid arthritics who could be sensitized to NDMA, a weaker sensitizer than DNCB, to which normal response were obtained. The normal responses to DNCB may have been because these authors did not differentiate between sero-positive and sero-negative patients (39% of the patients were sero-negative for rheumatoid factor), and our results show that sero-negative rheumatoid arthritics respond normally to DNCB.

Why patients with sero-positive rheumatoid arthritis and rheumatoid arthritis with Sjøgren's syndrome should not respond to DNCB is difficult to explain. The finding of normal Mantoux responses shows that the memory cells are intact, and able to mount a delayed hypersensitivity reaction in response to antigenic challenge, which suggests that unresponsiveness to DNCB may be due to a defect on the afferent side of the immunological response. This could be due to failure of immunologically competent small lymphocytes to come in contact with the area of skin exposed to DNCB, or alternatively the small lymphocytes having been exposed to DNCB, may fail to proliferate on reaching the local lymph node. Alternatively, unresponsiveness may be due to a defect on the efferent side of the immunological response. Thus, elevation of the RNA/DNA ratios of peripheral blood lymphocytes which has been shown to be due to an increase in the mean lymphocyte diameter, suggests that patients having elevated ratios have either a circulating population of large lymphocytes, perhaps transformed in response to antigenic stimuli, and/or a decrease in the number of circulating small lymphocytes possibly due to sequestration peripherally in the synovium. The finding that the groups of patients responding poorly to DNCB were the same as those with elevated lymphocyte RNA/DNA ratios suggests that the inability to become sensitized to DNCB may be due to such mechanisms. Finally, it is known that macrophages in patients with rhematoid arthritis have diminished phagocytic ability, and are unable to process protein antigens properly. Furthermore, they also respond poorly to chemotactic agents (Holt, 1970). All these factors could possibly account for failure to respond to DNCB, and obviously merit further study.

Diminished secondary responses to tetanus toxoid immunization occurred in patients with sero-positive rheumatoid arthritis and rheumatoid arthritis complicated by Sjøgren's syndrome. The reason for this is not apparent, but it is of interest that Barr *et al.* (1964) noted similar responses in patients with autoimmune thyroiditis. Conceivably patients who have high titres of humoral autoantibody have a diminished capacity to produce other immunoglobulins just as they have diminished albumin synthesis. On the other hand high or normal antitoxin titres to tetanus and diphtheria toxoids have been reported in patients with alcoholic cirrhosis where hypoalbuminaemia and general protein deficiency are evident (Havens, Shaffer & Hopke, 1951; Havens, Myerson & Klatchko, 1957; Cherrick *et al.*,

1959; Buchanan *et al.*, 1962). Moreover, antibody production is known to be normal in states of malnutrition (Gelfand, 1971. Personal communication).

Anti-A_1 isohaemagglutinin titres were reduced in patients with the sicca syndrome and in patients with sero-positive rheumatoid arthritis. Anti-A_2 levels were reduced in patients with the sicca syndrome, rheumatoid arthritis with Sjøgren's syndrome, and sero-positive rheumatoid arthritis. Other workers have noted decreased titres of anti-A and anti-B isohaemagglutinins in rheumatoid arthritic sera (Rawson, Abelson & McCarty, 1961; Rawson & Abelson, 1964; Kornstad, Guldberg & Kornstad, 1970). As isohaemagglutinins are exclusively IgM immunoglobulins (Kunkel, 1960). it is possible that the immune system is exclusively producing IgM rheumatoid factor and the presence of normal isohaemagglutinin titres in sero-negative rheumatoid arthritics is in agreement with this suggestion.

ACKNOWLEDGMENTS

We are grateful to Dr George Wallace (Director of West of Scotland Regional Blood Transfusion Service) for estimating isohaemagglutinin titres, and Dr David Gall (Burroughs Wellcome Ltd.) for estimating tetanus antitoxin titres.

We would like to acknowledge financial assistance of the Arthritis and Rheumatism Council of Great Britain and the University of Glasgow.

K.W. is an MRC Clinical Research Fellow and G.N. is a CIBA Research Fellow.

REFERENCES

ANDERSON, J.R., BUCHANAN, W.W. & GOUDIE, R.B. (1967) *Autoimmunity—Clinical and Experimental.* Charles C. Thomas, Springfield, Illinois.

BARR, M., BUCHANAN, W.W., DONIACH, D. & ROITT, I.M. (1964) Antibody response to tetanus toxoid in patients with autoimmune thyroiditis. *Scot. Med. J.* **9,** 295.

BECK, J.S. (1961) Variations in the morphological patterns of autoimmune nuclear fluorescence. *Lancet,* **i,** 1203.

BLOCH, K.J., BUCHANAN, W.W., WOHL. M.J. & BUNIM, J.J. (1965) Sjøgren's syndrome a clinical, pathological and immunological study of 62 cases. *Medicine* (Baltimore) **187,** 231.

BUCHANAN, W.W., CROOKS, J., KOUTRAS, D.A., MELROSE, A.G. & GOUDIE, R.B. (1962) The association of autoimmune thyroiditis and cirrhosis of the liver. *Scot. Med. J.* **7,** 22.

CHERRICK, G.R., POTHIER, L., DUFOUR, J.J. & SHERLOCK, S. (1959) Immunologic response to tetanus toxoid inoculation in patients with hepatic cirrhosis. *New Engl. J. Med.* **261,** 340.

COULSON, A.S. & CHALMERS, D.G. (1964) Separation of viable lymphocytes from human blood. *Lancet,* **i,** 468.

CREGER, W.P., CHOY, S.H. & RANTZ, L.A. (1951) Experimental determination of the hypersensitive diathesis in man. *J. Immunol.* **66,** 445.

DACIE, J.V. & LEWIS, S.M. (1968) Published J. & A. *Practical Haematology* (4th ed.). Churchill Ltd. (London).

DAVIDSON, J.N. (1957) Some critical comments on the chemical estimation of nucleic acids in tissues. *Exp. Cell. Res.* Supp. **4,** 260.

EPSTEIN, W.L. & JESSAR, R.A. (1959) Contact-type delayed hypersensitivity in patients with rheumatoid arthritis. *Arthr. and Rheum.* **2,** 178.

FLECK, A. & MUNRO, H.N. (1962) The precision of ultraviolet absorption measurements in the Schmidt-Thannhauser procedure for nucleic acid estimation. *Biochem. Biophys, Acta.* **55,** 571.

GLEN, A.C.A. (1967) Measurement of DNA and RNA in peripheral blood lymphocytes. *Clin. Chem.* **13,** 299.

GLENNY, A.T. & STEVENS, M.D. (1938) Laboratory control of tetanus prophylaxis. *J. Roy. Army Med. Cps.* **70,** 308.

GREENWOOD, R. & BARR, M. (1960) Circulating antibody production in rheumatoid arthritis. *Ann. Phys. Med.* **5,** 258.

HAVENS, W.P. JR, MYERSON, R.M. & KLATCHKO, J. (1957) Production of tetanus antitoxin by patients with hepatic cirrhosis. *New Engl. J. Med.* **257,** 637.

HAVENS, W.P. JR, SHAFFER, J.M. & HOPKE, C.J. JR (1951) The production of antibody by patients with chronic hepatic disease. *J. Immunol.* **67,** 347.

HOLT., P.J.L. (1970) Annual General Meeting of the Heberden Society, London, November, 1970.

KORNSTAD, L., GULDBERG, D. & KORNSTAD, A.M.G. (1970) Isohaemagglutinin anti-A and anti-B in rheumatoid arthritis and ankylosing spondylitis. *Ann. Rheum. Dis.* **29,** 421.

KUNKEL, H.G. (1960) Macroglobulins and Antibodies. In: *The Plasma Proteins*, Vol. I. (Ed. by F.W. Putnam) Academic Press (N.Y. and Lond.).

LARSON, D.L. & TOMLINSON, L.J. (1953) Quantitative antibody studies in man III. Antibody response in leukaemia and other malignant lymphomata. *J. Clin. Invest.* **32,** 317.

LEA, A.J. (1964) An association between the rheumatic diseases and the reticulosis. *Ann. Rheum. Dis.* **3,** 480.

LEVENTHAL, B.G., WALDORF, D.S. & TALAL, N. (1967) Lymphocyte function in Sjøgren's syndrome. *J. clin. Invest.* **46,** 1338.

MACSWEEN, R.N.M., ANDERSON, J.R. & MILNE, J.A. (1967) Histological appearances of the thymus in systemic lupus erythematosus and rheumatoid arthritis. *J. Path. Bact.* **93,** 611.

MEISELAS, L.E., ZINGALE, S.B., LEE, S.L., RICHMAN, S. & SIEGEL, M. (1961) Antibody production in the rheumatic diseases. The effect of Brucella antigen. *J. clin. Invest.* **40,** 1872.

RAWSON, A.J. & ABELSON, N.M. (1964) An isohaemagglutinin deficiency in the relatives of rheumatoid patients. *Arthritis Rheum.* **7,** 391.

RAWSON, A. J., ABELSON, N.M. & MCCARTY, D.J. (1961) The isohaemagglutinin partition in rheumatoid arthritis. *Arthritis Rheum.* **5,** 463.

RITCHIE, D.M., BOYLE, J.A., MCINNES, J.M., JASANI, M.K., DALAKOS, T.G., GRIEVESON, P. & BUCHANAN, W.W. (1968) Clinical studies with an articular index for the assessment of joint tenderness in patients with rheumatoid arthritis. *Quart. J. Med.* **37,** 393.

ROBERTSON, M.D.J., HART, F.D., WHITE, W.F., NUKI, G. & BOARDMAN, P.L. (1968) Rheumatoid lymphadenopathy. *Ann. Rheum. Dis.* **27,** 253.

ROPES, M.W., BENNETT, G.A., COBB, S., JACOX, R. & JESSAR, R.A. (1958) 1958 Revision of diagnostic criteria for rheumatoid arthritis. *Bull. Rheum. Dis.* **9,** 175.

SHEARN, M.A., EPSTEIN, W.V. & ENGLEMAN, E.P. (1963) Antibody response to Brucella antigen in patients with rheumatoid arthritis. *Proc. Soc. exp. Biol. (N.Y.)* **113,** 1001.

STEINBROKER, O., TRAEGER, C.H. & BATTERMAN, R.C. (1949) Therapeutic criteria and rheumatoid arthritis. *J. Amer. med. Ass.* **140,** 659.

TALAL, N. & BUNIM, J.J. (1964) The development of maligant lymphoma in the course of Sjøgren's syndrome. *Amer. J. Med.* **36,** 529.

TALAL, N., SOKOLOFF, L. & BARTH, W.F. (1967) Extra salivary lymphoid abnormalities in Sjøgren's syndrome (Reticulum cell sarcoma, 'pseudolymphoma', macroglobulineamia). *Amer. J. Med.* **43,** 50.

VAUGHAN, J.H. & BUTLER, V.P. (1962) Current status of the rheumatoid factor. *Ann. Intern. Med.* **56,** 1.

WALDORF, D.S., SHEAGREN, J.N., TRAUTMAN, J.R. & BLOCK, J.B. (1966) Impaired delayed hypersensitivity in patients with lepromatous leprosy. *Lancet* **ii,** 773.

WHALEY, K. & BUCHANAN, W.W. (1969) Clinical aspects of autoimmunity. *Triangle*, **9,** 61.

WHALEY, K., CHISHOLM, D.M., GOUDIE, R.B., DOWNIE, W.W., DICK, W.C., BOYLE, J.A. & WILLIAMSON, J. (1969) Salivary duct autoantibody in Sjøgren's syndrome: correlation with focal sialadenitis in the labial mucosa. *Clin. exp. Immunol.* **4,** 273.

WILLIAMSON, J., CANT, S., MASON, D.K., GREIG, W.R. & BOYLE, J.A. (1967) Sjøgren's syndrome in thyroid disease. *Brit. Ophthal.* **51,** 721.

ZIFF, M., BROWN, P., LOSPALLUTO, J., BADEN, J. & MCEWAN, C. (1956) Agglutination and inhibition by serum globulin in the sensitised sheep cell agglutination reaction in rheumatoid arthritis. *Amer. J. Med.* **20,** 500.

THE MEASUREMENT OF DNA AND RNA IN HUMAN LYMPHOCYTES IN CONDITIONS OF ALTERED IMMUNOLOGICAL ACTIVITY

Alastair C.A. Glen

VOLUME II

CONTENTS OF VOLUME 2

SECTION 4

The lymphocyte and renal transplantation

4.1 Introductory review

The immune basis of allograft rejection has been established beyond question and an extensive literature on this topic has accumulated (Brent and Medawar, 1967; Elves, 1966). Not unexpectedly in the light of present understanding of immune reactions, antibody as well as cell bound mechanisms have been considered responsible for the rejection process. An early observation by Gorer (1937), the appearance of circulating iso-agglutinins following tumour transplantation, stimulated the search for anti-graft antibodies and claims of the importance of humoral mechanisms in renal transplantation have been made (Stetson, 1963; Altman, 1963; Milgrom, Litvac, Kano and Witebsky, 1966). On the other hand the active role of the lymphocyte in the processes of rejection has been firmly established since transplantation immunity can be transferred by lymph node cells (Mitchison, 1954), spleen cells (Billingham, Brent and Medawar, 1954), and peripheral blood leucocytes (Billingham, Silvers and Wilson, 1962).

The apparently conflicting claims of the importance of humoral mechanisms and cell bound immunity in rejection phenomena are less at variance when it is appreciated that rejection of a graft may take more than one form. An understanding of the pathology of graft rejection has evolved and although based on experimental work in rats (Strober and Gowans, 1965) and on studies of canine renal

allografts (Porter, Joseph, Rendall, Stolinski, Hoehn and Calne, 1964, and others), reviewed by Porter (1967), the process is probably as follows.

Within a few hours of the host blood circulation being established in the graft, host lymphocytes are exposed to graft antigens either by traversing the graft itself or in the local lymph nodes. In either case these lymphocytes reach the local lymph node or the spleen and give rise to a dividing line of cells, the immunoblasts. Over the succeeding few days the progeny of the immunoblasts are released via efferent lymphatics and the blood stream to reach the graft. There, these so called immunocytes interact probably with the endothelium of peritubular capillaries and gradual invasion of renal parenchyma with progressive tissue damage ensues. This is the pathology of acute renal allograft rejection occurring in the absence of immunosuppressive drugs, within two weeks of the time of transplant. It is principally a cell mediated process. A proportion of rejected kidney grafts show only a minor cellular infiltrate. In these cases vascular damage is more conspicuous and there may be fibrinoid necrosis of arteriolar walls with platelet thrombi in glomerular capillaries. Here the presence of immunoglobulins, IgG and IgM as well as components of complement in the lesions, make it likely that antigen-antibody complexing is important for this type of rejection and may well initiate the formation of platelet thrombi (Mowbray, 1966). Although of necessity this description has

derived from animal experiments, there is evidence that renal allografts in man are rejected by the same mechanisms (Starzl, 1964; Porter, 1967). The use of immunosuppressive drugs is considered to modify principally the time scale of these events although Mowbray (1970) considers that in patients on adequate doses of steroids and azathioprine, humoral mechanisms are more important than cell mediated effects in human renal allografts.

The nature of the mononuclear cells which invade the graft in rejection is of special interest. Porter (1967) has classified the cells and recognises three types. Firstly, large lymphoid cells with cytoplasm packed with RNA in the form of ribosome clusters, the larger of these cells are the immunoblasts, the smaller the immunocytes; secondly, macrophages characterised by evidence of pinocytosis, the presence of lysosomes and small vesicles; and lastly, plasma cells. Early in the course of graft rejection immunocytes and immunoblasts predominate while later a greater proportion of macrophages and plasma cells are found. Hall (1967) in a comprehensive series of experiments on sheep with skin allografts demonstrated that immunocytes were produced in the lymph node draining the site of the graft and appeared in efferent lymph. Since these cells drain via major lymphatic trunks and appear ultimately in the blood they may be detectable by chemical measurements on isolated blood mononuclear cells.

Recently a report has appeared of morphological abnormalities

in the blood lymphocytes in man in response to renal allograft (Parker and Mowbray, 1971). There are also reports of functional abnormalities of blood. Inhibition of leucocyte migration under standard conditions has been shown to be a measure of cell mediated hypersensitivity (Soborg and Bendixen, 1967) and this method has revealed the presence of strong sensitization to kidney and histocompatibility antigens in renal allograft rejection (Smith, Eddleston, Dominguez, Evans, Bewick and Williams, 1969). The phytohaemagglutinin response of the blood lymphocytes in patients with a renal allograft on immunosuppressive therapy is impaired and there may be some lessening of this impairment prior to an episode of kidney rejection (Tennenbaum, St. Pierre and Cerilli, 1968). Another author however has reported no change in the phytohaemagglutinin response prior to rejection (Joseph, 1966), but it appears that spontaneous transformation of lymphocytes in culture is increased when the cells are obtained from a patient prior to a rejection episode (Tennenbaum, St. Pierre and Cerilli, 1968). The changes in spontaneous transformation are supported by the observations of an increase in the rate of RNA synthesis (Parker, Ellis, Cameron, Ogg and Mowbray, 1970) and of DNA synthesis (Hersh, Butler, Rossen and Morgan, 1970) in peripheral blood lymphocytes at times of rejection. Spontaneous transformation is believed to indicate a sensitization of the lymphocytes to antigen or antigens and the impaired phytohaemagglutinin response may have

resulted from the effect of immunosuppression. This last suggestion is uncertain since in the published work the impaired response is not usually present early in the life of the graft at a time when the immunosuppresive drugs are used in greatest dose.

On the assumption that a renal allograft has been achieved with an adequate, but less than perfect tissue match, one of the main problems in the management of the recipient is the balancing of suppression of graft rejection processes with the danger of impairment of the patient's response to infection. This problem is magnified when rejection of the graft is suspected and immunosuppressive therapy has to be increased. Berenbaum (1967), in a review of the reports of the Kidney Transplant Registry, points out that difficulties associated with drug toxicity and infection account for about one third of graft failures and are also factors in most instances of death in the recipient. Immunosuppression is usually monitored by maintaining the white cell count within the 5,000 to 10,000 cells per mm^3 range. This method is at best empirical and there is a great need for a method which will allow better control of immunosuppression and give an early indication of impending graft rejection.

Both azathioprine and prednisone, the established immunosuppressive drugs used in renal transplantation in man, are inhibitors of nucleic acid synthesis in lymphocytes (Berenbaum, 1967; Tormey, Fudenberg and Kamin, 1967), and measurement of lymphocyte DNA and RNA content is therefore of potential value in

monitoring the effect of these drugs. In this section a series of these measurements on the blood lymphocytes from nineteen patients with a renal allograft is presented with the double objective of observing the effect of immunosuppression on the chemical analyses and of testing the hypothesis that changes occur in the blood lymphocytes in rejection in advance of changes in renal function, the most reliable sign of impending rejection (Chisholm, Papadimitriou, Kulatilake and Shackman, 1969).

Thoracic duct drainage prior to renal transplantation has had a limited trial as an adjunct to immunosuppressive therapy (Murray, Wilson, Tilney, Merril, et al., 1968 ; Sarles, Remmers, Fish, et al., 1970). In a separate study, the opportunity was taken to monitor the chemistry of the lymphocytes obtained from the thoracic duct in man as immunosuppressive drugs, azathioprine and prednisone were commenced. The serial measurements obtained should be helpful in the interpretation of results of the blood lymphocyte analyses in patients on this regime. The technique of thoracic duct fistula (Tilney and Murray, 1968) with continuous drainage of the lymph and return of the cell free lymph to the patient intravenously, provides massive numbers of lymphocytes of the order of ten thousand million per day, free from contamination with other leucocytes. It was possible therefore to study the chemistry of human thoracic duct lymphocytes without the constraint of limited material.

Considerable controversy existed at the time of the study over

the nature of the RNA species present in lymphocytes especially in the early stages of the response of the cell to phytohaemagglutinin. Although the bulk of RNA in lymphocytes is ribosomal RNA, two groups of workers (Cooper and Rubin, 1965; Kay, 1966) considered that the RNA rapidly synthesised in response to phytohaemagglutinin was not principally ribosomal or ribosomal precursor RNA. There is now much more information about the process of biosynthesis of RNA and the molecular species involved, a topic which has been reviewed by Burdon (1971). RNA destined to be ribosomal RNA is transcribed in the nucleolus from DNA as a high molecular weight material recognised experimentally by its sedimentation during high speed centrifugation as 45s RNA. This material is processed before it appears in the cytoplasm where it is found as two distinct molecular species, the 18s and 28s components of ribosomal RNA. Although it is now recognised that the lymphocytes, even when stimulated by phytohaemagglutinin, conform to the usual mammalian cell process for RNA maturation (Monjardino and MacGillivray, 1968; Cooper and Kay, 1969) the earlier work of Cooper and Rubin (1965) was the stimulus to the investigation to be described.

Cooper and Rubin (1966) described a heterogeneous RNA, extractable from lymphocytes after short term, i.e. less than three hours, exposure to phytohaemagglutinin and recognisable as non-ribosomal RNA by pulse labeling and chase of the label with actinomycin D. The nature of such RNA was then unknown but its

presence in cells stimulated by phytohaemagglutinin suggested that the rapidly labelled RNA might be a messenger RNA. Their work was restricted by limited material and non-lymphocyte RNA was required as a carrier in their RNA extraction procedures. The experiments described in this thesis provide an answer to the question of the nature of lymphocyte RNA in the resting and stimulated state.

4.2 Material and Methods

Clinical material.

Serial measurements of blood lymphocytes in patients with a renal allograft.

This is a study of nineteen patients, recipients of twenty renal allografts, all but one carried out in the three years period up to the end of 1970. There were four live donors and sixteen cadaver donor kidneys in the series; the recipients were nine males and nine females, one male receiving a second graft. The mean age of the patients was thirty-one years with a range of from seventeen years to forty-six years. In addition, some results from a patient studied after this period were included since they illustrated a type of rejection noted in the main study, but less well documented. This patient was a female aged eighteen years

who received a live donor renal allograft. Details of all these patients including an assessment of the tissue match according to the criteria of Botha (1969) are included in Appendix 10.

Immunosuppression was maintained with azathioprine given as an initial dose of 3 mg per kg and then regulated according to the white cell count, and prednisone 200 mg per day, started at the time of the transplants and reduced daily in steps of 10 mg to a maintenance dose of from 10 mg to 30 mg per day. Definite clinical rejection episodes were diagnosed in the event of an increased serum creatinine and a decreased creatinine clearance present for twenty-four to forty-eight hours. Other factors such as decreased urine volume and renal tenderness were inconsistent findings. Such episodes were treated by an intravenous infusion of 1 g prednisolone given over a two hour period and repeated on the following day if necessary. Two patients received x-irradiation (150 rads) to the area of the allograft during rejection crises.

Samples of venous blood were taken from the patients, always between 8.30 a.m. and 10.30 a.m., for the lymphocyte analyses as well as the general monitoring of the patient by the usual series of tests. In the initial stage of the project, blood sampling for lymphocyte analyses was infrequent and for this reason and because of early graft failure in some cases, less than five blood samples were obtained during the life of each of four grafts. In the remaining fourteen allografts 289 lymphocyte analyses were undertaken during 141 graft months, i.e. each patient had

on average an analysis every two weeks. In practice, the blood sampling was more frequent early in the life of the graft when the immunosuppressive therapy was being adjusted and it became apparent that sampling twice per week would be advantageous.

Studies on thoracic duct lymphocytes; serial measurements of the lymphocyte RNAP/DNAP ratio and analysis of the nature of lymphocyte RNA.

The four subjects in this study were patients maintained by intermittent haemodialysis prior to receiving allografts in the transplantation unit of the Peter Bent Brigham Hospital. The regimen before renal transplantation was not the conventional one and suppression of the immune response was carried out as follows: thoracic duct lymphocytes were drained continuously from a fistula beginning five days (in one patient nine days) before transplantation; azathioprine (2.5 mg daily per kilogram of body weight) was given starting forty-eight hours before operation, and prednisone (1.5 mg daily per kilogram of body weight) twenty-four hours before kidney transplantation. These drugs were continued for the lifetime of the allograft, dosages being adjusted as dictated by the patient's clinical state. The lymph was allowed to flow freely and was collected in sterile citrate-phosphate-dextrose bags (Fenwal Laboratories); the cells were separated from the lymph by

centrifugation at 150 gav. The cells were retained and the supernatant lymph fluid returned to the patient intravenously.

Methods.

Serial analyses of blood lymphocyte DNAP, RNAP and RNAP/DNAP ratio were carried out by the gelatin and cotton wool cell separation technique (Section 2.3) followed by the standard chemical technique (Section 2.8). The cytological methods used were those described in Section 2.5.

In the serial measurements of the DNA and RNA content of thoracic duct lymphocytes a much simplified cell separation procedure was adopted which produced excellent lymphocyte preparations. Prior to estimation of the nucleic acid content of these lymphocytes, they were freed of red cells by osmotic shock, fifteen seconds in ice cold distilled water, and analysed either freshly harvested or after short term storage at -40C (Section 2.15).

Tissue typing.

The tissue typing procedures were carried out in the Glasgow Royal Infirmary following the microdrop technique of Terasaki and McLelland (1964) and assaying for leucocyte antigens. The results

are listed according to the tissue match using the classification of Botha (1969).

Short term lymphocyte cultures.

Thoracic duct lymphocytes were obtained from anephric patients just prior to renal transplantation whose blood urea nitrogen levels were kept low by frequent haemodialysis. The lymph contained about 98% small lymphocytes with variable contamination with erythrocytes. Lymph, collected in plastic citrate-phosphate-dextrose bags, was centrifuged at 4C at 600 gav for ten minutes and the cell button washed twice with Medium 199 (Grand Island Biological Co.). Lymphocyte cultures were set up in Medium 199 with 3% autologous serum at a concentration of 20×10^6 lymphocytes per ml. PHA-P (Difco), diluted according to specifications of the manufacturer, was used at a final concentration of 0.3 ml per 100 ml of suspension and the same volume of sterile saline was used in control tubes. Uridine H^3 was added to the cultures as will be detailed.

Isolation of nuclei.

Lymphocyte nuclei were isolated by a method similar to that of Pogo, Allfrey and Mirsky (1966). All procedures following

harvesting of cells were carried out at 0C to 4C unless stated otherwise. After the cell harvesting centrifugation 0.01M citric acid (29 ml per tube) was added to the cell button and the resulting suspension agitated using a snugly fitting teflon pestle. Suspensions were then centrifuged at 5000 r.p.m. for five minutes in a Sorval RC2B centrifuge (3000 gav) and the citric acid treatment repeated. After a final wash with 10 ml of 0.34 M sucrose, a drop of the suspension was removed and stained with azure C to monitor the presence of clean lymphocyte nuclei. The nuclei were then collected by another centrifugation (3000 gav) and RNA extracted from them.

RNA extraction, fractionation and counting.

Lymphocyte nuclei or whole lymphocytes were homogenised with a teflon pestle in 10 ml to 12 ml of 0.3% sodium dodecyl sulfate containing 0.14 M sodium chloride and 0.05 M sodium acetate (pH 5.1). After a few strokes, an equal volume of 90% phenol containing 0.1% hydroxyquinoline was added and the mixture homogenised further. The suspension was immediately shaken at 65C for ten minutes, shaken at room temperature for fifteen minutes, and centrifuged (17,000 gav) for fifteen minutes at 4C. The clear aqueous layer was then removed and the RNA precipitated with 2.5 volumes of ethanol containing 2% potassium acetate. RNA was reprecipitated once with ethanol. An aliquot of an

aqueous solution of the RNA containing 100 µg RNA was layered on a 5 ml 10% to 40% linear sucrose density gradient, and centrifuged for fifteen to sixteen hours at 24,000 rev/min in the SW 50 head of the Beckman L2 ultra-centrifuge. Gradients were passed through the cell of a Gilford model 2000 recording spectrophotometer and collected in fractions of eight drops each. Each preparation produced about sixteen fractions. After addition of one drop of 5M perchloric acid to each vial, the fractions were hydrolysed at 80C for fifteen minutes, cooled, and scintillation solution (10 ml) added to each vial. The scintillation fluid was of the following composition: Xylene 500 ml, Dioxane 1500 ml, Ethylene glycol monomethyl ether 1500 ml, PPO 35 g, POPOP 1.75 g, Napthalene 280 g. Uridine H^3, generally labelled, was the isotope used in the experiments and was obtained from New England Nuclear Corporation at a specific activity of 5C/mM. Radioactivity was determined in a Beckman CPM 100 liquid scintillation system. Quench curves were prepared for the counting conditions used and a counting efficiency of between 14 and 18 per cent established throughout.

RESULTS

4.3 Blood lymphocyte DNA content in patients with a renal allograft

The mean lymphocyte DNA content for all the observations in the seventeen patients studied was 0.824 µg DNA phosphorus per 10^6 cells with a standard deviation of 0.165. This was slightly higher than the mean DNA content of the lymphocytes found in normal untreated individuals, mean value 0.732 µg DNA phosphorus per 10^6 cells, and this difference was significant by t-test at the five per cent level. There was a significant difference in variance for this comparison ($F = 2.10$, $p = < 0.05$) and the t-test result was therefore confirmed by Welsh's test for 'd' which upheld the result ($n_1 = 20$, $n_2 = 299$, $d = 2.11, 1.0$, $p = < 0.05$).

When the DNA values were examined after allocation of the patients to four clinical groups, as will be defined later, the results were as follows: no infection, no rejection, 0.824; unequivocal infection, 0.836; unequivocal rejection, 0.805; excessive immunosuppression, 0.856 µg DNA phosphorus per 10^6 cells. There were no significant differences between the groups and it is therefore valid to regard any changes in the lymphocyte RNAP/DNAP ratio between groups as evidence of alteration in lymphocyte RNA content.

4.4 The blood lymphocyte RNAP/DNAP ratio in patients with a renal allograft in relation to the blood lymphocyte count

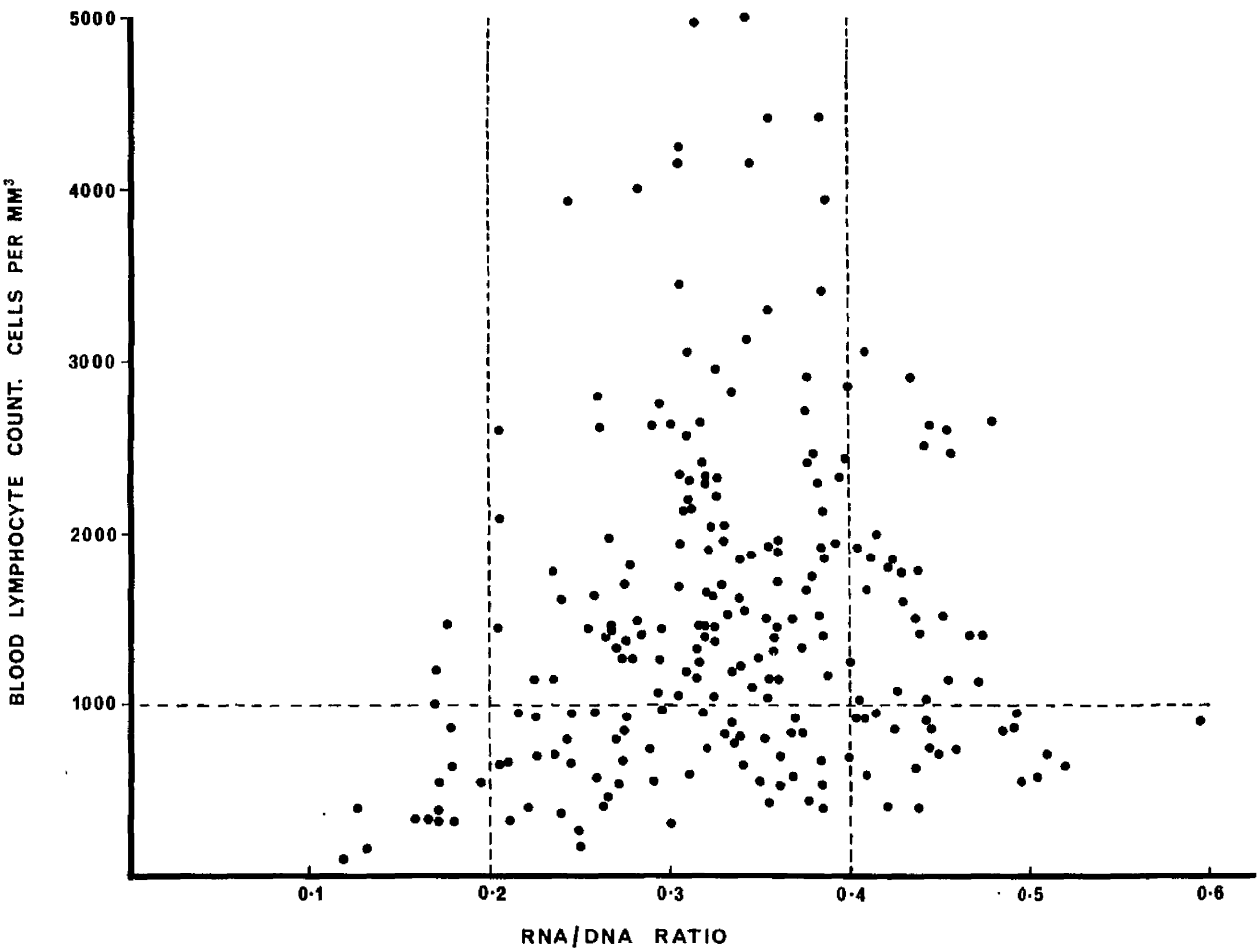
The results of two hundred and ninety-nine analyses of both the blood lymphocyte RNAP/DNAP ratio and the blood lymphocyte count are shown in Figure 13.

In normal individuals not receiving immunosuppressive therapy, the accepted values for the normal blood lymphocyte count is from 1500 to 3500 cells per mm^3. Figure 13 shows eighty-four samples with a lymphopenia of 1000 cells per mm^3 or less. Ten samples showed a lymphocytosis with a lymphocyte count of 3500 cells per mm^3 or greater.

It can be seen from Figure 13 that the blood lymphocytes from patients with a renal allograft and receiving immunosuppressive treatment can have RNAP/DNAP ratio results outwith the range found in normal individuals of 0.20 to 0.40. Strictly, the two standard deviation normal range is 0.22 to 0.37 (Section 3.4). The range 0.20 to 0.40 gives convenient values close to the three standard deviation limits of normal. On fifteen occasions the lymphocyte RNAP/DNAP ratio was low and on fifty-four occasions it was elevated when compared to the lymphocytes of normal untreated individuals. Examination of Figure 13 would suggest that a low lymphocyte RNAP/DNAP ratio is associated with lymphopenia while a high lymphocyte RNAP/DNAP ratio may be associated with a normal or low lymphocyte count.

Figure 13

Blood lymphocyte RNAP/DNAP ratio and lymphocyte count in patients with a renal transplant, all results.



These results were obtained by serial analyses of the blood lymphocytes from nineteen patients with a renal allograft studied over a period of three years. The broken lines indicate limits set for the recognition of lymphopenia (less than 1,000 lymphocytes per mm^3) and an abnormal lymphocyte RNAP/DNAP ratio (outwith the range 0.20 to 0.40).

4.5 The relationship between the results of the analyses of blood lymphocyte count and RNAP/DNAP ratio and the clinical state in patients with a renal allograft

In order to arrive at a range of values for the lymphocyte analyses in immunosuppressed patients with good graft function and to show possible differences in the lymphocyte response to infection, rejection or excessive immunosuppression, four clinical states were defined.

(A) No infection or rejection.

To qualify for this group, patients had to be free of clinical or laboratory evidence of generalised or local infection or graft rejection at the time of or within ten days of analysis. In addition, the creatinine clearance had to be at least 50 ml/min. If the patient had more than one rejection episode in the past they were not included in this group.

(B) Unequivocal infection.

To be included in this group the patient had to exhibit clinical and laboratory evidence of viral, fungal or bacterial infection. The possibility of the presence of rejection excluded patients from this group.

(C) Unequivocal rejection.

Inclusion in the group required that clinical suspicion of graft rejection, acute or chronic, should be confirmed by a substantial elevation of the plasma creatinine maintained for twenty-four hours. A substantial rise of plasma creatinine was defined as an increase of 0.5 mg per 100 ml when the plasma creatinine was under 2.0 mg per 100 ml, and a rise of 1.0 mg when the initial plasma creatinine was greater than 2 mg per 100 ml. The presence of infection excluded patients' results from this group.

(D) Excessive immunosuppression.

Patients were included in this group if they had had a blood leucocyte count of less than 4000 cells per mm^3, and no evidence of rejection or significant infection. A leucocyte count of less than 4000 cells per mm^3 is recognised as an early sign of excessive immunosuppressive therapy, especially of azathioprine.

The blood lymphocyte counts and the lymphocyte RNAP/DNAP ratios for these groups are summarised in Table 22. Thirty-seven results from six patients comprised Group A. Nineteen results from seven patients comprised Group B. Seventeen results from ten patients comprised Group C and nine results from three patients

Table 22

The results of blood lymphocyte counts and the lymphocyte RNAP/DNAP ratio in four categories; A) no infection or rejection, B) unequivocal infection, C) unequivocal rejection, D) excessive immunosuppression, all in patients with a renal allograft.

Category	Number	Blood lymphocyte count cells per mm^3		Lymphocyte RNAP/DNAP ratio	
A No infection, no rejection	37	Mean SD	2224 1056	Mean SD	0.300 0.047
B Unequivocal infection	19	Mean SD	1792 1028	Mean SD	0.354 0.066
C Unequivocal rejection	17	Mean SD	888 536	Mean SD	0.422 0.063
D Excessive immunosuppression	9	Mean SD	632 403	Mean SD	0.253 0.071

The categories A, B, C and D are defined in Section 4.5 of the text.

Table 22 (cont'd)

Analysis by F-test

Comparison	Degrees of freedom	F	Probability
Blood lymphocyte count			
A v B	36, 18	1.05	N.S.
A v C	36, 16	3.88	< 0.001
A v D	36, 8	6.86	< 0.001
B v C	18, 16	3.68	< 0.01
Lymphocyte RNAP/DNAP			
A v B	36, 18	1.97	N.S.
A v C	36, 16	1.80	N.S.
A v D	36, 8	2.29	N.S.
B v C	18, 16	1.09	N.S.

A = No infection, no rejection

B = Unequivocal infection

C = Unequivocal rejection

D = Excessive immunosuppression

Table 22A

Analysis of the mean values of the results from Table 22.

Where there is significant difference in variance the d-test has been used in place of the t-test.

Comparison	Degrees of freedom	t	d	Probability
Blood lymphocyte count				
A v B	54	1.13	-	<0.30 >0.20
A v C	36, 16	3.76	6.15,0.6	*<0.01
A v D	36, 8	3.80	6.16,0.6	*<0.01
B v C	18, 16	3.52	3.36,0.6	*<0.01
Lymphocyte RNAP/DNAP				
A v B	54	3.54	-	*<0.001
A v C	52	8.15	-	*<0.001
A v D	44	2.42	-	*<0.02 >0.01
B v C	34	3.23	-	*<0.01 >0.001

*Differences are significant.

Probability values are calculated from the statistic d where differences in variance have required this to be used.

A = No infection, no rejection

B = Unequivocal infection

C = Unequivocal rejection

D = Excessive immunosuppression

were included in Group D.

Results from patients with neither rejection nor infection are taken as the normal group in this study of renal allografts. The results are very similar to published values for the blood lymphocyte count and the observed lymphocyte RNAP/DNAP ratio in normal untreated individuals, although a tendency to slight lymphopenia is noted in patients with treated allografts. Serial analyses from individual patients show a striking stability of the RNAP/DNAP ratio in patients included in this group. This is illustrated by the results obtained in patient BL displayed in Figure 15.

Blood lymphocyte counts in the four clinical states.

A useful intercomparison may be made between the four clinical states defined above, applying Student's t-test to the mean value for each group (Table 22). In the presence of infection the mean blood lymphocyte count is lower than when there is no infection, but this has not been shown to have statistical significance. When unequivocal rejection is present there is a statistically significant lymphopenia ($p = < 0.001$). Not unexpectedly since leucopenia defines the group, lymphopenia was present in the Group D patients (excessive immunosuppression).

Blood lymphocyte RNAP/DNAP ratio in the four clinical groups.

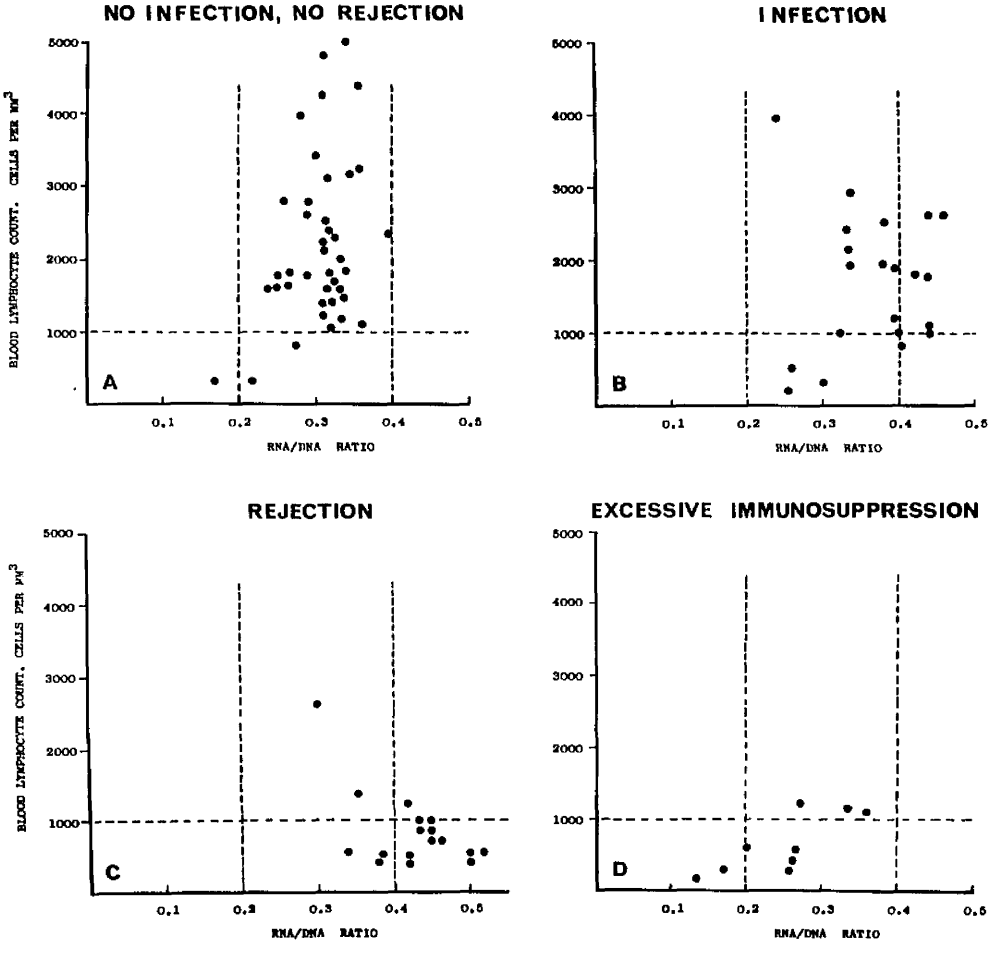
The lymphocyte RNAP/DNAP ratio results for the patients are listed in Table 22. Group B (infection group) and Group C (rejection group) each showed a significantly greater mean value for the lymphocyte RNAP/DNAP ratio than Group A (no infection or rejection), $p = \leq 0.001$ in each case. Although the degree of elevation of the mean lymphocyte RNAP/DNAP ratio was significantly greater in the presence of rejection than in the infection group ($p = \leq 0.01$), there is considerable overlap of observed values for the ratio in these two groups. The findings of a reduction in the lymphocyte RNAP/DNAP ratio in the presence of over-immuno-suppression (Group D) is of relevance later in differentiation of the causes of lymphopenia.

When the value of the lymphocyte RNAP/DNAP ratio and the blood lymphocyte count are considered together in the same four clinical groups a clearer pattern emerges as may be seen from examination of Figure 14. This shows the individual values for the relationship between the blood lymphocyte count and the lymphocyte RNAP/DNAP ratio separately plotted for each of the four groups. These results are now summarised.

When neither infection or rejection are present (Group A) the blood lymphocyte count is variable, only occasionally low. The lymphocyte RNAP/DNAP ratio is not elevated above 0.40 in these patients, rather it may be occasionally low and then usually in

Figure 14

Blood lymphocyte RNAP/DNAP ratio and lymphocyte count in patients with a renal allograft grouped according to clinical state.



The results plotted are from individuals allotted to the categories A - no infection, no rejection; B - unequivocal infection; C - unequivocal rejection; D - excessive immunosuppression. These categories are defined in Section 4.5.

association with a low blood lymphocyte count. When infection is present (Group B) a low blood lymphocyte count tends to be associated with a low lymphocyte RNAP/DNAP ratio and a high lymphocyte count with a normal or high lymphocyte RNAP/DNAP ratio. In contrast with this are the results from patients with unequivocal rejection (Group C). Here the usual values are a low blood lymphocyte count, less than 1000 per mm^3, and an elevated lymphocyte RNAP/DNAP ratio usually greater than 0.40. In the presence of excessive immunosuppression as evidenced by a general leucopenia (Group D), it appears that a very low lymphocyte RNAP/DNAP ratio may be present when the blood lymphocyte count is also very low.

4.6 The value of the lymphocyte analyses in the diagnosis of rejection

In the previous section it was shown that a low blood lymphocyte count and an elevated lymphocyte RNAP/DNAP ratio are common features of rejection. If the changes in the blood lymphocytes found in unequivocal rejection are to have diagnostic value, the incidence of misleading results must be shown to be low. Possible false negative results are considered first. There were two normal results from the same patient for both the blood lymphocyte count and the lymphocyte RNAP/DNAP ratio in the seventeen

analyses during unequivocal rejection (Figure 14). During a further three rejection episodes each involving different patients there was a lymphopenia but with a lymphocyte RNA/DNA ratio within the upper normal limit of 0.40. These last results were however one, three and fourteen days after treatment of rejection with 1 g of prednisolone, so there remained only two results with a lymphocyte count of more than 1000 per mm^3 and a lymphocyte RNAP/DNAP ratio of less than 0.40 during untreated unequivocal rejection out of a total of fourteen. Thus, a low incidence, i.e. 14 per cent of false negative results in untreated unequivocal rejection has been found in the three year study period. Special features of the rejection in the patient who had these normal results will be discussed later together with results of an additional patient, obtained after the three year study was complete but included since this patient also showed normal lymphocyte chemistry at a time of unequivocal rejection.

Table 23 details all the instances when results were obtained for the lymphocyte analyses which suggested rejection. Over the period of study, such results, i.e. those which showed a low blood lymphocyte count less than 1000 cells per mm^3 and a lymphocyte RNAP/DNAP ratio of more than 0.40, were found during thirteen distinct periods in ten patients. Nine of these patients did have a clinical rejection episode. Of the individual nineteen abnormal results, rejection followed in fourteen with an average interval of twenty-three days. The interval between finding the abnormal

Table 23

The relation in time of results for the lymphocyte analyses which suggested rejection, i.e. a blood lymphocyte count of 1000 cells per mm^3 or less and a RNAP/DNAP ratio greater than 0.40, and previous or subsequent rejection in patients with a renal allograft.

Patient	Days since transplant	Blood lymphocyte cells per mm^3	RNAP/DNAP	Days since previous rejection	Days to next rejection
AP	7	560	0.50	1	1
AP	(20	410	0.44	12	0
AP	(23	864	0.43	15	0
HB	(21	900	0.60	8	7
HB	(30	570	0.42	2	0
CG	27	950	0.42	12	4
CM	(68	860	0.43	64	44
CM	(96	1000	0.43	92	16
CM	170	700	0.45	68	28
CM	240	980	0.50	41	0
JM	426	940	0.41	0	26
MS	125	660	0.44	12	29
RM	188	658	0.52	184	1
JM	70	860	0.42	0	0
JO	243	900	0.44	0	3
MC	(96	860	0.49	67	129
MC	(195	864	0.45	166	30
MC	(218	708	0.51	189	7
MC	(225	714	0.46	196	1

Results bracketed together are regarded as belonging to a single episode.

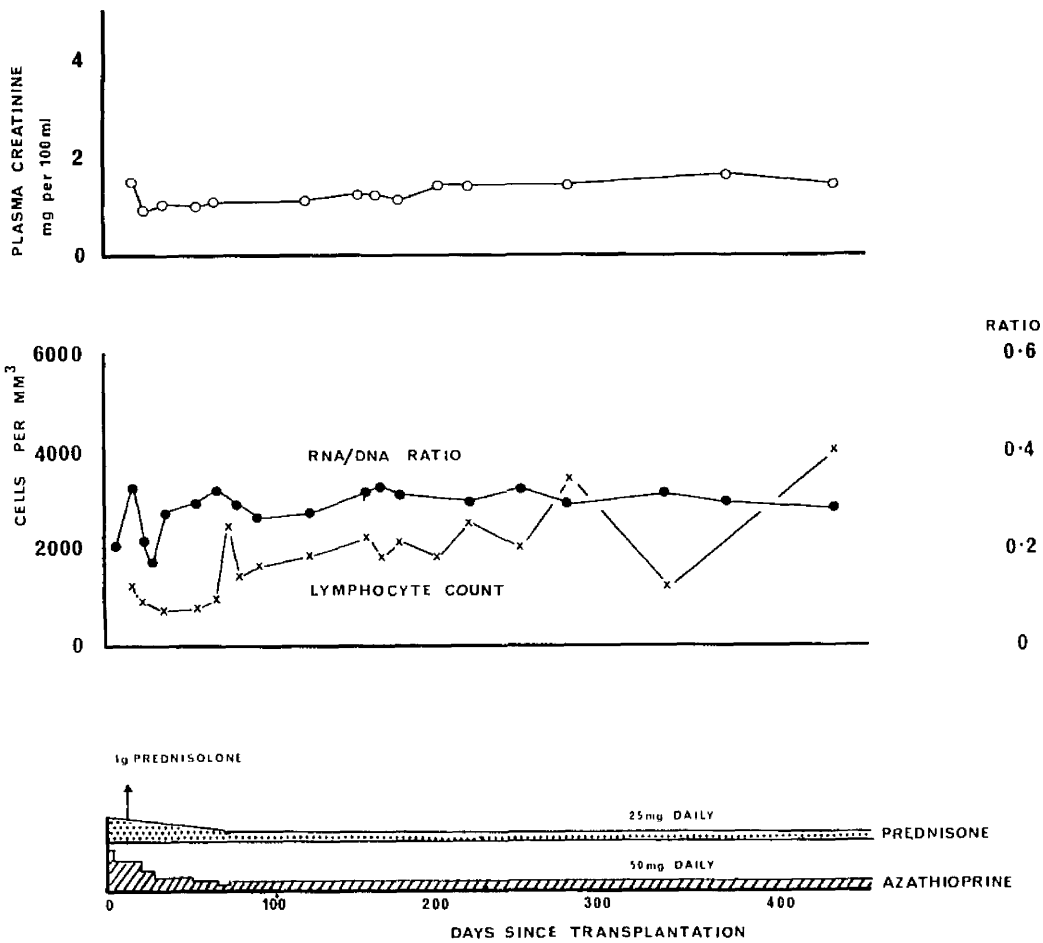
result and the clinical rejection episode (listed in Table 23) was variable, in one case the lymphocyte abnormality appeared one hundred and twenty-nine days in advance of rejection and was shown still to be present thirty, seven and one day before gradual function impairment of the graft was apparent and treatment initiated. Serial results from this patient who was latterly considered to be suffering from chronic rejection are shown in Figure 16. The remaining five abnormal results were not followed by a recognisable rejection episode. Two of those may be discounted since the results were obtained one and two days after treatment of a previous rejection with 1 g prednisolone. The incidence of false positive results in the diagnosis of rejection was thus three in nineteen, or 16 per cent.

It is appropriate to present now the results of serial analyses of the lymphocyte in patients with a renal allograft in order to illustrate the different lymphocyte responses encountered. Although many measurements were made in these patients only the lymphocyte count, the lymphocyte RNAP/DNAP and the plasma creatinine together with the immunosuppressive therapy are included in the figures.

Figure 15 shows the results from a patient (BL) who had an excellent result from transplantation with no major rejection episodes. The limits of variation of the lymphocyte RNAP/DNAP are small. This is in contrast to Figure 16 which has been referred to earlier, illustrating chronic rejection. Here the lymphocyte

Figure 15

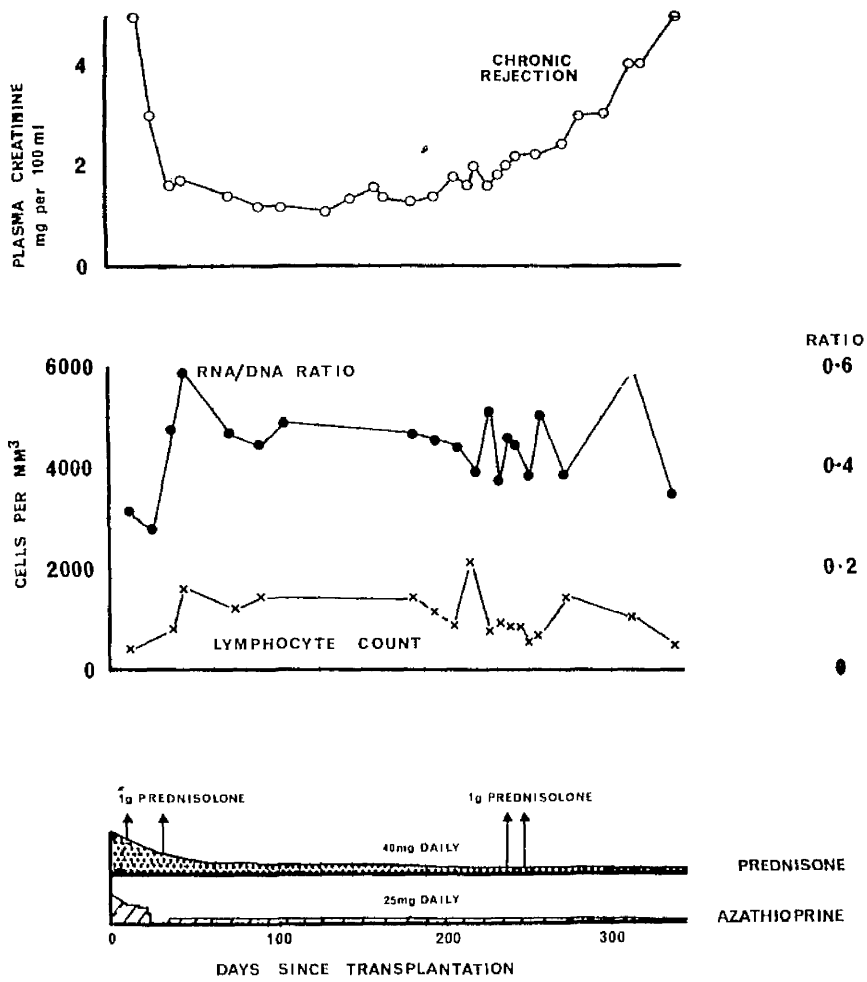
Serial analyses in renal transplantation; studies on a patient with an excellent clinical result.



The blood lymphocyte analyses, RNAP/DNAP ratio and count are plotted together with the plasma creatinine and details of the immunosuppressive therapy. This patient (BL) had one early rejection episode marked by the administration of 1 g prednisolone and thereafter no evidence of rejection. A herpes zoster infection was present from day 60 to day 80.

Figure 16

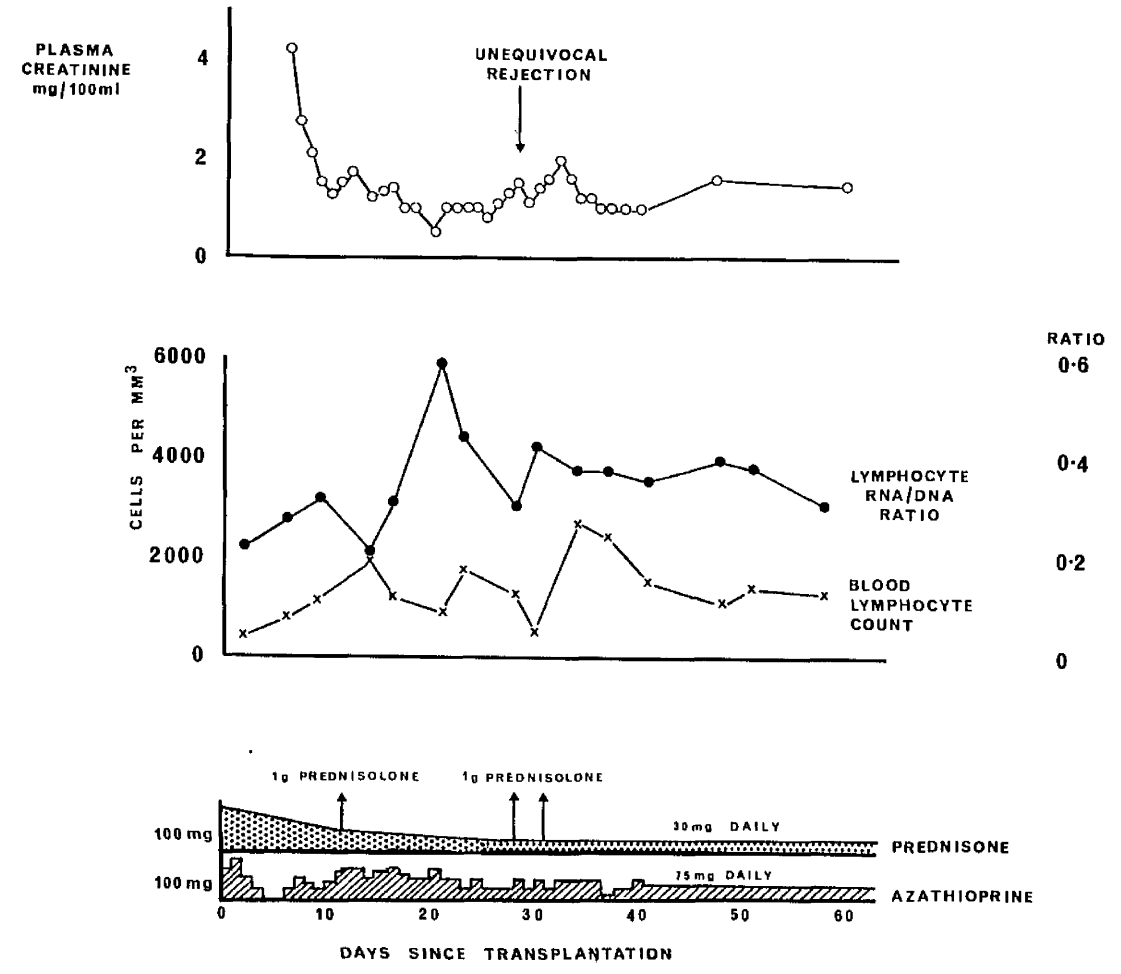
Serial analyses in renal transplantation; studies on a patient with chronic rejection.



The blood lymphocyte analyses, plasma creatinine and details of the immunosuppressive therapy are plotted for a patient (MM) who developed chronic rejection. The lymphocyte RNAP/DNAP ratio was elevated and remained elevated over 100 days before renal function deteriorated and a chronic rejection state recognised.

Figure 17

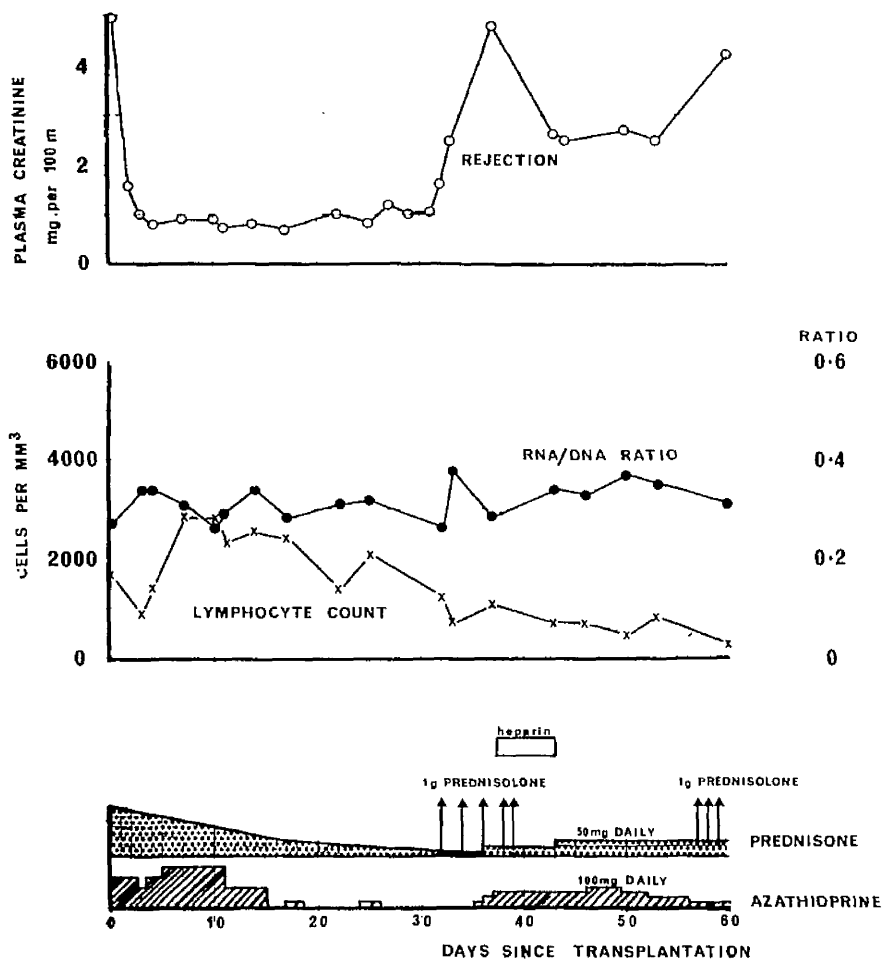
Serial analyses in renal transplantation; studies on a patient with acute rejection.



The blood lymphocyte analyses, plasma creatinine and details of the immunosuppressive therapy are plotted for a patient (HB) who developed an acute rejection episode, recognised by changes in renal function on day 28. The lymphocyte RNAP/DNAP ratio was elevated and a lymphopenia present seven days in advance of changes in renal function.

Figure 18

Serial analyses in renal transplantation; studies on a patient with normal lymphocytes during rejection.



The blood lymphocyte analyses, plasma creatinine and details of the immunosuppressive therapy are plotted for a patient (CD) who developed renal graft rejection on day 32 and failed to respond to the usual measures to reverse the rejection. The lymphocyte RNAP/DNAP results remained normal during this period. These were atypical findings.

abnormality was apparent early in the life of the graft and when the transplanted kidney was finally removed after just over one year in the recipient there were almost no recognisable tubules or glomeruli present on microscopic examination of the allograft tissue.

An example of the results of serial monitoring of the blood lymphocytes during a period of acute reversible rejection is shown in Figure 17. In this illustration lymphopenia with a markedly elevated lymphocyte RNAP/DNAP ratio was present at least seven days in advance of the decision to treat for acute rejection. The normal lymphocyte RNAP/DNAP ratio just at the time of rejection was the only atypical feature of the results illustrated and was not found in other studies. The earliest rise in the plasma creatinine on which the decision to increase the immunosuppression was based, occurred five days after the changes in the lymphocytes were established. Four such examples, in different patients, of prior abnormality of the blood lymphocytes, have been adequately documented, thus in patients HB, CMcN, RMcK and MMcC the lymphocyte abnormality was present seven, forty-four, one, and one hundred and twenty-nine days in advance of the change in renal function. The last illustration of serial analyses shows a sequence of events which was much less common than events illustrated in Figures 15 and 17, and was seen in two patients only. In both these patients there was early reduction of immunosuppression because of sensitivity of the patient to azathioprine or to the azathioprine and prednisone combination. About two weeks after the reduction

of azathioprine therapy to a low level there was evidence of rejection which was not resolved by the usual measures. The lymphocyte RNAP/DNAP remained below 0.40 throughout the period. In one of the two patients the donor kidney had to be removed on day forty-six and in the second (illustration in Figure 18) the creatinine clearance fell to 10 ml per minute with a plasma creatinine of greater than 4 mg per 100 ml.

4.7 Lymphopenia in patients with a renal transplant and on immunosuppressive therapy and its importance in the differentiation of over-suppression and rejection

Lymphopenia was a more frequent observation than leucopenia in this study. Of the eighty-four samples with a lymphocyte count of less than 1000 cells per mm^3 twenty-one had a severe lymphopenia with a count of less than 500 lymphocytes per mm^3. Leucopenia, defined as a blood white cell count of less than 4000 cells per mm^3, in contradistinction to lymphopenia, was present in only eleven samples in the study and only three times was severe lymphopenia and leucopenia present together. This dissociation of a low lymphocyte count and a low total leucocyte count is illustrated by white cell counts in the samples with severe lymphopenia. The mean white cell count of these lymphopenic bloods was 6516 cells per mm^3.

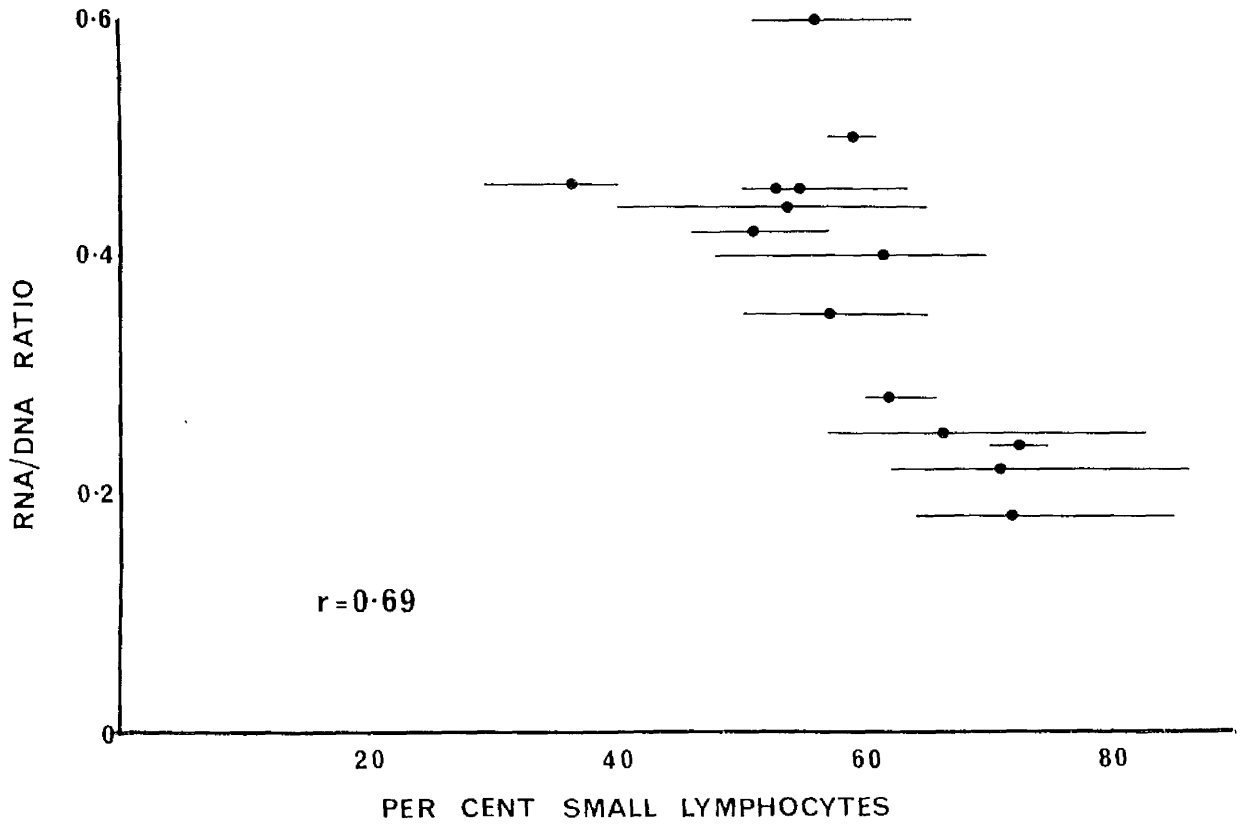
The mean blood lymphocyte RNAP/DNAP value from patients showing excessive immunosuppression recognised by a leucopenia is listed in Table 22, and the individual results displayed in Figure 14. When lymphocytes specifically are reduced in number, and there is no rejection, the mean lymphocyte RNAP/DNAP is very low at 0.197 (SD 0.054), indeed lower than the value of 0.253 found in a general leucopenia. This difference of the means is just significant ($p = < 0.05$). The observed range of values for the RNAP/DNAP ratio in severe lymphopenia without rejection is 0.12 to 0.27, and when patients with less severe lymphopenia are considered, i.e. those with a blood lymphocyte count up to 1000 cells per mm^3, the range of observed values for the lymphocyte RNAP/DNAP ratio extends upwards to 0.37. This value for the lymphocyte RNAP/DNAP ratio is lower than that considered appropriate for the diagnosis of graft rejection and it appears therefore that a lymphocyte RNAP/DNAP ratio of less than 0.40 distinguishes the lymphopenia which may be the result of immunosuppressive drug action from the lymphopenia associated with rejection.

4.8 The nature of the blood lymphocyte in patients with a renal allograft

The nature of the blood lymphocytes in patients with a renal allograft was investigated by examination of their morphology on a stained slide. Three observers were asked to classify the lymphocytes as either large, medium, or small allocating as few cells as possible to the intermediate grading. Small lymphocytes were defined as mononuclear cells which approximated to the size of the red cells, and had a nucleus which occupied more than half of the cell diameter. The observers carried out their examination independent of each other and of the chemical measurements. Fifteen blood samples were examined in this way and Figure 19 shows the results of the correlation between the lymphocyte RNAP/DNAP ratio and the percentage of blood lymphocytes classified as small lymphocytes, determined as the mean value for the three observers. The coefficient of linear correlation for the comparison was 0.69, and this was significant at the one per cent level ($t = 3.44$). When the observed values of percentage of small lymphocytes for each individual observer was correlated with the cell chemistry, the correlation coefficients, 0.68, 0.66 and 0.43, were poorer than that derived from mean observations. The t values were respectively 3.34, 3.19, 1.72 and only the first two were significant, $p = < 0.01$ in both cases. Figure 19 shows the considerable observer

Figure 19

Correlation of the blood lymphocyte RNAP/DNAP ratio and the percentage of small lymphocytes determined on a stained blood film.



The percentage of small lymphocytes was calculated as the mean of three independent observations and the observed range indicated by horizontal lines through the mean values. The coefficient of linear correlation for the percentage small lymphocytes and the lymphocyte RNAP/DNAP ratio was 0.69.

variation in the individual values. As was anticipated, a high lymphocyte RNAP/DNAP ratio was associated with a low percentage of small lymphocytes. An attempted correlation of the lymphocyte chemistry and the percentage of large lymphocytes was less successful than that reported above.

4.9 The relationship between the donor kidney match and the RNAP/DNAP ratio of the blood lymphocytes

Botha (1969) has examined methods of classifying the degree of tissue match between donor and recipient in human allografts and listed six main categories. These categories are best listed in a table (Table 24). It can be seen from the table that Grade A is perfect matching by present techniques and Grades B, C, D, E and F are in order of decreasing compatibility judged mainly by the presence of antigens in the donor leucocytes, not present in cells of the recipient.

The grade of match of the allograft for each of the patients according to the Botha system are included in Appendix 10. These data allowed comparison of the blood lymphocytes RNAP/DNAP ratio for patients of differing grades of tissue match. Certain precautions were required in the preparation of the data. The average of the values recorded for the lymphocyte RNAP/DNAP in

Table 24

Grades of tissue match in human allografts (Botha, 1969).

Grade of Match	Number of leucocyte antigens	
	Donor + Recipient −	Donor − Recipient +
A	0	0
B	0	1 or more
C +	1	0
C	1	1 or 2
C −	1	3 or more
D	2	0 or more
E	3	0 or more
F	recipient has preformed antibodies to donor leucocytes.	

Table 25

The relation between the donor kidney match, rejection and the lymphocyte RNAP/DNAP ratio.

Tissue match	Patient	Duration of graft months	Rejection episodes	Creatinine clearance at 1/1/71 ml/min	Mean monthly lymphocyte RNAP/DNAP ratio	
					individual	group
A	MC	14	0	86	0.36	0.36
	MS	9	2u	60	0.35	
B	BL	19	0	93	0.30	0.30
	HB	3	1u 1p	60	0.34	
	CG	3	3u	64	0.33	
C	JM	13	0	37	0.28	0.33
	JO	11	2p	111	0.37	
	AP	3	1u	78	0.34	
	RF	8	1p	96	0.32	
D	MM	8	2u	Irreversible rejection	0.43	0.37
	CM	10	2u	77	0.36	
	JL	1	1u	Irreversible rejection	0.36	
E	SM	<1	1u	Infection	0.25	0.33
	RM	13	1u	26	0.34	

Tissue match is according to Botha (1970). The rejection episodes are classed as unequivocal (u) and probable (p) as defined in the text. The mean lymphocyte RNAP/DNAP ratios for each patient are calculated as follows. Individual analyses are grouped monthly and the average monthly ratio calculated. The mean ratio tabulated is the average of these monthly ratios.

each patient was calculated month by month and the mean of these monthly ratios included as part of Table 25. This method of calculation reduced the bias which might arise when analyses were repeated frequently at times of possible rejection. Unequivocal rejection recorded in the table is defined in Section 4.5 and probable rejection when the rise in plasma creatinine although sustained, does not meet the criteria of a substantial rise laid down.

There is no simple relationship between the tissue match and the mean lymphocyte RNAP/DNAP ratio and it appears likely that the lymphocyte RNAP/DNAP ratio has been influenced by factors other than the tissue match. The highest mean ratio occurred in a patient with a D match who developed an irreversible rejection, the lowest ratio 0.25 was in a patient with a D match who died of over-immunosuppression and infection. Three patients, one from each of Group A, B and C had no rejection episodes during the period of study, but their mean lymphocyte RNAP/DNAP ratio values were 0.36, 0.30 and 0.28 respectively. The first of these patients had repeated surgery for recurrent ureteric fistulae.

4.10 The nucleic acid content of human thoracic duct lymphocytes

Table 26 shows the nucleic acid content of lymphocytes obtained

Table 26

The nucleic acid content of human thoracic duct lymphocytes obtained from the initial drainage of a thoracic duct fistulae prior to renal transplantation.

Patient	Number of samples	µg DNAP per 10^6 cells	µg RNAP per 10^6 cells	RNAP/DNAP ratio
1	2	0.91	0.20	0.22
2	2	0.65	0.18	0.28
3	2	0.63	0.11	0.17
4	2	0.64	0.14	0.22
Mean value	10	0.72	0.16	*0.22 SD 0.045

*Comparison of the mean value for the thoracic duct lymphocyte RNAP/DNAP ratio with the values for seventy-four estimations of blood lymphocyte RNAP/DNAP ratio in normal individuals show a significantly lower ratio in thoracic duct lymphocytes ($t = 3.14$ $p = < 0.01$). The variance of these two groups was not significantly different ($F = 1.49$, $p = > 0.05$).

early in the thoracic duct drainage of four patients before immunosuppressant drugs were administered. The mean DNA content, 0.72 μg DNAP per 10^6 cells, was similar to that found in normal blood lymphocytes by the same chemical methodology (Section 3.4). The mean value for the RNA content, 0.16 μg RNAP per 10^6 cells and the RNAP/DNAP ratio 0.22, was lower than that found in blood lymphocytes and was significantly lower when compared by t-test with the seventy-four results for normal individuals by all methods ($p = \leq 0.01$).

4.11 The effect of immunosuppressant drugs, azathioprine and prednisone on the RNAP/DNAP ratio of human thoracic duct lymphocytes

In this study serial measurements were made in four patients of the RNAP/DNAP ratio of lymphocytes isolated from the thoracic duct at a time of institution of immunosuppressive therapy and also for a period after renal transplantation. Table 27 summarises the results of these determinations and serial results in one patient, typical of all four, are illustrated in Figure 20. The figure shows an unmistakable rise in the RNAP/DNAP ratio of the thoracic duct lymphocytes as azathioprine and then prednisone therapy are commenced. The tendency for the lymphocyte chemistry to return to normal on continual therapy is also illustrated. Statistical analysis of the results for all four patients (Table 27) shows that

Table 27

Effect of immunosuppressive therapy on thoracic duct lymphocyte RNAP/DNAP ratio.

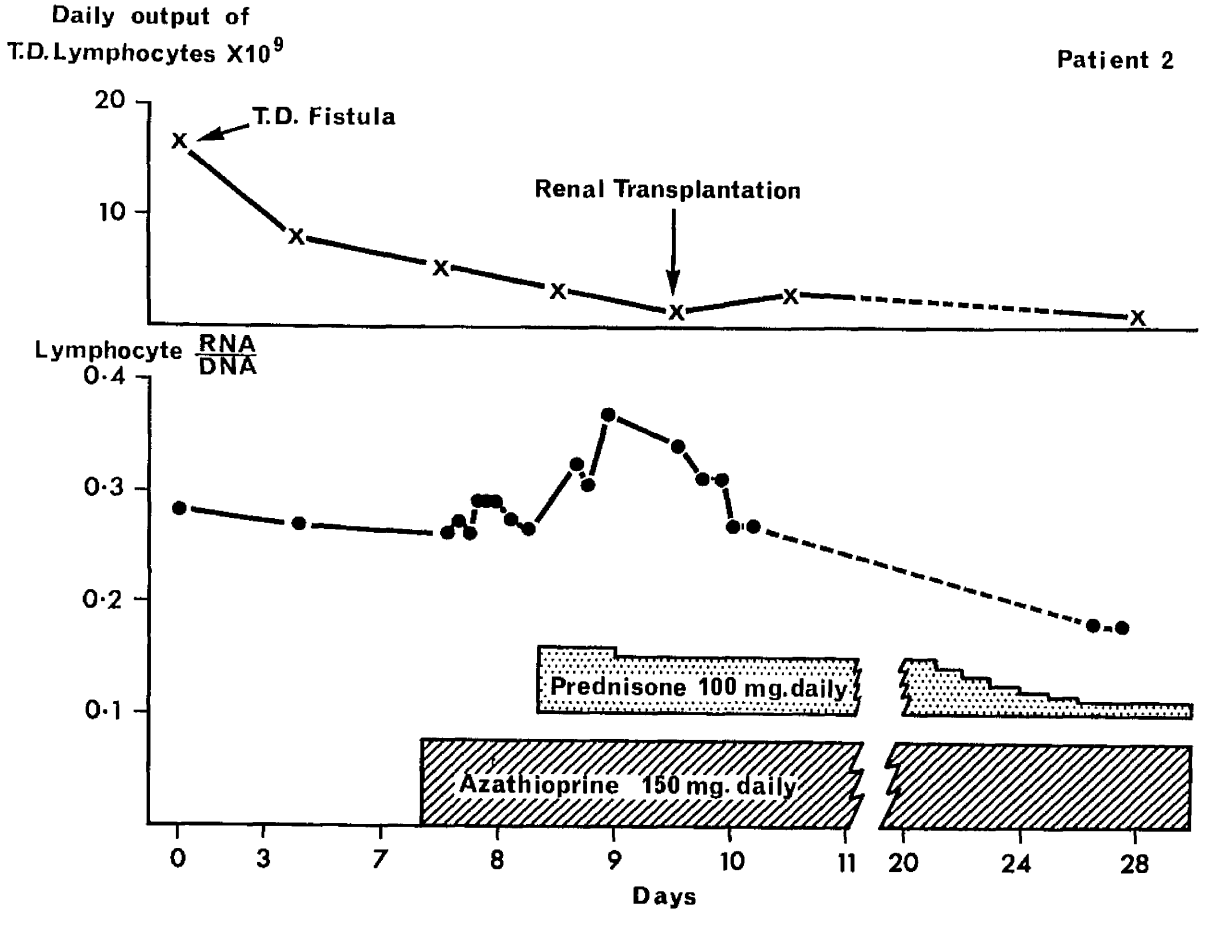
The tabulated results are the RNAP/DNAP ratios of samples of lymphocytes from the thoracic duct obtained - a) prior to renal transplant and immunosuppressive treatment; b) during the 24 hours which followed initiation of azathioprine treatment; c) during the 24 hours which followed initiation of prednisone treatment, azathioprine continued; d) after the transplant operation, with continued immunosuppression.

Patient	Pre-treatment period (a)	24-hour period following initiation of azathioprine therapy (b)	24-hour period following initiation of azathioprine therapy (c)	Post-transplant period (d)
1	0.22 (mean of 2)	0.29 (1 analysis)	0.37 (1 analysis)	0.27 (mean of 2)
2	0.28 (mean of 2)	0.28 (mean of 8)	0.33 (mean of 3)	0.27 (mean of 6)
3	0.20 (mean of 5)	0.23 (mean of 6)	0.30 (mean of 4)	0.33 (mean of 3)
4	0.24 (mean of 4)	0.30 (mean of 3)	0.29 (mean of 2)	Spontaneous early closure of thoracic duct fistula
Mean result for four patients	0.235 S.D. 0.033	0.275 SD 0.030	0.323 SD 0.035	0.290 SD 0.035
Comparison with pre-treatment values by Student's t-test		Significant difference $p = < 0.01$	Significant difference $p = < 0.001$	Significant difference $p = < 0.05$

Variances were not significantly different by F-test.

Figure 20

Serial analyses on human thoracic duct lymphocytes.



The figure shows the daily output of thoracic duct lymphocytes from a thoracic duct fistula established as an immunosuppressive measure nine days before renal transplantation. Serial determinations of the thoracic duct lymphocyte RNAP/DNAP ratio were made before immunosuppression, as azathioprine was commenced and then prednisone therapy was instituted. Measurements are also shown nineteen days after the transplant operation.

immunosuppressive therapy was associated with a significant increase in the RNAP/DNAP of the thoracic duct lymphocytes over the pre-treatment levels. The increase averaged 17 per cent with azathioprine and 37 per cent with combined prednisone and azathioprine therapy in the twenty-four hour period following the start of treatment. Some individual measurements of RNAP/DNAP were considerably higher than this, as much as 105 per cent over the pre-treatment level when patient 3 was first given prednisone (0.20 to 0.41). A constant finding illustrated in Figure 20 was the progressive fall in the output of thoracic duct lymphocytes with continued drainage. However, there was no consistent change in RNAP/DNAP when early and late stages of the pre-treatment period were studied. Following transplantation the RNAP/DNAP was followed in two of the four subjects. In patient 2, the RNAP/DNAP value had fallen to the pre-treatment level sixteen days after transplantation, while in patient 3 the value was still elevated thirty days after the transplant. The latter patient showed signs of rejecting the graft.

4.12 The nature of lymphocyte RNA

The ribonucleic acid of whole lymphocytes.

Lymphocytes obtained from the thoracic duct of a patient on

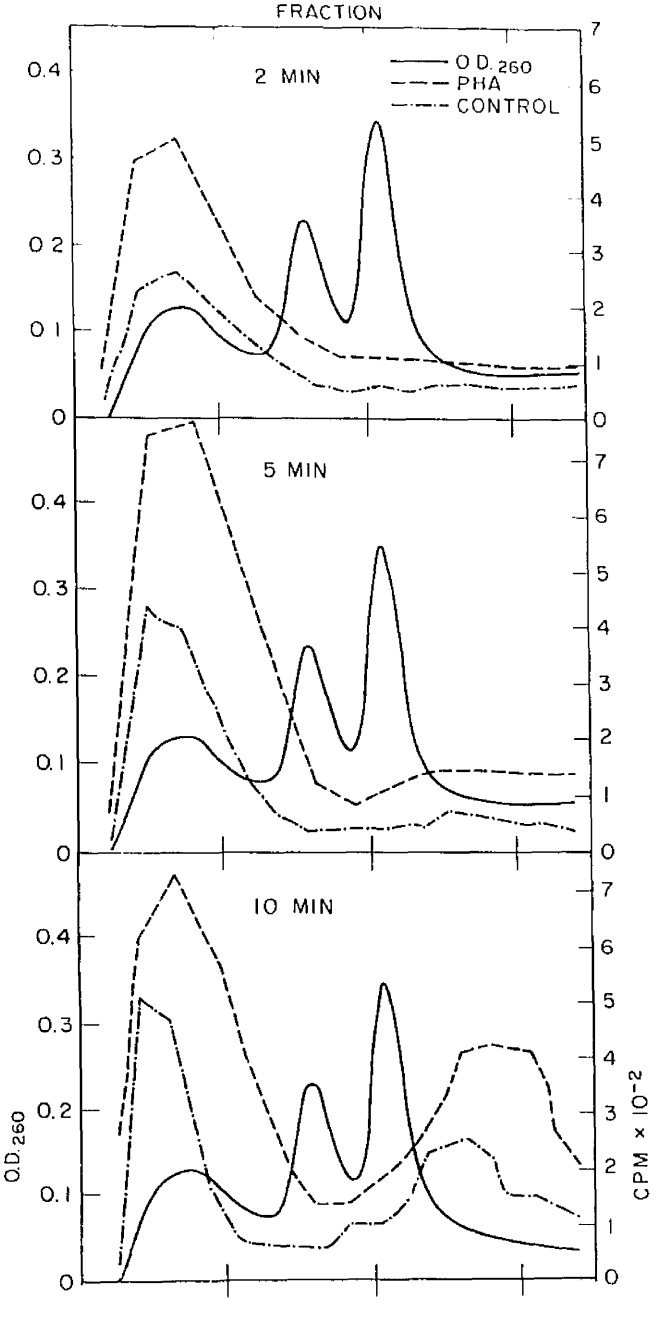
intermittent haemodialysis were set up in culture using Medium 199 at a concentration of twenty million lymphocytes per ml. After addition of phytohaemagglutinin (PHA) to one half of the cells and saline to the other as control, the suspensions were immediately distributed among ten culture tubes, five PHA and five control, each containing 250 x 10^6 lymphocytes. The tubes were closed and incubated for eighty minutes at 37C. For the final two, five, ten, twenty and thirty minutes of the incubation labelled Uridine was added to each tube as Uridine H^3, 4 μc per ml. At the end of the eighty minutes incubation all cultures were harvested and the RNA extracted from whole lymphocytes. The extracted RNA was analysed by sucrose gradient centrifugation as described, measuring optical density and radioactivity of the fractions separated by the gradient.

The results of the analysis of whole lymphocyte RNA are presented in Figure 21. Optical density measurements for control and phytohaemagglutinin stimulated culture were very similar and for simplicity only one line is plotted in the figures. Previous experience with this type of gradient allows recognition of the three optical density peaks in the positions anticipated for 4s, 18s and 28s RNA. This would indicate that the usual mammalian cell RNA components are present in human thoracic duct lymphocytes, namely transfer RNA and the two components of ribosomal RNA.

The kinetics of Uridine labelling in RNA is illustrated by the radioactivity measurements in Figure 21. Within two minutes of exposure of lymphocytes to the labelled RNA precursor, radioactive

Figure 21

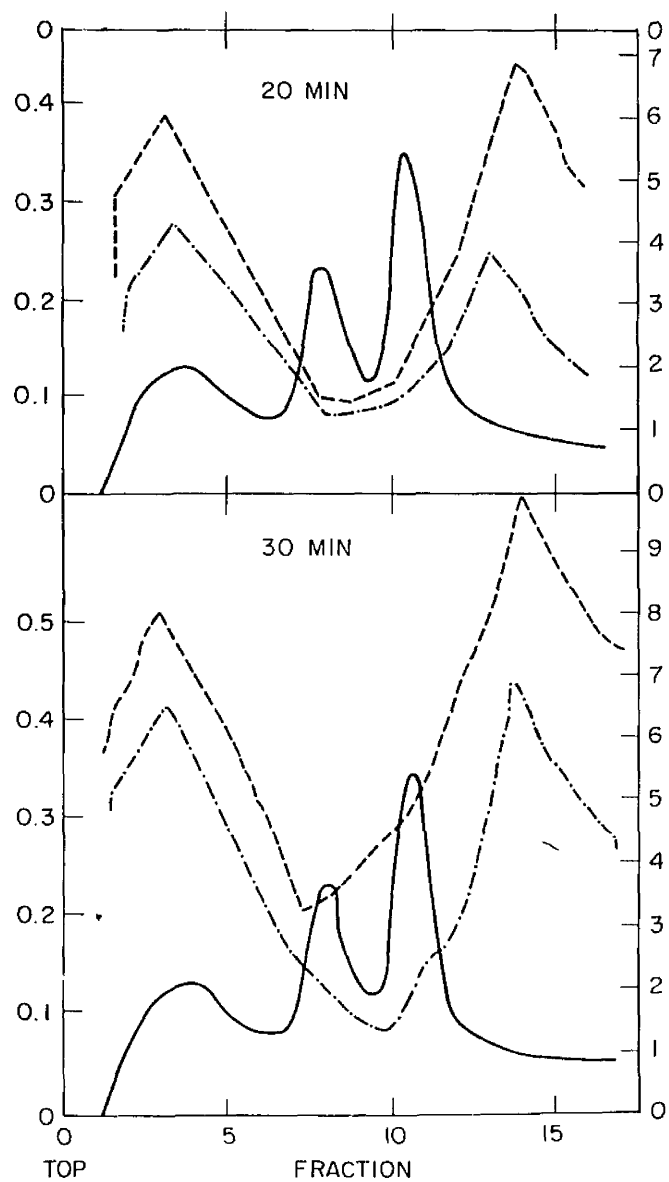
Labeling kinetics of whole lymphocyte RNA with and without phytohaemagglutinin (PHA). Continued as Figure 21A overleaf.



Thoracic duct lymphocytes were set up in a series of cultures and incubated as described in the text. Half were controls and half were incubated with PHA for 80 minutes. For the final 2, 5, 10 (and in Figure 21A) 20 and 30 minutes of the incubation Uridine-H^3 was present at a concentration of 4 µc/ml. At the end of 80 minutes the cultures were harvested and the RNA of the lymphocytes analysed by density gradient centrifugation. The distribution of the RNA on the gradient was analysed by ultra-violet absorption at 260 nm (continuous line) and by radioactivity (counts per minute) shown as interrupted lines.

Figure 21A

Labeling kinetics of whole lymphocyte RNA continued.



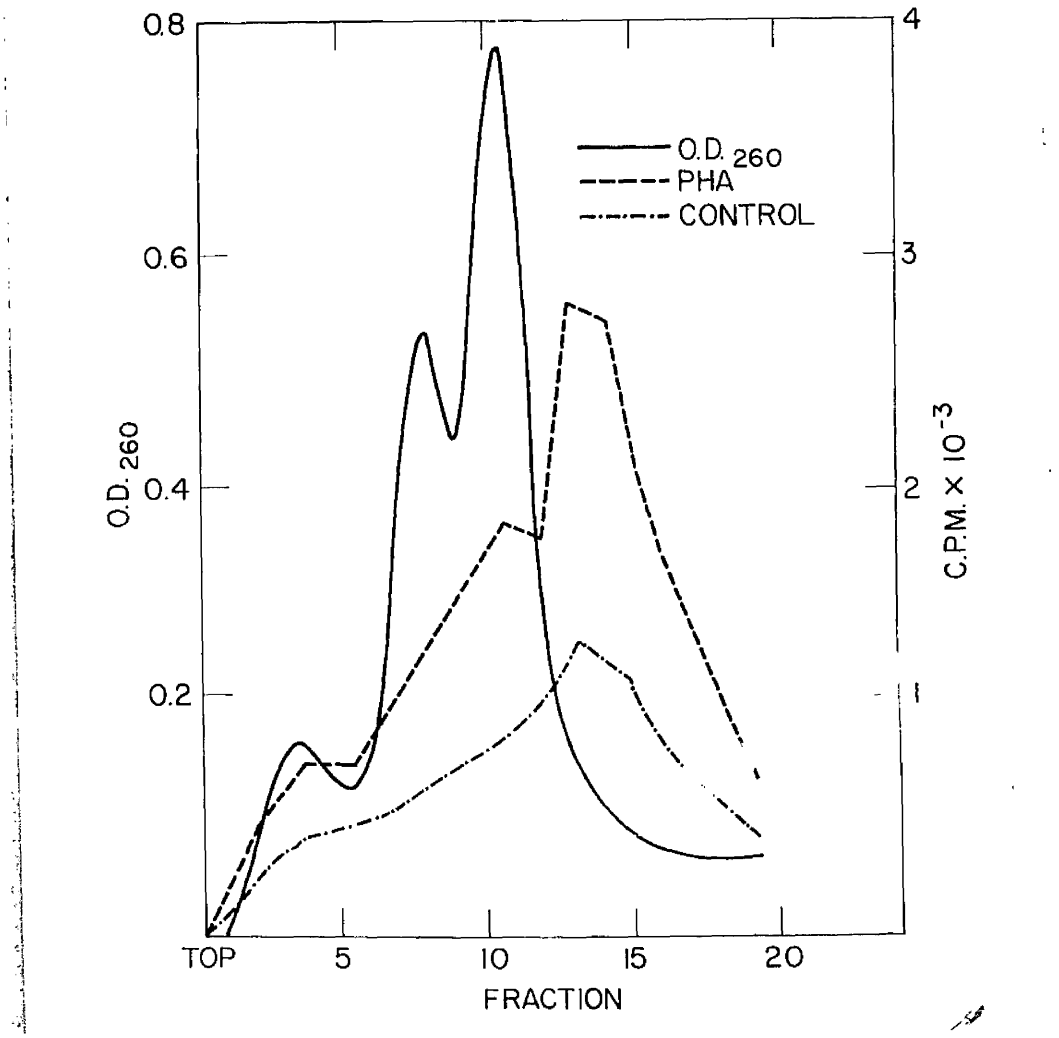
Legend as for Figure 21. Optical density denoted by continuous line is calibrated on the left and radioactivity, the broken lines, is calibrated in counts per minute on the right. The heavy molecular weight RNA species appear in the fractions to the right in the diagrams.

RNA of low sedimentation coefficient (4s to 6s) was present and phytohaemagglutinin had stimulated its synthesis by more than one hundred per cent over the control. Labelling in the 4s to 6s fraction increased, and by five minutes after addition of the isotope was almost twice the two minute value. No further increase in labelling of this fraction occurred during the remaining twenty-five minutes exposure to isotope. Until ten minutes after the addition of Uridine H^3 no significant labelling occurred in the 40s to 50s RNA region, i.e. the part of the gradient occupied by newly synthesised ribosomal RNA in the form of heavy molecular weight ribosomal precursor RNA. The peaks of radioactivity in the 40s to 50s region thereafter increased at all intervals up to thirty minutes, and the extent of labelling of RNA was always greater in the phytohaemagglutinin tubes than in the control tubes. This 50s RNA was not however present in sufficient quantity to give an optical density peak.

In a separate experiment two lymphocyte cultures were set up as before and incubated for a total of one hundred and thirty-five minutes with and without phytohaemagglutinin. After an incubation period of one hundred and five minutes Uridine H^3 was added, 4 μc per ml, and incubation continued up to the one hundred and thirty-five minutes total incubation time. The cultures were then harvested, this time the lymphocyte nuclei were isolated and the nuclear RNA extracted, fractionated and counted. The results showing the optical density pattern and the radioactive counts for

Figure 22

Density gradient separation of RNA from lymphocyte nuclei with and without PHA.



Human thoracic duct lymphocytes were incubated in cultures with and without PHA for a total of 135 minutes. During the last 30 minutes of the incubation the cells were exposed to Uridine-H^3 at 4 µc per ml. The nuclei were isolated prior to extraction of their RNA and its analyses by gradient centrifugation. The continuous line is optical density at 260 nm and the broken lines the radioactivity for PHA and control samples. High molecular weight RNA is found in fractions to the right in the figure

nuclear RNA from phytohaemagglutinin and control cultures appear in Figure 22.

It may be seen that although the three major RNA species are represented in the optical density peaks the low molecular weight 4s component is less abundant in the extract of nuclei than in the previous whole cell RNA extract. High molecular weight RNA is present in nuclei, recognisable by the incorporation of Uridine H^3 in the area of high molecular weight material to the right of the 28s optical density peak. This radioactivity was again more marked in the phytohaemagglutinin stimulated cultures.

In further experiments reported elsewhere (Jacob, Cooper, Glen and Munro, 1969) the high molecular weight and rapidly synthesised RNA is seen to be precursor to ribosomal RNA since on exposure of a culture to excess of unlabelled Uridine and to Actinomycin D which inhibits RNA synthesis, with continuation of the incubation for one hour, label is lost from the high molecular weight region and appears mainly in the 28s region, i.e. the region of ribosomal RNA.

4.13 Discussion

Blood lymphocyte analyses in renal transplantation.

Lymphocyte DNA content.

The evidence that the mean DNA content (DNAP content) of the lymphocyte is slightly greater in patients who have a renal allograft than in normal individuals does not affect comparisons of the lymphocyte RNAP/DNAP ratios reported since there were no differences in the mean DNAP content of the lymphocytes between the various clinical groups. This observation does however support the view that in the presence of a substantial immunological stimulus the proportion of lymphocytes which have synthesised or are synthesising DNA prior to cell division is increased. This has been discussed earlier in Section 3.26.

Lymphocyte RNAP/DNAP ratio.

Certain types of grafted tissue, for example skin allografts in rabbits, appear to be rejected principally by a cell mediated reaction, that is an infiltration of large lymphoid cells which results in damage and destruction of the cells of the graft. The proliferation of lymph node cells in areas later identified as

paracortical areas of the nodes (Scothorneand MacGregor, 1955) suggests that T-lymphocytes respond to the stimulus of the graft, but other cells are also involved. Detailed studies of the lymph nodes in rabbits with skin grafts show an increase in a variety of cells including lymphoblasts, plasma cells, plasma cell precursors as well as lymphocytes (André, Schwartz, Mitus and Damashek, 1962A). The presence of plasma cells would indicate that antibody as well as cell mediated processes are involved in rejection.

Some evidence that there are abnormal mononuclear cells present in the blood at times of graft rejection has been introduced and the work reported in this thesis has confirmed in man that a significant abnormality in the circulating mononuclear cells is frequently present when a renal graft is being rejected. Previous studies of skin allografts have shown that the lymphoid cells which invade the graft are metabolically active with prominent nucleoli, and abundant free ribosomes in their cytoplasm (Wiener, Spiro and Russell, 1964). Histochemically they have a high RNA content (Scothorne and Tough, 1952). The levels of RNAP/DNAP ratio observed in the blood lymphocytes during allograft rejection were sometimes greater than 0.50, equalling the highest levels reached in any condition. Only in neoplastic disease with secondary metastases were similar levels reached, notable since graft rejection and the response to neoplastic disease have common

features, both involving cellular immune reactions (Meuwissen, Stutman and Good, 1969).

It is evident from reviews of renal allograft rejection in man that the rejection process in patients on standard immunosuppression can take several forms, and Porter (1967) has defined these as follows:

1. Acute and immediate

This occurs when there is a major blood group incompatibility between host and donor or when the donor is presensitized. Rejection usually takes place within minutes of transplantation and there is red cell sludging in the glomeruli.

2. Acute and early

Rejection occurs two to ten days after transplantation and is characterised by dense cellular infiltration and rupture of the peritubular capillaries. This is largely cell mediated and renal shutdown follows tubular necrosis.

3. Acute and later

In the period from eleven days onward rejection occurs and is characterised by coating of arteries, arterioles and glomerular capillaries with immunoglobulins and complement. Platelet aggregates are seen in glomerular capillaries with thrombi in larger vessels. Cellular infiltrate may or may not be a feature. Acute renal shutdown seems to be due to the glomerular platelet aggregates.

4. Insidious and late

This type of rejection is characterised by subendothelial accumulations of IgM and complement on the glomerular capillary basement membranes with slow impairment of renal function.

The information on the timing of rejection recorded in Table 23 reveals that the majority of rejection episodes included in the present study would qualify as Porter's class 3 rejections. There is a single episode occuring on day seven and falling within the time schedule for an acute early cell mediated rejection. The possibility that some rejections were 'insidious and late' cannot be ruled out on the basis of day of onset alone.

If, as seems likely, the majority of the rejection episodes were of the type three variety a difficulty arises in determining the degree of cell mediated reaction to the graft and the extent that platelet aggregation and vascular damage has contributed to the rejection. This is important since platelet aggregation is thought to be initiated by antigen-antibody complexing in the glomeruli and small vessels of the graft and therefore results from an antibody response. It is appropriate here to consider the evidence already introduced that the 'acute and later' rejection episodes in treated human renal allografts are triggered either by a cellular or an antibody response.

Extensive studies with many species of animal, reviewed for

example by Moller and Moller (1967), have shown that humoral antibodies can by themselves be responsible for rejection of grafts which have a high sensitivity to antibodies, mainly cells belonging to the lymphatic system, 19s antibodies are more potent than 7s antibodies in this respect. On the other hand repeated experiments based on transfer of sensitized lymphoid cells have shown that cells can evoke rejection of transplanted tissues in situations where serum antibodies are ineffective (Mitchison, 1954; Billingham, Brent and Medawar, 1954).

Mowbray (1966) considers that the dose levels of azathioprine which can be used in human renal transplant patients are insufficient to prevent an antibody response to graft antigens and he believes that antigen-antibody complexing initiates platelet aggregation in small vessels of the graft and hence the rejection episode. Rejection episodes are treated with prednisone (or prednisolone) and this may be effective since both steroids and 6-mercaptopurine have been shown to prevent immune complex induced platelet aggregation (Mowbray, 1966). Cell mediated graft rejection in Mowbray's view is rare in treated human renal allografts. Biopsy of the renal graft in patients on immunosuppression during acute rejection occuring more than eleven days after transplantation frequently shows changes in the vasculature with platelet thrombi and the later consequence, fibrinoid necrosis in arteriolar walls, but a cellular infiltrate of lymphoid cells is also nearly always present despite the suppressing action of azathioprine (Porter, 1967).

Hurd and Ziff (1968) have shown in animal work that the effect of 6-mercaptopurine, the active metabolite of azathioprine, is to decrease the number of monocytes and large proliferating lymphocytes in the blood concurrently with the decrease in mononuclear cells in inflammatory sites. In an independent study of the in vivo action of azathioprine it was shown that rejection of skin grafts in rabbits occurs when the drug fails to prevent the proliferation of large mononuclear cells in the lymphoid tissue (André, Schwartz, Mitus and Damashek, 1962B). One obvious interpretation of the elevation in lymphocyte RNAP/DNAP ratio observed frequently in the patients rejecting their grafts is that immunocytes or immunoblasts on route for the allograft are present in peripheral blood and are contributing to the mean RNA content of the isolated cells. This would imply that the cell mediated reaction is important in the majority of the rejection episodes.

Parker and Mowbray (1971) have published a detailed investigation of the uptake of labelled Uridine in tissue culture by peripheral blood mononuclear cells from patients with renal allografts. They used the same type of gelatin sedimentation method for isolation of the blood mononuclear cells as the present study (Coulson and Chalmers, 1967) and their finding of increased intensity of RNA synthesis in the blood mononuclear cells prior to and following rejection would appear to corroborate the lymphocyte RNAP/DNAP ratio results. However, Parker and Mowbray attribute the increase RNA synthesis to the contribution from large atypical

cells present in the blood at the time of rejection. These cells on the basis of histochemistry, light and electron microscopy are considered to be myeloid rather than lymphoid cells and have been tentatively identified as promyelocytes.

The data on the absolute count and histochemistry of the atypical cells in the blood have been further detailed by Parker (1969), the maximum count observed was 60 per ml of defibrinated blood, that is on an estimated lymphocyte count of 1000 per mm^3, six per cent of the blood mononuclear cells. A simple calculation shows that a tenfold increase in the RNA content of six per cent of the cells would be required to raise the mean RNAP/DNAP ratio of the mononuclear cells from the normal 0.30 to 0.46 a value commonly found in rejection in the present study. The atypical promyelocytes observed by Parker and Mowbray and noted in the course of the present study do not have strikingly basophilic cytoplasm and although the nuclei are sites of rapid RNA turnover it is held that cells other than these are responsible for the increased RNAP/DNAP ratio in the blood mononuclear cells at the time of rejection. It is still of course uncertain what is implied by an elevated lymphocyte RNAP/DNAP ratio in terms of cellular or antibody mediated rejection, but a possible suggestion is that an elevated ratio signifies a more intense cellular immune response. The increase in spontaneous transformation of lymphocyte in vitro at times of rejection documented earlier would support this interpretation.

The results presented here propose the simultaneous determination of the blood lymphocyte count and the mean lymphocyte RNAP/DNAP ratio as a method of monitoring the effectiveness of immunosuppression in patients with a renal allograft. The method may distinguish graft rejection from excessive immunosuppression although both are associated with a low lymphocyte count. Lymphopenia may be present in both infection and rejection in patients on standard immunosuppressive therapy, but only in rejection is the combination of lymphopenia with an elevated lymphocyte RNAP/DNAP ratio found. In contrast, lymphopenia in the presence of infection may be associated with a distinctly low lymphocyte RNAP/DNAP ratio and presumably there may then be excessive immunosuppression. This difference between infection and rejection would appear to be the result of the more intense cellular response produced by the presence of the renal graft. Comparison of the results for infection and rejection in Figure 15B and 15C will clarify this point and show the necessity of measuring the lymphocyte count and RNAP/DNAP ratio together.

The conclusion that the mean lymphocyte RNAP/DNAP ratio is significantly reduced in excessive immunosuppression has not, to the author's knowledge, been previously reported. The belief that these findings reflect the high proportion of small lymphocytes circulating in the blood is bourne out by the study of the relationship between the lymphocyte RNAP/DNAP ratio and the percentage of small lymphocytes on a stained peripheral blood smear.

From these findings, and since lymphopenia is usually present, it appears that in patients suffering from excessive immunosuppression there is a dearth of the large lymphocytes.

It is difficult to understand why lymphopenia should be a feature of graft rejection. Although in classical acute and early rejection of a renal graft the donor kidney is infiltrated with a large number of lymphocytes (Porter, 1967), these can hardly be sufficiently numerous to deplete the entire lymphoid system of cells. Hall (1967) and Prendergast (1964) consider that the mononuclear cells entering a graft represent a random selection from the mononuclear population of the blood. It is likely that immunosuppressive therapy reduces the turnover of lymphoid cells and the rate at which replacement cells reach the blood. Experimental verification of this has been produced by Hurd and Ziff (1968) and it may be that cells leaving the blood and entering kidney tissue during rejection are only slowly replaced and a lymphopenia develops. When there is lymphopenia and a high mean RNAP/DNAP ratio, the cells present must show a greater than normal proportion of large lymphocytes and since there is a low blood lymphocyte count it would appear that the blood is depleted of small lymphocytes during rejection. Because of the known reactivity of the lymphoid system during rejection it may be wrong to consider this state as a general depletion, and perhaps the immunocompetent small lymphocytes are instead deployed as 'graft rejection cells' to use the term of Wiener and colleagues (1964). It would be logical to consider this

a T-lymphocyte depletion.

The value of an elevation in the lymphocyte RNAP/DNAP ratio in predicting graft rejection in a patient with a renal allograft is only seen when there is an accompanying lymphopenia. When both the high lymphocyte RNAP/DNAP ratio and low count were found a rejection episode took place after a variable interval (averaging twenty-three days) in nine out of the ten patients who showed these results. In seven episodes the interval was less than ten days, more akin to a delayed hypersensitivity response although immunosuppression might be expected to influence this. All but one of the nine rejections were reversible acute rejection, the single exception was of the chronic irreversible type.

Rejection occurred in two patients while the lymphocytes remained unequivocally normal and it is striking that in both of these patients the usual measures to reverse the rejection were ineffective. One patient improved only temporarily on heparin therapy combined with intravenous prednisolone. In the other patient who came to nephrectomy the donor kidney showed considerable vascular damage with proliferation of the intima and occlusion by thrombosis. The infiltrate of round cells, although present, was not a striking feature of the histology. Despite attempts to demonstrate antibody to HLA antigens in these patients none have been demonstrated.

The results of serial analyses of the blood lymphocytes in patients with a renal allograft, some of which are illustrated in

Figures 15 to 18, show several interesting features. Figure 15 shows typical results from a patient who has had excellent function in the grafted kidney for over two years at the time of writing. After the initial few weeks of the life of the graft the ratio has remained between 0.25 and 0.35, very different from the results shown in the succeeding figure (Figure 16). In Figure 15 the period from day sixty to eighty is of interest since during this time the patient had a herpes zoster infection which ran a typical course. A small reduction in azathioprine dosage was the only measure taken. The figure shows a sharp increase in the blood lymphocyte count at first but only a minor rise in the lymphocyte RNAP/DNAP ratio occurred.

The results illustrated in Figure 16 from a patient who developed chronic rejection have been described earlier. They were unique in this study. Support for the view that the early and marked changes in the lymphocyte RNAP/DNAP ratio illustrated represent an immunological reaction came from parallel determinations of the immunoglobulin levels in the patient. Exceptionally high levels of IgM, tenfold increases over normal levels, were found from an early stage in the life of the graft. These results are not reported because of difficulties in obtaining satisfactory absolute values, but the levels were undoubtedly exceptionally high. Such high levels in association with rejection have been reported (Zuhlke, Deodhar, Nakamoto and Kolft, 1967). Stocker and his colleagues studied cytoxic

antibodies in patients with a renal allograft on immunosuppressive therapy and noted a high incidence of cytoxic antibody in the IgM fraction (Stocker, McKenzie and Morris, 1969). But perhaps the important point for this study is that the high immunoglobulin levels and the abnormal lymphocyte RNAP/DNAP ratios found in the patient were the only indication that all was not well until some weeks later when deterioration of renal function was established.

Figure 17, an illustration of acute reversible rejection with prior abnormality of lymphocyte chemistry was a sufficiently common finding to make lymphocyte analyses potentially a clinically helpful test but since patients were followed who show no significant lymphocyte changes during rejection (Figure 18) although less commonly in the experience to date, certain caution in interpretation is required. Chisholm et al. (1969) have examined the value of eight simple laboratory determinations in blood and urine in patients after renal transplantation. Although significant changes occurred in several of these in relation to graft rejection, their experience was that tests based on serum and urine creatinine determinations were the most reliable indications of rejection. Other measurements have been made, for example urine enzyme analyses (Ballantyne, Wood and Meffan, 1968), but it is doubtful if they are superior to measurements of serum and urine creatinine, their predictive value is no better than the simpler measurements. In four individuals with adequate documentation in the present study the lymphocyte abnormality was

present prior to the change in renal function, while in two patients with rejection there was no lymphocyte abnormality. It is considered likely that the nature of the rejection process differs in these two groups.

The comparison of measurements on stained blood films with the chemistry of the lymphocytes isolated from the same blood shows an inverse relation between the percentage of small lymphocytes and the average RNA content expressed as the ratio RNAP/DNAP. This is useful confirmation that the method of cell isolation has not greatly distorted the distribution of cells. The variation of individual observers in the determination of percentage small lymphocytes is considerable and determination of the percentage of small lymphocytes on a blood film would require checking by independent observers for reliability. The measurement of cell volumes electronically, for example by the Coulter counter, is a possible alternative.

Although earlier studies could demonstrate no evident correlation between graft failure and the grade of tissue match, more recently when account is taken of undetermined loci in the HLA system the correlation of a good match and good prognosis for the graft has been established (Hors, Feingold, Fradelizi and Dausset, 1971). Failure to show a relationship between the lymphocyte RNAP/DNAP ratio and the compatibility of donor and recipient by HLA testing in groups of

patients suggests that other factors such as immunosuppression have been more important in determining the ratio.

Human thoracic duct lymphocytes.

The nucleic acid content of human thoracic duct lymphocytes has not been recorded in the literature. The findings reported in Table 26 show a mean lymphocyte DNA content of 0.73 µg DNAP per 10^6 cells.

The thoracic duct lymphocytes have a RNAP/DNAP ratio which is lower than the average ratio in normal blood lymphocytes. This is as anticipated from the knowledge of the high proportion of small lymphocytes in the thoracic duct lymph of various species (Elves, 1966) ranging from seventy to ninety-five per cent in different mammals.

Interpretation of the results shown in the section which reports serial monitoring of the thoracic duct lymphocytes in patients requires a knowledge of the effect of azathioprine and prednisone on nucleic acid metabolism and on the lymphocyte population as a whole.

Azathioprine inhibits synthesis of both DNA and RNA by the action of its metabolite 6-mercaptopurine which forms 6-mercaptopurine ribotide. This acts by mimicking inosinic acid and inhibiting the early reactions leading to the synthesis of

inosinic acid, the so called end product inhibition of the synthesis of phosphoribosylamine. 6-mercaptopurine ribotide also interferes with inter-conversions of the purine ribonucleotides (Berenbaum, 1967). The net effect of azathioprine is to inhibit nucleic acid synthesis. It is unclear which of the two, DNA or RNA synthesis, is affected most but it is likely that where active cell division is minimal the effect would be greatest on RNA synthesis. Although the effect of azathioprine on human thoracic duct lymphocytes reported here is a minor one, the RNAP/DNAP ratio of the lymphocytes showed a significant increase as azathioprine was first administered. This change is difficult to understand unless azathioprine alters the distribution of small and large lymphocytes perhaps by inhibiting release of the recirculating small lymphocytes from lymph nodes or, as in the case with steroids, small lymphocytes are more susceptable to the effects of the drug than are large lymphocytes.

The increase in the thoracic duct lymphocyte RNAP/DNAP ratio, as prednisone is first given to the patients, is considered to be an expression of changes in the cell types in the thoracic duct lymph at this time. Dougherty, Berliner and Berliner (1960) showed that hydrocortisone produces a preferential dissolution of the small lymphocyte, large lymphocytes being resistant to the steroid. It is clear from the literature that DNA as well as RNA synthesis is inhibited <u>in vitro</u> by moderate levels (30 µg per 100 ml) of prednisolone (Tormey, Fudenberg and Kamin, 1967). Depending on

the rate of inactivation of the drug this level may be within the tissue concentration produced by the therapeutic administration of 1 g prednisolone given intravenously over two hours, the technique used to treat acute rejection crises. It is relevant to the measurements carried out on patients on long term steroid therapy, for example some patients with rheumatoid arthritis, that an elevated lymphocyte RNAP/DNAP was not sustained on continued and more usual dose levels of prednisolone. Steroids do however depress lymphocyte function and cell mediated reactions of the delayed hypersensitivity type are depressed to a far greater extent by steroids than are antibody mediated reactions (Gabrielson and Good, 1967).

The nature of lymphocyte RNA.

The observations recorded in Section 4.12 on the nature of lymphocyte RNA require interpretation in the light of present understanding of the process of RNA biosynthesis reviewed recently by Burdon (1971). The rapid incorporation of labelled Uridine into material which sediments at 40s to 50s RNA (as was observed in lymphocytes) is typical of a cell synthesising ribosomal RNA. Although Cooper (1969) believes that lymphocytes may show an unusually rapid degradation of ribosomal RNA once it is formed, there is no reason to believe that the molecular species

synthesised are unusual. Other authors (Monjardino and MacGillivray, 1968) have recorded increased uptake of labelled precursor into ribosomal RNA under the influence of phytohaem-agglutinin and the label which is rapidly incorporated in the region of 4s RNA is now recognised as likely to be precursor to transfer RNA (Burdon, 1971). RNA extracted from whole lymphocytes has a high percentage of the nucleotides, guanylic acid and cytidylic acid, the two being 65 per cent of the total nucleotides present (Kiss, Astaldi and Airo, 1967). These percentages are typical of mammalian ribosomal RNA.

It was pointed out by Monjardino and MacGillivray (1968) that the unusual feature of the lymphocyte is its ability to respond to stimuli which may be specific antigen or non-specific such as phytohaemagglutinin. The cells respond by increasing their synthesis of ribosomal RNA thereby becoming equipped for accelerated protein synthesis and are therefore ideally suited for proliferation in a cellular immune or antibody response.

SECTION 5

Concluding Discussion

5.1 Concluding discussion

It is clear from what has been presented earlier that the average RNAP/DNAP ratio of the cells isolated by the gelatin technique is increased in certain diseases associated with immune reactions, a finding which supports and extends previous investigations of the nature of the blood lymphocytes during the immune response. Interest centres round two questions which have emerged during work, firstly what is the nature of the change which has produced the altered RNAP/DNAP ratio, and secondly is there a difference in degree of abnormality between immune reactions involving antibody production and immune reactions in which there is a cellular immune response.

The biochemical approach to the assessment of the lymphocytes, an advantage in producing an objective measurement of the cells, has meant that the morphology of the cells has been less intensively studied. Miller (1969) has listed the cell types likely to be present in the peripheral blood and of importance in immune reactions. Progenitor or stem cells are the first of these types and although these are increased following immune stimuli, they constitute less than one per cent of the mononuclear cells. Monocytes are of importance as mobile macrophages, participating in the cellular immune infiltrates of delayed hypersensitivity reactions, but they may be difficult to distinguish from the

quantitatively more important lymphocytes. Small lymphocytes are the most easily recognised of the circulating mononuclear cells and the proportion of these cells in the blood has been shown to fall when the RNAP/DNAP ratio of the gelatin isolated cells is increased. The proportion of mononuclear cells other than small lymphocytes must therefore increase in these circumstances and it is appropriate to consider the extent of the changes observed.

In response to quite minor stimuli, for example the injection of diptheria toxoid, pertussis antigen, or typhoid paratyphoid antigen, Pariser et al. (1952) and Crowther et al. (1969) have demonstrated up to six per cent of atypical mononuclear cells in the peripheral blood, usually around the sixth day following the stimulus. The present technique, RNAP/DNAP ratio determination, has revealed substantial changes in the lymphocyte population in rheumatoid arthritis, Sjøgren's syndrome, lupus erythematosus, neoplasm and during renal allograft rejection. The degree of change is best illustrated by considering a simple numerical example. If ten per cent of the lymphocytes double their RNA content, going from the normal RNAP/DNAP ratio of 0.30 to a ratio of 0.60, then the mean lymphocyte RNAP/DNAP ratio changes only from 0.30 to 0.33, that is $\frac{(90 \times 0.30) + (10 \times 0.60)}{100} = 0.33$.

It is similarly apparent that when the mean lymphocyte RNAP/DNAP increases to 0.50 a ninefold increase in the RNA content would be required in ten per cent of the cells. It seems likely then

that more than ten per cent of the cells are altered when the mean lymphocyte RNAP/DNAP ratio climbs to a high value and that a change in the proportion of large, medium and small lymphocytes in the blood accounts for this rather than the introduction of a small number of atypical cells. It is for this reason that the term 'lymphocyte RNAP/DNAP ratio' has been used throughout the study. In addition, the calculations throw some light on the sensitivity of the test and would suggest that changes in lymphocyte chemistry involving a small fraction of the cells could occur undetected.

One of the features which is apparent in a general view of the results is the association between reports of depressed blood lymphocyte response to phytohaemagglutinin *in vitro* and the observation of significant increase in the mean blood lymphocyte RNAP/DNAP ratio. This is the case in rheumatoid arthritis, Sjögren's syndrome and neoplasm, three out of the four conditions in which a substantial elevation in blood lymphocyte RNAP/DNAP ratio was found. Allograft rejection is left out here because of the complication of immunosuppression. In the fourth condition with an elevated blood lymphocyte RNAP/DNAP ratio, lupus erythematosus, the phytohaemagglutinin response is said to be normal but there must be reservations about this finding since it depends on observations by a single group of investigators, Patrucco et al. (1967), who also failed to show a significantly abnormal response in a smaller series of patients with rheumatoid

arthritis. If it is accepted that there may well be a correlation between an impaired phytohaemagglutinin response and an elevated lymphocyte RNAP/DNAP ratio, what does this imply? Davies (1969), in his review of the thymus and the cellular basis of immunity, is satisfied that the phytohaemagglutinin response is principally a function of thymus dependent cells, and it may be that the elevated lymphocyte RNAP/DNAP ratio in three at least of the above conditions has arisen from depletion of thymic dependent cells. The remaining lymphocytes would presumably be B-lymphocytes or perhaps T-lymphocytes already in the process of transformation and therefore not responsive to phytohaemagglutinin. For whatever reason it is clear that a considerable change in the lymphocyte population must occur to account for the high values of lymphocyte RNAP/DNAP ratio which have been observed.

Evidence that the thymus dependent T-lymphocytes are at least relatively depleted in rheumatoid arthritis has come from the detection of immunoglobulins bound to the surface of lymphocytes in rheumatoid arthritis. This technique outlined earlier (Papamichail et al., 1971) has shown that B-lymphocytes are a greater than normal proportion of the blood lymphocytes in the disease. What this means in terms of the relative importance of antibody and cellular immunity is uncertain but it is reasonable to question whether alterations in the proportions of B- and T-lymphocytes in rheumatoid arthritis and perhaps in other conditions would reflect on the mean lymphocyte RNAP/DNAP ratio. The

possibility that B-lymphocytes might have more RNA on average than T-lymphocytes remains open.

The second question, that of a difference in degree of change in the RNAP/DNAP ratio between cellular immune and antibody reactions, is inevitably linked to the first question, the nature of the change itself. Quite naturally one may not devise separate experimental tests of these responses in man and clues from the study of pathology are correspondingly more valuable.

The most reliable example of a cellular immune response occurs in the study of renal transplant patients. A single patient (AP in Table 23) had a rejection which met Porter's strict criteria (1967) for an early acute, and therefore cellular rejection. In this patient the lymphocyte RNAP/DNAP ratio reached a high value, 0.50, at the time of rejection. Other patients with a renal allograft had similar elevations of the ratio prior to rejection and probably illustrate cellular rejection, but since they do not meet Porter's criteria of acute and early rejection the data are less conclusive. Indirect evidence that many of these episodes are examples of cellular rejection has come from the two rejection episodes in which the lymphocyte RNAP/DNAP ratio remained normal. These suggest that two different types of rejection occur in patients on immunosuppressive therapy; the type with the evidence of metabolically active lymphocytes, that is the elevated RNAP/DNAP ratio, may be associated with cell mediated rejection. If however an elevated ratio implies, depleted T-lymphocytes as was

asserted earlier, then this depletion could have arisen from transformation of T-lymphocytes under the influence of graft antigens with subsequent accumulation of these cells in the graft.

Only minor changes are to be found in the lymphocyte RNAP/DNAP ratio when the immune response results principally in antibody production. The study of infection was not ideal in this respect since the timing of the blood samples was not always optimum and the response not necessarily specifically directed to antibody production. It is notable that the lymphocyte RNAP/DNAP ratio remained normal following typhoid paratyphoid immunisation, but more convincing was the observation that no significant elevation of the lymphocyte RNAP/DNAP ratio occurred in sicca syndrome. Here the main abnormality is in antibody production. It appears that antibody reactions do not produce sufficient change in the blood lymphocytes to alter their RNAP/DNAP ratio.

It is perhaps useful to try to summarise this discussion in the form of a working hypothesis and the following is proposed:

An elevated blood lymphocyte RNAP/DNAP ratio arises in the course of a cell mediated immune reaction due to a transformation-like response in the T-lymphocytes which are later gradually sequestered from the circulation. There is then a functional impairment of T-lymphocytes due to lack of uncommitted cells and

therefore a relative increase of B-lymphocytes in the blood.

The original aim of the work was to arrive at an objective means of assessment of the blood lymphocytes, and this has been achieved. The measurement of lymphocyte nucleic acids would be greatly increased in usefulness if it could be shown that a high lymphocyte RNAP/DNAP ratio distinguishes cellular immune reactions, but this point remains to be established. Increasing use of the immunofluorescent technique to identify B-lymphocytes would contribute here as would examination of the blood lymphocyte RNAP/DNAP ratio in experimental cell mediated reactions.

If measurement of the lymphocyte RNAP/DNAP ratio is to be developed into a routine test, a speeding up and simplification of the analytical techniques would be desirable. The rapid isolation of lymphocytes by flotation on a Ficoll-Isopaque mixture would be an obvious choice for cell isolation (Bøyum, 1968) and an automated technique for determination of nucleic acid by fluorimetry described by Van Dyke and Szustkiewicz (1968) holds promise of a rapid assay procedure.

CONCLUSIONS

Conclusions

6.1 The normal values in man for the lymphocyte nucleic acid content, using the most satisfactory cell separation technique, are as follows: DNA phosphorus, 0.60 to 1.00 μg per 10^6 cells
RNA phosphorus, 0.16 to 0.30 μg per 10^6 cells
RNAP/DNAP ratio, 0.22 to 0.37.

6.2 The mean blood lymphocyte DNA phosphorus 0.74 μg per 10^6 cells was similar in normal individuals and in patient groups. The value is close to the diploid amount for man. There were only minor exceptions to this rule, and in general it is valid to use the ratio RNAP/DNAP as a measure of the lymphocyte RNA content. This ratio is proposed as an objective assessment of the blood lymphocytes.

6.3 When groups of patients are considered the mean RNAP/DNAP ratio of the blood lymphocytes was shown to be significantly increased in patients with the following conditions; rheumatoid arthritis, Sjøgren's syndrome, lupus erythematosus and neoplasm.

6.4 The mean blood lymphocyte RNAP/DNAP ratio was increased in patients with infection, thyrotoxicosis and sicca syndrome.

This increase was less substantial than in conditions listed under 6.3. A statistically valid difference from normal could be shown in relation to only one group of normal results in patients with infection, only when LATS negative patients were eliminated in the thyrotoxic group, and not at all in patients with sicca syndrome.

6.5 Patients with no known immunological abnormality had normal results for the lymphocyte RNAP/DNAP ratio.

6.6 Factors which might influence the lymphocyte RNAP/DNAP ratio including severity of the disease, the blood lymphocyte count and therapy have been considered. In rheumatoid arthritis an elevated lymphocyte RNAP/DNAP ratio is most consistently related to severity of the disease.

6.7 Patients with a renal allograft on immunosuppressive therapy have a mean blood lymphocyte RNAP/DNAP ratio which is similar to that found in normal untreated individuals provided the patients are not showing evidence of infection, over-immunosuppression, or graft rejection.

6.8 The mean blood lymphocyte RNAP/DNAP ratio is frequently elevated in patients with a renal allograft when they show evidence of infection or graft rejection. These two may

usually be distinguished by the lymphopenia associated with graft rejection. Similarly, a clinically important distinction between lymphopenia associated with graft rejection and lymphopenia arising from over-immunosuppression is made by measuring the lymphocyte RNAP/DNAP ratio; the ratio is low when there is excessive immunosuppression and elevated when there is rejection.

6.9 While the lymphocyte RNAP/DNAP ratio may become abnormal some days in advance of an acute rejection reversible with steroid therapy, rejection may also occur in the presence of a normal blood lymphocyte RNAP/DNAP ratio. The experience has been that these latter rejections do not respond well to steroid treatment.

6.10 There is evidence drawn from the entire study that the lymphocyte RNAP/DNAP ratio may be more markedly elevated when there is a cellular immune response than when there is an antibody response alone.

REFERENCES

Adams, D.D., Purves, H.D. (1956) Abnormal responses in the assay of thyrotrophin. Proc. Univ. Otago med. Sch. 34, 11.

Alexander, W.R., Bremner, J.M., Duthie, J.J. (1960) Incidence of the anti-nuclear factor in human sera. Ann. rheum. Dis. 19, 338.

Allen, R.J.L. (1940) Estimation of phosphorus. Biochem. J. 34, 858.

Altman, B. (1963) Tissue transplantation, circulating antibody in the homotransplantation of kidney and skin. Ann. R. Coll. Surg. 33, 79.

Anderson, J.R., Buchanan, W.W., Goudie, R.B. (1967) In Autoimmunity, clinical and experimental. Thomas, Springfield, Illinois.

Anderson, J.R. (1963) Autoantibodies in diseases of man. Br. med. Bull. 19, 251.

André, Janine A., Schwartz, R.S., Mitus, W.J., Dameshek, W. (1962A) The morphologic responses of the lymphoid system to homografts. 1. First and second set responses in normal rabbits. Blood 19, 313.

André, Janine A., Schwartz, R.S., Mitus, W.J., Dameshek, W. (1962B) The morphologic responses of the lymphoid system to homografts. 2. The effects of antimetabolites. Blood 19, 334.

Armitage, P. (1971) Statistical methods in medical research. Blackwell, Oxford.

Barnes, J.M. (1940) The enzymes of lymphocytes and polymorphonuclear leucocytes. Brit. J. exp. Path. 21, 264.

Ballantyne, B., Wood, W.G., Meffan, P.M. (1968) Sequential analysis of urinary enzymes in early diagnosis of parenchymal damage after renal homotransplantation. Br. med. J. 2, 667.

Benezra, D., Grey, I., Davies, A.M. (1969) The relationship between lymphocyte transformations and immune responses. 2. Correlation between transformation and humoral and cellular immune responses. Clin. exp. Immunol. 5, 155.

Berenbaum, M.C. (1967) Immunosuppressive agents and allogenic transplantation. J. clin. Path. 20, 471.

Beutner, E.H., Jordon, R.E., Chorzelski, T.P. (1968) The immunopathology of phemphigus and bullous pemphigoid. J. invest. Derm. 51, 63.

Billingham, R.E., Silvers, W.K., Wilson, D.B. (1962) Adoptive transfer of transplantation immunity by means of blood borne cells. Lancet 1, 512.

Billingham, R.E., Brent, L., Medawar, P.W., Sparrow, E.M. (1954) Quantitative studies on tissue transplantation immunity. 2. The origin strength and duration of actively and adoptively acquired immunity. Proc. R. Soc. 143, 58.

Birbeck, M.S.C., Hall, J.G. (1967) Transformation in vivo of basophilic lymph cells into plasma cells. Nature (Lond.) 214, 183.

Bloch, K.J., Buchanan, W.W., Wohl, M.J., Bunim, J.J. (1965) Sjøgren's syndrome. A clinical, pathological and serological study of sixty-two cases. Medicine (Baltimore) 44, 187.

Bond, V.P., Cronkite, E.P., Fliedner, T.M., Schork, P. (1958) Deoxyribonucleic acid synthesising cells in peripheral blood in human beings. Science 128, 202.

Borell, U., Holmgren, H.J. (1948) The effect of methylthiouracil on the oxygen consumption of the thyroid, liver and kidneys. Endocrinology 42, 427.

Botha, M.C. (1969) Leucocyte-antigen matching in donor selection for heart transplantation. Lancet 2, 508.

Bøyum, A. (1968) Separation of leukocytes from blood and bone marrow. Scand J. Clin. Lab. Invest. 21, suppl. 97.

Braverman, L.E., Ingbar, S.H. (1962) Effects of propylthiouracil

and thiouracil on the metabolism of thyroxine and several of its derivatives by rat kidney slices in vitro. Endocrinology 71, 701.

Brent, L., Medawar, P.B. (1967) Cellular immunity and the homograft reaction. Br. med. Bull. 23, 55.

Broberger, O., Perlmann, P. (1959) Autoantibodies in human ulcerative colitis. J. exp. Med. 110, 657.

Bruton, O.G. (1952) Agammaglobulinaemia. Paediatrics 9, 722.

Buchanan, W.W., Alexander, W.D., Crooks, J., Koutras, D.A., Wayne, E.J., Anderson, J.R., Goudie, R.B. (1961) Association of thyrotoxicosis and auto-immune thyroiditis. Br. med. J. 1, 843.

Buchanan, W.W., Koutras, D.A., Crooks, J., Alexander, W.D., Brass, W., Anderson, J.R., Goudie, R.B., Gray, K.G. (1962) The clinical significance of the complement fixation test in thyrotoxicosis. J. Endocr. 24, 115.

Buchanan, W.W., Crooks, J., Alexander, W.D., Koutras, D.A., Wayne, E.J., Gray, K.G. (1961) Association of Hashimoto's thyroiditis and rheumatoid arthritis. Lancet 1, 245.

Buckton, K.E., Court-Brown, W.M., Smith, P.G. (1967) Lymphocyte survival in men treated with X-rays for ankylosing spondylitis. Nature (Lond.) 214, 470.

Burdon, R.H. (1971) Ribonucleic acid maturation in animal cells. In Brogress in nucleic acid research 11. Academic Press, New York.

Burke, G. (1968) Experimental production of long-acting thyroid stimulator *in vivo*. J. Lab. clin. Med. 72, 17.

Carneiro, L., Dorrington, K.J., Munro, D.S. (1966) Relation between long acting thyroid stimulator and thyroid function in thyrotoxicosis. Lancet 2, 878.

Ceriotti, G. (1955) Determination of nucleic acid in animal tissues. J. biol. Chem. 214, 59.

Chalmers, D.G. (1967) The use of gelatin in the separation of human peripheral blood lymphocytes. Research publication 68. Gelatin and Glue research association.

Chase, M.W. (1945) The cellular transfer of cutaneous hypersensitivity to tuberculin. Proc. Soc. exp. Biol (N.Y.) 59, 134.

Chisholm, G.D., Papadimitriou, M., Kulatilake, A.E., Shackman, R. (1969) The diagnosis of rejection of renal allografts in man. Lancet 1, 904.

Claman, H.N., Chaperon, E.A. (1969) Immunological complementation between thymus and marrow cells - A model for the two cell theory of immunocompetence. Transplant. Rev. 1, 92.

Clawson, C.C., Cooper, M.D., Good, R.A. (1966) Comparison of the fine structure of the bursa of Fabricius, the thymus and the germinal centre. Fed. Proc. 25, 309.

Cooper, I.A., Firkin, B.G. (1965) Deoxyribonucleic acid synthesising cells in the peripheral blood of patients with autoimmune disorders. Australas. Ann. Med. 14, 142.

Cooper, H.L. (1967) Personal communication.

Cooper, H.L. (1969) Ribosomal ribonucleic acid wastage in resting and growing lymphocytes. J. biol. Chem. 244, 5590.

Cooper, H.L., Rubin, A.D. (1965) RNA metabolism in lymphocytes stimulated by phytohaemagglutinin : initial responses to phytohaemagglutinin. Blood 25, 1014.

Cooper, H.L., Rubin, A.D. (1966) Synthesis of non ribosomal RNA by lymphocytes. Science 152, 516.

Cooper, H.L., Kay, J.E. (1969) Differential extraction of nuclear and cytoplasmic RNA from intact lymphocytes. Biochim. biophys. Acta 174, 503.

Coulson, A.S., Chalmers, D.G. (1964) Separation of viable lymphocytes from human blood. Lancet 1, 468.

Crowther, D., Fairley, G.H., Sewell, R.L. (1969) Lymphoid cellular responses in the blood after immunisation in man. J. exp. Med. 129, 849.

Cruickshank, J.M., Alexander, M.K. (1970) The effect of age, sex, parity, haemoglobin level and oral contraceptive preparations on the normal leucocyte count. Br. J. Haematol. 18, 541.

Davidson, J.N. (1957) Some critical comments on the chemical estimation of nucleic acids in tissues. Expl Cell Res. Supplement 4, 260.

Davidson, J.N., Leslie, I., White, J.C. (1951) Quantitative studies on the content of nucleic acids in normal and leukaemic cells from blood and bone marrow. J. Path. Bact. 63, 471.

Davidson, J.N., Smellie, R.M.S. (1952) Phosphorus compounds in the cell, 3. Biochem. J. 52, 599.

Davidson, J.N., Fraser, S.C., Hutchison, W.C. (1951) Phosphorus compounds in the cell, 1. Biochem. J. 49, 311.

Davies, A.J.S., Leuchars, E., Wallis, V., Marchant, R., Elliot, E.V. (1967) The failure of thymus derived cells to produce antibody. Transplantation 5, 222.

Davies, A.J.S. (1969) The thymus and the cellular basis of immunity. Transplant. Rev. 1, 43.

De Groot, L.J., Jaksina, Stephanie (1969) Observation on the role of circulating lymphocytes in thyroid auto-immunity. J. clin. Endocr. 29, 207.

Diehl, V., Henle, Gertrude, Henle, W., Kohn, Gertrude (1968) Demonstration of herpes group virus in cultures of peripheral leukocytes from patients with infectious mononucleosis. J. Virol. 2, 663.

Di George, A.M. (1965) Proceedings of the society for pediatric research. J. Paediat. 67, 907.

Digirolamo, A., Henshaw, E.C., Hiatt, H.H. (1964) Messenger ribonucleic acid in rat liver nuclei and cytoplasm. J. molec. Biol. 8, 479.

Doll, R., Kinlin, L. (1970) Immunosurveillance and cancer : epidemiological evidence. Br. med. J. 2, 420.

Doniach, Debora, Roitt, I.M. (1963) Auto-antibodies in disease. A. Rev. Med. 13, 213.

Dorrington, K.J., Munro, D.S. (1965) Immunological studies on the long acting thyroid stimulator. Clin. Sci. 28, 165.

Dougherty, T.F., Frank, J.A. (1953) The quantitative and qualitative response of blood lymphocytes to stress stimuli. J. Lab. clin. Med. 42, 530.

Dougherty, T.F., Berliner, M.L., Berliner, D.L. (1960) Hormonal influence on lymphocyte differentiation from RES cells. Ann. N.Y. Acad. Sci. 88, 78.

Duthie, J.J.R., Brown, P.E., Truelove, L.R., Barger, F.D., Lawrie, A.J. (1964) Course and prognosis in rheumatoid arthritis, a further report. Ann. rheum. Dis. 23, 193.

Eastham, R.D. (1970) In Clinical Haematology. John Wright & Sons, Bristol.

Einhorn, J., Fagraeus, Astrid, Jonsson, J. (1965) Thyroid antibodies after treatment I^{131} for hyperthyroidism. J. clin. Endocr. 25, 1218.

Elves, M.W. (1966) In The lymphocytes. Lloyd-Luke, London.

Epstein, W.L., Jessar, R.A. (1959) Contact-type delayed hypersensitivity in patients with rheumatoid arthritis. Arthr. rheum. 2, 178.

Epstein, W.L. (1967) Granulomatous hypersensitivity. Prog. Allergy 11, 36.

Everett, N.B., Caffrey, R.W., Rieke, W.O. (1964) Recirculation of lymphocytes. Ann. N.Y. Acad. Sci. 113, 887.

Ferguson-Smith, M.A. (1970) Personal communication.

Fleck, A., Munro, H.N. (1962) The precision of ultraviolet absorption measurements in the Schmidt-Thannhauser procedure for nucleic acid estimation. Biochim. biophys. Acta 55, 571.

Ford, W.L., Gowans, J.L. (1969) The traffic of lymphocytes. Seminars in Haematology 6, 67.

Fox, R.A., James, D.G., Scheuer, P.J., Sharma, O., Sherlock, Sheila, (1969) Impaired delayed sensitivity in primary biliary cirrhosis. Lancet 1, 959.

Frank, J.A., Dougherty, T.F. (1953) The assessment of stress in human subjects by means of quantitative and qualitative changes in blood lymphocytes. J. Lab. clin. Med. 42, 538.

Froland, S., Natvig, J.B., Berdal, P. (1971) Surface-bound immunoglobulin as a marker of B lymphocytes in man. Nature New Biology 234, 251.

Fulginiti, V.A., Hathaway, W.E., Pearlman, D.S., Blackburn, W.R., Reiquam, C.W., Githens, J.H., Claman, H.N., Kempe, C.H. (1966) Dissociation of delayed hypersensitivity and antibody synthesising capacities in man. Lancet 2, 5.

Gabrielsen, A.E., Good, R.A. (1967) Chemical suppression of adaptive immunity. Adv. Immun. 6, 91.

Glen, A.C.A. (1967) Measurement of DNA and RNA in human peripheral blood lymphocytes. Clin. Chem. 13, 299.

Glick, B., Chang, T.S., Jaap, R.G. (1956) The bursa of Fabricius and antibody production. Poultry Sci. 35, 224.

Glynn, L.E. (1965) Autoimmunity in rheumatoid arthritis. In Autoimmunity, Symposium of the 5th Congress of the International Academy of Pathology. Blackwell, Oxford.

Golob, E.K., Israsena, T., Quatrale, A.C., Becker, K.L. (1969) Effect of serum from cancer patients on homologous lymphocyte cultures. Cancer (Philad.) 23, 306.

Good, R.A., Kelly, W.D., Rotstein, J., Varco, R.L. (1962) Immunological deficiency diseases. Prog. Allergy 6, 187.

Gorer, P.A. (1937) The genetic and antigenic basis of tumour transplantation. J. Path. Bact. 44, 697.

Gowans, J.L. (1962) The fate of parental strain small lymphocytes in F1 hybrid rats. Ann. N.Y. Acad. Sci. 99, 432.

Gowans, J.L., McGregor, D.D. (1965) The immunological activities of the lymphocytes. Prog. Allergy 9, 1.

Gowans, J.L. (1959) The recirculation of lymphocytes from blood to lymph in the rat. J. Physiol. (Lond.) 146, 54.

Greig, W.R. (1966) The treatment of thyrotoxicosis with radio-active iodine. Scot. med. J. 11, 307.

Hale, A.J., Wilson, Sylvia J. (1961) The desoxyribonucleic acid content of the leucocytes in human blood, bone marrow and lymph glands. J. Path. Bact. 82, 483.

Hale, A.J., Wilson, Sylvia J. (1959) The DNA content of leukocytes in normal and in leukaemic human blood. J. Path. Bact. 77, 605.

Hale, A.J. (1963) The leucocyte as a possible exception to the theory of deoxyribonucleic acid constancy. J. Path. Bact. 85, 311.

Hall, J.G. (1967) Studies of the cells in the afferent and efferent lymph of lymph nodes draining the site of skin homografts. J. exp. Med. 125, 737.

Hall, J.G., Morris, B., Moreno, G.D., Bessis, M.D. (1967) The ultra-structure and function of the cells in the lymph following antigenic stimulation. J. exp. Med. 125, 91.

Hallinan, T., Fleck, A., Munro, H.N. (1963) Loss of ribonucleic acid into lipid solvents after acid precipitation. Biochim. Biophys. Acta 68, 131.

Hannestad, K. (1968) Rheumatoid factors reacting with autologous native γ G globulin and joint fluid γ G aggregates. Clin. exp. Immunol. 3, 671.

Harris, Jean, Vaughan, J.H. (1961) Transfusion studies in rheumatoid arthritis. Arthr. rheum. 4, 47.

Haserick, J. (1965) In report of the proceedings of the Chicago Dermatological Society. Arch. Dermatol. 92, 329.

Havard, C.W.H. (1969) The aetiology and management of thyrotoxicosis. Abstracts of World Medicine 43, 629.

Heiniger, H.J., Riedwyl, H., Giger, H., Sordat, B., Cottier, H. (1967) Ultrastructural differences between thymic and lymph node small lymphocytes of mice : nucleolar size and cytoplasmic volume. Blood 30, 288.

Hernberg, C.A. (1954) Thyrotoxicosis and the size of lymphocytes. Acta med. scand. 149, 37.

Hersh, E.M., Butler, W.T., Rossen, R.D., Morgan, R.O. (1970) Lymphocyte activation : a rapid test to predict allograft rejection. Nature (Lond.) 226, 757.

Hirschhorn, K., Ripps, Carolyn S. (1965) Lymphocyte interaction in vitro. In Iso-antigens and cell interactions. Wistar Institute Symposium Monograph 3. Wistar Institute Press, Philadelphia.

Hors, J., Feingold, N., Fradelizi, D., Dausset, J. (1971) Critical evaluation of histocompatibility in 179 renal transplants. Lancet 1, 609.

Hughes, L.E., Mackay, W.D. (1965) Suppression of the tuberculin response in malignant disease. Br. med. J. 2, 1346.

Hurd, E.R., Ziff, M. (1968) Studies on the anti inflammatory action of 6-mercaptopurine. J. exp. Med. 128, 785.

Hutchison, W.C., Munro, H.N. (1961) The determination of nucleic acids in biological materials. Analyst, Lond. 86, 768.

Irvine, W.J., Muir, A.R. (1963) An electron microscopic study of Hashimoto thyroiditis. Quart. J. exp. Physiol. 48, 13.

Iwao, N. (1968) Lymphocytes of the peripheral blood of carcinoma bearing patients with special reference to lymphocytopenia and function of the lymphocytes (Japanese). Shikoku Acta Med. 24, 564.

Jacob, S.T., Cooper, W.C., Glen, A.C.A., Munro, H.N. (1969) Synthesis of ribonucleic acid in human lymphocytes. In Proceedings of the 3rd Annual leucocyte culture conference. Appleton-Century-Crofts, New York.

Jager, B.V., Nickerson, M. (1947) The altered response of human beings to the intramuscular administration of typhoid vaccine during massive salicylate therapy. Am. J. Med. 3, 408.

Johns, P. (1970) The use of gelatin in the separation of lymphocytes. Gelatin and Glue research association. Research panel paper 76.

Joseph, N.H. (1966) Phytohaemagglutinin cultures of blood cells from renal homotransplant patients. Transplantation 4, 8.

Kay, J.E. (1966) RNA and protein synthesis in lymphocytes incubated with phytohaemagglutinin. In Proceedings of the Symposium :the biological effects of phytohaemagglutinin. Edited by M.W. Elves, Orthopaedic hospital management committee, Oswestry, England.

Keast, D. (1970) Immunosurveillance and cancer. Lancet 2, 710.

Kiss, K., Astaldi, G., Airo, O. (1967) The RNA nucleotide composition in human leucocytes from normal and leukaemic cases. Blood 30, 307.

Klein, G. (1970) Immunological factors affecting tumour growth. Br. med. J. 4, 418.

Kriss, J.P., Pleshakov, V., Chien, J.R. (1964) Isolation and identification of the long acting thyroid stimulator and its relation to hyperthyroidism and circumscribed pretibial myxoedema. J. clin. Endocr. 24, 1005.

Kristenson, A. (1949) The variation in size (the lymphocyte profile) of the lymphocytes circulating in the blood in some normal and pathologic conditions. Acta med. scand. 133, 157.

Lajtha, L.G., Oliver, R., Ellis, F. (1954) Incorporation of ^{32}P and Adenine ^{14}C into DNA by human bone-marrow cells in vitro. Br. J. Cancer 8, 367.

Lambert, P.H., Dixon, F.J. (1969) Pathogenesis of the glomerulonephritis of NZB/W mice. J. exp. Med. 130, 1093.

Landsteiner, K., Chase, M.W. (1942) Experiments on transfer of cutaneous sensitivity to simple compounds. Proc. Soc. exp. Biol. (N.Y.) 49, 688.

Lesiewska, J. (1967) Nucleic acids in the lymphocytes of the peripheral blood in the course of some infectious diseases in children. Folia histochem. and cytochem. 5, 297.

Leslie, I. (1955) The nucleic acid content of tissues and cells. In The Nucleic Acids, vol. 2. Eds. Chargaff, E., Davidson, J.N., Academic press, New York.

Leventhal, Brigid G., Waldorf, D.S., Talal, N. (1967) Impaired lymphocyte transformation and delayed hypersensitivity in Sjögren's syndrome. J. clin. Invest. 46, 1338.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. biol. Chem. 193, 265.

MacGregor, R.G.S., Richards, W., Loh, G.L. (1940) The differential leucocyte count. J. Path. Bact. 51, 337.

McKenzie, J.M. (1964) Neonatal Graves' disease. J. clin. Endocr. 24, 660.

Maxwell, J.D., Ferguson, Anne, McKay, A.M., Imrie, R.C., Watson, W.C. (1968) Lymphocytes in Whipples Disease. Lancet 1, 887.

Métais, P., Mandel, P. (1950) Teneur en acids desoxypentose-nucléique des leucocytes chez l'homme normal et à l'état pathologique. C.r. Seanc. Soc. Biol. 144, 277.

Metcalf, D. (1967) Lymphocyte kinetics in the thymus. In The lymphocyte in immunology and haemopoiesis. Arnold, London.

Meuwissen, H.J., Stutman, O., Good, R.A. (1969) Functions of the lymphocytes. Seminars in Haematology 6, 28.

Michie, W., Beck, J.S., Mahaffy, R.G., Honein, E.F., Fowler, G.B. (1967) Quantitative radiological and histological studies of the thymus in thyroid disease. Lancet 1, 691.

Milgrom, F., Litvak, B.I., Kano, K., Witebsky, E. (1966) Humoral antibodies in renal homograft. J. Am. med. Ass. 198, 266.

Miller, J.F.A.P. (1969) The role of blood cells in immunity. Br. J. Haemat. 16, 331.

Miller, J.F.A.P., Mitchell, G.F. (1969) Thymus and antigen-reactive cells. Transplant. Rev. 1, 3.

Miller, J.F.A.P., Osoba, D. (1967) Current concepts of the immunological function of the thymus. Physiol. Rev. 47, 437.

Mitchison, N.A. (1954) Passive transfer of transplantation immunity. Proc. R. Soc. 142, 72.

Monjardino, J.P.P.V., MacGillivray, A.J. (1968) Rapidly labelled ribonucleic acid in short-term lymphocyte cultures *in vitro*. Biochem. J. 108, 22p.

Moller, G., Moller, Erna (1967) Humoral and cell-mediated effector mechanisms in tissue transplantation. J. clin. Path. 20, 437.

Mowbray, J.F. (1966) Methods of suppression of immune responses. Excerpta Med. International Congr. 137, 106.

Mowbray, J.F. (1970) The problem of rejection. Practitioner 205, 181.

Murray, J.E., Wilson, R.E., Tilney, N.L., Merril, J.P., Cooper, W.C., Birtch, A.G., Carpenter, C.B., Hager, E.B., Dammin, G.J., Harrison, J.H. (1968) Five years' experience in renal transplantation with immunosuppressive drugs : survival, function, complications and the role of lymphocyte depletion by thoracic duct fistula. Ann. Surg. 168, 416.

Nossal, G.J.V., Cunningham, A., Mitchell, G.F., Miller, J.F.A.P. (1968) Cell to cell interaction in the immune response. III. Chromosomal marker analysis of single antibody-forming cells in reconstituted, irradiated or thymectomized mice. J. exp. Med. 128, 839.

Nowell, P.C. (1960) Phytohaemagglutinin : an initiator of mitosis in cultures of normal human leucocytes. Cancer Res. 20, 462.

Oort, J., Turk, J.L. (1965) A histological and autoradiographic study of lymph nodes during the development of contact sensitivity in the Guinea Pig. Br. J. exp. Path. 46, 147.

Papamichail, M., Brown, J.C., Holborow, E.J. (1971) Immunoglobulins on the surface of human lymphocytes. Lancet 2, 850.

Pariser, S., Zucker, R.A., Meyer, L.M. (1952) Haematologic changes associated with the immune response in man. Acta med. scand. 144, 201.

Parish, C.R. (1971) Immune response to chemically modified flagellin. Evidence for a fundamental relationship between humoral and cell mediated immunity. J. exp. Med. 134, 21.

Parker, Joan R., Mowbray, J.F. (1971) Peripheral blood leucocyte changes during human allograft rejection. Transplantation 11, 201.

Parker, Joan R. (1969) Ph.D. Thesis, University of London.

Parker, Joan R., Ellis, F., Cameron, J.S., Ogg, C., Mowbray, J.F. (1970) Detection of rejection of human renal allografts by increased RNA synthesis in peripheral lymphocytes. Proc. European dialysis and transplant Ass. 7, 331.

Parker, J.W., Lukes, R.J. (1969) The variability of PHA induced transformation in repetitive studies. In proceedings of the Third Annual Leucocyte Culture conference. Appleton-Century-Crofts, New York.

Parker, R.C. (1961) Determination of cell viability by dye exclusion tests. In methods of tissue culture. Pitman, London.

Parrott, Delphine M.V., de Sousa, M.A.B., East, J. (1966) Thymus dependent areas in the lymphoid organs of neonatally thymectomized mice. J. exp. Med. 123, 191.

Patrucco, Aida, Rothfield, Naomi, Hirschhorn, K. (1967) The response of cultured lymphocytes from patients with systemic lupus erythematosus to DNA. Arthr. rheum. 10, 32.

Pearson, E.S., Hartley, H.O. (1954) Biometrika tables for statisticians. Oxford University press.

Pisciotta, A.V., Westring, D.W., Deprey, Celia, Walsh, B. (1967) Mitogenic effect of phytohaemagglutinin at different ages. Nature (Lond.) 215, 194.

Pogo, B.G.T., Allfrey, V.G., Mirsky, A.E. (1966) RNA synthesis and histone acetylation during the course of gene activation. Proc. natn. Acad. Sci. U.S.A. 55, 805.

Porter, K.A., Joseph, N.H., Rendall, J.M., Stolinski, C., Hoehn, R.J., Calne, R.Y. (1964) The role of lymphocytes in the rejection of canine renal homotransplants. Lab. Invest. 13, 1080.

Porter, K.A. (1967) Rejection in treated allografts. J. clin. Path. 20, 518.

Rabinowitz, Y. (1964) Separation of lymphocytes, polymorphonuclear leukocytes and monocytes on glass columns, including tissue culture observations. Blood 23, 811.

Raff, M.C. (1971) Surface antigenic markers for distinguishing T and B lymphocytes in mice. Transplant. Rev. 6, 52.

Reich, C., Reich, E. (1933) A study of a lymphocytic hemogram. Am. J. med. Sci. 186, 278.

Restifo, R.A., Lussier, A.J., Rawson, A.J., Rockey, J.H., Hollander, J.L. (1965) Studies on the pathogenesis of rheumatoid joint inflammation. III. Experimental production of arthritis by the intra articular injection of purified 7s gamma globulin. Ann. Intern. Med. 62, 285.

Richterich, R. (1959) Clinical Chemistry. Academic Press, New York and London.

Rieke, W.O., Schwartz, M.R. (1966) The proliferation and immunological potential of thoracic duct lymphocytes from normal and thymectomized rats. In The lymphocyte in immunology and haemopoiesis. Ed. J.M. Yoffey Arnold, London.

Rigas, D.A., Duerst, Marie L., Jump, Margaret E., Osgood, E.E. (1956) The nucleic acids and other phosphorus compounds of human leukemic leukocytes : Relation to cell maturity. J. Lab. clin. Med. 48, 356.

Robbins, S.L. (1967) Pathology. Saunders, Philadelphia.

Robinson, S.H., Brecher, G., Lourie, S.I., Haley, J.E. (1965) Leukocyte labeling in rats during and after continuous infusion of tritiated thymidine :implications for lymphocyte longevity and DNA reutilisation. Blood 26, 281.

Roit, I.M., Greaves, M.F., Torrigiani, G., Brostoff, J., Playfair, J.H.L. (1969) The cellular basis of immunological responses. Lancet 2, 367.

Ropes, M.W. (1959) Diagnostic criteria for rheumatoid arthritis, 1958 revision. Ann. rheum. Dis. 18, 49.

Rubin, A.D. (1967) The human lymphocyte in short term tissue culture. Postgrad. med. 41, 244.

Sakai, H. (1971) Personal communication.

Sarles, H.E., Remmers, A.R., Fish, J.C., Canales, C.O., Thomas, F.D., Tyson, K.R.T., Beathard, G.A., Ritzmann, S.E. (1970) Depletion of lymphocytes for the protection of renal allografts. Archs intern. Med. 125, 443.

Schmidt, G., Thannhauser, S.J.A. (1945) A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoprotein in animal tissues. J. biol. Chem. 161, 83.

Schneider, W.C. (1945) Phosphorus compounds in animal tissues. Extraction and estimation of deoxy-pentose nucleic acid and of pentose nucleic acid. J. biol. Chem. 161, 293.

Scothorne, R.J., MacGregor, I.A. (1955) Cellular changes in lymph nodes and spleen following skin homografting in the rabbit. J. Anat. 89, 283.

Scothorne, R.J., Tough, J.S. (1952) Histochemical studies of human skin autografts and homografts. Br. J. Plast. Surg. 5, 161.

Seal, S.H. (1959) Silicone floatation: a simple quantitative method for the isolation of free floating cancer cells from blood. Cancer 12, 590.

Seegal, B.C., Accinni, L., Andres, G.A., Christian, C.L., Erlanger, B.F., Hsu, K.C. (1969) Immunologic studies of auto-immune disease in NZB-NZW mice. J. exp. Med. 130, 203.

Sell, S., Asofsky, R. (1968) Lymphocytes and immunoglobulins. Prog. Allergy 12, 86.

Sellers, E.A., Awad, A.G., Schonbaum, E. (1970) Long-acting thyroid stimulator in Graves' disease. Lancet 2, 335.

Skoog, W.A., Beck, W.S. (1956) Studies on the Fibrinogen, Dextran and Phytohaemagglutinin methods of isolating leukocytes. Blood 11, 436.

Smetana, K., Janele, J., Malinsky, L. (1966) Nucleolar coefficient of lymphocytes in the peripheral blood of female patients with breast cancer. Neoplasma 13, 643.

Smith, M.J.H. (1959) Salicylates and metabolism. J. Pharm. Pharmac. 11, 705.

Smith, M.G.M., Eddleston, A.L.W.F., Dominguez, J.A., Evans, D.B., Bewick, M., Williams, R. (1969) Changes in leucocyte migration after renal transplantation. Br. med. J. 2, 275.

Snedecor, G.W., Cochran, W.G. (1967) Statistical methods. Iowa state university press, Iowa.

Soborg, M., Bendixen, G. (1967) Human lymphocyte migration as a parameter of hypersensitivity. Acta med. scand. 181, 247.

Starzl, T.E. (1964) In Experience in renal transplantation. Saunders, Philadelphia and London.

Stetson, C.A. (1963) The role of humoral antibody in the homograft rejection. Adv. Immun. 3, 97.

Stocker, J.W., McKenzie, I.F.C., Morris, P.J. (1969) IgM activity in human lymphocyte antisera after renal transplantation. Nature (Lond.) 222, 483.

Strober, S., Gowans, J.L. (1965) The role of lymphocytes in the sensitization of rats to renal homografts. J. exp. Med. 122, 347.

Tennenbaum, J.I., St. Pierre, R.L., Cerilli, G.J. (1968) Evaluation of Immunosuppressive therapy and clinical course in renal transplants by *in vitro* lymphocyte transformation. Transplantation 6, 986.

Terasaki, P.I., McLelland, J.D. (1964) Microdroplet assay of human serum cytotoxins. Nature (Lond.) 204, 998.

Thomson, A.E.R., Bull, Jennifer M., Robinson, Mary A. (1966) A procedure for separating viable lymphocytes from human blood, and some studies on their susceptibility to hypotonic shocks. Br. J. Haemat. 12, 433.

Tilney, N.L., Murray, J.E. (1968) Operative technique and physiologic effects of chronic thoracic duct fistula in man. Ann. Surg. 167, 1.

Topilsky, M., Siltzbach, L.E., Williams, M., Glade, P.R. (1972) Lymphocyte response in sarcoidosis. Lancet 1, 117.

Tormey, D.C., Fudenberg, H.H., Kamin, R.M. (1967) Effect of prednisolone on synthesis of DNA and RNA by human lymphocytes *in vitro*. Nature (Lond.) 213, 281.

Tsanev, R., Markov, G.G. (1960) Substances interfering with spectrophotometric estimation of nucleic acids and their elimination by the two wavelength method. Biochim. Biophys. Acta 42, 442.

Turk, J.L. (1967) Action of lymphocytes in transplantation. J. clin. Path. 20, 423.

Vallee, B.L., Hughes, W.L., Gibson, J.G. (1947) A method for the separation of leucocytes from whole blood by floatation on serum albumin. Blood, Special Issue No. 1, 82.

Van Dyke, K., Szustkiewicz, C. (1968) An automated sensitive specific and differential analysis of DNA and RNA. Automation in analytical chemistry. Technicon Corporation New York.

Weeke, B., Bendixen, G. (1969) Serum immunoglobulins and organ specific cellular hypersensitivity in ulcerative colitis and Crohn's disease. Acta med. scand. 186, 87.

Whitesell, F.B., Black, B.M. (1949) A statistical study of the clinical significance of lymphocytic and fibrocytic replacements in the hyperplastic thyroid gland. J. clin. Endocr. 9, 1202.

Whitmore, G.F., Stanners, C.P., Till, J.E., Gulyas, S. (1961) Nucleic acid synthesis and the division cycle in X-irradiated L-strain mouse cells. Biochim. biophys. Acta 47, 66.

Whipple, G.H. (1907) A hitherto undescribed disease characterised anatomically by deposits of fat and fatty acids in the intestinal

and mesenteric lymphatic tissues. Bull. Johns Hopkins Hosp. 18, 382.

Wiener, J., Spiro, D., Russell, P.S. (1964) An electron microscopic study of the homograft reaction. Am. J. Path. 44, 319.

Williams, R.H. (1965) In Textbook of Endocrinology. Saunders, Philadelphia.

Wilson, R.E., Hagar, E.B., Hampers, C.L., Corson, J.M., Merril, J.P., Murray, J.E. (1968) Immunologic rejection of human cancer transplanted with renal allograft. New Engl. J. Med. 278, 479.

Wintrobe, M.M. (1967) In Clinical Haematology, 6th ed. Kimpton, London.

Wiseman, B.K. (1931) Criteria of the age of lymphocytes in the peripheral blood. J. exp. Med. 54, 271.

Yoffey, J.M., Courtice, F.C. (1970) Lymphatics lymph and the lymphoid complex. Academic Press, London.

Ziff, M., Brown, P., Lospalluto, J., Baden, J., McEwan, C. (1956) Agglutination and inhibition by serum globulin in the sensitized sheep cell agglutination reaction in rheumatoid arthritis. Amer. J. Med. 20, 500.

Zucker-Franklin, Dorothea (1969) The ultrastructure of lymphocytes. Seminars in Haematology 6, 4.

Zucker-Franklin, Dorothea, Franklin, E.C., Cooper, N.S. (1962) Production of macroglobulins *in vitro* and a study of their cellular origin. Blood 20, 56.

Zuhlke, V., Deodhar, S.D., Nakamoto, S., Kolft, W.J. (1967) Serum immunoglobulin levels following human renal allotransplantation, a preliminary report. Transplantation 5, 135.

Zytko, J., de Lamirande, G., Allard, G., Cantaro, A. (1958) Ribonucleases of rat liver. Biochim. biophys. Acta 27, 495.

Abbreviations

The recommendations of the Biochemical Journal (Instructions to authors, 1969) have been used throughout the text. The less common abbreviations have been explained where they appear, and are also listed here for reference.

C	Centigrade
DNA	Deoxyribose nucleic acid
E	Extinction coefficient
EDTA	Ethylene-diamine-tetra-acetic acid
ESR	Erythrocyte sedimentation rate
gav	Average centrifugal force in gravity units
LATS	Long acting thyroid stimulator
LE	Lupus erythematosus
PHA	Phytohaemagglutinin
POPOP	1,4-Di-(2-(5 phenyloxazolyl)) -benzene
PPO	2,5-Diphenyloxazole
PVP	Polyvinyl Pyrrolidone
RNA	Ribose nucleic acid
s	The Svedberg unit (10^{-13} sec.)
SD	Standard deviation

ACKNOWLEDGEMENTS

Acknowledgements

I wish to express my appreciation for the support and encouragement I have received from those in charge of the departments in which the work for this thesis was undertaken, in particular Dr. E.B. Hendry of the biochemistry department, Western Infirmary, Glasgow, Professor H.N. Munro of the department of nutrition, Massachusetts Institute of Technology, and Dr. I.A. Anderson and Dr. A.P. Kenny of the biochemistry department, Victoria Infirmary.

I wish also to thank physicians and surgeons in Glasgow and elsewhere who so readily gave me advice and permission to study patients under their care and would mention here Professor T. Anderson, Mr. P.R.F. Bell, Dr. J.D. Briggs, Dr. W.W. Buchanan, Professor A.C. Kennedy, Dr. A.L. Linton, Professor E.M. McGirr, Mr. A.J. Mack, Mr. H.C. Reid and Dr. W.C. Watson.

It is a pleasure to acknowledge the contribution made by collaborators in the studies and would especially mention here Dr. W.C. Cooper and Dr. S.T. Jacob in the work on the nature of lymphocyte RNA, Dr. J. Thomson in the investigation of lupus erythematosus, Dr. M.K. Jasani and Dr. K. Whaley in the investigations of rheumatoid arthritis and Sjøgren's syndrome, Dr. J.F.B. Smith and Dr. W.R. Greig in the work on patients with thyrotoxicosis and Dr. Anne Ferguson in the measurements in

Whipple's disease.

Some data recorded in the thesis in support of a diagnosis or for comparison with results of nucleic acid chemistry has come from general or specialised laboratories and I am grateful to Professor J.R. Anderson for immunopathological data, to Dr. J.F.B. Smith for the LATS assays, to Dr. Heather Dick for data on the tissue matching of transplant patients, also to Dr. E.B. Hendry for biochemical data and to Dr. R.W. Payne for total white cell counts on these patients.

Mrs. Erica Kerr undertook the technical work of the blood lymphocyte nucleic acid analyses for the latter part of the study on renal transplantation and I greatly appreciate her careful work.

My thanks is due in considerable measure to Mr. H. Gray and his staff in the photographic department, Victoria Infirmary, for their work in preparing the figures, and also to Mr. A.D. McLaren of the statistics department, the University of Glasgow, for guidance on statistical matters.

I also wish to record my thanks to Professor R.B. Goudie for his constructive criticism and advice during the preparation of the manuscript. The task of typing the manuscript was undertaken by Mrs. Janette Cameron whose patience and skill I greatly appreciate.

Financial support from two sources, Grant DA-49-139-Md-02560 from the United States Army and the Burroughs Wellcome Co., is gratefully acknowledged.

APPENDICES AND PUBLICATIONS

Publications

Data recorded in this thesis have appeared in the following publications:

1. Glen, A.C.A. (1967) Measurement of DNA and RNA in human peripheral blood lymphocytes. Clin. Chem. 13, 299.

2. Glen, A.C.A., Jasani, M.K. (1968) Lymphocyte DNA and RNA content in rheumatoid arthritis. Ann. rheum. Dis. 27, 170.

3. Jacob, S.T., Cooper, W.C., Glen, A.C.A., Munro, H.N. (1969) Synthesis of ribonucleic acid in human lymphocytes. In Proceedings of the 3rd Annual leucocyte culture conference. Appleton-Century-Crofts, New York.

4. Ferguson, Anne, Maxwell, J.D., Imrie, R.C., Glen, A.C.A. (1969) Modern Gastroenterology. Proceedings of the VIII International Congress of Gastroenterology. Stuttgart.

5. Glen, A.C.A., Cooper, W.C., Boak, J.L., Murray, J.E., Munro, H.N. (1970) DNA and RNA measurements on thoracic duct lymphocytes of patients undergoing renal transplantation. Transplantation 9, 83.

6. Smith, J.F.B., Glen, A.C.A., Greig, W.R. (1971) The nucleic acid content of lymphocytes in thyrotoxicosis. Clin. exp. Immunol. 8, 911.

7. Whaley, K., Glen, A.C.A., MacSween, R.N.M., Deodhar, S., Dick, W.C., Nuki, G., Williamson, J., Buchanan, W.W. (1971) Immunological responses in Sjøgren's syndrome and rheumatoid arthritis. Clin. exp. Immunol. 9, 721.

8. Glen, A.C.A., Bell, P.R.F., Briggs, J.D., Paton, A.M., Calman, K.C., Linton, A.L. (1971) Lymphocyte monitoring by blood lymphocyte counts and RNA/DNA determination in patients with a renal allograft. Transplantation 12, 434.

APPENDIX 1

Nucleic acid analyses in lymphocytes separated by the gelatin method from the blood of normal adults, data on individual results.

The lymphocyte DNA content, (DNA phosphorus per 10^6 cells) RNA content (RNA phosphorus per 10^6 cells) and the calculated RNAP/DNAP ratio are recorded for 39 normal individuals with sex, age and data on immunisation i.e. the number of conditions for which the individual has been actively immunised and allergies i.e. the number of known allergies in the individual.

Initial	MR	MC	DG	MC	DH	DA	RD	RG	JV	RC	JT	LM	CB	LG	JT	MG	AB
Sex	F	F	M	M	M	M	M	M	M	M	F	F	F	F	M	F	F
Age	56	22	31	25	26	31	26	35	27	18	20	59	57	31	28	44	21
Immunisations	3	9	4	6	6	6	4	3	7	8	4	3	1	4	3	4	4
Allergies	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DNAP per 10^6 cells μg	0.97	0.65	0.70	0.91	0.67	0.68	0.78	1.05	0.55	0.83	0.68	0.58	0.62	0.95	0.74	0.65	0.63
RNAP per 10^6 cells μg	0.36	0.24	0.25	0.32	0.23	0.23	0.27	0.36	0.18	0.26	0.21	0.17	0.18	0.27	0.21	0.18	0.17
RNAP/DNAP	0.37	0.36	0.36	0.35	0.35	0.34	0.34	0.34	0.33	0.33	0.31	0.30	0.29	0.29	0.29	0.28	0.28

APPENDIX 1 (continued)

Initial	HA	MW	RW	MG	MW	JI	EL	CW	IH	AS	DM	AG	RD	RM	HM	IS	AM	FM
Sex	F	F	M	M	F	F	F	M	F	F	M	M	M	M	F	F	F	M
Age	23	47	18	26	24	18	53	29	22	77	66	29	28	27	57	55	52	3(
Immunisations	3	2	3	3	5	4	1	1	4	0	5	5	8	5	1	3	1	3
Allergies	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DNAP per 10^6 cells μg	0.82	0.65	0.67	1.15	0.64	0.70	0.75	0.65	0.77	0.63	0.70	1.09	0.64	0.61	0.60	0.71	0.71	0.84
RNAP per 10^6 cells μg	0.23	0.15	0.18	0.30	0.17	0.19	0.19	0.16	0.19	0.16	0.17	0.27	0.16	0.15	0.13	0.15	0.16	0.17
RNAP/DNAP	0.28	0.27	0.27	0.26	0.26	0.26	0.26	0.25	0.25	0.25	0.25	0.25	0.24	0.22	0.22	0.22	0.21	0.21

APPENDIX 1 (continued)

Initial	HC	DW	FJ	JM
Sex	M	M	F	M
Age	53	30	20	23
Immunisations	1	3	4	1
Allergies	0	0	0	0
$DNAP_6$ per 10^6 cells	0.69	0.88	0.73	0.75
$RNAP_6$ per 10^6 cells	0.14	0.17	0.12	0.12
RNAP/DNAP	0.20	0.19	0.17	0.16

APPENDIX 2

Nucleic acid analyses of lymphocytes separated by the gelatin and cotton wool method from the blood of normal adults; data on individual results.

The lymphocyte DNA content (DNA phosphorus per 10^6 cells), RNA content (RNA phosphorus per 10^6 cells) and the calculated RNAP/DNAP ratio are recorded for twenty individuals with sex, age and data of immunisations, i.e. the number of conditions for which the individual has been actively immunised; also allergies, i.e. the number of known allergies in the individual. The blood lymphocyte count in lymphocytes per cubic mm is also recorded for nine patients.

Initial	AW	RS	LG	RB	RI	HE	CC	IA	JC	FM	JM	LT
Sex	M	F	M	F	M	F	M	M	F	F	M	M
Age	23	27	32	27	32	26	20	64	26	18	30	47
Immunisations	3	4	3	-	-	4	3	4	4	3	-	8
Allergies	1	0	0	-	-	0	0	0	1	0	-	0
Blood lymphocyte count	2380	2200	-	-	-	1800	1550	-	2430	2680	-	7100
DNAP per 10^6 cells	0.78	0.76	0.63	0.59	0.59	0.65	0.67	0.72	0.70	0.89	0.72	0.95
RNAP per 10^6 cells	0.27	0.26	0.21	0.19	0.19	0.21	0.21	0.22	0.22	0.28	0.21	0.28
RNAP/DNAP	0.35	0.34	0.33	0.32	0.32	0.32	0.32	0.31	0.31	0.31	0.30	0.30

APPENDIX 2. (continued)

Initial	EB	HM	MF	EM	MF	AP	AG	WD
Sex	F	F	M	F	F	M	M	M
Age	22	27	42	25	28	29	32	27
Immunisations	4	3	7	3	5	-	3	-
Allergies	0	1	1	0	0	-	0	-
Blood lymphocyte count	1930	-	1920	-	-	-	-	-
$DNAP_6$ per 10^6 cells	0.66	1.01	0.73	0.79	0.59	0.71	0.71	0.79
$RNAP_6$ per 10^6 cells	0.19	0.29	0.20	0.21	0.16	0.17	0.16	0.17
RNAP/DNAP	0.29	0.29	0.28	0.27	0.27	0.24	0.22	0.21

APPENDIX 3

Nucleic acid analyses in lymphocytes separated by the gelatin method from the blood of patients with either probable or definite rheumatoid arthritis as defined by Ropes, 1959.

Age is in years, the ESR by the Westergren method in mm per hour, SCAT is the sensitised sheep cell agglutination titre, the presence of subcutaneous nodules is indicated, and the drug therapy at the time of analysis is recorded as either Sal (Salicylates) or St (Steroids). The lymphocyte DNA content is in μg DNA phosphorus per 10^6 cells and the RNA content in μg RNA phosphorus per 10^6 cells, with the calculated RNAP/DNAP ratio shown.

Initial	JS	MB	LL	AB	CC	MF	DMcA	JK	AW	SD	JG	JC	AP
Sex	F	F	F	F	F	F	M	F	M	F	F	F	F
Age	67	54	62	30	64	52	68	51	58	54	25	52	46
ESR	28	60	88	66	68	52	90	47	71	20	46	58	42
SCAT	1/256	1/32	1/256	1/256	1/64	1/512	1/256	1/128	1/128	-	1/4096	1/1024	1/12
Nodules	+	-	-	-	-	+	+	-	+	+	-	-	-
Drug therapy	nil	Sal St	Sal St	Sal	Sal	Sal	Sal	Sal	Sal	Sal St	nil	Sal	Sal
Definite or Probable RA	D	D	D	D	P	D	D	D	D	D	D	D	D
DNAP/10^6 cells	0.72	0.71	0.91	0.62	0.90	0.86	0.75	0.67	0.67	0.78	0.47	0.81	0.9
RNAP/10^6 cells	0.33	0.32	0.40	0.27	0.38	0.36	0.31	0.27	0.27	0.31	0.18	0.32	0.3
RNAP/DNAP	0.46	0.46	0.44	0.44	0.43	0.42	0.41	0.40	0.40	0.40	0.39	0.39	0.3

APPENDIX 3 (continued)

Initial	JH	AS	JC	AF	BR	MN	JK	CMcC	HL	EF	AB	HC	JS
Sex	M	F	F	F	F	F	F	F	F	F	F	F	F
Age	59	27	57	45	29	66	78	43	50	74	52	71	63
ESR	43	36	19	40	56	50	119	40	54	66	28	60	16
SCAT	1/2024	-	1/512	1/64	< 1/16	1/128	1/1024	1/256	1/1024	1/128	-	1/64	1/64
Nodules	+	-	+	-	-	-	-	-	+	-	-	-	-
Drug therapy	nil	Sal	Sal St	Sal	nil	Sal St	St	nil	Sal St	Sal	Sal	nil	Sal St
Definite or Probable RA	D	P	D	D	P	D	D	D	D	D	D	D	D
DNAP per 10^6 cells	0.70	0.90	0.93	0.78	0.59	0.79	0.89	0.59	0.65	0.59	0.57	0.71	0.72
RNAP per 10^6 cells	0.25	0.31	0.32	0.26	0.19	0.26	0.29	0.19	0.20	0.19	0.18	0.21	0.21
RNAP/DNAP	0.36	0.35	0.34	0.33	0.33	0.33	0.33	0.33	0.32	0.31	0.31	0.30	0.29

APPENDIX 3 (continued)

Initial	JC	JMcD	AE	JS	ST	JA	JT	IA	MK
Sex	F	F	F	F	F	F	M	F	F
Age	62	55	56	70	51	50	25	49	30
ESR	36	15	19	14	43	17	42	37	38
SCAT	1/32	-	-	-	-	1/64	-	-	-
Nodules	-	-	-	-	-	-	-	-	-
Drug therapy	Sal	Sal St	Sal St	St	Sal	Sal St	St	nil	Sal
Definite or Probable RA	P	D	P	P	P	P	P	P	P
DNAP per 10^6 cells	0.61	0.62	0.95	0.73	1.03	0.63	0.73	0.60	0.65
RNAP per 10^6 cells	0.18	0.18	0.26	0.20	0.29	0.17	0.19	0.16	0.15
RNAP/DNAP	0.29	0.29	0.28	0.28	0.28	0.27	0.27	0.27	0.24

APPENDIX 4

Nucleic acid analyses in lymphocytes separated by the gelatin and the gelatin and cotton wool method from the blood of patients with sicca syndrome and Sjogren's syndrome.

Age is in years, the ESR by the Westergren method is in mm per hour, SCAT is the sensitised sheep cell agglutination titre, the white cell count is the total count in peripheral blood cells per cu mm, drug therapy at the time of analysis is recorded as Sal (Salicylates), St (Steroids), Mef (Mefenamic acid), Ind (Indomethacin); the presence of Xerostomia and Kerato-conjunctivitis sicca is also recorded as well as the presence of rheumatoid joint changes allowing a diagnosis of sicca syndrome Si or Sjogren's syndrome to be listed. The lymphocyte DNA content is in µg DNA phosphorus per 10^6 cells and the RNA content in µg RNA phosphorus per 10^6 cells, with the calculated RNAP/DNAP ratio shown.

APPENDIX 4 (continued)

Initial	JL	JS	DMcA	JK	SD	MG	MA	JK	JD	EF	AR	JC	JS	ML	IS	IM
Sex	F	F	M	F	F	F	F	F	F	F	F	F	F	F	F	F
Age	65	67	68	51	54	55	67	78	63	74	58	62	70	67	66	76
ESR	23	28	90	47	20	35	64	119	35	66	73	55	14	18	9	0
SCAT	-	1/256	1/256	1/128	-	1/256	1/256	1/1024	-	1/128	1/32	1/32	-	1/32	-	1/32
White cell count	5400	7400	-	9100	5200	4800	7900	7600	9200	7400	5300	5200	6950	6300	9600	4900
Drug therapy	nil	nil	Sal	Sal	Sal St	nil	nil	St	Ind	Sal	nil	Sal	St	nil	Mef	nil
Rheumatoid joint disease	-	+	+	+	+	-	+	+	-	+	-	-	+	-	-	-
Xerostomia	+	-	+	-	+	+	+	+	+	-	+	+	+	+	+	+
Kerato-conjunctivitis sicca	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Diagnosis Si or Sj	Si	Sj	Sj	Sj	Sj	Si	Sj	Sj	Si	Sj	Si	Si	Sj	Si	Si	Si
DNAP$_6$ per 10^6 cells	0.65	0.72	0.75	0.67	0.78	0.54	0.91	0.89	0.87	0.59	0.80	0.61	0.73	0.79	0.75	0.87
RNAP$_6$ per 10^6 cells	0.35	0.33	0.31	0.27	0.31	0.21	0.31	0.29	0.29	0.19	0.25	0.18	0.20	0.22	0.19	0.22
RNAP/DNAP	0.55	0.46	0.41	0.40	0.40	0.38	0.34	0.33	0.33	0.31	0.31	0.29	0.28	0.28	0.25	0.25

APPENDIX 5

Nucleic acid analyses in lymphocytes from the blood of patients with thyrotoxicosis.

Sex is recorded, age is in years, thyroid radio iodine (I^{131}) uptake is in percentage of dose at 24 hours, normal range 15 to 40%; protein bound iodine is in µg per 100 ml, L.A.T.S. bioassay is as recorded in the text, the blood lymphocyte count is in cells per mm^3, DNAP content is in µg DNA phosphorus, RNAP content in µg RNAP phosphorus, both per 10^6 cells. The RNAP/DNAP ratio is also listed. Treatment is recorded as I^{131} or I^{125}, a therapeutic dose of radio iodine; C (Carbimazole) and PT (partial thyroidectomy). The post treatment lymphocyte RNAP/DNAP ratio and count is also listed for most patients.

APPENDIX 5 (continued)

Initial		SF	JH	MW	AM	SB	HP	JT	MW	JA	IB	MM	ML	AN	MA	MH	WF
Sex		F	F	F	F	F	F	M	F	F	F	F	F	F	F	F	F
Age		63	45	37	64	43	40	36	22	70	48	61	43	39	28	32	45
I^{131} uptake		76	52	57	59	45	46	69		62	77	63	53	58		88	71
PBI^{127}						17.1	8.0	20.6	12.6	15.3		8.0			12.5		15.0
L.A.T.S.		-	+	-	-	+	+	-	+	+	-		-	+	-	-	+
Lymphocyte count						2400	1220	3820		1610		1060		1720	2850	2000	2600
$DNAP_6$ per 10^6 cells		0.62	0.55	0.64	0.70	0.62	0.70	0.65	0.57	0.65	0.70	0.90	0.55	0.79	0.87	0.69	0.84
$RNAP_6$ per 10^6 cells		0.35	0.23	0.25	0.27	0.22	0.25	0.22	0.20	0.22	0.23	0.28	0.17	0.25	0.28	0.21	0.26
RNAP/DNAP		0.56	0.42	0.40	0.40	0.36	0.35	0.34	0.34	0.34	0.32	0.32	0.32	0.31	0.31	0.31	0.31
Treatment		I^{131}	C	PT	I^{125}	I^{131}	I^{131}	C	C	I^{125}	I^{131}	I^{131}	I^{125}	PT	C	PT	I^{125}
Post Treatment	L.A.T.S.	-	+	-	-	-	-	-	+	-	-	-	-	+	-	-	+
	Lymphocyte count	960				2480	1950			1300		1320					
	RNAP/DNAP	0.32	0.36	0.30	0.42	0.28	0.36	0.44	0.38	0.47	0.36	0.32	0.29	0.50	0.36	0.40	0.37

APPENDIX 5 (continued)

Initial		JM	MO	MD	WM	MD	MS	JS	RS	RC	AM	HP	EH	EP	MR	JE	MG	MH	DJ
Sex		M	F	F	F	F	F	F	M	F	F	F	F	F	F	M	F	F	F
Age		28	46	60	66	45	54	56	49	44	45	55	54	47	49	45	44	48	57
I^{131} uptake		54	43	60	47	52	56	53	54	65	59	63	72	70	92	50	67	66	67
PBI^{127}		17.5				10.4	17.0	9.1	9.8	12.0	20.0		18.8		16.6		16.2	13.2	
L.A.T.S.		-	+	+	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-
Lymphocyte count			1410	2080	1960	2660	2000	4000	2590		2520		1980			4640	1430		1980
DNAP per 10^6 cells		0.60	0.81	0.86	0.58	0.79	0.88	0.98	0.66	0.85	0.69	0.89	0.85	0.61	0.73	0.72	0.77	0.89	0.85
RNAP per 10^6 cells		0.19	0.25	0.25	0.17	0.23	0.26	0.28	0.18	0.24	0.19	0.25	0.24	0.17	0.20	0.19	0.20	0.22	0.19
RNAP/DNAP		0.31	0.30	0.30	0.29	0.29	0.29	0.29	0.29	0.28	0.28	0.28	0.28	0.28	0.28	0.27	0.26	0.25	0.22
Treatment		C	I^{131}	I^{125}	I^{125}	I^{131}	I^{125}	I^{131}	C	C	I^{125}	I^{131}	I^{125}	I^{131}	I^{131}	C	C	C	I^{131}
Post Treatment	L.A.T.S.	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
	Lymphocyte count		2900		2420				1880							2820		1800	
	RNAP/DNAP	0.32	0.38	0.28	0.30	0.43	0.38	0.38	0.31	0.33		0.36	0.37	0.32		0.28	0.32	0.29	0.37

APPENDIX 6

The nucleic acid content of the blood lymphocytes in patients with lupus erythematosus.

Age is in years. The classification according to Haserick (1965) is recorded which depends on the presence of systemic manifestations of lupus erythematosus, skin lesions and positive L.E. test in the blood. Treatment is recorded as St (Steroids), Sal (Salicylates), T (Thymectomy) and Mep (Mepacrine). DNAP, RNAP are in µg of RNA phosphorus per 10^6 cells.

Initial	HB	AG	JP	IW	EF	MB	ED	EM
Sex	F	F	M	F	F	F	F	F
Age	43	55	53	64	45	18	20	29
Systemic manifestations	+	+	-	+	-	-	-	-
Skin lesions	+	+	+	+	+	+	+	+
LE test	+	+	+	+	+	+	+	-
Haserick classification	3	3	2	3	2	2	2	1
Treatment	St,T	Sal	0	St	St	Mep	0	0
DNAP per 10^6 cells	0.72	0.78	0.66	0.79	0.83	0.61	0.94	0.62
RNAP per 10^6 cells	0.41	0.33	0.27	0.32	0.29	0.19	0.24	0.16
RNAP/DNAP ratio	0.57	0.43	0.41	0.41	0.35	0.32	0.26	0.26

APPENDIX 7

Nucleic acid analyses in the blood lymphocytes of patients with infection.

The infections are indicated as follows - Pn (pneumonia), RF (rheumatic fever), Cor (Coryza), Br (bronchitis), R (Reiter's syndrome), Inf (influenza). DNAP is in µg DNA phosphorus per 10^6 lymphocytes, RNAP is in µg RNA phosphorus per 10^6 lymphocytes.

Initial	JG	DS	AS	HA	JP	FG	AM	MS	PO	JG
Sex	M	M	F	F	M	M	F	F	M	M
Age	20	28	27	23	61	17	31	63	54	18
Infection	Pn	Pn	RF	Cor	Br	R	Pn	Inf	Br	Pn
Infection duration - days	7	5	14	3	>60	42	-	8	>60	10
DNAP per 10^6 cells	0.63		0.90	0.81	0.76	0.63	0.64	0.71	0.83	0.64
RNAP per 10^6 cells	0.23		0.31	0.28	0.25	0.20	0.20	0.21	0.21	0.14
RNAP/DNAP ratio	0.36	0.36	0.35	0.34	0.33	0.32	0.31	0.29	0.25	0.22

APPENDIX 8

Nucleic acid analyses in blood lymphocytes from patients with neoplasm.

Local neoplasm, L, or the presence of metastases, M, is indicated. Lymphocyte DNA content and RNA content are in µg RNA or DNA phosphorus per 10^6 cells.

Initial	MB	AC	ET	AB	MH	CM
Sex	F	F	F	F	F	F
Age	62	72	61	69	68	75
Local or Metastases	M	M	M	M	L	L
Blood lymphocyte count	1504	3465	1034	1452	3312	1596
DNAP per 10^6 cells	0.54	0.74	0.86	0.71	0.78	0.63
RNAP per 10^6 cells	0.27	0.37	0.41	0.33	0.25	0.16
RNAP/DNAP ratio	0.51	0.49	0.47	0.46	0.31	0.26

APPENDIX 9

Individual results of the nucleic acid analyses in patients from the list of miscellaneous conditions. DNA is in μg DNA phosphorus per 10^6 cells and RNA in μg RNA phosphorus per 10^6 cells.

Condition	DNAP μg per 10^6 cells	RNAP μg per 10^6 cells	RNAP/DNAP ratio
Acute leucaemia	0.66	0.26	0.40
Ulcerative colitis	0.66	0.25	0.38
Ulcerative colitis	0.62	0.20	0.33
Macroglobulinaemia	0.65	0.23	0.36
Crohn's disease	0.63	0.24	0.38
Crohn's disease	0.44	0.13	0.29
Sarcoidosis	0.98	0.32	0.33
Salicylate poisoning	0.98	0.28	0.33
Whipple's disease	0.62	0.22	0.36
Whipple's disease	0.54	0.11	0.20
Pemphigus	0.74	0.22	0.30
Colonic histiocytosis	0.45	0.13	0.30
Colonic histiocytosis	0.68	0.21	0.28
Post TAB immunisation	0.70	0.20	0.29
Post TAB immunisation	0.79	0.21	0.27
Post TAB immunisation	1.01	0.29	0.29
Acne	0.80	0.23	0.29

APPENDIX 9 (continued)

Condition	DNAP µg per 10^6 cells	RNAP µg per 10^6 cells	RNAP/DNAP ratio
Osteoporosis	0.71	0.19	0.27
Osteomalacia	0.43	0.10	0.24
Light chain myeloma	0.57	0.13	0.22
Chronic lymphatic leucaemia	1.42	0.37	0.26
Chronic lymphatic leucaemia	0.72	0.14	0.19
Chronic lymphatic leucaemia	0.72	0.15	0.20

APPENDIX 10

Details of the patients included in the study of renal allografts.

Age is in years at time of allograft, donor is listed as Cadaver (Cr) or Live (L), tissue match is according to Botha (1969), creatinine clearance is in ml per minute as at 1st January, 1971, d signifies where patient has died and r where the donor kidney has been rejected and removed.

Initial	MA	HB	HB	MC	CD*	MD	RF	CG	JL	BL	MM	SM	JM	CM	RM	JM	GO_1	GO_2	AP	MS
Sex	M	M	F	F	F	M	M	F	M	M	F	F	M	F	F	M	M	M	M	F
Age	33	45	38	27	18	34	32	32	30	17	35	46	39	20	17	38	24	24	40	19
Donor Live or Cadaver	Cr	Cr	Cr	L	L	Cr	Cr	Cr	Cr	L	Cr	Cr	Cr	Cr	Cr	Cr	Cr	Cr	Cr	L
Tissue match	D	-	C	A	A	-	D	C	E	B	D	E	-	D	E	C	-	C	C	A
Creat. Clearance	61	67	60	86	10	d	96	64	r	93	d	d	71	77	26	37	r	111	78	60

* Results for this patient were obtained outwith the period ending 1st January, 1971, but were included for reasons given in the text.

TRANSPLANTATION
Vol. 12, No. 6
Copyright © 1971 by The Williams & Wilkins Co.
Printed in U.S.A.

LYMPHOCYTE MONITORING BY BLOOD LYMPHOCYTE COUNTS AND RNA/DNA DETERMINATION IN PATIENTS WITH A RENAL ALLOGRAFT[1]

A. C. A. GLEN, P. R. F. BELL, J. D. BRIGGS, A. M. PATON, K. C. CALMAN, AND A. L. LINTON

Department of Biochemistry, Victoria Infirmary, Glasgow, the University Department of Surgery, and the Renal Unit, Western Infirmary, Glasgow, Scotland

SUMMARY

Two hundred and ninety-nine analyses of both blood lymphocyte count and lymphocyte ribonucleic acid/deoxyribonucleic acid (RNA/DNA) ratio in 17 patients with a renal allograft are reported. In the absence of infection, patients on standard immunosuppressive regimes have normal results for the lymphocyte RNA/DNA ratio, although they may have lymphopaenia. Unequivocal infection and rejection may usually be distinguished by the lymphopaenia associated with rejection, although in both the lymphocyte RNA/DNA ratio is elevated. A clinically important distinction between lymphopaenia in rejection and lymphopaenia resulting from overimmunosuppression is made by measuring the lymphocyte RNA/DNA ratio. The ratio is low in oversuppression and elevated in rejection. The combined measurements of lymphocyte RNA/DNA ratio and lymphocyte count have a predictive value in more than one-half of the rejection episodes which show abnormality at least 5 days earlier than measurements which depend on renal function.

On the assumption that a renal allograft has been achieved with an adequate, but less than perfect, tissue match, one of the main problems in the management of the recipient is the balancing of suppression of graft rejection processes with the danger of impairment of the patient's response to infection. This problem is magnified when rejection of the graft is suspected and immunosuppressive therapy has to be increased. Berenbaum (*2*), in a review of the reports of the Kidney Transplant Registry (*14–16*), points out that the difficulties associated with drug toxicity and infection account for about one-third of graft failures and are also factors, in most instances, of death in the recipient (*3, 5*).

Immunosuppression is usually monitored by maintaining the white cell count within the 5,000–10,000 cells/mm^3 range. This method is at best empirical and there is a great need for a method which will allow better control of immunosuppression and give an early indication of impending graft rejection.

Lymphocytes are closely implicated in the pathology of graft rejection and are readily accessible in blood. Both azathioprine and prednisone, the established immunosuppressive drugs used in renal transplantation in man, are inhibitors of nucleic acid synthesis (*2, 23*), so that measurement of lymphocyte DNA and RNA content is of potential value in monitoring the effect of these drugs. In addition, previous experience with the lymphocyte nucleic acid determination points to the mean blood lymphocyte RNA/DNA ratio as a reproducible measurement (*6*) correlated with the mean lymphocyte diameter (*7*) and related in theory and in practice to immunological activity in man (*8, 25*). A range for the blood lymphocyte RNA/DNA ratio in normal persons has been established (*6*) and confirmed in an independent laboratory (H. Sakai et al., personal communication).

There is already in the literature evidence of functional abnormality of the blood lymphocytes

[1] Address requests for reprints to: Dr. A. C. A. Glen, Biochemistry Department, Victoria Infirmary, Glasgow, S.2., Scotland.

in relation to allograft rejection and immunosuppression. Spontaneous transformation of blood lymphocytes in vitro increases at a time of immunological activity directed against the graft and effective immunosuppression impairs the phytohaemagglutinin response (*22*). Changes in recipient leucocyte migration, using a standard technique, can be demonstrated in relation to renal allograft rejection (*21*) and an increase in the rate of RNA (*18*) and DNA synthesis (*11*) in peripheral blood lymphocytes has been demonstrated at times of rejection. Because of these changes in the behaviour of lymphocytes during periods of immunological activity, a study of the qualitative and quantitative changes in lymphocytes following renal transplantation may provide a reliable means of monitoring immunosuppression and forecasting graft rejection. This paper presents the results of monitoring blood lymphocyte count and RNA/DNA ratio in a series of 17 patients following renal transplantation.

MATERIALS AND METHODS

Clinical material. This study of 17 patients, recipients of 18 renal allografts, was carried out in the 3-year period up to the end of 1970. There were 3 live donors and 16 cadaver donor kidneys in the series; the recipients were 9 males and 8 females, 1 male receiving a second graft. The mean age of the patients was 31 years, with a range of from 17 to 46 years.

Immunosuppression was maintained with azathioprine given as an initial dose of 3 mg/kg and then regulated according to the white cell count. Prednisone, 200 mg/day, was started at the time of the transplants and reduced daily in steps of 10 mg to a maintenance dose of from 10 to 30 mg/day. Definite clinical rejection episodes were diagnosed in the event of an increased serum creatinine and a decreased creatinine clearance present for 24–48 hr. Other factors such as decreased urine volume and renal tenderness were inconsistent findings. Such episodes were treated by an i.v. infusion of 1 g of prednisolone given over a 2-hr period and repeated on the following day if necessary. Two patients received X-irradiation (150 rads) during rejection crises.

Samples of venous blood were taken from the patients, always between 8:30 and 10:30 AM, for the lymphocyte analyses as well as the general monitoring of the patient by the usual series of tests. In the initial stage of the project, blood sampling for lymphocyte analysis was infrequent and, for this reason and because of early graft failure in some cases, fewer than 5 blood samples were obtained during the life of each of 4 grafts. In the remaining 14 allografts, 289 lymphocyte analyses were undertaken during 141 graft months, i.e., each patient had, on the average, an analysis every 2 weeks. In practice, the blood sampling was more frequent early in the life of the graft when the immunosuppressive therapy was being adjusted and it became apparent that sampling twice/week would be advantageous.

Laboratory methods. Blood samples from patients were analysed by recording the total white count by standard Coulter counter techniques and the differential white cell count, on a stained slide. Lymphocyte nucleic acid analysis was carried out after lymphocyte isolation from blood by a modification (*6*) of the Coulson and Chalmers gelatin sedimentation method (*18*). An additional step from the published technique (*6*) was included at the stage when the lymphocytes were in suspension and freed from the bulk of the red cells. This was to incubate the suspension with washed cotton wool for 15 min at 37 C. Lymphocytes were recovered from the wool thereafter by gentle pressure. This step improved the purity of the preparation and reduced polymorphonuclear cell contamination to under 5%; residual red cells were removed by osmotic shock.

The analyses of DNA and RNA in the lymphocyte suspensions, using a minimum of 3×10^6 cells, were carried out by a modified Schmidt Thannhauser (*11*) procedure described elsewhere (*6*).

Although the lymphocyte DNA and RNA content/10^6 cells was noted, taking account of the lymphocyte count in the cell suspension, the results in this paper are recorded as the mean lymphocyte RNA/DNA ratio. The ratio RNA/DNA of the cell is a valid index of its RNA content provided it can be shown that the mean DNA content of the cell is constant in the population under study and is not being influenced by active DNA synthesis or polyploidy.

RESULTS

The results of 299 analyses from patients are plotted in Figure 1. In normal persons not re-

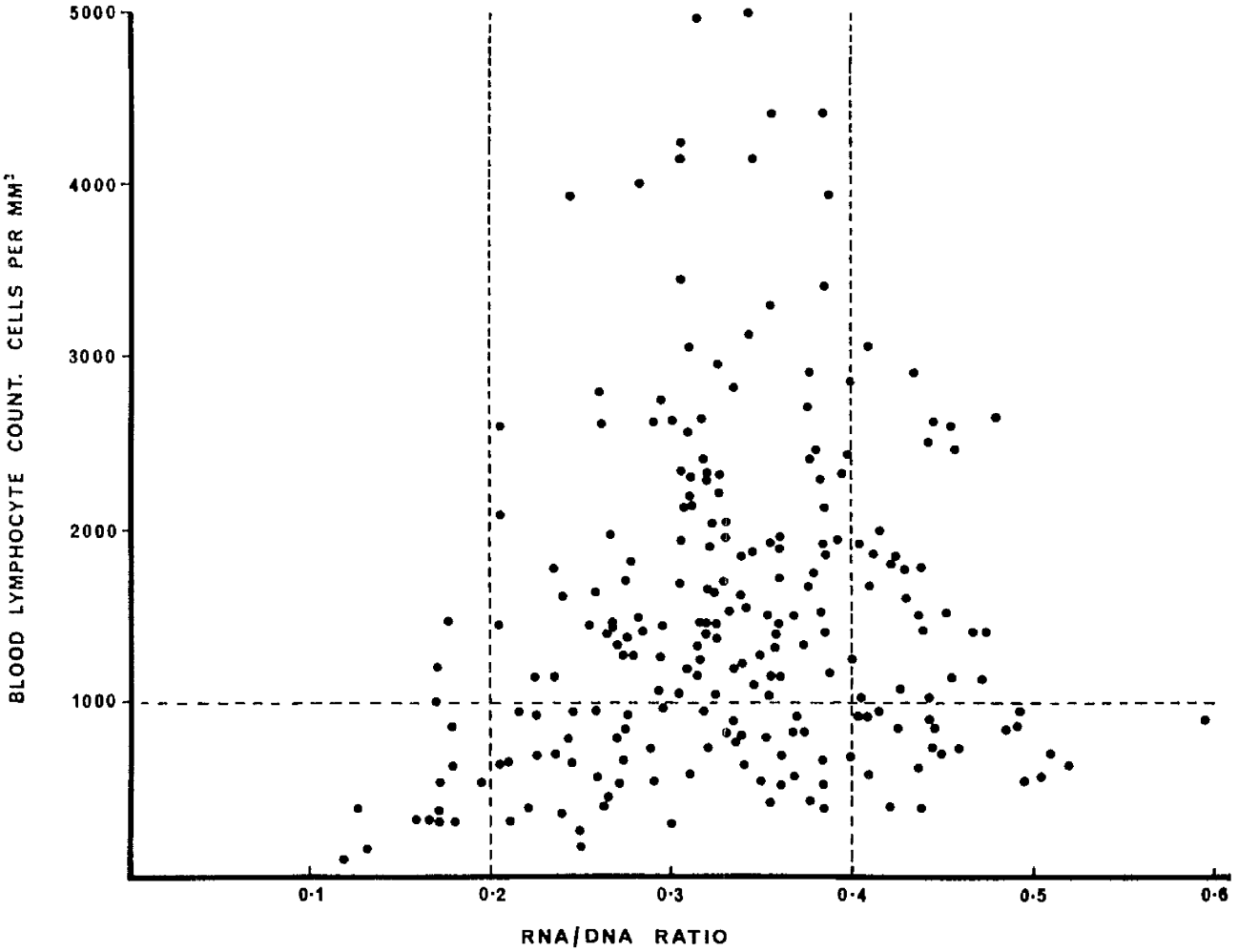


FIGURE 1. The relationship between blood lymphocyte count in cells per cubic millimeter and the lymphocyte RNA/DNA ratio in patients with a renal allograft on immunosuppressive therapy. The dotted lines show limits of normal for the lymphocyte RNA/DNA ratio in untreated persons without a graft. A blood lymphocyte count of 1,000 cells/mm³ or less defines a lymphopaenia.

ceiving immunosuppressive therapy, the accepted value for the normal blood lymphocyte count is from 1,500 to 3,500 cells/mm³. Figure 1 shows 84 samples with a lymphopaenia of 1,000 cells/mm³ or less. Ten samples showed a lymphocytosis with a lymphocyte count of 3,500 cells/mm³ or greater.

It can be seen also from Figure 1 that the blood lymphocytes from patients having a renal allograft and receiving immunosuppressive treatment can have RNA/DNA ratio results outside the range found in normal persons of 0.20–0.40. On 15 occasions the lymphocyte RNA/DNA ratio was low and on 54 it was elevated when compared to the lymphocytes of normal untreated persons. Examination of Figure 1 would suggest that a low lymphocyte RNA/DNA ratio is associated with lymphopaenia while a high lymphocyte RNA/DNA ratio may be associated with a normal or low lymphocyte count.

In order to arrive at a range of values for the lymphocyte analyses in immunosuppressed patients with good graft function and to show possible differences in the lymphocyte response to infection, rejection, or excessive immunosuppression, four clinical states were defined.

A. No infection or rejection. To qualify for this group patients had to be free of clinical or laboratory evidence of generalised or local infection or graft rejection, at the time of, or within 10 days of, analysis. In addition, the creatinine clearance had to be at least 50 ml/min. If the patients had more than one rejection episode in the past they were not included in this group.

B. Unequivocal infection. To be included in

this group the patient had to exhibit clinical and laboratory evidence of viral, fungal, or bacterial infection. The possibility of the presence of rejection excluded patients from this group.

C. Unequivocal rejection. Inclusion in this group required that clinical suspicion of graft rejection, acute or chronic, should be confirmed by a substantial elevation of the plasma creatinine maintained for 24 hr. A substantial rise of plasma creatinine was defined as an increase of 0.5 mg/100 ml when the plasma creatinine was under 2.0 mg/100 ml, and a rise of 1.0 mg when the initial plasma creatinine was greater than 2 mg/100 ml. The presence of infection excluded patients' results from this group.

D. Excessive immunosuppression. Patients in this group had a blood leucocyte count of less than 4,000 cells/mm^3. Evidence of rejection or significant infection resulted in exclusion from the group.

The blood lymphocyte counts and the lymphocyte RNA/DNA ratios for these groups are presented in Table 1. Thirty-seven results from 6 patients comprised group A, 19 results from 7 patients comprised group B, 17 results from 10 patients comprised group C, and 9 results from 3 patients were included in group D.

Results from patients with neither rejection nor infection are taken as the normal group in this study of renal allografts. The results are very similar to published values for the blood lymphocyte count and the lymphocyte RNA/DNA ratio (*6*) in normal untreated persons, although a tendency to slight lymphopaenia is noted in patients with treated allografts. Serial analyses from individual patients show a striking stability of the RNA/DNA ratio in those patients included in this group.

A useful intercomparison may be made between the 4 clinical states defined previously, applying Student's t test to the mean value for each group (Table 2). The blood lymphocyte count results are considered first. In the presence of infection, the mean blood lymphocyte count is lower than when infection is absent, but this has not been shown to have statistical significance. When unequivocal rejection is present, there is a statistically significant lymphopaenia ($P < 0.001$). Not unexpectedly, lymphopaenia was present in the group D patients (excessive immunosuppression). On examination of the lymphocyte chemistry, group B (infection group) and group C (rejection group) each showed a significantly greater mean value for the lymphocyte RNA/DNA ratio than group A (no infection or rejection) ($P < 0.001$ in each case). The degree of elevation of the mean lymphocyte RNA/DNA ratio was significantly greater in the presence of rejection ($P < 0.01$). There is, however, considerable overlap of observed values for the ratio in groups B and C (infection and rejection). The finding of a reduction in the lymphocyte RNA/DNA ratio in the presence of overimmunosuppression is of relevance later in relation to the cause of lymphopaenia.

When the value of the lymphocyte RNA/DNA ratio and the blood lymphocyte count are considered together in the same 4 groups a clearer pattern emerges as may be seen from examination of Figure 2. This shows the individual values for the relationship between the blood lymphocyte count and the lymphocyte RNA/DNA ratio separately plotted for each of the 4

TABLE 1. Intercomparison of the results of the blood lymphocyte count and the lymphocyte RNA/DNA ratio in the four categories

Category	Blood lymphocyte count (mean cells/mm^3 ± SD)	Lymphocyte RNA/DNA ratio (mean ± SD)
A. No infection, no rejection	2,224 ± 1,056	0.300 ± 0.047
B. Unequivocal infection	1,792 ± 1,028	0.354 ± 0.066
C. Unequivocal rejection	888 ± 536	0.422 ± 0.063
D. Excessive immunosuppression	623 ± 403	0.253 ± 0.071

TABLE 2. Analysis by t-test

Measurement	Comparison	P
Blood lymphocyte count	No infection, no rejection versus infection	NS
	No infection, no rejection versus rejection	<0.001
	No infection, no rejection versus excessive immunosuppression	<0.001
	Infection versus rejection	<0.01
Lymphocyte RNA/DNA ratio	No infection, no rejection versus infection	<0.001
	No infection, no rejection versus rejection	<0.001
	No infection, no rejection versus excessive immunosuppression	<0.02
	Infection versus rejection	<0.01

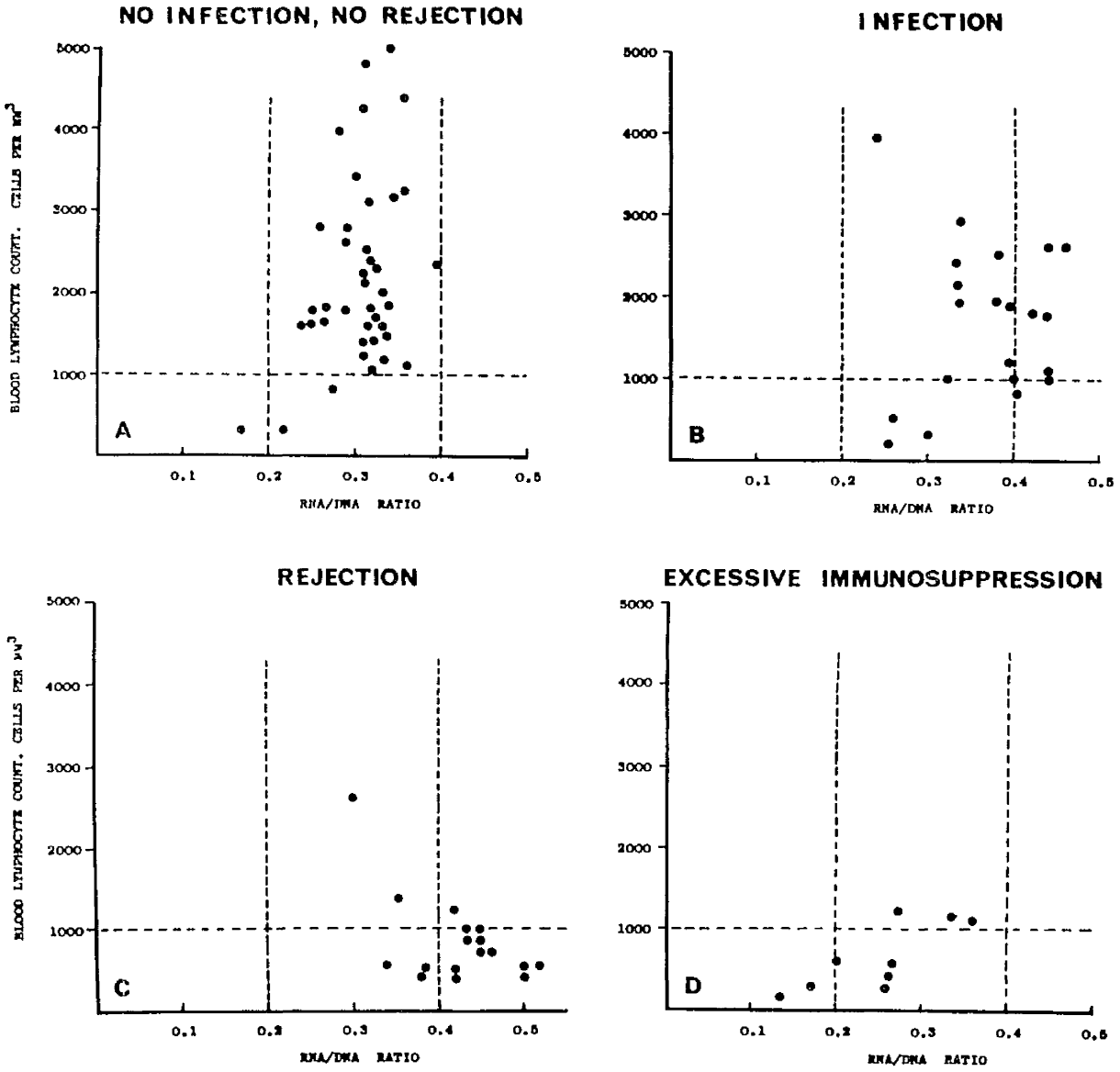


FIGURE 2. The relationship between the blood lymphocyte count in cells per cubic millimeter and the lymphocyte RNA/DNA ratio in patients with a renal allograft and on immunosuppressive therapy in four clinical categories: A, no infection, no rejection; B, unequivocal infection; C, unequivocal rejection; and D, excessive immunosuppression. These categories are defined in the text.

groups. The results may be summarised as follows.

When both infection and rejection are absent (group A) the blood lymphocyte count is variable, only occasionally low. The lymphocyte RNA/DNA ratio is not elevated above 0.40 in these patients; rather, it may be occasionally low and then usually in association with a low blood lymphocyte count. When infection is present (group B), a low blood lymphocyte count tends to be associated with a low lymphocyte RNA/DNA ratio and a high lymphocyte count with a normal or high lymphocyte RNA/DNA ratio. In contrast with this are the results from patients with unequivocal rejection (group C). Here the usual values are a low blood lymphocyte count, less than 1,000/mm^3, and an elevated lymphocyte RNA/DNA ratio, usually greater than 0.40. In the presence of excessive immunosuppression, as evidenced by a general leucopaenia (group D), it appears that a very low lymphocyte RNA/DNA ratio may be present when the blood lymphocyte count is also very low.

If the changes in the blood lymphocytes found in unequivocal rejection are to have diagnostic value, the incidence of misleading results must be shown to be low. Possible false negative results are considered first. There were two normal

results from the same patient for both the blood lymphocyte count and the lymphocyte RNA/DNA ratio in the 17 analyses during unequivocal rejection. During a further 3 rejection episodes, each involving different patients, there was a lymphopaenia but with a lymphocyte RNA/DNA ratio within the upper normal limit of 0.40. These last results were, however, 1, 3, and 14 days after treatment of rejection with 1 g of prednisolone, so there remained only 2 results with a lymphocyte count of more than 1,000/mm^3 and a lymphocyte RNA/DNA ratio of less than 0.40 during untreated unequivocal rejection out of a total of 14. Special features of the rejection in the 1 patient who had these normal results are discussed later. Thus, a low incidence, i.e., 14% of false negative results in untreated unequivocal rejection, has been found.

Over the whole time of study, results were obtained during 12 distinct periods in 10 patients who showed a low blood lymphocyte count and a lymphocyte RNA/DNA ratio of more than 0.40 and therefore suggested the possibility of a subsequent rejection episode. Nine of these patients did have a clinical rejection episode. Of the individual 19 abnormal results, rejection followed in 14 with an average interval of 23 days. The interval between finding the abnormal result and the clinical rejection episode was variable; in one case the lymphocyte abnormality appeared 129 days in advance of rejection and was shown still to be present 30, 7, and 1 day before gradual functional impairment of the graft was apparent and treatment was initiated. The remaining 5 abnormal results were not followed by a recognisable rejection episode. Two of these may be discounted since the results were obtained 1 and 2 days after treatment of rejection with 1 g of prednisolone. The incidence of false positive results in the diagnosis of rejection was thus 3 in 19, or 16%.

An example of the results of serial monitoring of the blood lymphocytes during a period of acute reversible rejection is shown in Figure 3. In this illustration lymphopaenia with a markedly elevated lymphocyte RNA/DNA ratio was present at least 7 days in advance of the decision to treat for acute rejection. The earliest rise in the plasma creatinine, on which the decision to increase the immunosuppression was based, occurred 5 days after the changes in the lymphocytes were established. Four such examples, in different patients, of prior abnormality of the blood lymphocytes have been documented.

Lymphopaenia was a more frequent observation than leucopaenia in this study. Of the 84 samples with a lymphocyte count of less than 1,000 cells/mm^3, 21 had a severe lymphopaenia with a count of less than 500 lymphocytes/mm^3. Leucopaenia, defined as a blood white cell count of less than 4,000 cells/mm^3, in contradistinction to lymphopaenia, was present in only 11 samples in the study, and only 3 times were severe lymphopaenia and leucopaenia present together. Thus, the average white cell count in the samples with less than 500 lymphocytes/mm^3 was 6,516 cells/mm^3.

The mean blood lymphocyte RNA/DNA value from patients showing classical overimmunosuppression by virtue of a leucopaenia not associated with rejection is listed in Table 1, and the individual results are displayed in Figure 2. In the presence of severe lymphopaenia, and not associated with rejection, the mean lymphocyte RNA/DNA is lower at 0.197 (SD 0.054) than the value of 0.253 found in leucopaenia. This difference of the means is just significant ($P < 0.05$). More important is the observed range of values for the ratio in severe lymphopaenia not associated with rejection, i.e., 0.12–0.27. When patients with less severe lymphopaenia are considered, i.e., those with a blood lymphocyte count of less than 1,000 cells/mm^3, the range of observed values for the lymphocyte RNA/DNA ratio extends upward to 0.37. This value for the lymphocyte RNA/DNA ratio is lower than that considered appropriate for the diagnosis of graft rejection and it appears, therefore, that a lymphocyte RNA/DNA ratio of less than 0.40 distinguishes the lymphopaenia which may be the result of immunosuppressive drug action from the lymphopaenia associated with rejection.

Measurement of the mean DNA content of the lymphocytes isolated from peripheral blood was not a main objective in the study, but a cell count was carried out to check that in each case the yield of cells was sufficient for analysis. A series of values for the mean DNA content of the cells was therefore obtained using manual counting in Neubauer chambers as a reference. Individual values thus determined are inaccurate, but average values of multiple samples have more validity. The following values were found for the mean lymphocyte DNA content in the various groups all expressed as μg DNA

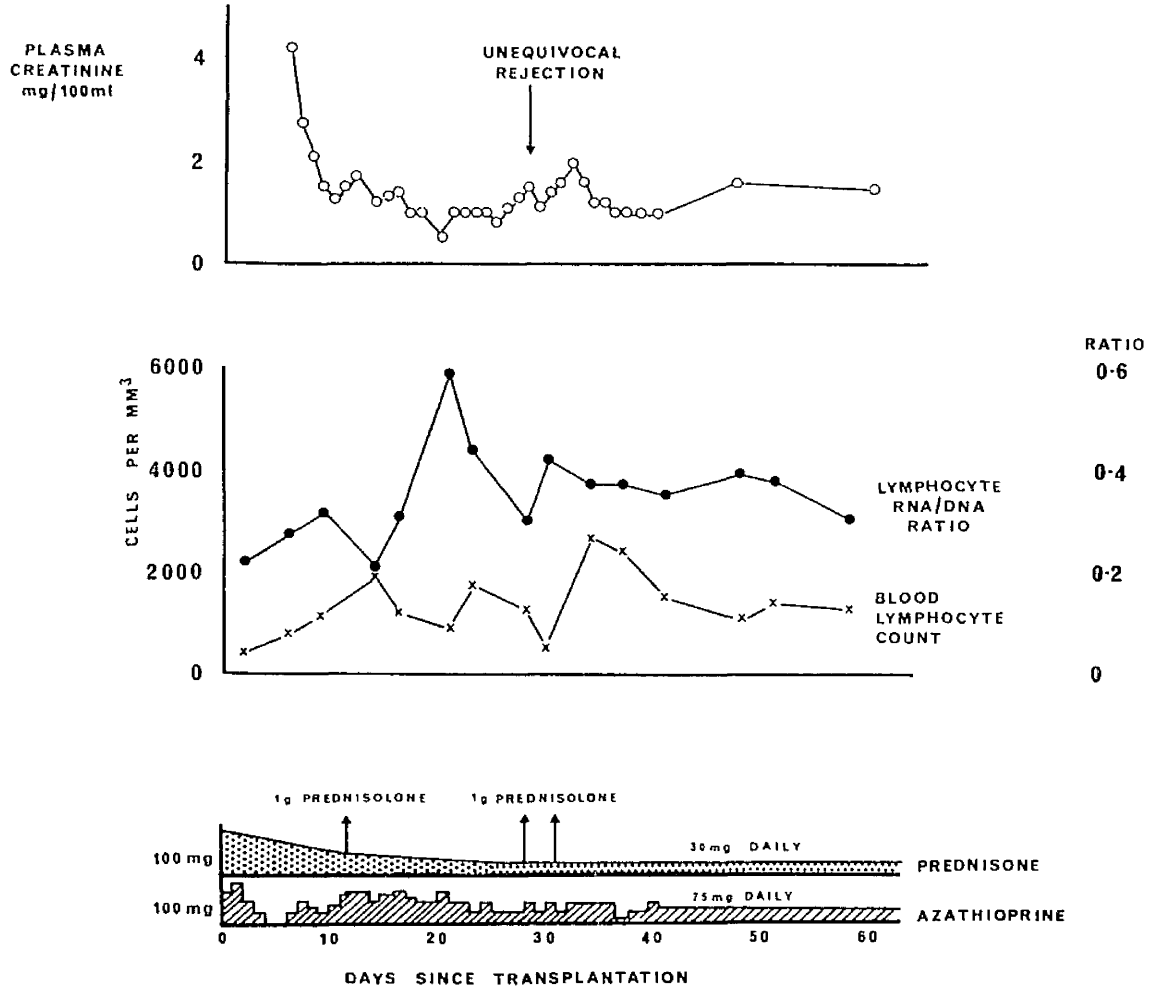


FIGURE 3. The course of an acute rejection episode showing serial changes in plasma creatinine, blood lymphocyte count, lymphocyte RNA/DNA ratio, and prednisone and azathioprine therapy. Treatment of rejection episodes with i.v. prednisolone in 1-g dose is indicated by an arrow.

phosphorus/10^6 cells: no infection, no rejection, 0.824; unequivocal infection, 0.836; unequivocal rejection, 0.805; excessive immunosuppression (leucopaenia), 0.856; and severe lymphopaenia, 0.837. As there was no significant difference between the mean values for all groups it is valid to regard changes in the lymphocyte RNA/DNA ratio between the groups as evidence of a difference in the mean lymphocyte RNA content.

When the groups were combined, the mean lymphocyte DNA content was 0.824 with a standard deviation of 0.165 μg of DNA phosphorus/10^6 cells. This was slightly higher than the mean DNA content of lymphocytes found in normal untreated persons, i.e., 0.732 μg of DNA phosphorus/10^6 cells, a difference of the mean which was significant at the 5% level.

DISCUSSION

The features of cell-mediated graft rejection, notably the proliferation of blast cells in the lymph node which drains the area of the graft and the subsequent migration of lymphoid cells to the graft to participate in its destruction, have been established experimentally (*1, 10, 24*). It was anticipated from this experimental work that atypical mononuclear cells would be found in the peripheral blood as part of the immunological response to the graft, and this has been confirmed in man by the previously reported functional abnormalities of the lymphocyte (*11, 18, 21, 22*) as well as by the changes in the lymphocyte RNA/DNA ratio in relation to renal allograft rejection reported here. The diagnostic usefulness of the various tests showing alteration in the circulating blood lymphocytes

in graft rejection is limited by the appearance of the so called hyperbasophilic mononuclear cells in response to a variety of stimuli including injected antigen (*4, 17*), virus (*13*), and bacterial (*26*) infection. The present paper proposes the simultaneous determination of the blood lymphocyte count and the mean lymphocyte RNA/DNA ratio as a method of monitoring the effectiveness of immunosuppression in patients with a renal allograft. The method distinguishes graft rejection from excessive immunosuppression, even when the oversuppression is complicated by infection.

Lymphopaenia may be present in both infection and rejection in patients on standard immunosuppressive therapy. Only in rejection, however, is the combination of lymphopaenia with an elevated lymphocyte RNA/DNA ratio found. In contrast, lymphopaenia in the presence of infection may be associated with a distinctly low lymphocyte RNA/DNA ratio. This difference between infection and rejection may be the result of the more intense cellular response produced by the presence of the renal graft. In some infections in immunosuppressed patients the lymphocyte RNA/DNA ratio is increased but here there is no lymphopaenia. Comparison of the results for infection and rejection in Figure 2, B and C, will clarify this point and show the necessity of measuring the lymphocyte count and RNA/DNA ratio together.

It is difficult to understand why lymphopaenia should be a feature of graft rejection. Although in classical acute rejection of a renal graft the donor kidney is infiltrated with a large number of lymphocytes (*19*), these can hardly be sufficiently numerous to deplete the entire lymphoid system of cells. Hall (*10*) and Prendergast (*20*) consider that the mononuclear cells entering a graft represent a random selection from the mononuclear population of the blood. It is likely that immunosuppressive therapy reduces the turnover of lymphoid cells and the rate at which replacement cells reach the blood; it may be that cells leaving the blood and entering kidney tissue during rejection are only slowly replaced and a lymphopaenia develops.

The observation that the lymphocyte RNA/DNA ratio, and, hence, the mean lymphocyte RNA content, is reduced when overimmunosuppression is present is of interest since azathioprine, a 6-mercaptopurine analogue, and corticosteroids are believed to inhibit RNA and DNA synthesis (*2*). However, the effect on the mean lymphocyte RNA/DNA ratio is more likely to be attributable to a change in cell population than to a direct action on the nucleic acid content of individual lymphocytes. Azathioprine is thought to interfere with the turnover of the large active mononuclear cells (*12*), reducing their numbers, and this would account for the lowered mean lymphocyte RNA/DNA ratio in overimmunosuppressed patients. In a previous study of thoracic duct lymphocytes in man (*9*), an increase in the mean cell RNA/DNA ratio was noted as azathioprine and prednisone were first administered prior to transplantation. The altered ratio was transient and drug treatment alone is unlikely to be the explanation of the changes observed in the blood lymphocytes during rejection since most of the results obtained were during maintenance therapy with a moderate drug dosage. Indeed, on successful treatment of rejection with 1 g of prednisolone, the lymphocyte abnormality is resolved (Fig. 3).

Porter (*19*) has detailed the pathological changes which occur during rejection of a renal allograft in man. He considers that rejection which occurs in the first 11 days after transplantation, excluding immediate rejection, is largely a cell-mediated process and that, in later rejection, vascular changes and immunoglobulin deposition with platelet and fibrin thrombi are more prominent features of the pathology. In the study of unequivocal rejection reported here all but one of the episodes were acute or chronic rejections occurring 11 days or more after the date of the transplant. Lymphocyte abnormality was detected in most of these rejection episodes. The one patient who had persistently normal lymphocytes in the presence of unequivocal rejection did have predominantly vascular changes in the donor kidney and cellular infiltrate was slight. It is apparent, however, that the blood lymphocytes are usually abnormal even when rejection is more than 11 days from transplantation. The patient who showed the longest period of abnormality prior to graft rejection developed an insidious progressive rejection which did not respond to treatment, and over a period of 2 months the creatinine clearance fell from 40 ml/min to less than 2 ml/min.

The evidence that the mean DNA content of the lymphocyte is slightly greater in patients who have a renal allograft than in normal per-

sons does not affect comparisons of the lymphocyte RNA/DNA ratios reported since there was no difference in the mean DNA content of the lymphocytes between the various clinical groups.

In conclusion, the usefulness of this method of lymphocyte monitoring is in the distinction which may be made between graft rejection and overimmunosuppression in lymphopaenic patients. The abnormality of the lymphocyte in rejection was present 5 days or more before evidence of renal damage became apparent in more than one-half of the fully documented cases. The appearance of an elevated lymphocyte RNA/DNA ratio in association with lymphopaenia is therefore an indication for close supervision of the patient. Normal lymphocyte counts and RNA/DNA ratios do not guarantee that patients will be free of rejection, but do contribute information on the degree of immunosuppression.

Acknowledgements. Acknowledgement is made of the contribution by many persons, both clinical and laboratory, to the management of the patients in this study. Due special mention for their practical contributions are Miss E. Linklater, who carried out all the more recent lymphocyte analyses, and Dr. R. W. Payne and his staff in the Haematology Department of the Victoria Infirmary for their interest, as well as their help, in undertaking the blood leucocyte counts. Financial support by the Burroughs Wellcome Co. is gratefully acknowledged.

REFERENCES

1. Andrew, J. A.; Schwartz, R. S.; Mitus, W. J.; Dameshek, W. 1962. Blood *19:* 313.
2. Berenbaum, M. C. 1967. J. Clin. Pathol. *20:* 471.
3. Calne, R. Y.; Evans, D. B.; Herbertson, B. M.; Joysey, V.; McMillan, R.; Maginn, R. R.; Millard, P. R.; Pena, J. R.; Salaman, J. R.; White, H. J. O.; Withycombe, J. F. R.; Yoffa, D. E. 1968. Brit. Med. J. *1:* 405.
4. Crowther, D.; Hamilton Fairly, G.; Sewell, R. L. 1969. J. Exp. Med. *129:* 849.
5. Dunea, G.; Nakamoto, S.; Stratton, R. A.; Figueroa, J. E.; Versaci, A. A.; Shibagaki, M.; Kloff, N. J. 1965. Brit. Med. J. *1:* 7.
6. Glen, A. C. A. 1967. Clin. Chem. *13:* 299.
7. Glen, A. C. A.; Jasani, M. K. 1968. Proc. Assoc. Clin. Biochem. *5:* 99.
8. Glen, A. C. A.; Jasani, M. K. 1968. Ann. Rheum. Dis. *27:* 180.
9. Glen, A. C. A.; Cooper, W. C.; Boak, J. L.; Murray, J. E.; Munro, H. N. 1970. Transplantation *9:* 83.
10. Hall, J. G. 1967. J. Exp. Med. *125:* 737.
11. Hersh, E. M.; Butler, W. T.; Rossen, R. D.; Morgan, R. O. 1970. Nature *226:* 757.
12. Lemmel, E. M.; Hurd, E.; Ziff, M. 1970. Fed. Proc. *29:* 431.
13. Lesiewska, J. 1967. Folia Histochem. Cytochem. *5:* 297.
14. Murray, J. E.; Gleason, R.; Bartholomay, A. 1964. Transplantation *2:* 660.
15. Murray, J. E.; Gleason, R.; Bartholomay, A. 1965. Transplantation *3:* 294.
16. Murray, J. E.; Gleason, R.; Bartholomay, A. 1965. Transplantation *3:* 684.
17. Pariser, S.; Zucker, R. A.; Meyer, L. M. 1952. Acta. Med. Scand. *144:* 201.
18. Parker, J. R.; Ellis, F.; Cameron, J. S.; Ogg, C.; Mowbray, J. F. 1970. Proc. Europ. Dial. Transpl. Assoc. *7:* 331.
19. Porter, K. A. 1967. J. Clin. Pathol. *20:* 518.
20. Prendergast, R. A. 1964. J. Exp. Med. *119:* 377.
21. Smith, M. G. M.; Eddleston, A. L. W. F.; Dominguez, J. A.; Evans, D. B.; Berwick, M.; Williams, R. D. 1969. Brit. Med. J. *2:* 275.
22. Tennenbaum, J. I.; St. Pierre, R. L.; Cerilli, G. J. 1968. Transplantation *6:* 986.
23. Tormey, D. C.; Fudenberg, H. H.; Kamin, R. M. 1967. Nature *213:* 281.
24. Turk, J. L. 1967. J. Clin. Pathol. *20:* 423.
25. Whaley, K.; Glen, A. C. A.; Dick, W. C.; Nuki, G.; Buchanan, W. W. Clin. Exp. Immunol. (in press).
26. Wood, T. A.; Frenkel, E. P. 1967. Amer. J. Med. *42:* 923.

Received 10 May 1971.
Accepted 17 June 1971.

TRANSPLANTATION
Vol. 9, No. 2
Copyright © 1970 by The Williams & Wilkins Co.
Printed in U.S.A.

DNA AND RNA MEASUREMENTS ON THORACIC DUCT LYMPHOCYTES OF PATIENTS UNDERGOING RENAL TRANSPLANTATION[1]

A. C. A. GLEN[2], W. C. COOPER[2], J. L. BOAK[3], J. E. MURRAY[3], AND H. N. MUNRO[2]

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and Department of Surgery, Peter Bent Brigham Hospital, Harvard Medical School, Boston, Massachusetts 02115

SUMMARY

The ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) content of thoracic duct lymphocytes was examined serially prior to and during immunosuppressive therapy in four patients receiving renal allografts. Before drug treatment, the mean values for the nucleic acid content of the thoracic duct lymphocytes were 0.72 pg of DNA phosphorus/cell and 0.16 pg of RNA phosphorus/cell. In all patients the mean RNA/DNA ratio of the lymphocytes increased transiently as azathioprine or prednisone was administered. The increases, which averaged 17% with azathioprine and 37% with combined prednisone and azathioprine in the 24 hr following initiation of the drugs, were statistically significant. In short-term tissue culture, the response of thoracic duct lymphocytes on exposure to phytohemagglutinin was determined by nucleic acid measurement. Evidence is presented of an impaired transformation response of lymphocytes from patients being treated with azathioprine.

The circulating lymphocyte is involved directly in several basic immunological reactions, one of which is the rejection of allografts (7). We have measured ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in thoracic duct lymphocytes (TDL) of patients undergoing renal homotransplantation with two general objectives in mind: to assess the functional status of the TDL serially, during treatment of the patient with immunosuppressive drugs and fol-

[1] Send reprint requests to Dr. H. N. Munro, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

[2] Supported by Grant DA-49-193-Md-02560 from the United States Army.

[3] Supported by Grants AI-06091, AI-04606, and HE-01771 from the United States Public Health Service and by Grant MD-2061 from the United States Army Research and Development Command. Grateful appreciation is also extended to the John A. Hartford and Avalon Foundations.

lowing renal transplantation, and to assess the in vitro responsiveness of the patient's lymphocytes to phytohemagglutinin (PHA).

Human TDL, available to us as a result of thoracic duct drainage of patients undergoing renal transplantation, permitted us to use greater quantities of lymphocytes in these experiments than would be feasible with blood lymphocytes. Therefore, it was possible to perform multiple experiments on lymphocytes from the same patient and, since the thoracic duct fistula was open during the initiation of immunosuppressive treatment, serial studies of lymphocytes were possible during this period. The TDL were 95–100% small lymphocytes, almost uncontaminated with other blood cell types; thus, it was not necessary to remove contaminating leukocytes. A disadvantage of using TDL from transplantation subjects who are anephric and are maintained by hemodialysis is that the lymphocytes so obtained cannot be considered normal in all respects; that is, it is known that such lymphocytes may not transform as readily as do lymphocytes from normal persons (*15*). However, these lymphocytes do respond to mitogens and we have found no significant metabolic abnormalities in these cells.

Morphological studies of the TDL in individuals with increased immunological activity, as evidenced by elevated γ-globulin levels (*17*), show an increased frequency of cells with developed endoplasmic reticulum, and there appears to be a morphological spectrum of lymphocytes with increasing ribosome clustering and development of endoplasmic reticulum (*18*). Approximately 75% of lymphocyte RNA is ribosomal RNA, so that an increase in the average RNA content of lymphocytes essentially reflects a rise in ribosomal population. Changes in nuclear RNA are quantitatively insignificant. Thus, measurement of the mean RNA content of lymphocytes in the thoracic duct would quantitate the protein synthetic potential of these cells and, hence, to some extent, reflect their immunological activity. The relationship between immunological activity and the average RNA content of the lymphocytes is complex (*6*), but appears to follow, at least in part, immunological changes. In peripheral blood lymphocytes, the average DNA content of the lymphocyte has been shown to be constant both in normal individuals and in those in whom immunological activity is increased (*6*). Therefore, the RNA/DNA ratio of lymphocytes is a measure of their RNA content and is known to be elevated in conditions of increased immunological activity, e.g., rheumatoid arthritis (*6*), lupus erythematosis, Sjögren's disease, and allograft rejection (Glen, unpublished data). Morphological changes, implying changes in the RNA/DNA ratio of lymphocytes, are a well documented feature of the immune response (*1*, *10*), so that a change in the ratio may have clinical application to patients receiving allografts whose lymphocytes are involved in rejection of the foreign tissue.

In this study, nucleic acid measurements have also been used to follow the response of the patients' lymphocytes in culture to stimulation by phytohemagglutinin. The use of lymphocyte RNA/DNA ratio has practical advantage over enumeration of transformed lymphocyte as an index of cell response. This procedure permits assessment of alterations in the lymphocyte metabolic response to PHA in the patients on immunosuppressive therapy.

MATERIALS AND METHODS

The four subjects in this study were patients maintained by intermittent hemodialysis prior to receiving allografts in the transplantation unit of the Peter Bent Brigham Hospital. The regimen before renal transplantation was directed at suppression of the immune response as follows: TDL were drained continuously from thoracic duct fistula beginning 5 days (in one patient, 9 days) before transplantation; azathioprine (2.5 mg daily/kg of body wt) was given, starting 48 hr before operation; and prednisone (1.5 mg daily/kg of body wt) was given, 24 hr before kidney transplantation. These drugs were continued for the lifetime of the allograft, dosages being adjusted as dictated by the patients' clinical status. Dosage schedules and other details of clinical management of the patients have been described (*16*). The lymph was allowed to flow freely and was collected in sterile bags with citrate as the anticoagulant (Fenwal Laboratories); the cells were separated from the lymph by centrifugation at 150 *g*.

Prior to estimation of DNA and RNA, samples of lymphocytes were freed of red cells by osmotic shock and analysed using a modified Schmidt-Thannhauser procedure (*4*, *5*). The lymphocytes, usually 10×10^6 cells/tube, were analysed either when freshly harvested from the lymph or after short-term storage at −40 C in 0.01 M sodium acetate buffer, pH 5.2. A decay curve was used to compensate for the small losses of nucleic acids during storage (amounting at most to 15% of the RNA after 30 days).

The same methods of measurement for DNA and RNA were used to follow a series of short-term in vitro cultures of the TDL, which were analysed serially in duplicate. The cultures were set up with sterile precautions using saline-washed, nonosmotically shocked fresh lymphocytes in tissue culture medium 199 (Grand Island Biological Co.). The lymphocyte concentration was $1–3 \times 10^6$ cells/ml. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added to the culture medium with 3% serum, usually the patient's own, obtained prior to any drug treatment. In some experiments fetal calf serum was substituted for autologous serum. When PHA (Difco PHA-P, Difco Laboratories, Detroit, Michigan) was used, 1.25 μl of the solution, reconstituted to the manufacturer's specifications, were added per milliliter of culture. The closed culture tubes were incubated at 37 C. Red cells present in the cultures contributed an error of about 5% to the RNA assays; however, no correction was made for this since all the cultures had about the same degree of red cell contamination.

RESULTS

Composition of fresh TDL. Table 1 shows the nucleic acid composition of lymphocytes obtained early in the thoracic duct drainage of four patients before immunosuppressants were administered. The mean DNA content (0.72 pg of DNA phosphorus/cell), the diploid amount for man (*9*), was similar to that found in blood lymphocytes by the same analytical method (*5*). The mean values for the RNA content (0.16 pg of RNA phosphorus/cell) and for the

TABLE 1. DNA and RNA content of human TDL measured before drug treatment

Patient	No. of samples	Cell content (pg/cell)		RNA/DNA
		DNA phosphorus	RNA phosphorus	
1	2	0.91	0.20	0.22
2	4	0.65	0.18	0.28
3	2	0.63	0.11	0.18
4	2	0.64	0.16	0.22
Mean TDL	10	0.72	0.16	0.22
Mean blood lymphocytes for comparison[a]	40	0.73 ± 0.12[b]	0.19 ± 0.06	0.26 ± 0.06

[a] This row of data was obtained from measurements on normal human blood lymphocytes (*5*). The mean RNA/DNA ratio, 0.26, is slightly higher than the mean ratio (0.22) of the four patients, but the difference between the means is not statistically significant.

[b] Mean ± SD.

TABLE 2. Effect of immunosuppressive therapy on TDL RNA/DNA ratio[a]

Patient	Ratio at			
	Pretreatment period (a)	24-hr period following initiation of azathioprine therapy (b)	24-hr period following initiation of prednisone therapy (c)	Posttransplant period (d)
1	0.22 (mean of 2)	0.29 (1 analysis)	0.37 (1 analysis)	0.27 (mean of 2)
2	0.28 (mean of 2)	0.28 (mean of 8)	0.33 (mean of 3)	0.27 (mean of 6)
3	0.20 (mean of 5)	0.23 (mean of 6)	0.30 (mean of 4)	0.33 (mean of 3)
4	0.24 (mean of 4)	0.30 (mean of 3)	0.29 (mean of 2)	Spontaneous early closure of thoracic duct fistula
Mean result for four patients	0.235	0.275	0.323	0.290
Comparison with pretreatment values by Student's *t* test		Significant difference $P < 0.01$	Significant difference $P < 0.001$	Significant difference $P < 0.05$

[a] The tabulated results are the RNA/DNA ratios of samples of lymphocytes from the thoracic duct obtained (a) prior to renal transplant and immunosuppressive treatment, (b) during the 24 hr which followed initiation of azathioprine treatment, (c) during the 24 hr which followed initiation of prednisone treatment, with azathioprine continued, and (d) after the transplant operation, with continued immunosuppression.

RNA/DNA ratio (0.22), while lower than those found in blood lymphocytes (*5*), were not significantly different by Student's *t* test.

Table 2 summarizes the results of serial determination of the RNA/DNA ratio of TDL at the time of institution of immunosuppressive therapy and also for a period after renal transplantation. These results are also illustrated in Figures 1 and 2. Statistical analysis of the results for all four patients (Table 2) shows that immunosuppressive therapy increased the RNA/DNA of the TDL significantly over the pretreatment levels. The increase averaged 17% with azathioprine and 37% with combined prednisone and azathioprine therapy in the 24-hr period following the start of treatment. Some individual measurements of the RNA/DNA were considerably higher than this, as much as 105% over the pretreatment level when patient 3 was first given prednisone (Fig. 2; 0.20–0.41). A constant finding illustrated in Figures 1 and 2 was the progressive fall in the output of TDL with continued drainage. However, there

was no consistent change in the RNA/DNA when early and late stages of the pretreatment period were studied. The progressive rise of the RNA/DNA of the lymphocytes at the initiation of immunosuppressive treatment is also evident. Following transplantation, the RNA/DNA was followed in 2 of the 4 subjects. In patient 2 (Fig. 1), the RNA/DNA value had fallen to the pretreatment level 16 days after transplantation, while in patient 3 (Fig. 2), the value was still elevated 30 days after the transplant. The latter case showed signs of rejecting the graft.

Changes in TDL during culture. The TDL were also studied in short-term tissue culture, using cells obtained both during the pretreatment period and after azathioprine treatment (but before predisone was started). Parallel cultures gave satisfactory duplication. The mean variation (difference between duplicate cultures expressed as percentage of the mean value) was 1.7% for DNA measurements and 3.5% for RNA analyses. For the RNA/DNA, the mean variation was 4.4%. These values are close to the expected variation for the method of analysis (*5*).

The response of the TDL to PHA was examined before and after azathioprine therapy by comparing cultures grown with and without mitogen. A representative experiment is illustrated in Figure 3, and a comparison of the cultures at 32 hr is drawn up in Table 3. Results at 32 hr were selected for Table 3 since the RNA content of the cultures reached a plateau at about that time.

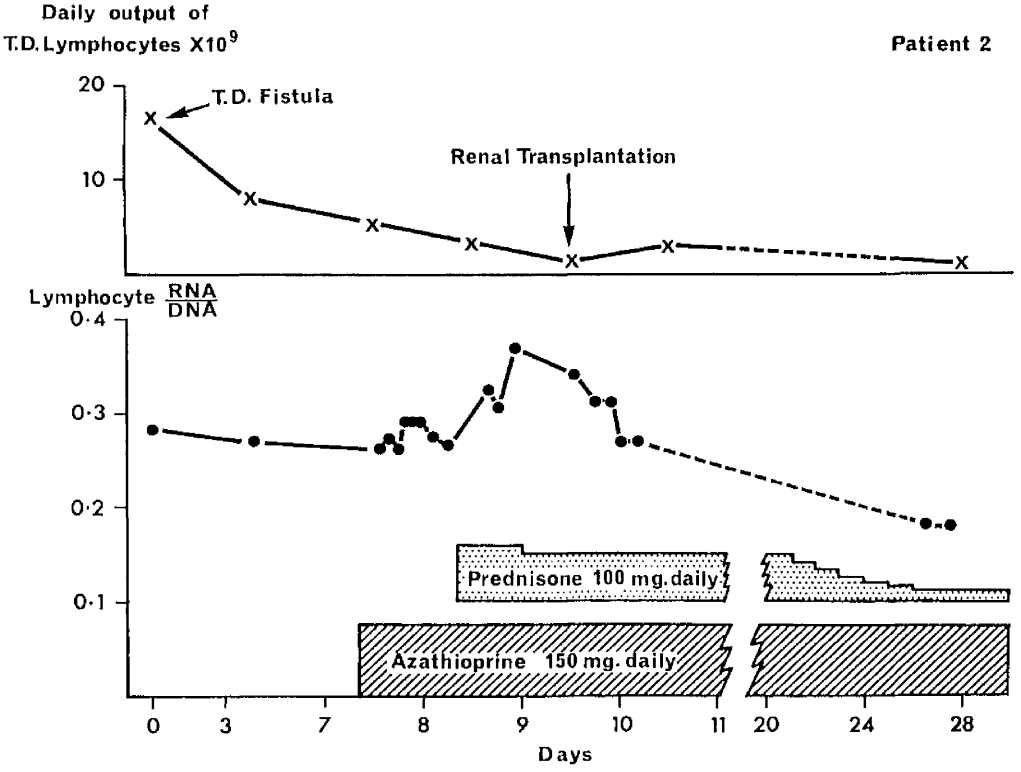


FIGURE 1. The daily output and RNA/DNA ratio of the TDL of patient 2 undergoing continuous duct drainage, immunosuppressive therapy, and renal transplantation. This figure is representative of the four patients studied, with the exception of patient 3 (Fig. 2), whose final ratio was higher, rather than lower, than her pretreatment ratio.

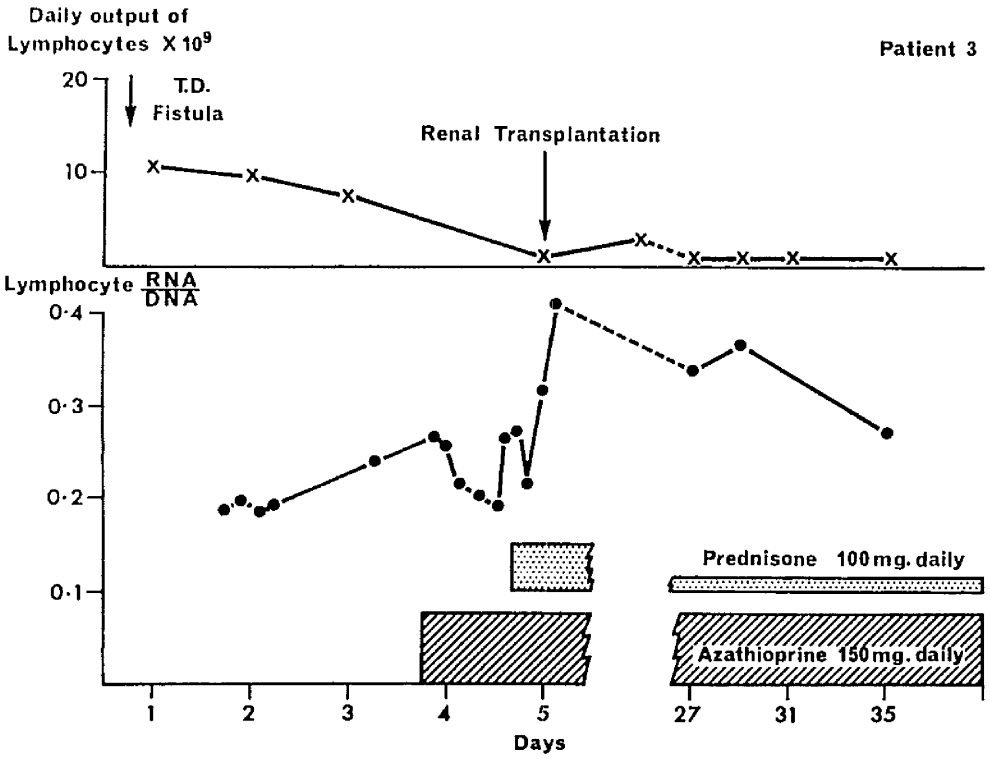


FIGURE 2. The daily output and RNA/DNA ratio of the TDL of patient 3 undergoing continuous duct drainage, immunosuppressive therapy, and renal transplantation.

DNA analyses. A consistent fall in the DNA content of all the cultures occurred with incubation, an illustration of the cell death and dissolution which take place in lymphocyte cultures. At 32 hr, the DNA level had fallen to between 74 and 80% of the initial level.

RNA analyses. Net accumulation of RNA, despite falling cell numbers indicated by the DNA analysis, occurred during the culture of lymphocytes (PHA-stimulated) obtained prior to azathioprine, in two of three patients. At 32 hr, the average RNA content of the cultures for the three patients was 10% above the starting level in the presence of PHA, and 30% below the starting level in the absence of PHA. In contrast, for cultures of lymphocytes obtained after initiation of azathioprine treatment, the total RNA content of the cultures had fallen slightly even with PHA stimulus and had fallen more dramatically in the absence of PHA (compare Fig. 3, A and B). At 32 hr, the average RNA content for the cultures after azathioprine was 58% of the starting level in the absence of PHA and 89% of the starting level with PHA.

RNA/DNA ratio. Figure 3, C and D illustrates the change in the average lymphocyte RNA/DNA ratio during culture of the cells obtained in one patient, prior to and after azathioprine therapy. While the RNA/DNA ratio of the cells cultured in the absence of mitogen apparently fell more rapidly if the lymphocytes were from a patient on azathioprine treatment, more significant is the difference between the response to PHA in the pre- and postazathioprine cultures. Analysis of the cultures at 32 hr for three patients, comparing the PHA-stimulated culture with the corresponding control cultures, shows an

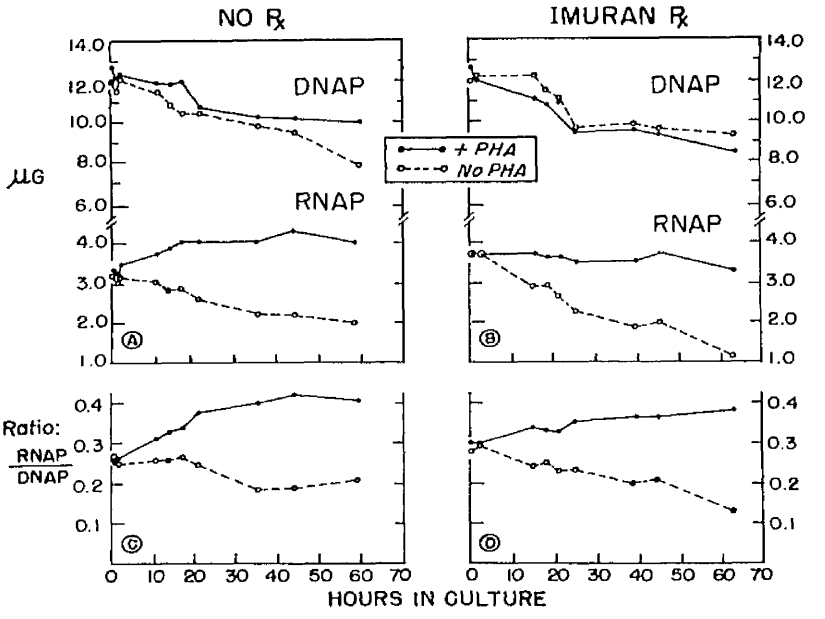


FIGURE 3. An assessment of short-term tissue culture of TDL by measurement of DNA phosphorus (DNAP), RNA phosphorus (RNAP), and the RNA/DNA ratio. A, DNA and RNA measurements on cells obtained before the patient received azathioprine treatment, cultured in the presence of PHA (●) and in the absence of PHA (○). B, DNA and RNA on cultured cells obtained during azathioprine treatment. C, The RNA/DNA ratio of the cells in culture, calculated from A. D, The RNA/DNA ratio of the cells in culture, calculated from B.

TABLE 3. The effect of azathioprine therapy on the PHA response of TDL[a]

Measurement[b]	Responses of patient not on immunosuppression		Responses of patient on azathioprine	
	Control culture	Culture with PHA	Control culture	Culture with PHA
DNA content of culture	81, 79, 64 (74)	76, 83, 78 (78)	74, 78, 88 (80)	90, 76, 75 (80)
RNA content of culture	79, 73, 60 (71)	117, 129, 85 (110)	58, 54, 62 (58)	87, 98, 83 (89)
RNA/DNA ratio of cultured cells	98, 94, 84 (92)	163, 158, 117 (146)[c]	79, 69, 94 (81)	97, 128, 98 (108)[d]

[a] Thoracic duct cells obtained from patients before immunosuppression and also after 24 hr on azathioprine therapy were set up in tissue culture. Eight cultures were incubated for each patient. For the presuppression period, there were duplicate cultures with PHA added and duplicate controls without PHA; similarly, for the azathioprine period, there were two PHA-treated cultures and two controls. The cultures were incubated at 37 C for 32 hr and analysed for their DNA and RNA content. Results are expressed as the mean of duplicate cultures for each patient, together with the mean response for three patients in parentheses.

[b] Preincubation content = 100.

[c] Control versus PHA, significant difference by *t* test, $P < 0.05$.

[d] Control versus PHA, no significant difference by *t* test, $P > 0.05$.

average difference of 54% (146–92) between the cultures obtained before azathioprine treatment and 27% (108–81) between those after azathioprine administration. The *t*-test of the RNA/DNA analysis from three patients at 32 hr reveals a significant difference between the PHA culture and the control culture when the cells were obtained prior to immunosuppressive therapy (Table 3). Culture prepared from cells obtained after azathioprine treatment did not show this significant difference between control culture and PHA-stimulated culture.

DISCUSSION

The response of the TDL to the initiation of immunosuppressive therapy by increase in the RNA/DNA is regarded as an expression of changes in the cell types in thoracic duct lymph. Dougherty et al. (*2*) showed that hydrocortisone produces preferential dissolution of the small lymphocyte, the large lymphocyte being relatively resistant to the steroid. A similar sensitivity of the small lymphocyte to azathioprine, a derivative of 6-mercaptopurine, would explain the observed change in the RNA/DNA ratio. The observed increase in the average RNA content of the lymphocyte has application in the interpretation of change in the lymphocyte, whether in blood or lymph, in relation to the immune response. In the clinical setting, misinterpretation of a rise in the lymphocyte RNA/DNA, as immunosuppressive drugs are instituted or increased, can be avoided. A rise in the lymphocyte RNA/DNA ratio in the absence of any change in immunosuppression is likely to result from an immune response, the appearance of active "intermediate" cells being a known accompaniment of immunological activity (*17*). A similar increase in the RNA/DNA ratio of the circulating lymphocyte may be expected to occur during the rejection of an allograft.

In two patients, the lymphocyte was followed after transplantation. Patient 3 (Fig. 2) showed evidence of graft rejection and her lymphocyte RNA/DNA remained elevated. It is likely that the increased sensitivity of the patient to strong donor histocompatibility antigens caused production and release into the circulation of a population of large, young lymphocytes; perhaps the enlarged pyroninophilic cells were associated with rejection of allografts in general (*7*) and with kidney allografts in particular (*11*). Six months following the allograft, patient 3 was readmitted to the hospital with clinical signs of a rejection crisis. The dosages of azathioprine and prednisone were increased, and the rejection was aborted. Thus, serial determination of the lymphocyte RNA/DNA may be of potential value in monitoring the immune status of patients who have received kidney allografts. This is being currently tested in a study of the blood lymphocyte RNA/DNA as a gauge of impending renal allograft rejection.

The small lymphocyte of blood or lymph is not an "end stage" cell, as formerly believed, but can enlarge and start dividing when appropriately stimulated (*12*). In vitro stimulation of lymphocytes by PHA and other mitogens has made it possible to study these cells as they differentiate into large blastoid cells which undergo mitosis. The degree of PHA response in vitro can be cor-

related with the effectiveness of suppression of delayed hypersensitivity (*8, 13*), of which allograft rejection is one manifestation.

Our culture experiments were monitored by analysis of DNA and RNA content. The advantage of this method is that change in cell number can be noted as well as changes in the nature of the cells present. Simple comparison of the RNA/DNA ratio would suffer from the problem of the more usual measurement, e.g., percentage of transformation or tritiated thymidine incorporation, for which changes in cell number are unknown.

Prior to azathioprine therapy (Table 3), TDL in tissue culture responded to PHA by increasing their RNA content, one of the parameters associated with lymphocyte transformation. Sell et al. (*14*) showed DNA synthesis to be a late event in lymphocyte culture, occurring 36–48 hr after addition of PHA to lymphocytes. Our experiments show no net synthesis of DNA in response to PHA; rather, there is evidence of a reduction in cell numbers, but RNA synthesis is evident from the statistically significant increases in the RNA/DNA in a comparison of control and PHA-stimulated cultures.

The percentage of transformation of cultured TDL used in the present experiments, although not precisely measured morphologically, was known (by scanning of stained preparations) to be less than the maximal percentage obtainable, which is about 80% of cultured normal blood lymphocytes 72 hr after addition of PHA. We believe that this less than maximal value agrees with the known blunted response to PHA of blood lymphocytes from uremic patients (*3*).

Cultured TDL that had been exposed in vivo to azathioprine did not respond well to PHA; their RNA/DNA ratio increased only slightly, compared with that of lymphocytes from the same patient before azathioprine was started (Fig. 3, C and D). Hersh and Oppenheim (*8*) reported that the in vitro response to PHA of blood lymphocytes of patients receiving intermittent therapy with 6-mercaptopurine was reduced from 71% before treatment to 1.5% during treatment. Percentage of transformation was used as the measurement. Tennenbaum et al (*15*) made similar observations in patients under immunosuppression. Our results confirm these observations by an objective chemical measurement and, further, draw attention to the changes in the nature of the cells in thoracic duct lymph which occur as immunosuppressive drugs are instituted.

REFERENCES

1. Crowther, D.; Farley, G. H.; Sewell, R. L. 1969. J. Exp. Med. *129:* 849.
2. Dougherty, T. F.; Berliner, M. L.; Berliner, D. L. 1960. Ann. N.Y. Acad. Sci. *88:* 78.
3. Elves, M. W. 1966. p. 215. *In* W. O. Rieke (ed.). *The lymphocytes.* Lloyd-Luke, London.
4. Fleck, A.; Munro, H. N. 1962. Biochim. Biophys. Acta *55:* 571.
5. Glen, A. C. A. 1967. Clin. Chem. *13:* 299.
6. Glen, A. C. A.; Jasani, M. K. 1968. Ann. Rheum. Dis. *27:* 170.
7. Gowans, J. L. 1962. Ann. N.Y. Acad. Sci. *99:* 423.
8. Hersh, E. M.; Oppenheim, J. J. 1967. Cancer Res. *27:* 98.
9. Leslie, I. 1960. *In* J. N. Davidson and E. Chargaff (eds.). *The nucleic acids.* Vol. 2 Academic Press, New York.
10. Pariser, S.; Zucker, R. A.; Meyer, L. M. 1952. Acta Med. Scand. *144:* 201.

11. Porter, K. A. 1967. p. 518. In *Tissue and organ transplantation*. Brit. Med. Assoc., London.
12. Robbins, J. H. 1964. Science *146:* 1648.
13. Rubin, A. L.; Stenzel, K. H.; Hirschhorn, K.; Bach, F. 1964. Science *154:* 815.
14. Sell, S.; Rowe, D. S.; Gell, P. G. H. 1965. J. Exp. Med. *122:* 823.
15. Tennenbaum, J. I.; St. Pierre, R. L.; Cerilli, G. J. 1968. Transplantation *6:* 986.
16. Tilney, N. L.; Murray, J. E. 1967. Transplantation *5:* 1204.
17. Zucker-Franklin, D. 1963. J. Ultrastruct. Res. *9:* 325.
18. Zucker-Franklin, D. 1969. Seminars Hematol. *6:* 4.

Received 2 May 1969.
Accepted 13 October 1969.

Proceedings of 3rd Annual leucocyte culture conference
Appleton - Century - Crofts (1969)

Synthesis of Ribonucleic Acid in Human Lymphocytes

S. T. JACOB, W. C. COOPER, A. C. A. GLEN,* and H. N. MUNRO Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts

INTRODUCTION

The small lymphocyte of blood or lymph is an immunocompetent cell,[1] capable *in vitro* of synthesis of ribonucleic acid (RNA),[2] protein,[3] and deoxyribonucleic acid (DNA),[4] and of division.[5] When stimulated *in vitro* with the mitogen phytohemagglutinin (PHA), lymphocytes perform these functions in a greatly accelerated fashion. At the end of 72 hours of exposure to PHA, usually more than 70% of human lymphocytes in culture have become large, blast-like forms known as transformed lymphocytes. Although the mechanism of action of PHA is unknown, it is assumed that the *earliest* effects of this mitogen are the most important because they initiate the sequence of intracellular events whose ultimate expression is the fully transformed lymphocyte. Reported here are some early effects of PHA on lymphocyte RNA metabolism.

In these experiments, the answer was sought to the following question: "What are the size classes of RNA stimulated very early (within two hours) after addition of PHA to lymphocyte cultures?" Other reports sufficiently detailed to permit a tentative answer to this question have come from only two groups of workers.[2,6,7,8] Careful analysis of the data from these groups reveals the following points of agreement between them: 1) The resting lymphocyte synthesizes 4 to 6 S-RNA and a much smaller proportion of 18 and 28 S-RNA (ribosomal RNA: r-RNA). 2) Addition of PHA to lymphocytes in culture results in a time course pattern of RNA synthetic activity where increased synthesis of any given size species of RNA is a function of the time elapsed after addition of PHA. In

*Present Address, Biochemistry Department, Victoria Infirmary, Glasgow, Scotland.

short intervals (less than three hours) after addition of PHA to cultures, incorporation of labeled uridine into RNA occurs in two categories of RNA: 1) 4 to 6 S and polydisperse (about 6 to 35 S), both of which are stable to a chase with actinomycin-D and cold uridine.

Rubin, Cooper, and Kay concluded that PHA first enhances synthesis of nonribosomal RNA, which may either polydisperse or may sediment at 4 to 6 S, and that PHA causes increased synthesis of significant quantities of r-RNA only after six-hour incubation.* The latter conclusion, although perhaps generally accepted at this time, is not validated by the findings reported here. The authors' results indicate that within 90 minutes of incubation, PHA stimulates the synthesis of heavy (40 to 50 S) RNA, a significant portion of which is r-RNA precursor.

MATERIALS AND METHODS

Lymphocyte cultures

Thoracic duct lymphocytes were used from anephric patients whose blood urea nitrogen levels were kept low by frequent hemodialysis. The lymph leucocyte differential count was about 98% small lymphocytes; variable lymph contamination with erythrocytes occurred but did not alter the results. Lymph, collected in plastic citrate-phosphate-dextrose bags, was centrifuged at 4°C at 600 g for 10 minutes and the cell button washed twice with Medium 199. Lymphocyte cultures were set up in Medium 199 with 3% autologous serum at a concentration of 20 x 10^6 lymphocytes per ml. PHA-P (Difco), diluted according to specifications of the manufacturer, was used at a final concentration of 0.3 ml/100 ml suspension; the same volume of sterile saline was used in control tubes. Experiments are numbered 1, 2, and 3 to correspond with the figures. Other experimental details are described in the figure legends. Note that in Experiment 3, RNA was extracted from whole lymphocytes, not from nuclei.

Isolation of nuclei

All procedures following harvesting of cells were carried out at 0°C to 4°C unless stated otherwise. This method is similar to that of Pogo *et al.*[4] After the harvesting centrifugation,

**Data presented at this Conference by H.L. Cooper (Chapter 50) makes it clear that his group now finds that PHA does stimulate synthesis of r-RNA at early intervals after addition of the mitogen*

citric acid (0.01 M; 29 ml/tube) was added to the cell button and the resulting suspension agitated using a snugly-fitting motor-driven Teflon pestle in the tubes. Suspensions were then centrifuged (3,000 g) and the citric acid treatment repeated. After a final wash with 10 ml of 0.34 M sucrose, a drop of the suspension was removed and stained with azure C to monitor the presence of clean lymphocyte nuclei. (This procedure also removed all erythrocytes.) The nuclei were then collected by another centrifugation (3,000 g) and RNA extracted from them.

RNA extraction, fractionation, and counting

Lymphocyte nuclei or whole lymphocytes (see legend to Fig. 3) were homogenized with a Teflon pestle in 10 to 12 ml of 0.3% sodium dodecyl sulfate containing 0.14 M sodium chloride and 0.05 M sodium acetate (pH 5.1). After a few strokes, an equal volume of 90% phenol containing 0.1% hydroxyquinoline was added and the mixture homogenized further. The suspension was immediately shaken at 65°C for 10 minutes, shaken at room temperature for 15 minutes, and centrifuged (17,000 g) for 15 minutes at 4°C. The clear aqueous layer was then removed and the RNA precipitated with 2.5 volumes of ethanol containing 2% potassium acetate. RNA was reprecipitated once with ethanol. An aliquot of an aqueous solution of the RNA containing 100 μg RNA (about 2 OD units) was layered on a 5 ml, 10 to 40% linear sucrose density gradient, and centrifuged for 15 to 16 hours at 24,000 rev/min. Gradients were passed through the cell of a Gilford model 2,000 recording spectrophotometer and collected in fractions of eight drops each. Each preparation contained a total of about 16 fractions. After addition of one drop of 5 N perchloric acid to each vial, fractions were hydrolyzed at 80°C for 15 minutes, cooled, and Bray's solution (10 ml) added to each vial. Radioactivity was determined in a Beckman CPM-100 liquid scintillation system.

RESULTS

In comparison with control cultures, PHA in Experiment 1 (Fig. 1) stimulated incorporation of labeled uridine into high molecular weight (MW) RNA of lymphocyte nuclei by at least 100%. The peak of radioactivity in this experiment occurred at about 40 S, assuming a linear gradient. In Fig. 2, comparison of the heavy broken lines in parts A (control) and B (PHA) shows that the stimulatory effect of PHA occurred in 90 minutes, as compared with 135 minutes in Fig. 1. In Experiment 2, the labeling peak occurred at about 45 S in both PHA and control pulse tubes and

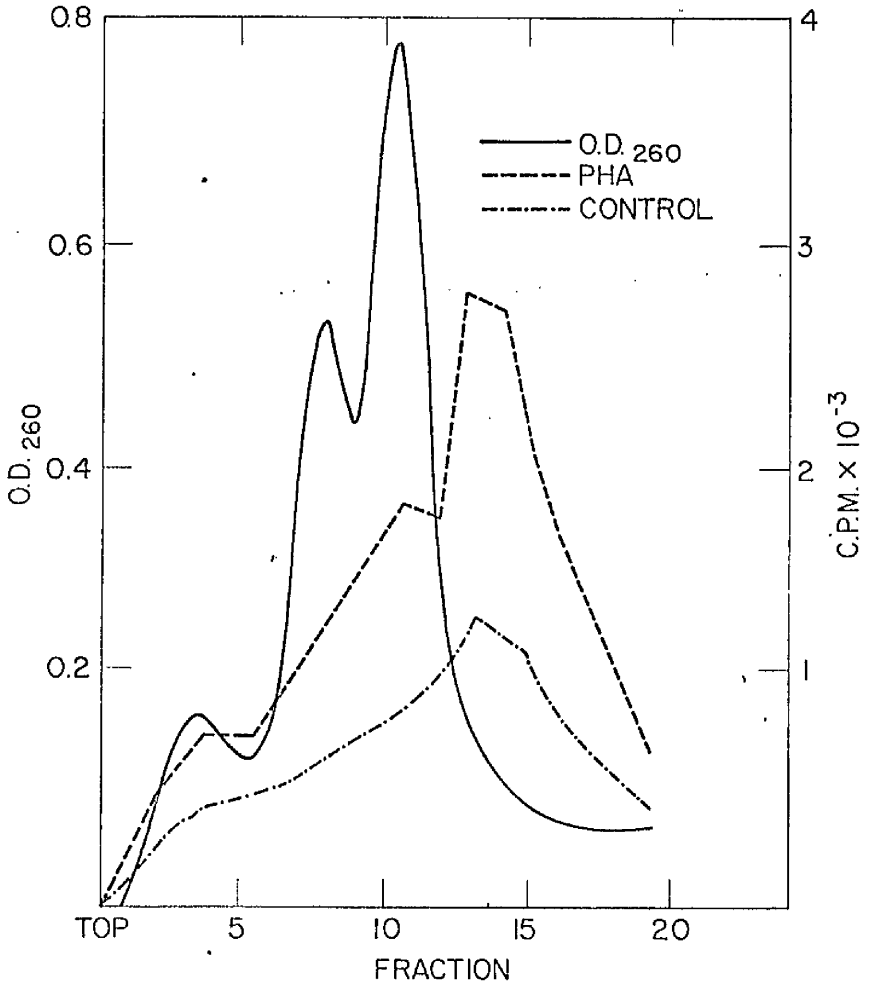


Fig. 1 Sucrose Gradient Sedimentation of Pulse-Labeled RNA from Nuclei of Lymphocytes Incubated for a Total of 135 minutes with and without PHA. A suspension of 20 x 10^6 lymphocytes per ml was divided into equal (25 ml) portions, one of which was treated with PHA-P, and the other with sterile saline. After an incubation period of 105 minutes, cultures were pulse labeled for an additional 30 minutes with uridine-5-T (4 μc/ml) (Nuclear Chicago Corporation; spec act 5 c/mM). Cultures were then harvested, nuclei isolated, and RNA extracted, fractionated, and counted. Solid line is OD_{260} of lymphocyte nuclear RNA (ordinate at left). Broken lines are counts/min for PHA and control nuclear RNA (ordinate at right).

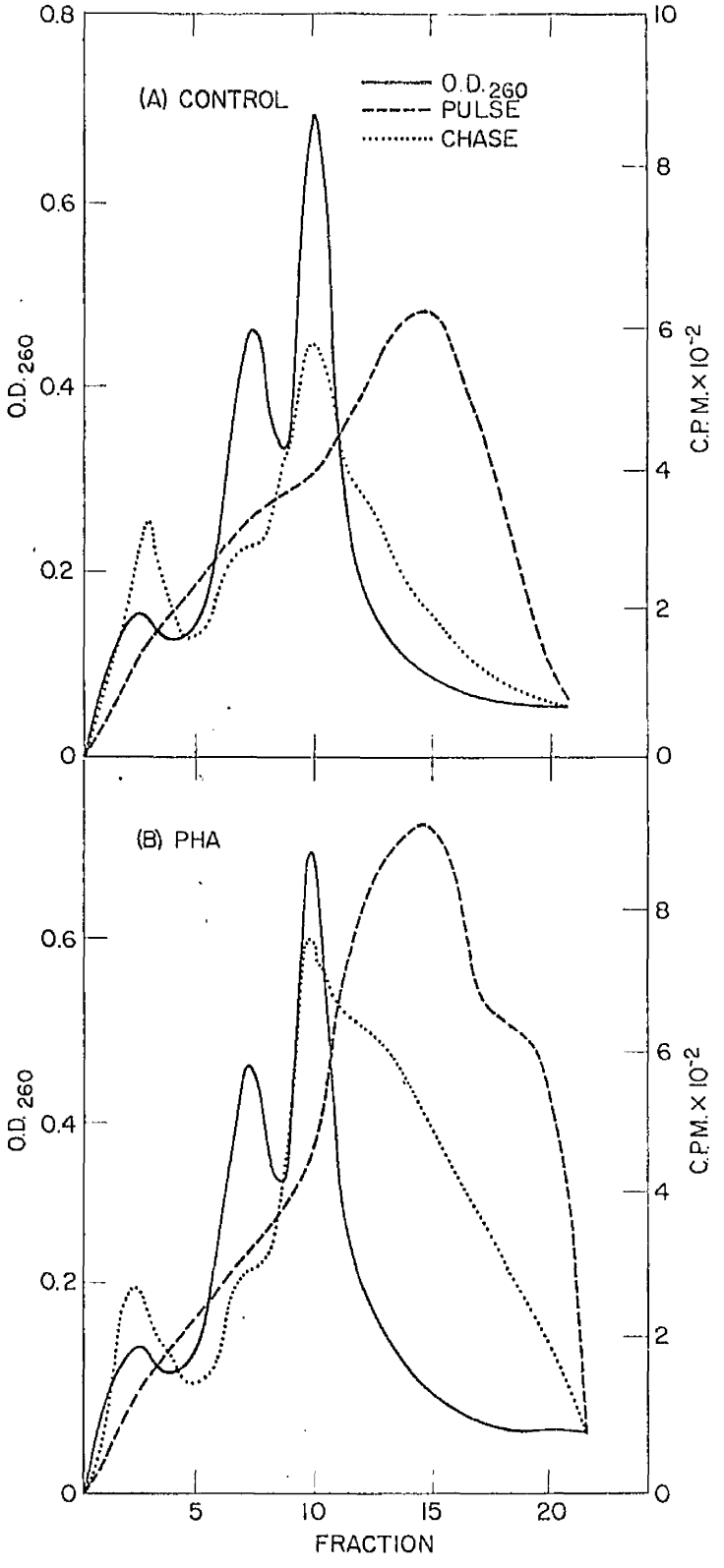


Fig. 2. Sucrose Gradient Sedimentation of Lymphocyte Nuclear RNA from Pulse-Chase Experiments. Data from two experiments (two patients) performed in the same manner have been combined in this illustration. Suspensions of 20 x 10^6 lymphocytes/ml (25 ml/tube) were incubated with sterile saline (A) or with PHA-P (B) for 90 min, during the last 30 min of which uridine-H^3 was also present (4 μc/ml). The pulse members of the pairs were then harvested, nuclei isolated, and RNA extracted, fractionated, and counted. Actinomycin-D (10 μg/ml) and cold uridine (0.1 mM) were added to the chase members of the pairs, and incubation was continued for another hour before harvesting. Solid lines are OD_{260} of lymphocyte nuclear RNA (ordinates at left). Broken lines are counts/min of RNA of pulse and chase tubes (ordinates at right).

the PHA pulse-labeling profile had a shoulder at about 50 S which was not present in the control tubes. The increase in extent of incorporation of labeled uridine into nuclear RNA in PHA cultures was between 50 and 100% that of controls in Fig. 2, which shows average values from two patients. Experiment 2 also demonstrates that the newly synthesized nuclear RNA is chased after one hour to reach a peak at 28 S in both control and PHA tubes. However, it appears that a smaller proportion of the high MW labeled RNA in the PHA tubes was chased to the 28 S region than was the case in the control tubes. In fact, the shape of the chased RNA curve in the controls (Fig. 2, part A) is roughly symmetrical around the 28 S OD peak, whereas the shape of the chased curve in the PHA tubes (Fig. 2, part B) is markedly skewed to the right of 28 S, indicating that a large portion of the 40 to 50 S-RNA synthesized in the presence of PHA was rather stable after a one-hour chase with actinomycin-D and cold uridine. Thus, synthesis of 45 S r-RNA precursor occurred in both control and PHA tubes, but in the latter, a significant proportion of the high MW RNA did not have the chase characteristics of r-RNA precursor. It is also apparent from Fig. 2 that some of the total radioactivity in the PHA pulse tubes (approximately 30%) was lost during the chase, and thus did not appear in the chase tubes. This phenomenon did not occur to any extent in the control experiment. Therefore, a portion of the newly made, high MW RNA is more labile in the presence of PHA than in its absence. It cannot be determined from the present data whether this lability is a function of the presence of PHA *per se*, or whether it represents greater lability of those species of high MW RNA synthesized in greater quantity in the presence of PHA.

Fig. 3 shows the kinetics of uridine-H^3 labeling of whole lymphocyte RNA (see legend to Fig. 3 for experimental details). Within two minutes' exposure of lymphocytes to the labeled RNA precursor, radioactive 4 to 6 S RNA was present, and PHA had stimulated its synthesis by more than 100% over the control. Labeling in the 4 to 6 S fraction increased, and by five minutes after addition of isotope was almost twice the two-minute value; no further increase in labeling of this fraction occurred during the remaining 25 minutes' exposure to isotope. No significant labeling occurred in the 40 to 50 S RNA region until 10 minutes after addition of uridine-H^3. The peaks of radioactivity in the 40 to 50 S region then increased at all intervals up to 30 minutes, and the extent of labeling of RNA was always greater in the PHA tubes than in the control tubes.

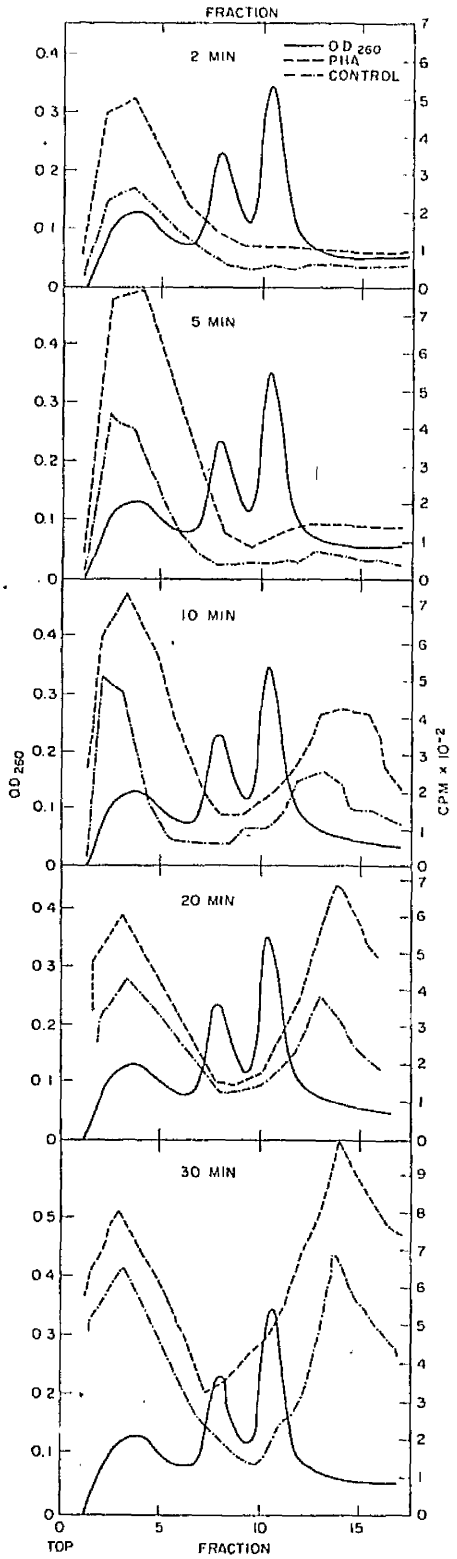


Fig. 3. Labeling-Kinetics of RNA in Lymphocytes With and Without PHA-P for 80 minutes. Lymphocyte cultures were set up as in Experiments 1 and 2. After addition of PHA-P or saline to equal portions, the lymphocyte suspensions were immediately distributed among 10 culture tubes (five PHA; five control), each containing 250 x 10^6 lymphocytes/ml. The tubes were closed and incubated at 37°C. For the final 2,5,10,20, and 30 minutes, respectively, (five parts of the figure, reading from top to bottom) of the 80-minute total incubation time of the five sets of tubes, uridine-H^3 (New England Nuclear Corporation; spec act 5 c/mM, generally-labeled) was added. Isotope was added in final concentration of 4 μc/ml suspension. At the end of 80 minutes all cultures were harvested and RNA was extracted from whole lymphocytes. RNA was fractionated and counted as in experiments 1 and 2.

DISCUSSION

The data indicate that PHA stimulates synthesis of both ribosomal and nonribosomal RNA within two hours after addition of the mitogen to lymphocyte cultures. This finding is contrary to the published reports of the two groups who have worked in this specific area.*

The authors believe that their method of rapid extraction of RNA with hot phenol from lymphocyte nuclei (the cytoplasm having been sheared away with a homogenizer) has permitted the obtainment of undegraded high MW RNA which more conventional methods of lymphocyte processing and RNA extraction do not obtain. Furthermore, it was not necessary in these experiments to risk introduction of artifact by addition of extraneous RNA either as an OD marker or as a carrier; the OD seen in the figures is that of the lymphocyte RNA itself.

At least three reasons explain the success of the method used in the present work to extract rapidly-labeled undegraded high MW RNA from PHA-stimulated lymphocytes. These are: 1) The radioactive high MW RNA of the nuclei is not diluted by cytoplasmic cold RNA, as occurs when RNA is extracted from whole lymphocytes. 2) Cytoplasmic ribonucleases, which might otherwise degrade the labeled RNA during the process of extraction, are removed with the cytoplasm before RNA extraction is begun. 3) The rapidity with which the hot phenol extraction is carried out limits the breakdown of high MW RNA during sample manipulation. That the RNA extracted in the present study is largely undegraded is evident from Figs. 1 and 2; the radioactivity after pulse-labeling is at peak values in the 40 to 50 S region of the gradients, and falls away in the direction of lower S values.

Since increased synthesis of RNA and of protein is demonstrably increased in lymphocytes exposed to PHA for only 15 minutes,[4] it is reasonable to assume that a prompt increase in synthesis of all macromolecules is necessary to maximize cellular RNA and protein synthetic activity. Such macromolecules should include r-RNA 45 S precursor and its processed products, 28 and 18 S-RNA. However, the 18 S particle probably moves into the cytoplasm immediately after synthesis and cleavage of 45 S-RNA.[9] Since only the lymphocyte nuclear RNA was extracted in Experiment 2, only the labeled 28 S product, and little labeled 18 S-RNA, was found after a one-hour chase.

In cultures incubated with or without PHA for 80 minutes (Fig. 3), kinetic studies showed uptake of uridine-H^3 into 4 to 6 S-RNA and into 40 to 50 S-RNA within 2 and 20 minutes, respectively,

*See previous footnote.

after addition of the isotope to the cultures (whole lymphocyte RNA extracted). The emerging peak of 4 to 6 S radioactivity increased to a maximum by five minutes and the 40 to 50 S peak continued to increase for the remaining 20 minutes of exposure to isotope. The low and high MW RNA peaks were at all times higher in the PHA than in the control cultures.

The rapid labeling of 4 to 6 S-RNA raises a question as to its nature. It does not appear to be transfer RNA (t-RNA) for the following reasons: 1) t-RNA is probably synthesized in the nucleus in mammalian cells studied.[10] The experiments indicate that the 4 to 6 S-RNA synthesized in lymphocytes, either resting or in the presence of PHA, has its origin in the cytoplasm (compare labeling pattern of Figs. 1 and 2 with that of Fig. 3). 2) Synthesis of 4 to 6 S-RNA occurs in resting lymphocytes,[2,8] and PHA rapidly enhances the synthesis of this RNA species. Kay has recently reported that gel-filtration analysis of this RNA species and the kinetics of its degradation by pancreatic ribonuclease suggest that much of the newly synthesized 4 to 6 S-RNA is not t-RNA.[7]

SUMMARY

RNA metabolism was studied in human thoracic duct lymphocytes exposed in cultures to PHA for two hours or less. After incubation with or without PHA and pulse-labeling with radioactive uridine, nuclei of the lymphocytes were isolated and their RNA extracted and fractionated. PHA-incubated lymphocytes synthesize 50 to 100% more 40 to 50 S-RNA than controls. Much of this high MW newly synthesized nuclear RNA appears to be ribosomal RNA precursor, since it is chased to a peak at 28 S. Labeling kinetics of whole lymphocyte RNA in cells exposed for 80 minutes to PHA shows that uridine-H^3 first appears in 4 to 6 S-RNA at two minutes, and in 40 to 50 S-RNA at 10 minutes after addition of label.

REFERENCES

1. Gowans, J.L., and McGregor, D.D. The immunological activities of lymphocytes. *Progr. Allergy* 9:1, 1965.
2. Rubin, A.D., and Cooper, H.L. Evolving patterns of RNA metabolism during transition from resting state to active growth in lymphocytes stimulated by phytohemagglutinin. *Proc. Nat. Acad. Sci.* 54:469, 1965.
3. Bach, F., and Hirschhorn, K. Gamma-globulin production by human lymphocytes *in vitro*. *Exp. Cell Res.* 32:592, 1963.

4. Pogo, B.G.T., Allfrey, V.G., and Mirsky, A.E. RNA synthesis and histone acetylation during the course of gene activation in lymphocytes. *Proc. Nat. Acad. Sci. 55*:805, 1966.
5. Yoffey, J.M., Winter, G.C.B., Osmond, D.G., and Meek, E.S. Morphological studies in the culture of human leucocytes with phytohaemagglutinin. *Brit. J. Haematol. 11*:488, 1965.
6. Cooper, H.L., and Rubin, A.D. RNA metabolism in lymphocytes stimulated by phytohemagglutinin: Initial response to phytohemagglutinin. *Blood 25*:1,014, 1965.
7. Kay, J.E. Effect of phytohaemagglutinin on lymphocyte ribonucleic acid synthesis. *Biochem J. Agenda Papers*, July, 1967, p. 33.
8. Kay, J.E. In *Proceedings of the Symposium: The Biological Effects of Phytohaemagglutinin*. Edited by M.W. Elves. Oswestry, England: Orthopaedic Hospital Management Committee, 1966.
9. Girard, M., Latham, H., Penman, S., and Darnell, J.E. Entrance of newly formed messenger RNA and ribosomes into HeLa cell cytoplasm. *J. Molec. Biol. 11*:187, 1965.
10. Penman, S. Ribonucleic acid metabolism in mammalian cells. *New England J. Med. 276*:502, 1967.

DISCUSSION SESSION

JOHN KAY, Bethesda, Md.: I feel a little bit misquoted on the subject of the 4 to 6 S-RNA. The 4 S-RNA that I was studying was located in the cytoplasm, I think, but that doesn't mean that it wasn't synthesized in the nucleus. Any labeling of 4 S-RNA which could occur in the cytoplasm would be labeling of the terminal trinucleotide of transfer RNA. I eliminated such a possibility by digestion of the RNA with venom phosphodiesterase, which didn't degrade the radioactive RNA. When I reported that a lot of labeled 4 S-RNA in the cytoplasm was not transfer RNA, I meant about one half of it was transfer RNA. The stimulation by PHA of the RNA which seemed to be transfer RNA was at least as big, if not bigger, than the stimulation of the RNA which could be distinguished from transfer RNA. What you call 45 S-RNA appears to be what we call polydisperse RNA. I would like to hear your evidence that this is in fact 45 S-RNA, because only a very little of it seemed to chase into ribosomal RNA!

ARNOLD RUBIN, New York, N.Y.: It also should be made clear, Dr. Cooper, whether you have any way of demonstrating that the 4 to 6 S was net synthesis. This is the point that Dr. Kay is trying to bring out.

W.C. COOPER: I didn't show the slides of the whole cell experiments that we did because many of them were unsuccessful. We did have tremendous peaks of labeling in 4 to 6 S in short times in whole cells and we don't think this is all due to terminal addition, because we believe the absolute amount of it is just too vast to be explained by this mechanism. We don't know what this 4 to 6 S is.

In answer to the question about the ribosome, in the control lymphocytes, the 45 S or the heavy RNA which peaked at 45 S, chased almost completely to 28 S after an hour of actinomycin and uridine. I thought that was clear on the slide. After PHA, which wasn't as good a chase and we presumed that this RNA which doesn't chase was heterodisperse.

HERBERT COOPER, Bethesda, Md.: The absence of a particular labeled species from the nuclear extract doesn't mean that it's not made in the nucleus. This is exemplified by the fact that you don't find any labeled 18 S-RNA, after your chase in the nuclear extract and, yet, even the most skeptical of us will admit that 18 S-RNA is made in the nucleus. So the absence of the 4 S label from your extract would also imply the same. Another point would be that it does look from your pulse-labeling studies and subsequent chase that, in fact, you do have essentially the same pattern we have in the pulse, that is, a super-imposition of a relatively small 45 S peak on a broad base of polydisperse material. The polydisperse material has been, to a good extent, lost following your chase. The 45 S material has gone into 28 S and as Dr. Kay pointed out, it does represent a rather small peak.