ANTICONVULSANT DRUGS IN IMMUNOSUPPRESSION AND CARCINOGENESIS

by

Tania C. Sorrell, M.B., B.S.
Department of Medicine, University of Adelaide

Submitted for the degree of Doctor of Medicine

May, 1974

This Thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge it does not contain material hitherto published or written by another person except when due reference to such material is made in the text.

TANIA C. SORRELL

ACKNOWLEDGEMENTS

This study was carried out in the Department of Medicine, University of Adelaide, with the aid of a post-graduate award from the National Health and Medical Research Council of Australia.

I have greatly appreciated the constant guidance and criticism of my supervisor, Dr. I.J. Forbes, (Reader in Medicine), and the helpful advice given me by members of other University Departments.

I am indebted to Dr. R.H.C. Rischbieth and to Dr. Norma Kent for allowing their patients to take part in the clinical trials. I also wish to acknowledge the excellent technical assistance of Mr. G.K. Mann, and to thank Dr. M.R. Wellby, Dr. R.J. Kimber, Dr. N.D. Hicks and Mr. C.S. Crisp for providing staff to assist me with the biochemical and haematological estimations and their statistical evaluation.

Data from control subjects were compiled in association with Mrs. K.T. Holmes and Dr. D.I. Grove. Reference to this work is made in the text.

Finally, I wish to thank Mr. J. Hadaway for the preparation of the photographs, and Mrs. Marrilyn Hammond for typing the manuscript.

TABLE OF CONTENTS

PREFACE

Understanding of basic immunological mechanisms has greatly increased in the past decade. It is clear that lymphocytes comprise a heterogeneous population of cells and that complex interactions between functionally distinct lymphocyte populations are central to immune responses. At the same time, the importance of immunological phenomena in human disease has been recognised, and a number of therapeutic agents has been shown to affect the lymphoid system.

Techniques used in animal research are being adapted to measure components of the normal human immune response, and to identify abnormalities within it. Immunological responsiveness is being investigated in diseases of the lymphoid tissue, particularly lymphoma and chronic lymphocytic leukaemia, with the aim of understanding the disease process and its pathogenesis.

The aim of this study was to evaluate and develop further a standard bracket of tests for such measurement, and to apply them to the investigation of a phenomenon which may contribute to the understanding of lymphoma, viz, the occurrence of lymphoma in patients treated with the anticonvulsant drug, phenytoin sodium, (diphenylhydantoin).

In particular, the role of immunodeficiency in the aetiology of lymphoma was investigated in this system.

Part of this work has been described in the following publication: Sorrell, T.C., Forbes, I.J., Burness, F.R., Rischbieth, R.H.C., (1971) Lancet 2, 1233.

SUMMARY

Criteria of normal immunological responsiveness were established for a control population of 177 subjects. B-cell function was assessed by quantitating serum concentrations of the immunoglobulins G, A, and M, primary antibody responses to Salmonella typhi and secondary antibody responses to Tetanus toxoid. In addition, sera were tested for the presence of antinuclear antibody, and complement (C_3) levels were determined. Indices of T-cell function included delayed hypersensitivity reactivity to intradermal and contact antigens, circulating lymphocyte counts, phytohaemagglutinin (PHA) - induced lymphocyte blastogenesis, and PHA-induced deoxyribonucleic acid (DNA) synthesis in the presence of autologous serum and foetal calf serum. DNA synthesis was also measured in the leukocytes of freshly drawn blood.

Conditions were established for the routine measurement of lymphocyte transformation in cultures of peripheral blood. Under these conditions lymphocyte DNA synthesis was related directly to the incorporation of tritiated thymidine into cell DNA. Comparisons were made between the measurement of lymphocyte transformation by morphological criteria, and the incorporation of radio-labelled thymidine into acid-precipitable DNA. There was a positive correlation between lymphocyte blastogenesis assessed by the microscopic examination of stained smears, and that measured by electronic pulse-height analysis of nuclear volume changes. Neither parameter could be correlated with PHA-induced DNA synthesis. Nuclear volume

analysis showed that transformation was maximal on different days of culture in different subjects, in contrast to PHA-induced DNA synthesis, which was maximal on the same day in different subjects. The microscopic assessment of blastogenesis is a subjective measurement, based on counting a small number of cells. It was concluded that the most practicable method for the measurement of lymphocyte transformation in blood cell cultures was by the incorporation of tritiated thymidine into DNA.

PHA-induced DNA synthesis was measured routinely in blood cell cultures. These were technically superior to lymphocyte enriched cultures because of the small volume of blood required and the lack of initial cell manipulation (some methods of cell separation are known to cause detectable impairment of lymphocyte function (Ling, 1968)). DNA synthesis and nuclear volume changes were measured in PHA-stimulated cultures containing lymphocytes of greater than 98% purity, or whole blood. In each case, there was no consistent relationship between the results of blood and lymphocyte cultures. This may have been due to technical factors, for example it is probable that at least part of a subpopulation of lymphocytes was removed during the lymphocyte separation procedure. Stimulation of lymphocyte DNA synthesis by other blood components has been reported, but only with blood component - lymphocyte ratios much smaller than those found in the peripheral blood of subjects used in this study. The curves obtained by varying serum concentrations, lymphocyte numbers, pH, and PHA concentrations, in

stimulated blood cell cultures were similar to those reported for lymphocyte-rich cultures. Mean DNA synthesis remained constant in a group of subjects over a 3 week period, although the results of individual subjects varied, in agreement with reported results in lymphocyte-rich cultures.

Immunological function was measured in two groups of patients on long-term therapy with the anticonvulsant drug, phenytoin sodium. At least one immunological defect was found in 60% of 63 general hospital patients (70% of female and 53% of male patients) compared with 7 of 15 patients treated with another anticonvulsant, carbamazepine, and 7 of 87 control subjects. One or more deficiencies were present in 38% of 52 intellectually retarded (I.R.) patients taking phenytoin, compared with 2 of 15 I.R. barbiturate-treated patients, and 3 of 45 I.R. control subjects. Eleven patients were studied before and at least two months after the commencement of phenytoin therapy. Mean IgA, measured in 10 patients, was significantly depressed (by 31%) after the commencement of therapy. Failure to make antibody to Salmonella typhi, and to manifest delayed hypersensitivity reactivity, was also observed after therapy had commenced.

Analysis of the data indicated that a number of factors influence the effect of phenytoin on immunological responsiveness. IgG concentrations were depressed in the I.R. patients, whereas IgA and IgM were depressed in the general hospital group. Antibody responses to Salmonella typhi, delayed hypersensitivity reactivity,

and lymphocyte responses to PHA were depressed in both groups of patients. These differences were not due to serum folic acid deficiency, but differences in phenytoin dosage and nutrition may have been factors. Analysis of the hospital series showed a sex influence on serum levels of IgA and IgM, and DNA synthesis (mean serum phenytoin levels being the same in males and females). IgA was depressed to a significantly greater extent in females than males, IgM was low only in males. PHA-induced DNA synthesis in autologous serum, and DNA synthesis in circulating leukocytes, were depressed in females, not males. Depression of PHA-induced synthesis in foetal calf serum was not significant, suggesting that a serum factor, probably phenytoin itself, was responsible for the depression of DNA synthesis observed in females. Antinuclear antibody was found in only 3% of the I.R. patients, and in none of the hospital patients treated with phenytoin.

Studies in vitro confirmed that phenytoin directly inhibits DNA synthesis in human lymphocytes. DNA synthesis was depressed by 50% in cultures containing phenytoin concentrations within the therapeutic range of 10-20 μg/ml. Depression of DNA synthesis by phenytoin varied inversely with the concentration of serum in cultures, suggesting that only the unbound form of phenytoin depresses DNA synthesis. Therapeutic concentrations of phenytoin caused a relatively small depression of DNA synthesis in the presence of high serum concentrations. Despite the effect of protein binding, this depression was significant in vivo, as DNA synthesis in

circulating leukocytes was impaired. In addition, depression of DNA synthesis in 10% autologous serum was significant, even though the maximum phenytoin concentration was not greater than 2 μg/ml. Higher concentrations of phenytoin depressed lymphocyte blastogenesis, RNA synthesis, protein synthesis, and cell counts in vitro. Kinetic studies suggested that phenytoin affected the early stages of lymphocyte activation in particular, causing a maximal depression of DNA synthesis when added within 8 hours of the initiation of culture.

Both T-cell and B-cell function, but especially T-cell function, were depressed in a patient (F.K.) who developed Hodgkin's disease 3 years after the commencement of phenytoin therapy. These tests were performed 10 months after the patient had received radiotherapy to a thoracic mantle field. In vitro, phenytoin induced a significant increase in DNA synthesis in lymphocytes from patient F.K., in contrast to the findings in control subjects. This response suggested an anamnestic response of lymphocytes to specific antigen.

It is suggested that immunosuppression is related to the role of phenytoin in carcinogenesis. Immunodeficiencies in phenytoin-treated subjects were similar to those in a study of 38 patients with untreated lymphoma. Deficiencies were present in 3 of 8 patients with Stage I Hodgkin's disease. The stimulation of lymphocyte DNA synthesis by phenytoin in patient F.K. supports the hypothesis of Kruger, that phenytoin-induced lymphoma is due to the combination of chronic antigenic stimulation and partial immunosuppression.

CONCLUSIONS

The following represent the main contributions to knowledge made by this study:

1. A standard bracket of tests has been described for the measurement of immunological capacity in man. Criteria of responsiveness were established, allowing discrimination between normal subjects and patients with immunological deficiencies.
2. A blood cell culture technique was adapted for the routine measurement of PHA-induced lymphocyte transformation.
3. Therapy with the anticonvulsant drug, phenytoin sodium, is commonly associated with the development of defects in B-cell and/or T-cell function. The type of immunological defect appears to depend on a number of factors, including sex, nutritional status and drug dosage, but not folic acid deficiency.
4. Evidence that phenytoin therapy is associated with an increased incidence of recurrent, severe infection was not obtained.
5. Depression of DNA synthesis by phenytoin is a major mechanism by which the drug causes immunosuppression.
6. The development of lymphoma in association with phenytoin therapy is a rare occurrence; immunosuppression alone seems inadequate to trigger the abnormal lymphoid tissue response. Study of a patient who developed Hodgkin's disease while receiving phenytoin revealed evidence of hypersensitivity to phenytoin as demonstrated by induction of DNA synthesis in vitro, despite a generalized depression of immunological function.

7. The data support the hypothesis of Kruger and Harris, that the combination of chronic antigenic stimulation and partial immunosuppression lead to the development of lymphoid tissue malignancy.

8. Depression of DNA synthesis by carbamazepine, studied in vitro, is significantly less than that caused by phenytoin, in parallel with the lesser immunosuppressive effect observed in vivo. Phenobarbitone, which is not immunosuppressive in vivo, causes insignificant depression of DNA synthesis in vitro.

CHAPTER I

REVIEW OF THE LITERATURE

1. INTRODUCTION

Morphologically, the lymphocyte is defined "rather by the absence of characteristics which other white cells possess than by positive attributes of its own" (Drinker and Yoffey, 1941). Functionally, the lymphocyte consists of a heterogeneous group of cells, whose activity is central to immune responses (Richter and Almgom, 1972).

It is clear from studies of experimental animals and to a lesser extent, of man, that immune responses involve complex interactions between lymphoid cells, their products, and phagocytic cells, and that there are two systems of functionally distinct lymphocytes involved in these responses.

Tests of human immunological function have been developed to identify and to quantitate abnormalities within the two systems. In this review, the primary immunological deficiencies have been used as a model for the classification of such defects.

Treatment with the anticonvulsant drug, phenytoin sodium, has been associated with the development of Hodgkin's disease and other lymphomas (Hyman and Sommers, 1966, Kruger and Harris, 1972). Depression of immunological function is known to occur in lymphoid tissue malignancy. Conversely, the presence of immunodeficiency may predispose to the development of neoplasia (Good, 1972). In this thesis, the relationship between anticonvulsant therapy, immuno-

suppression and carcinogenesis has been used as a model for study of the aetiology of lymphoid tissue malignancy.

2. ORIGIN AND MIGRATION OF LYMPHOCYTES

Lymphocytes are thought to be derived from a common haemopoietic stem cell (proposed by Maximow, 1909), descendents of which are seeded to embryonic marrow, spleen, thymus, and the bursa of Fabricius (or its equivalent). The line of cell differentiation is probably determined by the environment of the organ rudiment in which these cells proliferate (Moore and Owen, 1967). In the thymus and bursa, differentiation is dependent on a continuous stem cell supply, provided in adult life by the bone marrow (Playfair, 1971). Thymus-derived lymphocytes (T-cells) migrate via the blood to the peri-arteriolar regions of the spleen, the mid and deep cortical areas of the lymph nodes, and the internodular spaces of the gut-associated lymphoid tissue (Weissman, 1967, Parrott and De Sousa, 1971). They also form a large part of the recirculating pool of small lymphocytes (Davies, 1969). Peripheral T-cells have been distinguished in the mouse by the presence of the theta-antigen on the cell surface (Takahashi et al, 1971).

Plasma cell differentiation begins in the bursa of Fabricius in the chicken (Moore and Owen, 1965). The site of maturation of equivalent cells in man is not known (Watson, 1969); the gut-associated Peyer's patches, sacculus rotundus, and appendix have been proposed for the rabbit (Cooper et al, 1966) and the hamster (Bienenstock and Dolezel, 1971). Cells leaving the bursa

(or its equivalent) are distinguished by a high density of surface immunoglobulin receptors (Kincade et al, 1971, Miller et al, 1971), the presence of receptors for antigen-antibody complexes (Bianco et al, 1970, Miller et al, 1971), and in the mouse, by the presence of specific cell-surface antigen (MBLA, Raff et al, 1971). They migrate largely to the splenic red pulp and peripheral white pulp, the nodules and medulla of the lymph nodes, and the nodules of the gut-associated lymphoid organs (Parrott and De Sousa, 1971). Differentiation of B-cells into antibody-producing plasma cells occurs in the peripheral lymphoid tissues (Craddock et al, 1971), in response to antigenic stimulation (Cooper et al, 1972). This is accompanied by a decrease in the density of surface immunoglobulin receptors, and the appearance of the PC antigen in the mouse (Takahashi et al, 1971). The majority of B-cells are short-lived. They can migrate, and recirculate (Howard, 1972), although it is not yet certain whether a regular and continuous process of exchange of cells occurs in the periphery (Parrott and De Sousa, 1971).

3. THE FUNCTION OF THE THYMUS-DEPENDENT AND THE THYMUS-INDEPENDENT (T-CELL AND B-CELL) SYSTEMS

(a) The T-cell System

The function of the T-cell system has been elucidated mainly in the mouse and the chicken, by thymus extirpation and replacement experiments. T-cells are necessary for cell-mediated immune responses; specific immunity can be transferred from a sensitized to a non-sensitized animal by cells

but not antibody (Humphrey, 1967). Cell-mediated functions include delayed hypersensitivity (DHS, cell-mediated hypersensitivity) reactions (Arnason et al, 1962), defence against intracellular organisms (Suter and Ramseier, 1964), homograft rejection, graft versus host responses, and co-operation in humoral antibody responses to certain protein antigens (Miller, 1963). Evidence that the thymus plays the same role in man has come from studies of patients with the rare Di George syndrome.

Di George (1965) described a syndrome of severe runting and recurrent infection in an infant with congenital absence of the thymus and parathyroid glands, in association with failure of DHS reactivity to dinitrochlorobenzene and to Candida albicans, and inability to reject a skin homograft within the normal time. Circulating lymphocyte counts and serum immunoglobulin levels were normal. Some antibody responses are depressed in this disorder (Lischner et al, 1967), but not all (Kretschmer et al, 1968). In vitro responsiveness to phytohaemagglutinin (PHA) and to specific antigens is also depressed (Lischner et al, 1967). It has recently been shown that a high proportion of circulating lymphocytes may carry immunoglobulin determinants of the types characteristic of B-cells (heavy chain classes G, A, and M, vide infra), (Gajl-Peczalska et al, 1972). Immunological responsiveness has been restored in such patients by the transplantation of foetal thymus tissue (August et al, 1968, Cleveland et al, 1968).

(b) The B-cell System

The function of the B-cell system has been most readily studied in chickens, using hormonal or surgical ablation of the bursa of Fabricius (Szenberg and Warner, 1962, Cooper et al, 1965). B-cells are necessary for the maintenance of serum immunoglobulin levels, and for specific antibody production. Immunoglobulin is synthesized in the B-cell and its descendent, the plasma cell (Claman and Chaperon, 1969, Gudat et al, 1970, Uhr, 1970). B-cell function is deficient in the syndrome of infantile sex-linked agammaglobulinaemia, first described by Bruton (Bruton, 1952). Boys affected by this disease have recurrent, mainly bacterial, infections, and are hypogammaglobulinaemic, with subnormal or absent levels of all the major classes of immunoglobulin. They cannot develop normal antibody responses (Osoba, 1972), and their blood lymphocytes do not usually carry the immunoglobulin receptors of normal B-cells (Froland and Natvig, 1971, Grey et al, 1971). They can manifest DHS reactions, and reject skin homografts (Osoba, 1972).

(c) Cell Interactions

Specific synergistic interactions occur between T-cells and B-cells in the primary antibody response to sheep erythrocytes, to serum proteins, and to hapten-protein conjugates, in the mouse (Claman and Chaperon, 1969, Miller et al, 1971, Playfair, 1971). Activated T-cells (i.e. cells which have proliferated in response to contact with antigen), (Miller et al, 1971),

appear to initiate the sequence of events whereby antigen is presented to B-cells in an immunogenic form capable of stimulating antibody synthesis (Feldmann and Basten, 1971).

Cells with the characteristics of macrophages are necessary for this co-operative response to occur in vitro (Shortman et al, 1970), and in vivo (Feldmann, 1973). It has been proposed that macrophages modulate the influence of T-cells on the antibody response in vivo (Feldmann, 1973). Collaboration between T and B-cells is also necessary for an optimal secondary response to serum protein in the mouse (Miller et al, 1971). Specific interactions between T-cells and B-cells in cell-mediated immune responses are less clearly defined. It has been claimed that such interactions occur in graft versus host responses (Playfair, 1971), although recent evidence suggests that two differentiated classes of T-cells may be involved in the induction phase of this response (Asofsky and Cantor, 1971).

4. PRODUCTS OF T-CELLS AND B-CELLS

Immunological function can be measured by the determination of substances produced by T-cells and B-cells (or their descendents) in vivo (e.g. immunoglobulins, specific antibodies) and in vitro (e.g. lymphokines, antibodies). Such measurements are relatively crude tests, as they measure only the end-result of the complex sequence of events that constitutes an immune response; abnormalities may arise at any stage of this response. For example, the T-cell system contains cells (antigen reactive cells) which combine

with antigen by means of specific immunoglobulin-like receptors synthesized in the cell, and located at the cell surface (Bankhurst et al, 1971). Stimulation of specific T-cells by antigen is followed by the generation of clones of activated T-cells (Miller et al, 1971). Further proliferation leads to the development of memory cells, killer cells, cells which release soluble, non-antibody mediators, and cells which co-operate in certain antibody responses. Different classes of T-cell may take part in cellular and humoral immune responses (Segal et al, 1972) although different immunological activities may be performed by T-cells in different stages of differentiation (Cantor, 1971, Shortman et al, 1972).

(a) Products of T-cells

Culture of animal and human lymphocytes with sensitizing antigen has led to the detection of soluble factors (lymphokines) in the culture supernatants. These factors exert biological effects on different cell types, including macrophages; migration inhibition factor, agglutinating factor, phagocytosis-activating factor, chemotactic factor (Dumonde and Maini, 1971), factors enhancing macrophage adherence, spreading, and motility (Nathan et al, 1971), factors inhibiting macrophage spreading, and factors causing the release of biologically active materials from normal macrophages (Pick and Turk, 1972) have been described. Other lymphokines include lymphocyte mitogenic factor, inflammatory factor, cytotoxic and cytopathic factors, platelet-aggregating factor, lymph node activating factor,

polymorphonuclear leukocyte and eosinophil chemotactic factors, and an interferon-like factor. A number of these activities may be produced by the same substance. The non-specific plant lectins, phytohaemagglutinin and concanavalin A, can also stimulate the production of lymphokines by lymphocytes; inflammatory, mitogenic, migration-inhibitory, and cytopathic factors have been described. Lymphokines produced in response to antigenic stimulation are non-specific factors elicited by a specific immune response, and are not capable of transferring specific immunological reactivity from a sensitized to a non-sensitized animal (Dumonde and Maini, 1971).

Human blood leukocytes can be tested directly for three types of lymphokine activity in vitro - lymphocyte mitogenic factor, leukocyte migration inhibition factor, and lymphocyte cytotoxic factor. Supernatants from stimulated lymphocytes can be tested in addition for the presence of macrophage migration inhibition factor (using guinea pig macrophages) and inflammatory factor (using the local reactivity of guinea pig skin to intradermal injection), (Dumonde, 1970).

Measurement of migration inhibition by antigen stimulated lymphocytes (Rosenberg and David, 1970) or their supernatants (Rocklin et al, 1970, Fudenberg et al, 1971) is a reliable index of the ability to manifest DHS reactions in man. The relationship between antigen-induced lymphocyte transformation in vitro and DHS reactivity in vivo is less clearly defined.

Curtis et al (1970) found that lymphocyte transformation (measured by the incorporation of tritiated thymidine into deoxyribonucleic acid, DNA) correlated with the presence (but not the size) of DHS reactions, and that it also correlated with antibody titres developed in response to immunization with keyhole limpet haemocyanin. It has been reported by Zweiman et al (1969) that low concentrations of coccidioidin can elicit weak DHS reactions in the absence of an in vitro response, although this study demonstrated that there was a good correlation between the two tests at higher concentrations of antigen. Stimulation by antigen may initially involve a relatively small number of precommitted cells, which transform and release non-specific mitogenic factors, which themselves recruit non-sensitized cells, including B-cells (Fudenberg et al, 1971).

Transformation in response to antigen is depressed in both thymectomized and bursectomized chickens. It is likely that this parameter is measuring the ability of cells to react with, and be activated by, antigen, rather than distinguishing between the T-cell and the B-cell systems (Daguillard, 1972).

The literature regarding the response of lymphocytes to the non-specific stimulant, PHA, and to allogeneic leukocytes has been summarized by Daguillard, (1972). Both probably stimulate the T-cell, possibly T-cells in different stages of differentiation. The low transformation response of neonatally

thymectomised animals, and patients with the Di George Syndrome, can be restored by thymus grafts.

A significant number of immunoglobulin-bearing blast cells have been identified recently in cultures of PHA-stimulated human lymphocytes. It is possible that these are activated B-cells (Phillips and Roitt, 1973). At present, however, the lymphocyte response to PHA is accepted as a test of T-cell function.

There is evidence that sensitized lymphoid cells contain and elaborate other soluble factors (transfer factors) which confer specific immunological responsiveness when injected into non-sensitized recipients, or when added to non-sensitized lymphoid cells in culture. These factors may be informational polypeptides or polynucleotides which instruct previously uncommitted lymphocytes to become specifically hypersensitive, and thereby transfer the state of delayed hypersensitivity. (Lawrence, 1969.)

(b) Products of B-cells

Antibodies are protein molecules (immunoglobulins) consisting of pairs of polypeptide chains (two heavy chains and two light chains) united by disulphide bridges. Five classes of immunoglobulin have been identified in normal human serum; IgG, A, M, D and E. The type of heavy chain is characteristic for each class, but the two major types of light chain are found in all heavy chain classes. Within a given class of heavy chains,

subclasses have been distinguished by their antigenic determinants. Four subclasses of IgG have been identified, and two of IgA (Edelman and Gall, 1969). There are also at least two subclasses of IgM (Solomon and McLaughlin, 1973). Immunoglobulins with specific antibody reactivity are synthesized and secreted in large amounts by plasma cells. A single clone of plasma cells produces one immunoglobulin class of one specificity (Makela, 1970, Sell, 1970, Uhr, 1970) although there is some evidence that cells may switch from IgM to IgG production during an immune response (Edelman and Gall, 1969). In response to a single immunogen, a family of antibodies is generated, all capable of binding the immunogen, but varying in structure and affinity (Haber, 1968).

(1) Immunoglobulin M: IgM is characteristically produced early in the primary antibody response to an injected antigen in animals (Uhr, 1964), and in man (Lo Spalluto et al, 1962). It is confined to the intravascular compartment, where it is present as a pentamer (Tomasi, 1972). It comprises approximately seven percent of the total serum immunoglobulin (Hobbs, 1971). Certain antigens (usually particulate antigens, with a high carbohydrate content, e.g. Salmonella typhi O, red cell isoagglutinins) induce the formation of antibodies which are probably confined to the IgM class (Lo Spalluto et al, 1962).

(2) Immunoglobulin G: Molecules of the IgG class comprise approximately seventy-three percent of the total serum immuno-

globulin in adults. They are also present in the extravascular body fluids (Hobbs, 1971). They are usually monomeric, and arise during the latter part of the primary response to most injected antigens. IgG usually predominates during a secondary response (Lo Spalluto et al, 1962, Uhr, 1964). IgG molecules fix complement but do so less efficiently than IgM (Hobbs, 1971). Activation of the complement sequence is important in the opsonization of bacteria for immune phagocytosis by white cells, and for the killing of micro-organisms; it also generates factors mediating specific white cell chemotaxis (Bienenstock and Perey, 1972).

(3) Immunoglobulin A: Immunoglobulin A is found in serum and secretions. Up to sixty percent of the total IgA is synthesized by plasma cells in the lamina propria of the gut and respiratory systems (Hobbs, 1971). Serum IgA is usually monomeric, and comprises approximately 19 percent of total circulating immunoglobulin. Secretory IgA contains two IgA molecules united by a non-immunoglobulin secretory piece. The ratio of IgA to IgG in the body secretions is about 20:1 (Tomasi, 1972). It has been reported that local (oral/inhalational) immunization with a number of viruses or bacteria elicits the formation of both local and circulating antibody, (predominantly IgA), whereas parenteral immunization does not elicit a local response (Bienenstock and Perey, 1972).

(4) Immunoglobulin D: Immunoglobulin D occurs in very low concentrations in serum. Its function is not known.

(5) Immunoglobulin E: Immunoglobulin E (reaginic antibody) also occurs in low concentrations in serum (0.05 percent of the total immunoglobulin). It binds preferentially to basophils and to mast cells. IgE-containing cells are found beneath the respiratory and gastrointestinal mucous membranes, i.e. in a distribution similar to that of IgA, and may therefore play a part in local immunity. Interaction between specific antigen and cell-fixed IgE triggers allergic reactions (Ishizaka, 1971).

There is good evidence in animals that IgE is also important in host-parasite responses, especially to worms of the nematode class (Bienenstock and Perey, 1972); in man, elevated IgE levels have been found in patients with ascariasis, capillariasis, visceral larva migrans and acute trichinosis (Rosenberg et al, 1971).

5. CLASSIFICATION OF ABNORMALITIES OF THE IMMUNE RESPONSE IN CLINICAL MEDICINE

Immunodeficiency states have been classified on the basis of aetiological, structural or functional factors, or a combination of these.

None of these methods is completely satisfactory. Fudenberg et al (1971) have proposed a classification of the primary specific immunodeficiency states, based on the site of cellular defect, i.e., based on defects of the B-cell, the T-cell, or the stem

cell. Immunodeficiency arising from exogenous causes, or in association with infection or lymphoid tissue malignancy, was excluded from the classification. A similar, but more general classification, which includes the latter states, has been proposed by Osoba (1972) (see Table I-1).

In this system accessory cells (A) cells are predominantly macrophages. Specific T-cell defects, not included in the classification, have also been reported. Chilgren et al (1969) described three patients with mucocutaneous candidiasis, who failed to manifest DHS reactions to candida albicans and tuberculoprotein. The peripheral lymphocytes of these patients transformed normally in response to PHA, candida, and allogeneic cells. DHS reactivity was restored by the injection of normal lymphocytes. These studies suggested that there was a defect in lymphokine production, possibly the migration inhibitory factor, or that there was an inhibitory substance present. By isolating monocytes from human peripheral blood, Louie and Goldberg (1972) were able to define defects of cell-mediated immunity and monocyte function in a female with recurrent infections. This patient did not manifest delayed hypersensitivity reactions to candida antigens, and her lymphocytes failed to transform or to produce cytotoxic factor in the presence of candida, although they did produce macrophage migration inhibitory factor, (MIF) which activated homologous macrophages. Migration of the patient's peripheral monocytes was not inhibited in the presence of autologous MIF or homologous MIF from two of three healthy adults.

TABLE I - 1

CLASSIFICATION OF IMMUNODEFICIENCY DISEASES

1. Defects in the Function of Stem Cells
   - A. Stem cells of both the myeloid and lymphoid systems
     1. Reticular dysgenesis
   - B. Stem cells of the lymphoid system
     1. Combined immunodeficiency syndromes (Swiss-type agammaglobulinaemia, sex-linked lymphopenic agammaglobulinaemia)

II. Defects in the Function of Thymic and Bursal-type (Bone Marrow) Environments
   - A. Thymic environment
     1. III-IV pharyngeal pouch syndrome of diGeorge
     2. Ataxia telangiectasia
   - B. Bursal-type environment
     1. Sex-linked agammaglobulinaemia (Bruton-type)
     2. Late-onset agammaglobulinaemia ("acquired")

III. Defects in the Function of B, T and A cells
   - A. Temporary
     1. After immunosuppressive therapy
     2. Associated with viral infections
   - B. Persistent
     1. Associated with lymphoreticular malignancy (e.g. Hodgkin's disease, multiple myeloma, chronic lymphocyte leukaemia)
     2. Deficiencies of single immunoglobulin classes
     3. Wiskott-Aldrich syndrome

6. THE EFFECT OF IMMUNOSUPPRESSIVE DRUGS ON IMMUNOLOGICAL FUNCTION

(a) In vivo Studies

Immunological responsiveness has been studied in patients treated with immunosuppressive therapy for various neoplastic and non-neoplastic conditions. Levin et al (1964) studied 10 patients with metastatic carcinoma who were in good general condition. Treatment with 6-mercaptopurine, 2.5 mg/kg, was associated with persistence of pre-existing DHS reactivity, iso-haemagglutinin titres, and gamma globulin levels, but with loss of the ability to develop a primary antibody response, to develop DHS reactivity to a new antigen, and to reject skin homografts within the normal time. Skin grafts were rapidly rejected on cessation of therapy. Hersh et al (1966) studied the effect of 6-mercaptopurine or methotrexate on the primary antibody responses of 15 patients with acute lymphocytic leukaemia, 10 of whom were in remission throughout the period of drug therapy (5 days). There was a marked inhibition of the primary response to antigen given 24 hours after the commencement of therapy (antibody titres being tested 2 and 4 weeks post-immunization). Antibody responses to antigen given 24 hours after the cessation of therapy were present but sub-optimal, and responses to antigen given 72 hours after cessation of therapy were normal. The effect of cyclophosphamide on immunological responsiveness has been studied in patients with severe rheumatoid arthritis. Doses of 50-150 mg/day have been associated

with depression of circulating immunoglobulin concentrations, particularly IgM, and a significant decrease in the incorporation of radio-labelled thymidine into the DNA of lymphocytes stimulated with PHA, pokeweed mitogen, and specific antigens (Alepa et al, 1970, Winkelstein et al, 1972). However, the proportion of cells incorporating ^{3}H-thymidine into nuclear DNA was not altered (Winkelstein et al, 1972). PHA-induced lymphocyte transformation was not consistently depressed in ten patients receiving azathioprine for multiple sclerosis, tested before and after the commencement of therapy. Four intradermal antigens were used to test DHS reactivity in these subjects. Four subjects became non-reactive to one of these antigens after the commencement of therapy (Zweiman and Silberberg, 1971). Swanson and Schwartz (1967) studied 20 patients with autoimmune disease who were treated with azathioprine or amethopterin. Therapy with corticosteroids was stopped in these patients before the trial. In all cases the primary antibody response to keyhole limpet haemocyanin was abnormal. Titres were markedly reduced, or there was a prolonged induction phase with persistence of IgM antibody production and failure to switch to IgG. In two cases, antibody synthesis was enhanced. Secondary antibody responses to diphtheria toxoid were also abnormal, and 75 percent of patients failed to develop DHS reactivity to keyhole limpet haemocyanin.

The effects of immunosuppressive drugs on immune responses

in vivo have been more comprehensively studied in animals. It has been established that the immunosuppressive effect of certain antimetabolites and alkaloids, e.g. 5-fluorouracil, methotrexate, 6-mercaptopurine, vinblastine, and 6-thioguanine, is due mainly to impairment of the reproductive capacity of immunocompetent cells, and that these drugs are most effective when given a few days after the initiation of an immune response (when the cells are multiplying most rapidly, (Berenbaum, 1969)). Antimetabolites interfere with the synthesis of nucleic acids, thereby preventing cell division, proliferation, and protein (including antibody) synthesis. Studies of the purine analogue 6-mercaptopurine (Schwartz, 1965, 1967) showed that the observed effects of the drug on antibody formation in the rabbit were dependent on the timing of drug administration with respect to antigenic stimulus, the dosage of drug, the dose of antigen, the choice of antigen, and the route of administration. By varying these parameters the following results were obtained - suppression of both IgG and IgM antibody synthesis, selective suppression of IgG antibody, enhancement of IgM synthesis, or enhancement of both IgM and IgG antibody synthesis. In addition, under defined conditions, the acquisition of DHS to bovine gamma globulin could be suppressed by 6-mercaptopurine in rabbits which produced normal amounts of antibody, and Arthus reactions could be suppressed in rabbits with normal amounts of circulating antibody (Borel and Schwartz, 1964).

The above studies indicate that immunosuppressive therapy (in the doses used clinically in man) does not selectively depress the B-cell or the T-cell systems although manipulation of the test systems and drug dosage has produced selective deficiencies in animals. In addition, variable deficiencies have been defined within the two systems. The effects of immunosuppressive therapy have been incompletely worked out in man; the extent and type of immunological defects identified in patients treated with a given drug probably depend on factors found by Schwartz to affect the antibody response to 6MP in animals, (vide supra), and on additional factors, such as concomitant drug therapy. These variables require further study. Sophisticated techniques have been applied to the study of primary immunological deficiency syndromes in man, e.g. lymphokine production, direct identification of the proportion of T and B-cells, and measurement of lymphocyte responsiveness to a number of specific antigens. These methods have not been used in most studies of drug-induced immunosuppression.

(b) <u>In vitro Studies</u>

The effect of a number of immunosuppressive drugs on PHA-stimulated human lymphocytes has been studied in vitro. Azathioprine, actinomycin, aminopterin, 5-fluoro-2'-deoxyuridine (Ling, 1968) and 6-mercaptopurine (Smith, 1968) can inhibit DNA or RNA synthesis, and protein synthesis (which is nucleic-acid directed). Depression of protein synthesis has been observed

in cells cultured with azathioprine, 6-mercaptopurine, and 6-thioguanine, but not cyclophosphamide (Forbes and Smith, 1967). Cyclophosphamide is inactive in vitro because it is not converted to the active form as it is in vivo (Brock and Hohorst, 1963). The finding that the T and B-cell systems in man are not selectively depressed by immunosuppressive drugs is consistent with the fact that nucleic acid and protein synthesis are required for the proliferation of T-cells and B-cells, and for the elaboration of their products.

7. IMMUNOLOGICAL FUNCTION IN PATIENTS WITH LYMPHOID TISSUE MALIGNANCY

(a) Malignant lymphoma

(1) Nomenclature: Malignant lymphomas have been divided into four cytological groups - follicular lymphoma, Hodgkin's disease, lymphosarcoma and reticulum cell sarcoma. Subsequent studies have indicated that any cytological type of malignant lymphoma may have a follicular or diffuse pattern, and that the different cytological types of the so-called follicular lymphomas are probably more closely related to their non-follicular counterparts than to each other (Rappaport et al, 1956). A revised classification has therefore been proposed (Rappaport, 1966). However, most studies of immunological function in patients with lymphoma have utilized the older nomenclature, and for purposes of comparison it will be used in this Thesis.

(2) Hodgkin's Disease: It has been shown by a number of workers (Parker et al, 1932, Dubin, 1947, Lamb et al, 1962, Aisenberg, 1966) that cell mediated immune responses, particularly DHS reactivity, are depressed in patients with Hodgkin's disease. However, many of these patients had received radiotherapy or chemotherapy prior to being tested. It has since been confirmed that T-cell function as measured by DHS reactivity (Brown et al, 1967, Young et al, 1972), PHA-induced lymphocyte transformation (Brown et al, 1967, Sutherland et al, 1971), and circulating lymphocyte counts, are depressed in a significant number of patients before the commencement of treatment, and that primary antibody responses are usually intact (Brown et al, 1967). Brown et al (1967) and Young et al, (1972) found that immunological function was normal in patients with stage I disease as tested by their criteria. DHS reactivity and peripheral lymphocyte counts were depressed in patients with systemic symptoms, with advanced disease, and in patients with mixed cellularity and lymphocyte-depleted Hodgkin's disease (Young et al, 1972). These findings confirm the earlier conclusion of Sokal and Primikirios (1961), that DHS reactivity is depressed during periods of increased disease activity. Antibody responses become depressed late in the course of Hodgkin's disease (Kaplan, 1972). This may be due partly to general debility (Aisenberg, 1972) and to the effect of prior therapy (Kaplan, 1972).

(3) Non-Hodgkin's Lymphoma: Lymphosarcoma and reticulum cell sarcoma have not been well studied. It is probable that immunodepression occurs only in advanced disease (Aisenberg, 1972). Schrek et al (1969) studied the PHA responsiveness of lymph node cells from three untreated patients with lymphosarcoma, two of whom died within twelve months of testing. Lymphocyte transformation was low in these two subjects, and normal in the third. DHS reactivity to streptokinase was absent in 5 of 13 patients with untreated reticulum cell sarcoma compared with 4 of 25 controls in a study by Libansky (1969). These differences were not significant; the relationship between reactivity and the extent of disease was not stated. Studies by two groups have suggested that depression of secondary antibody responses (Libansky, 1965) and DHS reactivity (Lamb et al, 1962) are related to the condition of the patients rather than to a history of immunosuppressive therapy.

Burkitt's lymphoma, giant follicular lymphoma, and mycosis fungoides appear to be diseases without major immunological impairment (Aisenberg, 1972), although it has been reported that patients with disseminated Burkitt's lymphoma have significant depression of primary antibody responses to a polysaccharide (Vi) antigen, and that IgM levels tend to be low before the commencement of therapy (Ziegler et al, 1970).

8. THE RELATIONSHIP BETWEEN THE IMMUNOLOGICAL SYSTEM AND THE DEVELOPMENT OF MALIGNANCY

In 1959, Lewis Thomas proposed the theory that the phenomenon of homograft rejection, as defined by Medawar, might "represent a primary mechanism for natural defense against neoplasia", neoplastic cells being recognised by the immunological system as foreign (Thomas, 1959). This concept of "immunological surveillance" was developed by Burnet (Burnet, 1959, 1967, 1971) and has been modified by other workers (Fudenberg, 1971, Schwartz, 1972, Laroye, 1973).

A number of lines of evidence suggest that the immunological system is intimately involved in the development of neoplasia. The incidence of malignancy in primary immunodeficiency diseases is many times that of the general (age-matched) population (Gatti and Good, 1971, Good, 1972). Lymphoreticular malignancies have been identified in 10 percent of patients with infantile sex-linked agammaglobulinaemia, and severe combined immunodeficiency. Lymphoreticular malignancy or malignancy of other types has been identified in approximately the same proportion of patients with the Wiskott-Aldrich syndrome, ataxia telangiectasia, and the common variable form of immunodeficiency. It is surprising that the development of malignancy has not yet been described in patients with the DiGeorge syndrome, in whom cellular immune mechanisms are abnormal.

Evidence of immunodepression has been found in patients with malignancy of the lymphoid tissues (vide supra); this tends to parallel the severity of the disease. It has been reported that

immunological function is normal in patients with stage I Hodgkin's disease, suggesting that immunodepression is the result rather than the cause of the disease. It is possible, however, that subtle immunological defects do accompany the early manifestations of the disease, and that they may be of aetiological or pathogenic significance (Kaplan, 1972). Studies of patients with Hodgkin's disease are reported in this Thesis.

A number of chemicals, e.g. methylcholanthrene, have been found to be directly carcinogenic in mice. However, malignancy can occur in animals treated with immunosuppressants which are not themselves chemical carcinogens, e.g. through the activation of oncogenic viruses (Allison, 1970). The emergence of these tumours is consistent with a breakdown in the immunological surveillance mechanism. It has also been proposed that the combination of chronic antigenic stimulation and immunosuppression may lead to the development of malignancy. Krueger et al (1971) immunized mice with one of four antigens - the usually non-oncogenic lactic dehydrogenase elevating virus, vaccinia virus, complete Freund's adjuvant or HeLa cells. Malignant lymphoblastic lymphomas developed in 20 percent of BALB/c mice treated with azathioprine and antigen, as early as 3 months later, but not in mice treated with antigen or azathioprine alone. Lymph nodes showed changes of both antigen-induced proliferation and partial atrophy. Foci of atypical reticulum cells were present in 30 percent of atrophic nodes. The occurrence of virus particles in the lesions was not commented upon. It is possible that the immune

response, poorly controlled because of co-existent immunosuppression, was responsible for the activation of latent oncogenic viruses, as has been suggested by Schwartz (1972). A significantly increased incidence of epithelial and lymphoreticular malignancies has been described in human recipients of renal transplant homografts (Starzl et al, 1971), all of whom were subject to continuous antigenic stimulation and immunosuppressive therapy. Immunological mechanisms have also been implicated in human oncogenesis by the studies of Hellstrom et al (1968). These workers demonstrated that the serum of patients with neuroblastoma blocked the ability of sensitized lymphocytes to kill target neuroblastoma cells in vitro. They later demonstrated that this effect was no longer present in serum from which all immunoglobulins had been absorbed (Hellstrom et al, 1970). The role of blocking antibody in the production of lymphoid tumours has not been defined. It may be pertinent that epithelial tumours have not been identified in patients with severe B-cell deficiency, such as that found in infantile X-linked agammaglobulinaemia (Good, 1972).

9. IMMUNOLOGICAL SIDE-EFFECTS OF PHENYTOIN THERAPY

The hydantoin group of anticonvulsant drugs has been in clinical use since 1938 (Merritt and Putnam, 1938). Since then, over 100 cases of drug-associated lymphadenopathy have been reported (Anthony, 1970). Most of these patients have presented with the features of an hypersensitivity reaction, with fever, exanthemata, lymphadenopathy (especially of cervical nodes), hepatosplenomegaly, and eosinophilia (Saltzstein and Ackerman, 1959). However, the lymphadenopathy may be clinically and histologically indistinguishable from

Hodgkin's disease or other lymphomas (Hyman and Sommers, 1966, Anthony, 1970, Rausing and Trell, 1971), and has been classified by Gams et al (1968) on the basis of lymphoid tissue histology and clinical response to drug withdrawal. The lymphadenopathy usually regresses after cessation of therapy in patients whose nodes are histologically benign (category I), or malignant-looking (category II), although the patient may subsequently present with malignant lymphoma (category III). Initial histological and clinical features may be indistinguishable from true malignant lymphoma (category IV). Areas of reticulum cell hyperplasia (characteristic of that seen in association with hydantoin therapy), reticulum cell sarcoma, and cells with intermediate features, were identified in adjacent regions of a single lymph node by Rausing and Trell (1971), suggesting that the drug therapy may have been causally related to the development of the lymphoma.

Immunological abnormalities have been noted in three patients with hydantoin-associated malignant lymphoma. These have included M components in one case (Hyman and Sommers, 1966) and hypogammaglobulinaemia in two (Hyman and Sommers, 1966, Rausing and Trell, 1971). Hypogammaglobulinaemia has been described in one patient with lymphadenopathy which regressed after drug withdrawal (Bjornberg and Holst, 1967).

Circulating immunoglobulins may be abnormal in the absence of lymphadenopathy. Van Rootselaar and Westendorp Boerma (1968) studied 21 institutionalized epileptic patients receiving anti-

convulsant therapy. They found that the mean concentration of IgA was significantly low in 10 patients with idiopathic epilepsy, and normal in 11 patients post-traumatic epilepsy. However, details of therapy were not given. Hydantoin drugs induce the formation of antibodies directed against components of cell nuclei (antinuclear antibodies). In a study of 50 patients by Alarcon-Segovia et al (1972), antibody to soluble nucleoprotein was detected in the serum of 58 percent of cases. Antibodies to heat-denatured DNA, natural DNA, and to the Sm antigen were present in 12-20 percent of patients, and were more common in females.

10. DISCUSSION

The development and use of methods for the measurement of immunological status has resulted from increased understanding of the physiology of the lymphoid system. It is clear that immunological capacity can be usefully defined in terms of function of the B-cell and T-cell systems and that available techniques of measurement are not completely satisfactory. For example, measurement of serum immunoglobulin concentrations is a crude estimate of humoral immune function. It is of value as a complementary test, when parameters such as specific primary and secondary antibody responses, antibody responses in different immunoglobulin classes, and the proportion of cells with a high density of immunoglobulin receptors (B cells) can be measured as well. T-cell function is not satisfactorily measured by current methods, although the identification of T-cells and the measurement of lymphokines, particularly of macro-

phage migration inhibition factor, will be of value in the elucidation of defects in the T-cell system.

The development of tests of immunological function has been useful in the classification of primary immunodeficiency syndromes. More sophisticated methods have yet to be applied to patients with lymphoid tissue malignancy and patients treated with immunosuppressive drugs. The occurrence of lymphoreticular malignancy in patients treated with the anticonvulsant drug phenytoin sodium suggested that a study of this drug for immunosuppressive properties, using standardised tests of immune function, would be of value, particularly in the light of recent evidence and hypotheses concerning the role of immunosuppression, antigenic stimulation, and latent viruses in the aetiology of malignancy.

CHAPTER II

THE MEASUREMENT OF IMMUNOLOGICAL FUNCTION IN MAN

PART A

METHODS

1. INTRODUCTION

It has been known for many years that immune responses are depressed in diseases of lymphoid tissue (Parker et al, 1932, Dubin, 1947). More recently, severe immunodeficiency has been described in the thymus-dependent (T-cell) system (Di George Syndrome, Di George, 1965), and in the thymus-independent (B-cell) system (infantile X-linked agammaglobulinaemia, first described by Bruton, (Bruton, 1952)). It is the aim of tests of immunological capacity to characterize immunological failure in terms of dysfunction of the cells in these two systems.

Increasingly sophisticated tests are being developed for routine use. Basic requirements of tests of immunological function in man have been outlined by a number of workers (Bellanti and Schlegel, 1971, Forbes, 1971, Fudenberg et al, 1971, Whittingham and Mackay, 1971). The methods described in this Thesis are a modification of the procedure of Forbes. They have been used to establish criteria of normal immunological responsiveness in a South Australian population, for comparison with immunological function in patients treated with anticonvulsant drugs, and patients with lymphoma.

2. PROCEDURE

Immunological function was measured using the parameters

shown in Table II-1.

The routine performance of these tests is illustrated in Figure II-1.

(a) Venepuncture

Venous blood was obtained aseptically on days 1 and 17. Blood for cell culture (5 ml) was anticoagulated with preservative-free heparin (125 units, Weddel Pharmaceuticals Ltd., London). Blood for cell counts (2 ml) was anticoagulated with dipotassium ethylene diamine tetra-acetate (E.D.T.A., Disposable Products, Australia). Clotted blood (22 ml) was centrifuged at 1500g for 10 minutes. The serum was re-centrifuged and used immediately in cell cultures, or stored at $-20^{\circ}C$.

(b) Immunoglobulins and Complement

Serum concentrations of the immunoglobulin classes G, A, and M, and the C3 component of complement ($\beta^{1C}/_{1A}$), were measured using Behringwerke immunodiffusion plates.

(c) Antibody Responses

(1) Technique: Patients were immunized subcutaneously with monovalent Salmonella typhi suspension (S. typhi, Commonwealth Serum Laboratories, C.S.L., Australia), 0.1 ml, and with alum-precipitated Tetanus toxoid (5Lf, C.S.L.), 0.5 ml. Serum collected before, and two weeks after immunization, was tested for the presence of specific antibody to S. typhi H antigen, and to Tetanus toxoid.

(2) Determination of antibody to S. typhi: Serial dilutions of sera in physiological saline were incubated with specific

TABLE II-1.

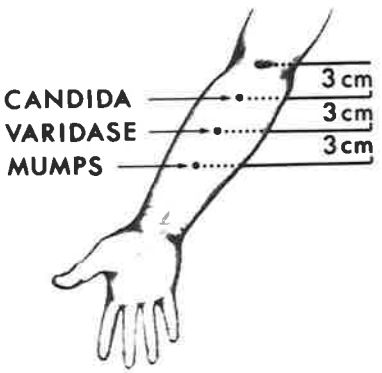
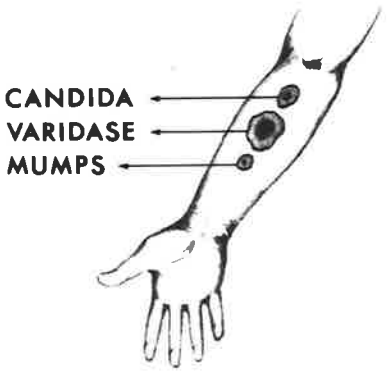
PARAMETERS OF IMMUNOLOGICAL FUNCTION

HUMORAL IMMUNOLOGICAL FUNCTION	CELLULAR IMMUNOLOGICAL FUNCTION
1. Serum Immunoglobulin concentration.	1. Delayed Hypersensitivity Reactions.
- classes G, A, M.	
2. Antibody Responses	2. Circulating Lymphocyte Count.
- primary.	
- secondary.	
3. Serum Complement Concentration.	3. Circulating Leukocyte DNA Synthesis.
4. Autoantibody.	4. PHA-induced Lymphocyte Transformation
	- in autologous serum.
	- in foetal calf serum.

FIGURE II-1

TESTS OF IMMUNOLOGICAL FUNCTION

For routine tests of immunological function the patient was seen on three occasions. The procedures performed at each visit are outlined in Figure II-1.

DAY 1	DAY 3	DAY 17
1. VENOUS BLOOD for ——— CELL CULTURE ——— WHITE CELL COUNT and DIFFERENTIAL COUNT ——— SERUM IgG, A, M complement Typhoid antibody Tetanus antibody Antinuclear factor **2. DELAYED HYPERSENSITIVITY**  	**1. IMMUNIZATION with** ——— S. TYPHI ——— TETANUS TOXOID **2. READ DELAYED HYPERSENSITIVITY REACTIONS**  	**1. VENOUS BLOOD for** ——— SERUM — Typhoid antibody Tetanus antibody *it*

S. typhi H (flagellar) antigen d (C.S.L.) at 56°C. for four hours. Controls contained saline and flagellar antigen. Results were expressed as the reciprocal of the highest dilution at which flocculation occurred.

(3) Determination of antibody to Tetanus toxoid: Tetanus toxoid (ultrafiltrate, 252 Lf/ml, C.S.L.) was coupled to tanned erythrocytes by the method of Gold and Fudenberg (1967). Human Group O, Rh positive erythrocytes were washed three times in physiological saline. The packed cells (1.0 ml) were incubated for 10 minutes at room temperature with albumin-saline (5%, 5 ml), soluble Tetanus toxoid (3 ml), and chromic chloride (0.1%, 3.0 ml). Controls contained albumin-saline and chromic chloride. The cells were washed in saline to remove excess chromic chloride. They were finally made up to a 10% suspension. Serial dilutions of serum were mixed on a semitransparent white tile with an equal volume of coated erythrocytes. Haemagglutination was determined microscopically after incubation at room temperature for seven minutes. Results were expressed as the reciprocal of the highest dilution at which haemagglutination occurred.

(4) Interpretation of results: Responses to S. typhi or to Tetanus toxoid were considered to be normal if antibody was detected in post-immunization sera.

(d) Autoantibody

Sera were tested for the presence of antinuclear factor by a double layer immunofluorescent technique (Weller and Coons,

1954). Fixed and unfixed cryostat sections (6 μ) of rat liver were incubated with serum (diluted 1:4 with Coons' buffer) for 30 minutes at 37°C., washed with several changes of Coons' buffer (0.01 molar, pH 7.1) containing 0.85% NaCl, covered with fluorescein-conjugated antihuman immunoglobulin for 30 minutes, washed, and mounted in buffer (pH 8.6) containing 50% glycerol.

(e) Delayed Hypersensitivity Reactions

(1) Definition: A reaction to a given antigenic stimulus is a manifestation of the activity of specific memory cells, generated during a previous immune response to that antigen.

(2) Intradermal skin tests: Three antigens were injected intradermally into the flexor aspect of the forearm (see Figure II-1). Candida albicans (1%, C.S.L.), streptokinase, 10 units, plus streptodornase, 2.5 units (Varidase, Lederle) in physiological saline, and killed mumps skin test antigen (Eli Lilly, Indianapolis), were injected in a volume of 0.1 ml.

Perpendicular diameters of erythema and induration were measured at 48 hours. Erythema and induration of at least 5 mm were regarded as positive for candida and streptococcal antigens. Erythema of 5 mm was regarded as positive for mumps. Non-reactive subjects were re-tested after an interval of two weeks.

(3) Sensitization with dinitrochlorobenzene (DNCB): This was attempted if subjects did not react to any of the intradermal skin tests on two occasions. DNCB (2000 μg in 0.1 ml of acetone)

was applied to the skin of the left forearm within a polythene ring (internal diameter 1.5 cm), and evaporated in a stream of warm air from a hair-dryer. Subjects were challenged with DNCB 10-14 days later. Two concentrations of DNCB, 50 µg and 25 µg, dissolved in 0.05 ml of olive oil-acetone (50% v/v) were smeared on the skin of the right forearm, over areas 2 cm in diameter. These sites were protected with clean, dry, gauze dressing, and assessed at 48 hours for the presence of erythema and induration of at least 5 mm in diameter, and for vesicle formation.

(4) Skin reactivity: The ability of the skin of otherwise non-reactive subjects to support an inflammatory response was assessed as described by Johnson et al (1971). Croton oil (10% solution in paraffin oil) 0.04 ml, was placed on a patch of non-wettable bandage, 1x1 cm, and secured with adhesive tape to the ventral aspect of the left forearm, 12 cm from the cubital fossa. The response was considered to be positive if vesicles were present in an area of erythema at least 10 mm in diameter, at 48 hours.

(f) Circulating Lymphocyte Counts

Leukocytes were counted in a Coulter counter, Model S. Differential counts were obtained by microscopic examination of 200 white cells on blood smears stained with May Grunwald-Jenner Giemsa solutions. Lymphocyte counts were computed from these values.

(g) Lymphocyte Transformation

(1) Definition: In this Thesis, the term lymphocyte transformation refers to measurable changes (morphological or metabolic) which occur in PHA-stimulated lymphocytes in culture.

Transformation was measured in blood cell cultures by the incorporation of tritiated thymidine (^{3}H-thymidine) into acid-precipitable DNA, using a modification of the method of Junge et al (1970).

(2) Equipment for cell culture: Glassware used in the preparation of cultures was siliconized (1% Siliclad, Clay-Adams, New York), washed and sterilized in an autoclave (see Appendix (i)). Culture tubes (Disposable Products, South Aust.) were sterilized, and dried under vacuum.

(3) Preparation of tissue culture solutions: Medium 199 for tissue culture (C.S.L., Aust., see Appendix (ii)), was buffered with N-2-hydroxyethyl-piperazine-N'-2-ethane sulphonic acid (HEPES, Calbiochem), and sterilized by millipore filtration (Appendix (iii)). The final pH of the medium was 7.5 (at 37°C).

A single batch of foetal calf serum (FCS, inactivated at 56°C for 30 minutes, C.S.L.), was used for patient studies.

Lyophilized PHA (Wellcome, Reagent grade, batch 3716) was reconstituted with sterile distilled water, and stored at 4°C for not more than one month before use.

(4) Preparation of blood cell cultures: Cultures were set up in triplicate. Each contained -

(a) blood suspension - heparinized blood (0.2 ml)

in HEPES-buffered medium 199 (0.4 ml) and serum (FCS or autologous serum), (0.4 ml).

(b) PHA solution - reconstituted PHA (0.02 ml) freshly diluted in medium 199 (3.0 ml).

The blood suspension was dispensed into culture tubes independently of the PHA solution, to prevent prior cell agglutination, which is known to occur in the presence of PHA (Ling, 1968).

(5) Incubation of cultures: Cultures were resuspended daily during incubation at 37°C. Lymphocyte transformation was measured at 96 hours, by the incorporation of ^{3}H-thymidine into acid-precipitable DNA.

(6) Measurement of DNA synthesis: Tritiated thymidine (^{3}H-methyl thymidine, 2.5 µCi, Specific activity 500 mCi/mMol, the Radiochemical Centre, Amersham, England) 0.1 ml, was added to cultures after 92 hours of incubation. Cultures were incubated for a further 4 hours at 37°C. At 96 hours, they were cooled rapidly to 4°C, and transferred to plastic centrifuge tubes (Camelec, Aust.) with cold physiological saline (0.9%, 2 ml). Cultures were centrifuged at 300g for 15 minutes, the supernatant fluid was removed, the erythrocytes lysed with acetic acid (3%, 4 ml), and the cell button washed in cold saline (4 ml). The DNA was precipitated in trichloroacetic acid (5%, 4 ml) at 4°C overnight. The precipitate was centrifuged at 1400g, washed once more in trichloroacetic acid, twice in methanol (4 ml), dried, dissolved in Soluene (0.5 ml,

Packard Instrument Company, Inc. Illinois), and transferred to glass vials with toluene-based scintillation fluid (see Appendix (iv)). Vials were cycled three times through a Packard liquid-scintillation spectrometer. The results were corrected to a counting efficiency of 100% using an automatic external standard. They were expressed as disintegrations per minute per culture (dpm/culture).

(h) DNA Synthesis in Circulating Leukocytes

This parameter was measured in triplicate blood cell cultures containing freshly-drawn, heparinized blood (0.2 ml), medium 199 (3.8 ml), and ^{3}H-thymidine (2.5 μCi, 0.1 ml). Cultures were incubated for four hours at 37°C, and processed as above.

PART B

CRITERIA OF NORMAL IMMUNOLOGICAL RESPONSIVENESS

The data presented here were compiled by myself, in association with Mrs. K.T. Holmes and Dr. D.I. Grove.

1. COMPOSITION OF THE CONTROL POPULATION

Studies of DNA synthesis were performed on 90 healthy adults. Other measurements were obtained from this population of normal adults (50 subjects), patients with vascular disease (20 subjects), untreated epilepsy (15 subjects), and various diseases not known to be associated with immunological abnormality (15 subjects). There was no clinical evidence of infection, and blood cell counts were normal, in control subjects.

The age and sex-distributions of control subjects are shown

for each parameter in Table II-1. For studies of DNA synthesis, the distribution of males and females was approximately equal for each age group. For other parameters, the number of males in the fifth decade (mean number, 12) exceeded the number of females (mean number, 4).

TABLE II-1

AGE AND SEX DISTRIBUTION OF CONTROL SUBJECTS

Parameter	No.	*M.	*F.	Mean age	Range
Immunoglobulins	87	48	39	34.2	14-78
Antibody response	136	74	62	31.4	15-76
Complement	54	?	?	24.2	10-55
DHS	100	55	45	36.0	14-78
Lymphocyte Ct.	83	41	42	29.7	16-60
Leukocyte DNA	90	51	39	30.6	14-65
Lymphocyte-	$^{+}$84	43	41	28.4	14-65
Transformation	$^{\phi}$82	45	37	27.8	14-65

*Number of males, females. Transformation in $^{+}$FCS, $^{\phi}$Autologous serum. (?) Sex distribution approximately equal.

2. STATISTICAL METHODS

(a) Continuous Distributions

The following parameters were tested for goodness of fit to a Gaussian curve, using Fisher's coefficients of skewness and

kurtosis (Snedecor, 1959):- serum concentrations of immunoglobulins and complement, and DNA synthesis in circulating leukocytes and in PHA-stimulated lymphocytes. The upper and lower limits of the control range were calculated at two standard deviations from the mean, and by using the non-parametric method of percentiles (Reed et al, 1971). The values in skewed distributions were converted to logarithms (to the base 10) and re-tested for goodness of fit to a Gaussian curve.

For Gaussian distributions, the difference between the mean values of males and females was tested for significance using Student's t-test. For non-Gaussian distributions, the difference between medians was tested for significance by the non-parametric Rank Sum test of Wilcoxon (Documenta Geigy, 1962).

The effect of age on PHA-induced lymphocyte transformation was studied by comparing the median values of subjects in the age groups 14-24, 25-34, and 35-65 years, using Wilcoxon's test.

(b) Discrete Distributions

The results of the following parameters were recorded as a positive or negative reaction or response, and expressed as the percentage of subjects with a positive reaction (or response):- primary and secondary antibody responses, autoantibodies, and DHS reactions. Differences in the results of males and females were tested for significance by Fisher's exact test (Bailey, 1968).

(c) Calculations

The statistical analyses of the data are shown in Appendix (v).

3. RESULTS

(a) Immunoglobulin Concentrations

The distributions of IgG and IgA concentrations are shown in Figure II-2; the means and standard deviations are shown in Table II-2. The distribution of values of IgM is shown in Figure II-3.

TABLE II-2

IMMUNOGLOBULIN CONCENTRATIONS

Group	IgG	IgA	IgM
All subjects	1190 ± 311	197 ± 77	*148
Males	1210 ± 297	212 ± 76	
Females	1161 ± 326	180 ± 75	

*Geometric mean

The values of IgG and IgA were normally distributed; IgM concentrations were log-normally distributed. All values were independent of the sex of the subjects. The range of values of IgG was 600-1900 mg/100ml, for IgA, it was 70-380 mg/100ml, and for IgM, it was 50-370 mg/100 ml.

(b) Serum Complement Concentrations

The distribution of values of the C3 component of complement is shown in Figure II-3. The group mean and standard deviation was 128 ± 38 mg/100ml. The exact sex-distribution of the subjects was not known. Therefore, comparisons between males

FIGURE II-2

FREQUENCY DISTRIBUTION OF IgG AND IgA

The frequency (expressed as a percentage of the total patients) is plotted against immunoglobulin concentration (mg/100ml).

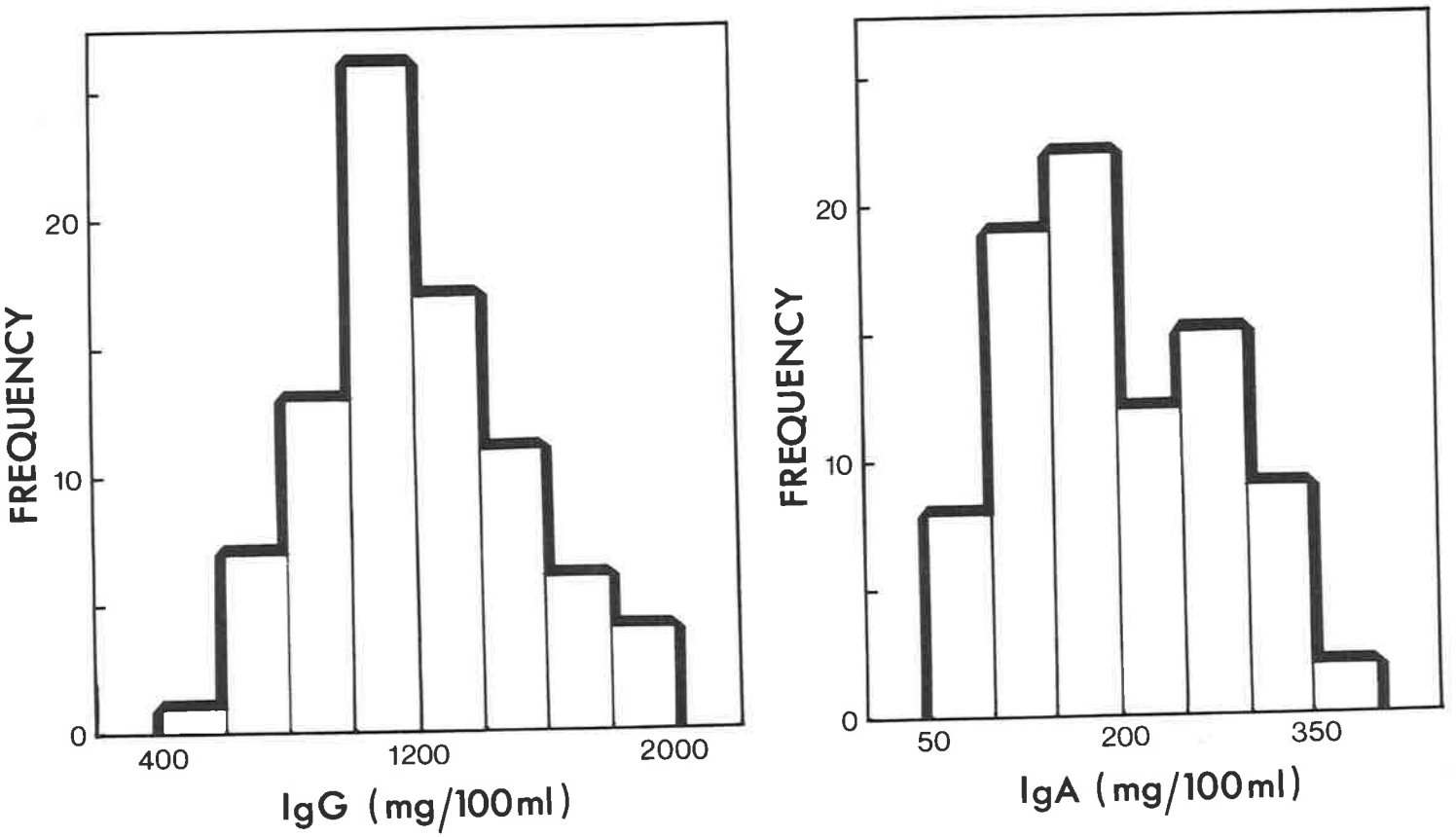
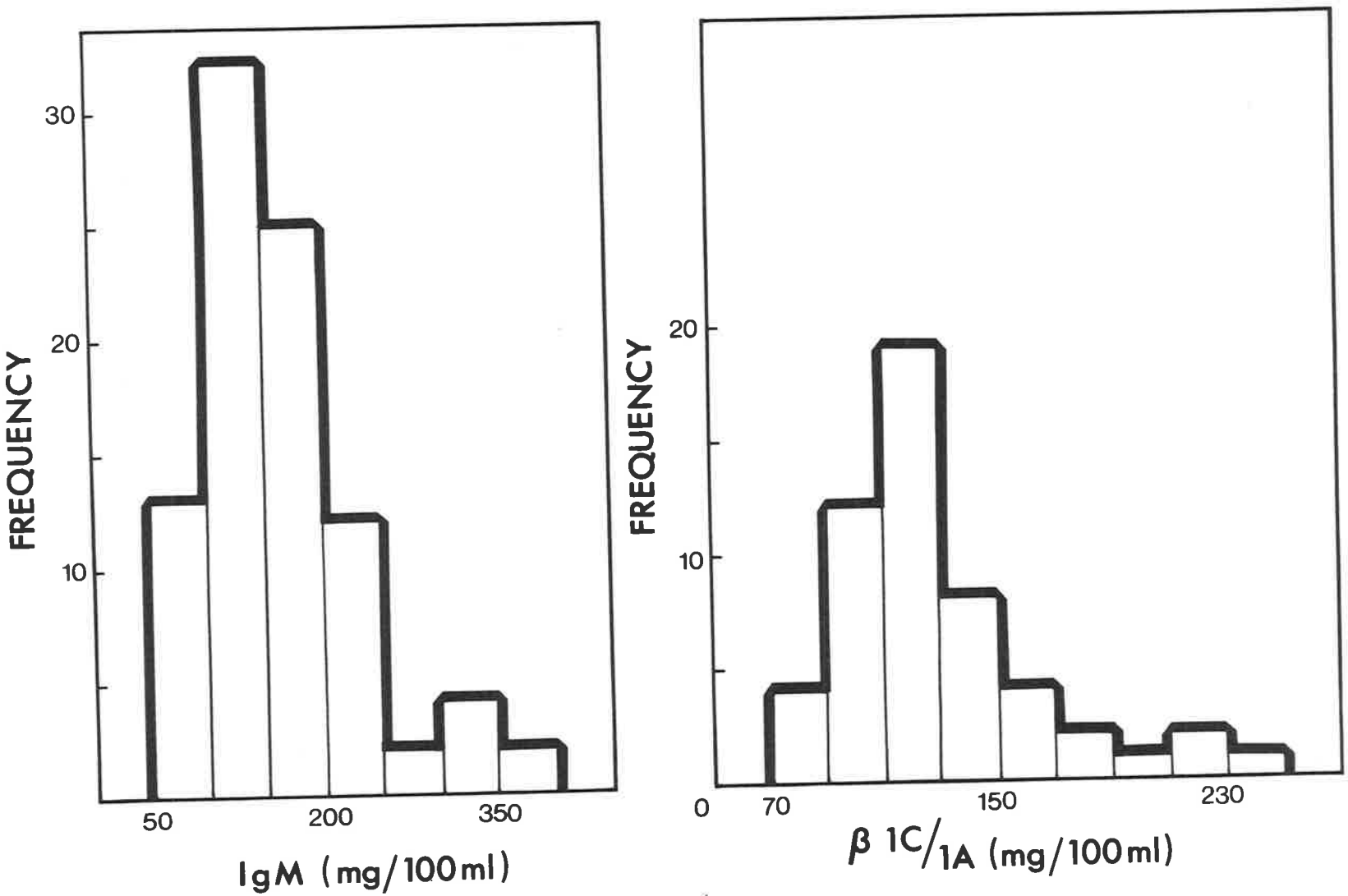

FREQUENCY
20
10
0
400
1200
2000
IgG (mg/100ml)
FREQUENCY
20
10
0
50
200
350
IgA (mg/100ml)

FIGURE II-3

FREQUENCY DISTRIBUTION OF IgM AND COMPLEMENT

The frequency (expressed as a percentage of the total patients) is plotted against the IgM and the complement concentration ($\beta^{IC}/_{IA}$). Results are given in mg/100ml.


FREQUENCY
30
20
10
50
200
350
IgM (mg/100ml)
FREQUENCY
20
10
0
70
150
230
β 1C/1A (mg/100ml)

and females were not made. The range of complement concentrations was 70-225 mg/100ml.

(c) Antibody Responses

The number of patients who developed antibody in response to immunization with S. typhi and Tetanus toxoid is shown in Table II-3. The total number of patients in the respective groups is shown in parentheses.

TABLE II-3

ANTIBODY RESPONSES

Antigen	All Responders	Males	Females
S. typhi	136 (136)	74 (74)	62 (62)
Tet. tox.	136 (137)	75 (75)	61 (62)

(d) Autoantibody

Antinuclear antibodies were detected in the serum of 1 of 50 control subjects.

(e) Delayed Hypersensitivity Reactions

The number of subjects reacting to each intradermal antigen and the number reacting to at least one of the three antigens, is shown in Table II-4. The percentage of positive reactions is shown in parentheses. One percent of patients failed to react to any antigen. The probability of three negative reactions occurring together was calculated from the reactions to individual antigens -

Probability = 36/100 X 19/100 X 19/100

= 1.3%

TABLE II-4

DELAYED HYPERSENSITIVITY REACTIONS

Group	Candida	*Sk/Sd	Mumps	1 Antigen
Males	32 (58)	45 (82)	45 (82)	53 (100)
Females	29 (71)	34 (79)	35 (80)	40 (98)
Total	61 (64)	79 (81)	80 (81)	93 (99)

*Streptokinase-streptodornase.

(f) Circulating Lymphocyte Counts

The distribution of results is shown in Figure II-4; mean values and standard deviations are shown in Table II-5.

TABLE II-5

LYMPHOCYTE COUNTS-MEANS AND STANDARD DEVIATIONS

	All Subjects	Males	Females
Mean	1926	1887	1964
Standard deviation	430	407	449

The distribution of results fitted a Gaussian curve, and values were independent of the sex of the subject. The control range of lymphocyte counts was 900-2900 cells per µl of blood.

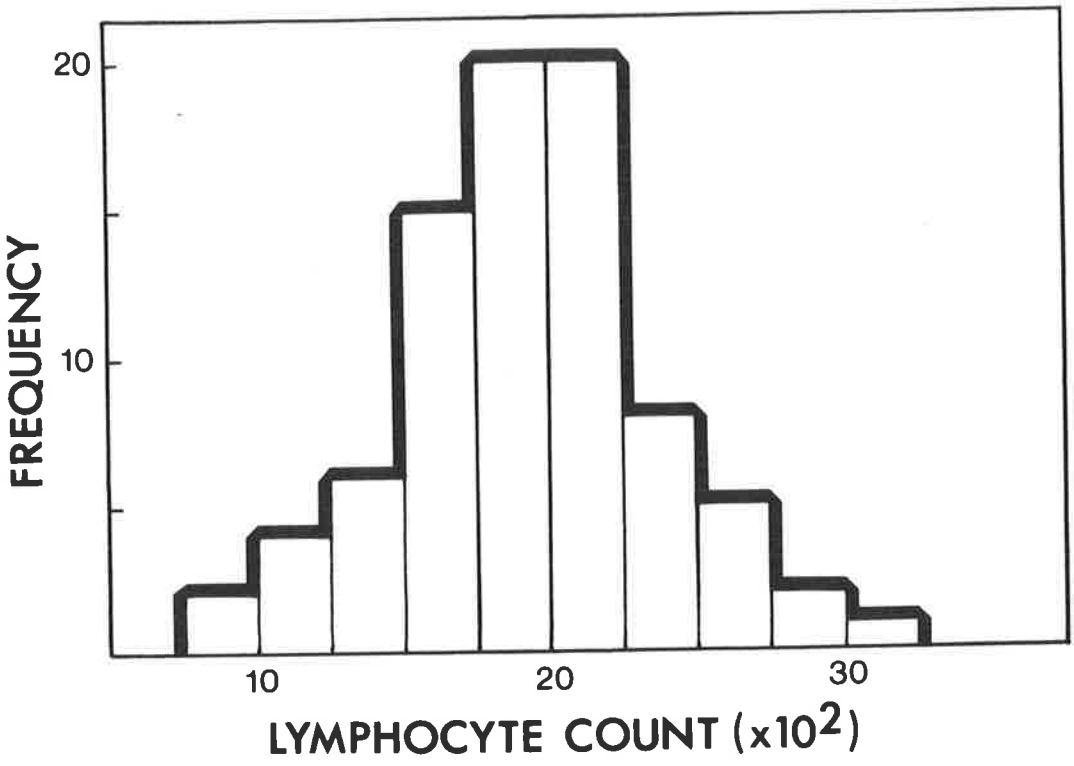
FIGURE II-4

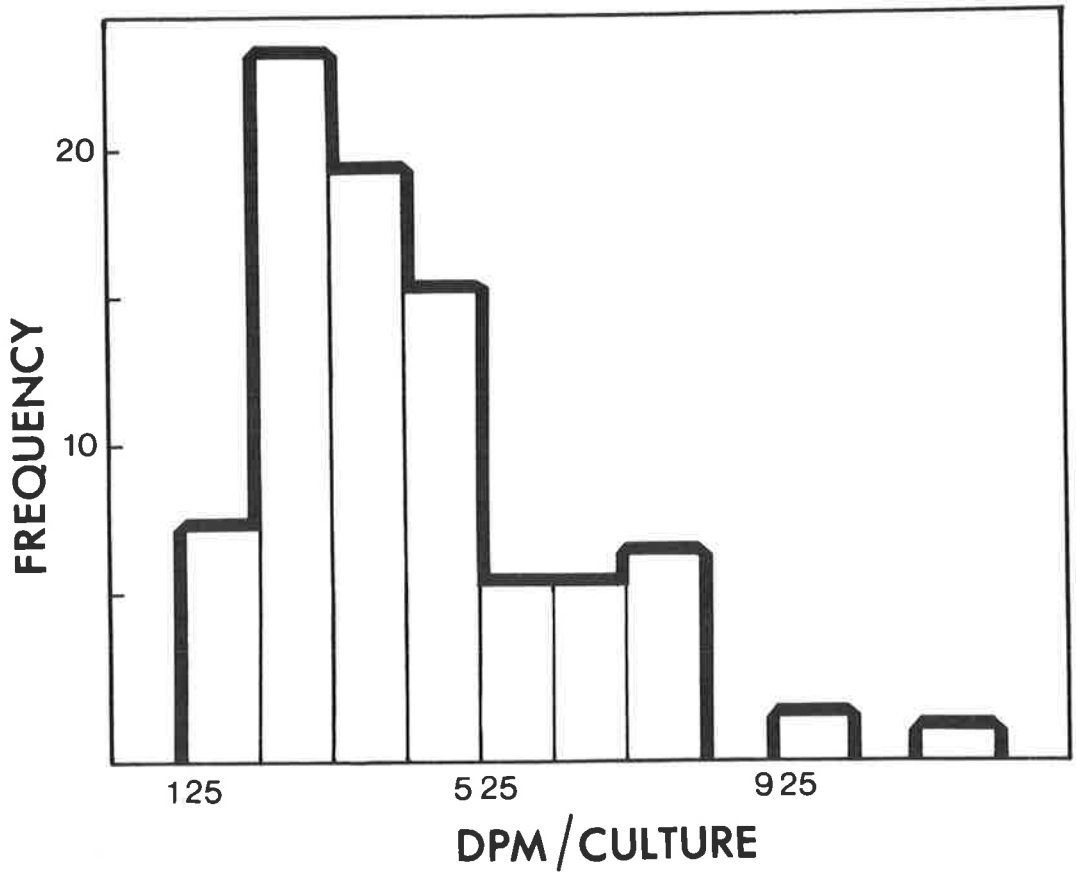
FREQUENCY DISTRIBUTION OF LYMPHOCYTE COUNTS AND DNA SYNTHESIS IN CIRCULATING LEUKOCYTES

Frequency : percentage of total subjects.

Lymphocyte count : cells per µl of blood.

DNA synthesis in circulating leukocytes : dpm/culture.


20
10
FREQUENCY
10
20
30
LYMPHOCYTE COUNT (x10²)


20
10
FREQUENCY
125
525
925
DPM/CULTURE

(g) DNA Synthesis in Circulating Leukocytes

The results have been graphed in Figure II-4. The distribution of results was log-normal; there was no significant difference between males and females. The geometric mean was 391, and the range of control values, 155-965 dpm/culture.

(h) Lymphocyte Transformation (DNA Synthesis)

(1) In the presence of autologous serum: The distribution of results is shown in Figure II-5; means and standard deviations are shown in Table II-6, and median values in the age-subgroups, in Table II-7.

TABLE II-6

LYMPHOCYTE TRANSFORMATION-MEANS AND STANDARD DEVIATIONS

	All Subjects	Males	Females
Mean	78.5	83.9	70.3
Standard deviation	39.0	36.5	37.8

TABLE II-7

EFFECT OF AGE ON LYMPHOCYTE TRANSFORMATION

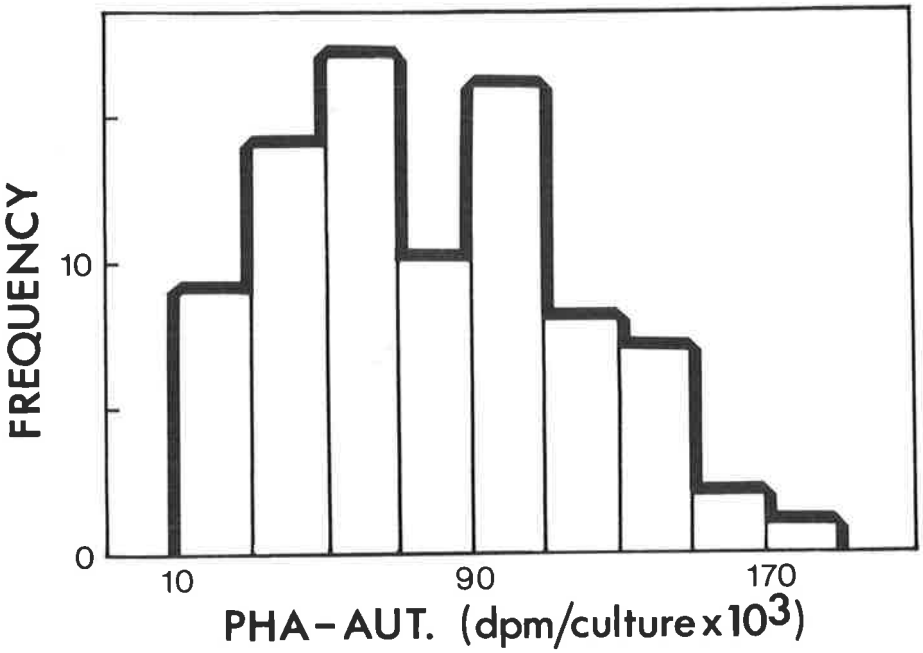
	14-24 yrs	25-34 yrs	Over 34 yrs
Number	33	34	17
Median	80.0	72.3	75.5

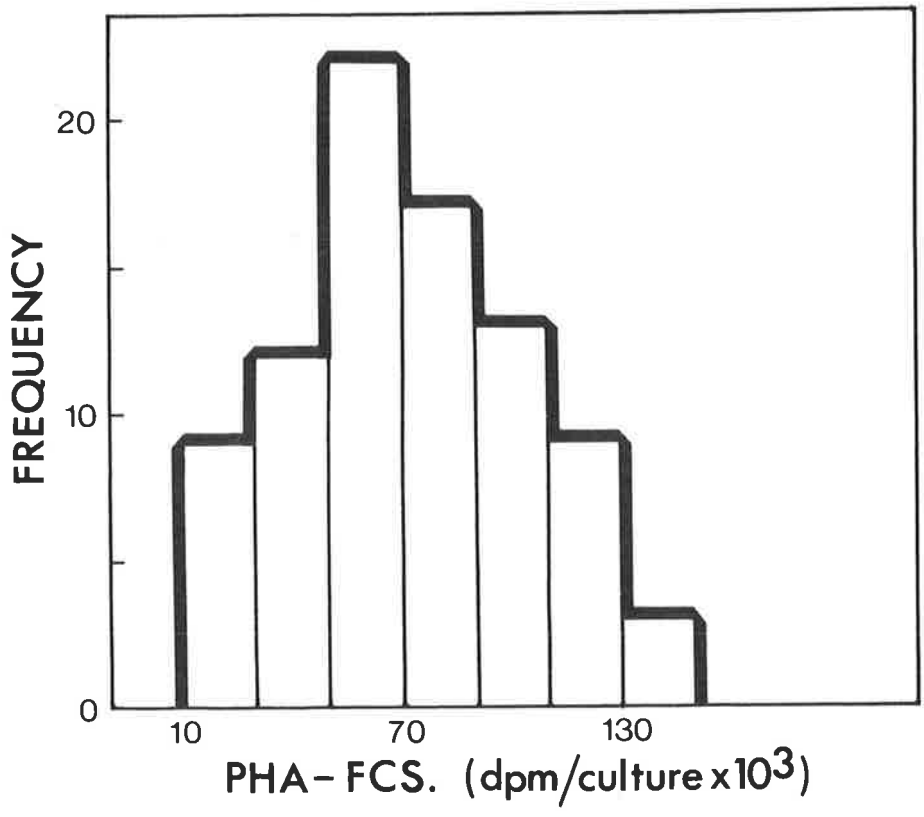
FIGURE II-5

FREQUENCY DISTRIBUTION OF PHA-STIMULATED DNA SYNTHESIS

(a) In medium supplemented with autologous serum (Aut)

(b) In medium supplemented with foetal calf serum (FCS)


FREQUENCY
10
0
10
90
170
PHA-AUT. (dpm/culture x 10³)


FREQUENCY
20
10
0
10
70
130
PHA-FCS. (dpm/culture x 10³)

The distribution of results fitted a Gaussian curve; values were independent of the age and sex of the subjects. The control range of values was 19.0–152.0 dpm/culture ($x10^3$).

(2) In the presence of foetal calf serum: The distribution of results is shown in Figure II–5; means and standard deviations are shown in Table II–8, and median values in the age subgroups, in Table II–9.

TABLE II–8

LYMPHOCYTE TRANSFORMATION–MEANS AND STANDARD DEVIATIONS

	All Subjects	Males	Females
Mean	70.7	73.2	64.3
Standard deviation	31.2	34.5	28.9

TABLE II–9

EFFECT OF AGE ON LYMPHOCYTE TRANSFORMATION

	14–24 yrs	25–34 yrs	Over 34 yrs
Number	37	28	17
Median	70.0	72.0	54.0

The distribution of results fitted a Gaussian curve; values were independent of the age and sex of the subjects. The control range of values was 15.0–130.0 dpm/culture ($x10^3$).

4. DISCUSSION

Criteria of normal immunological responsiveness were established in control subjects from a South Australian community, for comparison with responses of patients receiving anticonvulsant therapy, and patients with lymphoma.

IgG and IgA concentrations were normally distributed. IgM concentrations were log-normally distributed. These results confirm the findings of Rhodes et al (1969).

Other workers have found that the three immunoglobulin classes are log-normally distributed (Butterworth et al, 1967, Buckley and Dorsey, 1971, Hobbs, 1971). It has been shown that once adult levels are reached, serum immunoglobulin concentrations remain relatively stable in the individual patient (Allansmith et al, 1967), particularly IgA (West et al, 1962). There was no significant difference between levels in males and females in the present series. Hobbs (1971) found that IgM concentrations in females over the age of 7 years were 20-30% higher than in males. In a larger series, Buckley and Dorsey (1971) reported that IgM in white American females was 11.8% higher than in males, and that IgA was 24% lower. However, the major source of biological variation in immunoglobulin levels is due to the effect of ageing (Buckley and Dorsey, 1970(a)): in this series IgG levels decreased progressively after the third decade; IgM levels were significantly low by the sixth decade and IgA levels remained stable. In contrast Haferkamp et al (1966) found IgG and IgA concentrations in a smaller, homogeneous, Swiss

community increased significantly with age, whereas IgM remained stable. The numbers of elderly patients in the present study were too small for meaningful statistical analysis. The effect of biological variation can be minimised in intergroup comparisons by the use of age and sex matched controls (Buckley and Dorsey, 1970(b)).

In the present study, development of measurable serum antibody after a single immunization, measured at two weeks, was accepted as evidence of a normally functioning antibody-producing system. Good discrimination between control and disease states has been obtained using this criterion in studies of infection (Forbes, 1971), asthma (Grove et al, 1973), dystrophia myotonica (Grove et al, 1973) and multiple myeloma and paraproteinaemia (Grove et al, 1973).

All subjects in this study developed antibody to S. typhi. This antigen has been shown to elicit a primary immune response (predominantly IgM) in a South Australian population (Forbes, 1971). Over 99% of subjects responded to Tetanus toxoid, which elicits a secondary (predominantly IgG) response (Forbes, 1971). The data suggested that the ability to develop measurable antibody in response to antigenic challenge was independent of the age and sex of the subject. It has been reported that the mean antibody titre developed in response to bovine serum albumin in animals (Goullet and Kaufmann, 1964/5) and to Brucella vaccine in man (Rhodes et al, 1969), is significantly lower in old age. The response to flagellin derived from Salmonella Adelaide was studied in man by Rowley and Mackay (1969). They found that mean antibody titres were not affected by age, but

that levels of specific antibody (predominantly IgM) were significantly higher in females than in males. They also found that titres were lower in patients with gastrointestinal and cardiovascular disease than in healthy controls. Fifty general-hospital patients (20 with vascular disease, 15 with untreated epilepsy and 15 with other non-immunological disease) were included in the present study. One patient failed to make antibody to Tetanus toxoid. Responses of all other patients were normal by the criteria adopted in this study.

Serum complement (C3 component) concentrations were normally distributed in this study. The mean levels were in agreement with those published by Muller-Eberhard (1969).

Antinuclear antibody was detected in the serum of 1 of 50 normal subjects, all of whom were under 60 years of age. It has been reported that the prevalence of antinuclear factors in a random sample of the Welsh population is less than 5% (Jacobs et al, 1969). However, the prevalence of antinuclear antibody to human granulocyte nuclei, measured by immunofluorescence, has been shown to rise progressively after the age of 20 years, reaching approximately 55% in females, and 35% in males aged 70 years (Rowley et al, 1968).

Different combinations of antigens have been used by different workers to test for DHS reactions. Fudenberg et al (1971) recommended the use of five intradermal antigens to test for DHS reactions - tuberculin (purified protein derivative, PPD), candida, trichophyton, streptoccal antigens, and mumps. Brown et al (1967) used candida, histoplasmin, mumps, and PPD. Reactivity to a given

antigen depends on its prevalence in the community, e.g., in two North American populations 94% and 51% of healthy adults manifested DHS to candida albicans (Shannon et al, 1966, Brown et al, 1967), compared with 64% in this survey. Coccidioidin reactivity is known to be common in Californian subjects. Such antigens can usefully be tested for in particular communities (Fudenberg et al, 1971).

Forbes (1971) found that forty of forty-one subjects in a South Australian population reacted to at least one of candida, streptococcal antigen, and mumps. These antigens were therefore selected to study DHS reactions in the larger population reported in this study, in which 99 of 100 subjects were reactive.

Negative reactions to intradermal antigens may be due to lack of previous exposure to them. It has been suggested that sensitization with DNCB may be a better test for DHS, as most healthy subjects readily become sensitized to it (Aisenberg, 1966).

The delayed hypersensitivity reaction results from the activity of specific memory cells, previously generated by an immune response to the particular antigen. Reactivity to DNCB depends, in addition, on the development of hypersensitivity to a new antigen. The relative merits of the two tests have not been clearly defined. In a study of 20 normal subjects, Eilber and Morton (1970) found that 19 developed DHS to DNCB, and 18 gave a positive reaction to at least one of seven skin test antigens.

There is some evidence that the ability to react to DNCB may be lost earlier in disease than the ability to react to a

battery of skin test antigens (Chase, 1966), suggesting that the afferent (sensitization) limb of the immune response, which includes the development of specific memory cells, is affected earlier in disease than the efferent limb. However, Eilber and Morton (1970) studied cancer patients who were apparently well six months after surgical removal of the tumour; they found that, pre-operatively, 92% of these patients reacted to DNCB and 55% to at least one skin test antigen. They suggested that reactivity to DNCB is a useful means of predicting the clinical course of patients with resectable tumour. One interpretation of their findings is that the afferent limb of the immune response remained intact, and that memory cells, developed previously in response to a naturally encountered antigenic substance, were qualitatively or quantitatively depressed in these patients. It is possible that loss of reactivity to skin test antigens is evidence of less severe immunodepression than loss of reactivity to DNCB under these conditions, i.e., the discriminatory power of the two tests is different.

The problem of prior sensitization was overcome in the present study by re-testing non-reactive patients after an interval of two weeks. Subjects were tested with DNCB only if they failed to react to any intradermal antigens on two occasions, as sensitization to DNCB has caused severe local reactions (Fudenberg et al, 1971).

Circulating lymphocyte counts were normally distributed in this series. Mean counts were not significantly different in males and females. Zacharski et al (1971) found that the lymphocyte counts

of 507 healthy American adults were log-normally distributed and that they were independent of the age and sex of the subjects. It has been suggested that counts are depressed during summer months (Meyer et al, 1972). It is also known that immunization may temporarily lower lymphocyte numbers (Ammann, 1972). Lymphocyte counts in this series were determined before antigenic challenge but seasonal variations were not taken into account.

DNA synthesis in circulating leukocytes has been studied by autoradiography (Bond et al, 1958) and by liquid scintillation spectrophotometry (Crowther et al, 1969, Horwitz et al, 1970). In this series the incorporation of ^{3}H-thymidine into DNA was measured by liquid scintillation methods; values were log-normally distributed and ranged from 130-1135 dpm/culture. The percentage of leukocytes incorporating ^{3}H-thymidine did not exceed 0.06 in Bond's series; this percentage remained stable in healthy subjects (Rubini et al, 1961, see Chapter III).

The measurement of DNA synthesis in circulating leukocytes was included under cellular immune responses in Table II-1. However, the cells synthesizing DNA have not been identified as thymus-dependent lymphocytes. They may be immature lymphoid cells released from marrow or peripheral lymphoid tissues, particularly in response to infection or immunization (Wood and Frenkel, 1967, Crowther et al, 1969) monocyte precursors (Gump and Fekety, 1967), or both (Horwitz et al, 1970). At least a proportion of activated cells in the blood of subjects immunized with flagellin bind specific

antigen (Dwyer and Mackay, 1970). Crowther et al (1969) studied the appearance of DNA-synthesizing cells in human blood after immunization with S. typhi-paratyphi. The peak response was obtained 5-7 days post-immunization for both primary and secondary immune responses. The incorporation of ^{3}H-thymidine was elevated only between days 3 and 9 after the immunizing injection. It was decided to measure DNA synthesis before any antigenic stimulation in the present study.

PHA-stimulated lymphocyte transformation was measured by the incorporation of ^{3}H-thymidine into acid-precipitable DNA. Indices of DNA synthesis in the presence of autologous serum and of foetal calf serum, were normally distributed. There was a wide range of values in control subjects, confirming the results obtained by other workers in leukocyte-rich cultures (Richter and Naspitz, 1967, McIntyre and Cole, 1969, Parker and Lukes, 1969). Whole blood cultures contained a fixed amount of blood; white cell rich cultures contained a fixed number of leukocytes (Richter and Naspitz, 1967). Variations in the number of lymphocytes and other blood elements probably contributed to the spread of results (see Chapter IV). Studies on the effect of ageing on lymphocyte transformation have produced conflicting results. Pisciotta et al (1967) using a morphological assessment, found a linear decrease in indices of mitosis and transformed cells in patients aged from 13 to 98 years. Pentycross (1969) studied 64 healthy subjects aged up to 80 years. He found that morphological indices of transformation were relatively low in subjects under the age of 26 years and that they

did not decrease in the elderly. Sutherland et al (1971) reported that the percentage of transformed cells did not change with age (up to 50 years) in 21 healthy subjects, but that there was a linear decrease in PHA-induced DNA synthesis with increasing age, in 14 subjects aged up to 58 years. The present study was based on 3 groups of healthy subjects in the age range 14 to 65 years. There was no significant variation in the medians of the three groups. Pentycross also noted that most of his patients with low scores were females. There was no significant difference between males and females in the present study or in the results of Pisciotta. Transformation indices do vary in the individual subject (see Chapter IV).

Comparison of the data from this, and other, control populations, emphasises the need to establish criteria of normal immunological responsiveness in the community under study. DNA synthesis in PHA-stimulated lymphocytes and in circulating leukocytes, and immunoglobulin and complement concentrations varied with respect to ranges and distribution curves in the different series. This may have been due to differences in techniques, to environmental or genetic factors, or to other determinants of biological variation, e.g. the age and sex distribution of the study group. DHS reactions to individual antigens also varied in the different communities.

The regimes used in the present study have been chosen on the basis of technical and clinical practicability, and on their ability to yield meaningful data on immunological competence. Isolated defects of cellular or humoral immunity can be defined more

precisely using more sophisticated techniques. The tests described do, however, provide a screening test capable of discriminating between normal and abnormal immunological capacity.

CHAPTER III

COMPARISON OF METHODS FOR QUANTITATING LYMPHOCYTE TRANSFORMATION

1. INTRODUCTION

Phytohaemagglutinin-stimulated lymphocytes undergo metabolic and morphological changes during transformation in culture. The techniques of autoradiography (Cleaver, 1967) and gas-flow or liquid scintillation spectrophotometry (Ling, 1968) have been used to measure DNA synthesis in transformed lymphocytes, using ^{3}H- or ^{14}C-thymidine as the DNA precursor. Transformation has also been assessed by the microscopic examination of stained smears (Yoffey, 1965) and by determination of the volume of stimulated cell-nuclei (Stewart and Ingram, 1967). The following three methods have been compared in this study:

(a) The incorporation of ^{3}H-thymidine into acid-precipitable DNA, measured by liquid scintillation spectrophotometry.

(b) The percentage of transformed cells in culture, estimated by the microscopic examination of stained smears.

(c) The volume of transformed cell-nuclei, measured by electronic pulse-height analysis.

2. THE INCORPORATION OF ^{3}H-THYMIDINE INTO DNA

(a) Determination of Optimal Labelling Conditions

Blood cell cultures were established and processed as described in Chapter II. The specific activity of the ^{3}H-thymidine used throughout the study was 500 mCi/mMol. The effect of

increasing the concentration of radioactivity added for the final four hours of culture is shown in Figure III-1. The system became saturated at ^{3}H concentrations of 2.5 μCi/culture. Identical curves were obtained on repetititon of the experiment, and in cultures containing autologous serum.

The concentration of added radioactivity used in subsequent experiments and in all routine studies was 2.5 μCi/culture.

(b) Routine Measurement of DNA Synthesis in Blood Cell Cultures - Expression of Results

As the lymphocyte is the transforming cell in stimulated cultures (reviewed by Naspitz and Richter, 1968, Ling, 1968), the relationship between the number of lymphocytes initially present in the culture, and the incorporation of ^{3}H-thymidine into cellular DNA on day 4 of culture, was studied. This was done by varying the number of lymphocytes in blood cell cultures of individual subjects and by attempting to correlate initial lymphocyte counts with DNA synthesis in routine cultures from 46 normal subjects.

(1) The effect of varying lymphocyte concentration on DNA synthesized in blood cell cultures: This series of experiments was designed to vary the lymphocyte component of blood cell cultures by factors from one-eighth to four times the concentration in peripheral blood.

Method

Blood suspension containing approximately four times the

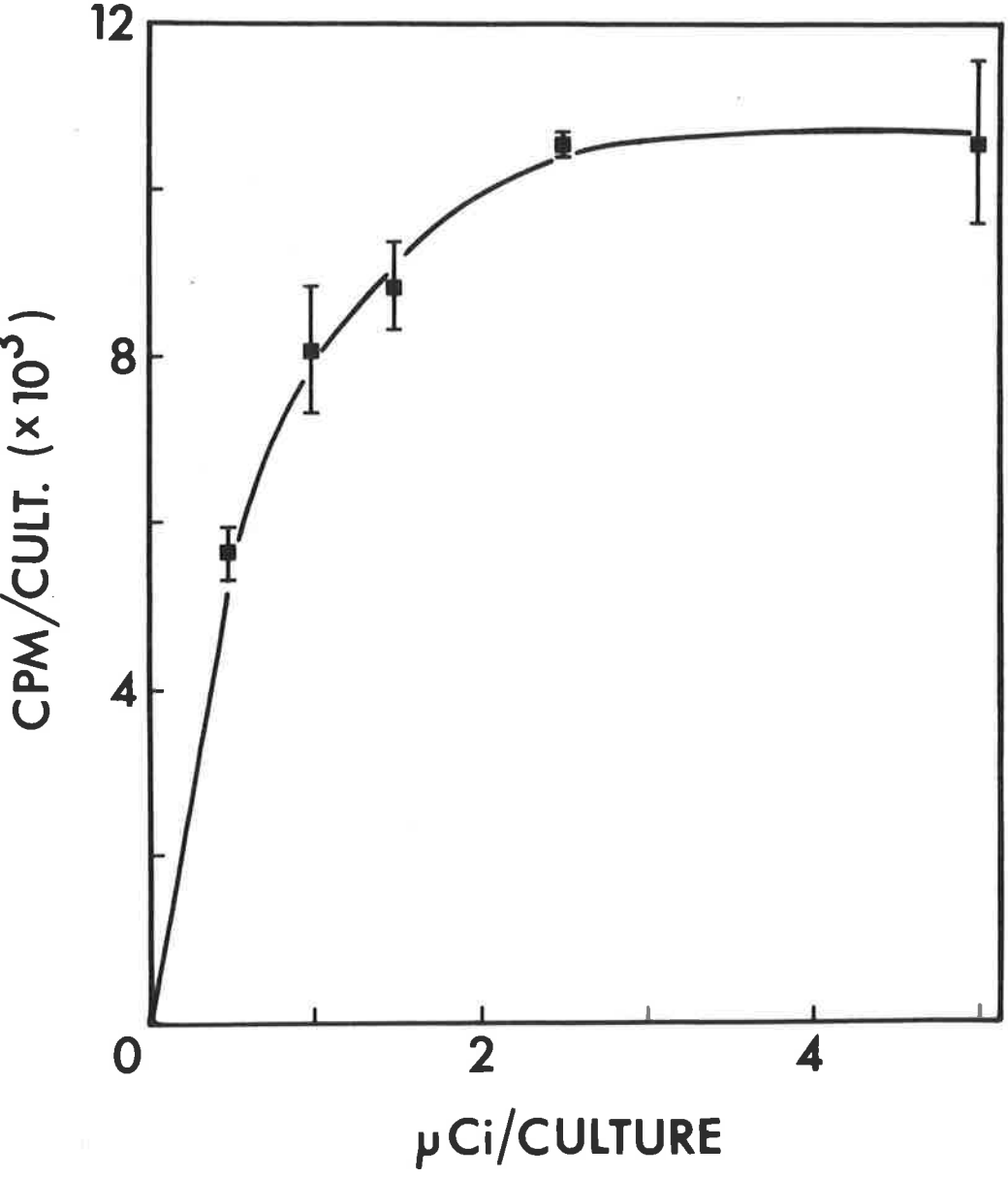
FIGURE III-1

^{3}H-THYMIDINE - DETERMINATION OF OPTIMAL LABELLING CONDITIONS

Cultures were stimulated with PHA in the presence of foetal calf serum.

The concentrations of added radioactivity (µCi/culture) are plotted against the amount of ^{3}H-thymidine incorporated into DNA (cpm/culture).

Counting efficiency of spectrometer: 34%.


12
8
4
0
2
4
CPM/CULT. (x10^3)
μCi/CULTURE

usual number of lymphocytes (400% suspension) were prepared by centrifuging heparinized blood (16ml) in a capped syringe (nozzle downward) at 300g for 10 minutes. Part of the red-cell concentrate (5.4ml) was transferred to a sterile bottle; the remainder of the cells in the syringe were resuspended and recentrifuged with the syringe inverted. The upper layer of plasma (6.6ml) was collected into a sterile bottle by syringing through a bent hypodermic needle. The remainder of the cells in the syringe was resuspended and transferred to a sterile bottle as the 400% suspension. The 200% lymphocyte suspension was prepared as above, except that 8ml of blood was initially centrifuged. Packed erythrocytes (1.8ml) and plasma (2.2ml) were withdrawn from the syringe as described. Heparinized blood was diluted with packed erythrocytes and plasma obtained from the above procedure, to give 50%, 25%, and 12.5% lymphocyte suspensions, as shown in Table III-1. All calculations were based on a blood haematocrit value of 45%.

TABLE III-1

PREPARATION OF DILUTE LYMPHOCYTE SUSPENSIONS

Lymphocytes (%)	Blood (ml)	Packed R.B.C.* (ml)	Plasma (ml)
50	2.00	0.90	1.10
25	1.00	1.36	1.66
12.5	0.50	1.58	1.92

*Red Blood Cells.

The appropriate blood cell suspension (1.0ml) was mixed with dilute PHA solution (3.0ml) in the culture tube.

Leukocyte counts were determined on each sample of cell suspension using a Coulter Counter, Model S; differential counts were determined from stained blood smears; lymphocyte counts were computed from these figures.

Five experiments were performed on 4 normal donors, and one patient with aspiration pneumonia. The cell population density ranged from 0.3 - 1.9 x 10^5 lymphocytes per ml of culture.

Results

The total ^{3}H-thymidine incorporation was plotted against lymphocyte population density using logarithmic scales. The results from two subjects are shown in Figure III-2. In each case, the hypothetical incorporation in a culture containing 1 x 10^5 lymphocytes per ml, was calculated by extrapolation from the incorporation in cultures containing approximately 1 x 10^5 lymphocytes/ml. All values of incorporation of ^{3}H-thymidine could be expressed as fractions of this hypothetical value and all graphs could be plotted from this hypothetical origin. (Lawton, 1967). The linear relationship between total incorporation and cell population density, can be expressed by the equation

$$\text{Total incorporation } It = Im\,(1+kC),$$

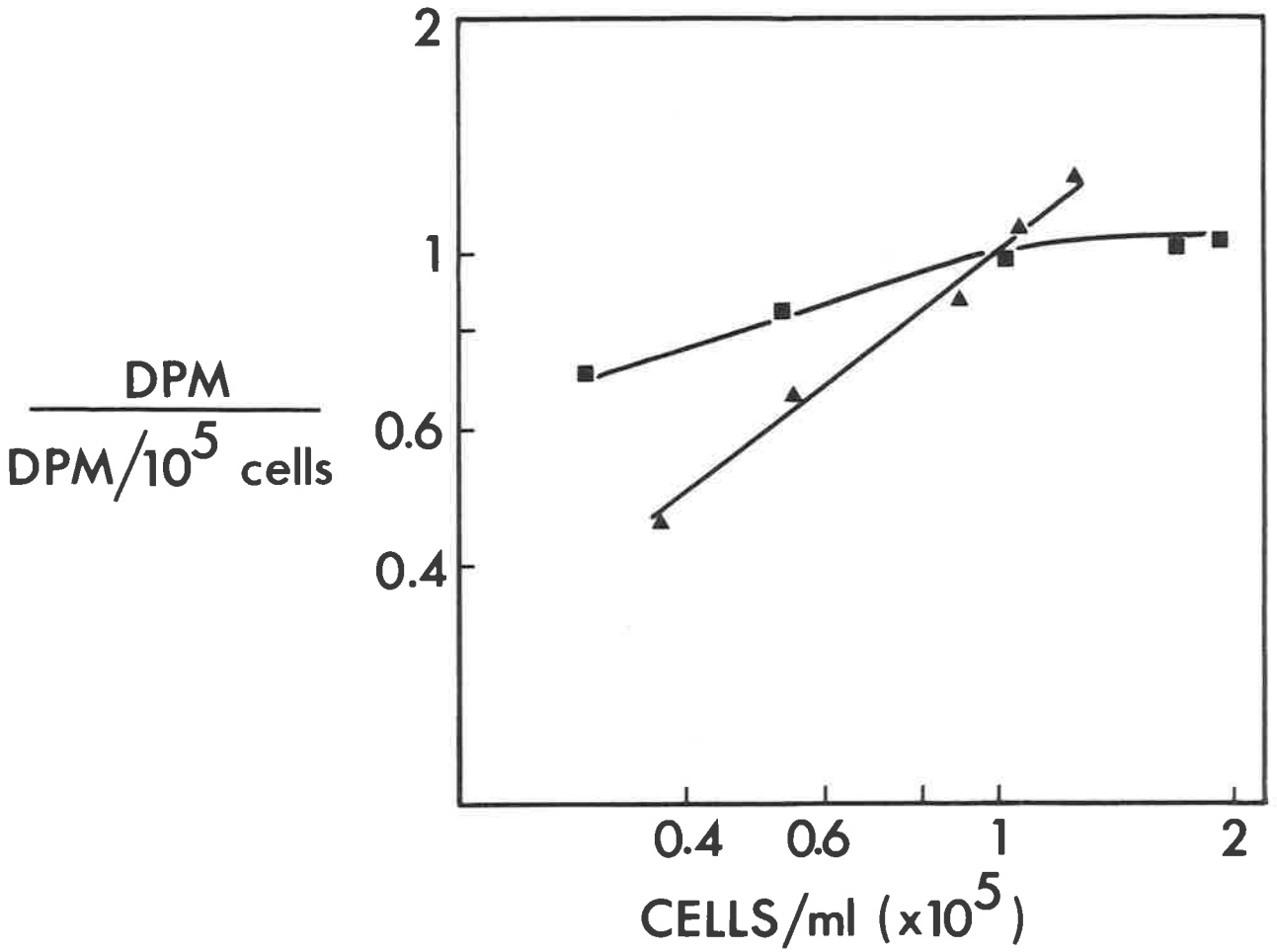
where Im is the calculated figure for incorporation in a culture containing 1 x 10^5 cells/ml, C is the number of cells (x10^5)/ml

FIGURE III-2

THE RELATIONSHIP BETWEEN LYMPHOCYTE POPULATION DENSITY AND DNA SYNTHESIS

The derivation of the graphs is explained in the text. The results of patients with the greatest and least responses are shown.

All cultures were incubated in the presence of foetal calf serum.


DPM
DPM/10^5 cells
2
1
0.6
0.4
0.4
0.6
1
2
CELLS/ml (x10^5)

in excess of 10^5, and k is the equation constant. The value of k is given by the slope of the straight line section of the graph, using an arithmetic plot. If the ^{3}H-thymidine incorporation increased in direct proportion to the lymphocyte population density, the value of k would be 1. However, in the five cases studied, the value of k ranged from 0.30 to 0.77. The values of k, and the maximum population densities for which they were applicable, are shown in Table III-2.

TABLE III-2

EFFECT OF LYMPHOCYTE NUMBER ON PHA-STIMULATED DNA SYNTHESIS

Subject	Sex	Diagnosis	'k' value	*Maximum density
M.D.	M	normal	0.73	1.20
A.H.	F	normal	0.33	**N.D.
R.P.	M	normal	0.77	N.D.
T.S.	F	normal	0.30	N.D.
D.M.	M	aspiration pneumonia	0.30	1.25

* cells/ml of culture x 10^5

** not determinable over the range of blood volumes studied.

(2) The relationship between lymphocyte counts and transformation in normal subjects: PHA-stimulated blood cell cultures from normal subjects were incubated in medium supple-

mented with autologous serum (46 subjects) or FCS (53 subjects). The correlation coefficients between lymphocyte population density and ^{3}H-thymidine incorporation were 0.09 and 0.03 respectively. Neither value was significant, i.e. there was no correlation between the initial lymphocyte number, and the transformation response to PHA. However, when ^{3}H-thymidine incorporation (cpm/culture) was plotted against ^{3}H-thymidine incorporation (cpm/10^{6} lymphocytes in the original culture) there was a linear relationship (see Figure III-3). The correlation coefficients were 0.851 for cultures containing autologous serum, and 0.790 for cultures containing F.C.S. Both values are significant at 0.1% level.

The equations for the regression lines ($y = ax+b$) were:

(a) Autologous serum.

$$y = 2.17\ x + 2.3$$

(b) F.C.S.

$$y = 1.96\ x + 6.9$$

where a = slope, n = intercept on the ordinate.

(3) Determination of the range of lymphocyte population density in control subjects and its effect on the expression of results: In the 53 control subjects the minimum number of lymphocytes per ml of culture was 0.69×10^{5}, and the maximum number was 1.3×10^{5}. Extrapolation from the two graphs in Figure III-2 shows that for this range of lymphocyte concentrations expressing results as cpm/10^{6} lymphocytes

FIGURE III-3

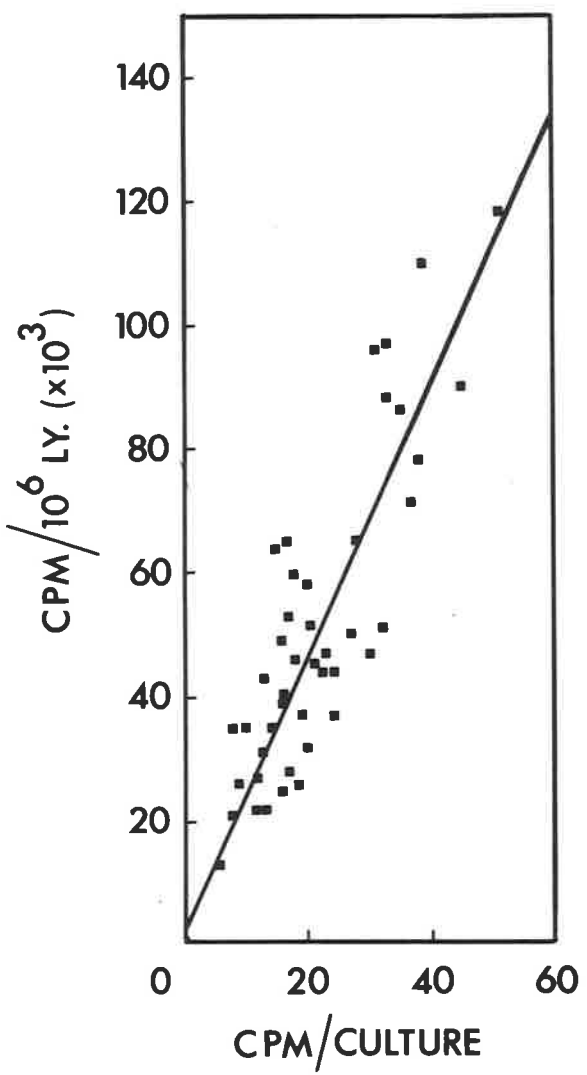
PHA - INDUCED DNA SYNTHESIS

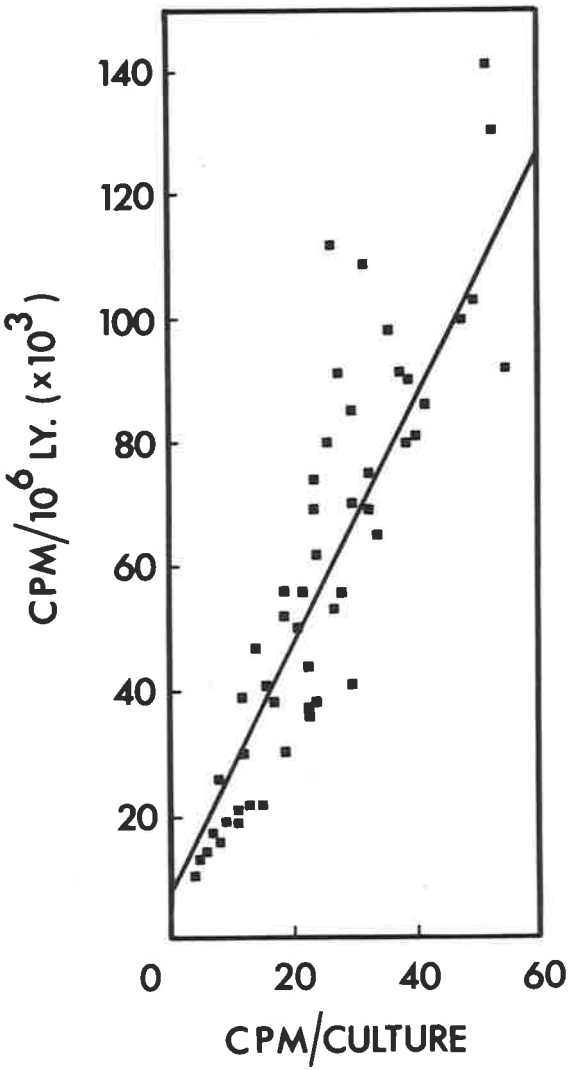
DNA synthesis (cpm/culture x 10^3) is plotted against

DNA synthesis (cpm/10^6 lymphocytes in the initial culture x 10^3)

Left hand graph : DNA synthesis in medium supplemented with autologous serum.

Right hand graph : DNA synthesis in medium supplemented with foetal calf serum.


140
120
100
80
60
40
20
0
20
40
60
CPM/10^6 LY. (×10^3)
CPM/CULTURE


140
120
100
80
60
40
20
0
20
40
60
CPM/10^6 LY. (×10^3)
CPM/CULTURE

would only increase cpm/culture by factor of 1.2 - 1.8 times, i.e. the results of lymphocyte transformation were not significantly affected by lymphocyte number because there was only a small range of lymphocyte counts in cultures of normal blood cells. For routine studies of lymphocyte transformation results were corrected to a counting efficiency of 100% and expressed as dpm/culture.

3. DETERMINATION OF THE VOLUME OF TRANSFORMED CELL NUCLEI - ANALYSIS OF THE METHOD

(a) Method

Blood cell cultures were stimulated with PHA as described in Chapter II. Controls contained no PHA. At 96 hours of incubation cell growth was arrested, and cultures processed to disperse leukocyte clumps and lyse erythrocytes, by a modification of the method of Stewart and Ingram (1967). The cytoplasm was stripped from the cells by vigorously shaking each culture (4ml) with filtered counting fluid (40ml, see Appendix (vi)). The nuclear volume distribution was obtained by pulse height analysis, using a 128- channel analyser (Nuclear Data) coupled to a Coulter counter, Model B (Coulter Electronics). The Coulter counter settings were determined as described in Appendix (vii), and readings from the multichannel analyser were standardized before each experiment using latex particles (diameter 5.02 microns, Appendix (vii)). Volume analysis was performed three times on each sample; results were printed when the number of

counts in the peak channel (modal channel) reached 10^3. The counts from each channel were averaged, and the final curve corrected to 10^3 counts in the modal channel. Representative curves for PHA-stimulated cultures and controls are shown in Figure III-4. The difference in area between PHA-stimulated and control cultures was obtained by weighing the area shaded on standard paper, and converting the results back to mm^2. The modal volume of the particles was calculated after standardizing the channel analyser with mushroom spores or latex particles of fixed diameter.

(b) Results

(1) Comparison between lymphocyte and blood cell suspensions: Nuclear volume analysis has previously been applied to lymphocyte-rich cell cultures (Stewart and Ingram, 1967, Muniz et al, 1970). The volume distribution curves obtained with freshly prepared lymphocyte and blood cell cultures were therefore compared. Lymphocyte suspensions of 98% purity were prepared by cotton wool filtration, as described in Chapter IV, Part A. The curves are shown in Figure III-5; values are plotted arithmetically. Clearly defined peaks of similar size distribution were obtained in each case. Debris from lysed erythrocytes did not affect the leukocyte curve.

(2) Time course study of changes in nuclear volume:

(a) Comparison of lymphocyte and blood cell cultures

Cultures of purified lymphocytes and blood cells were incubated with PHA. The results of nuclear volume analysis

FIGURE III-4

LYMPHOCYTE TRANSFORMATION - NUCLEAR VOLUME CHANGES

Solid line - PHA stimulated culture.

Dotted line - non-stimulated culture.

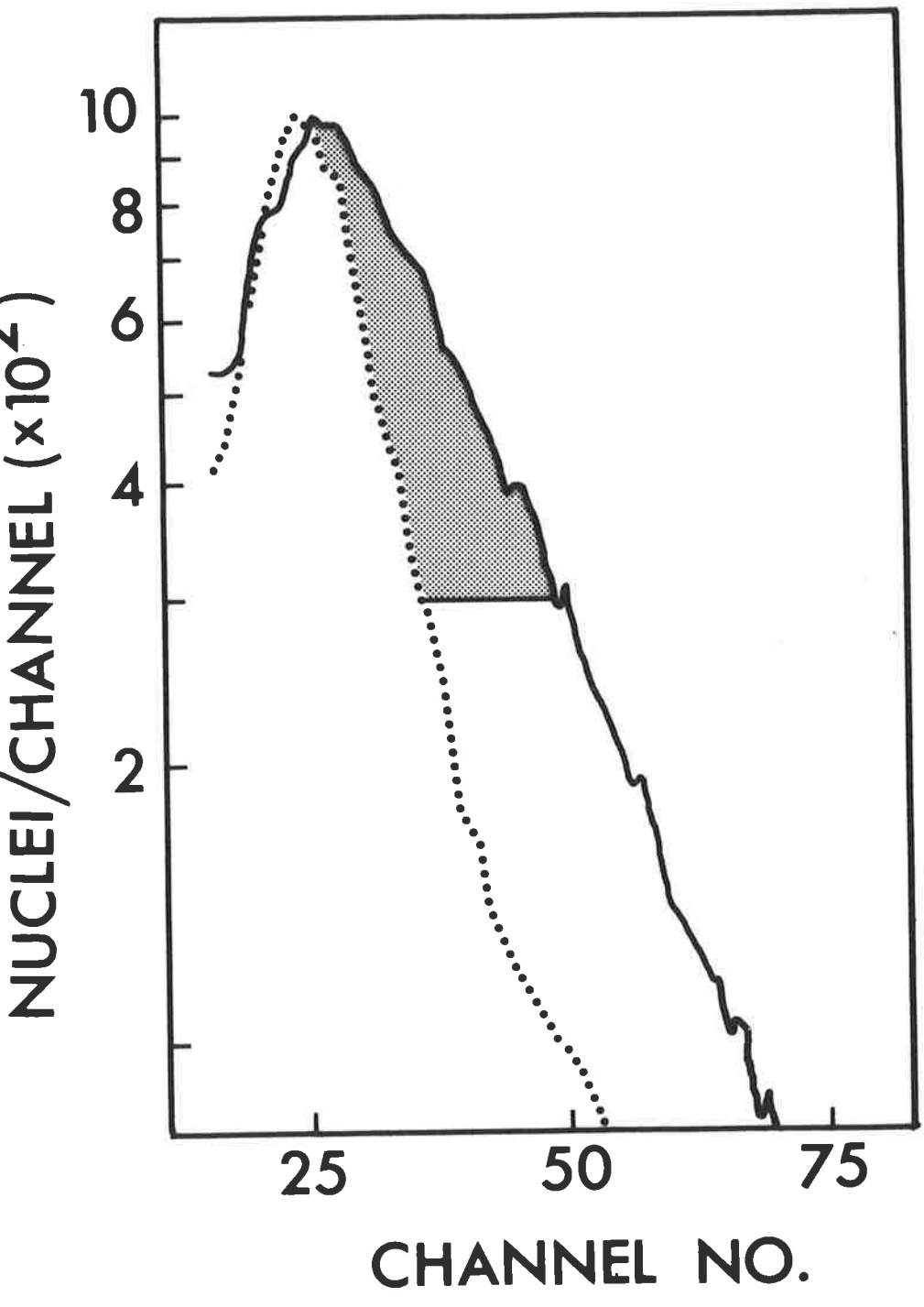

10
8
6
4
2
NUCLEI/CHANNEL (x10^2)
25
50
75
CHANNEL NO.

FIGURE III-5

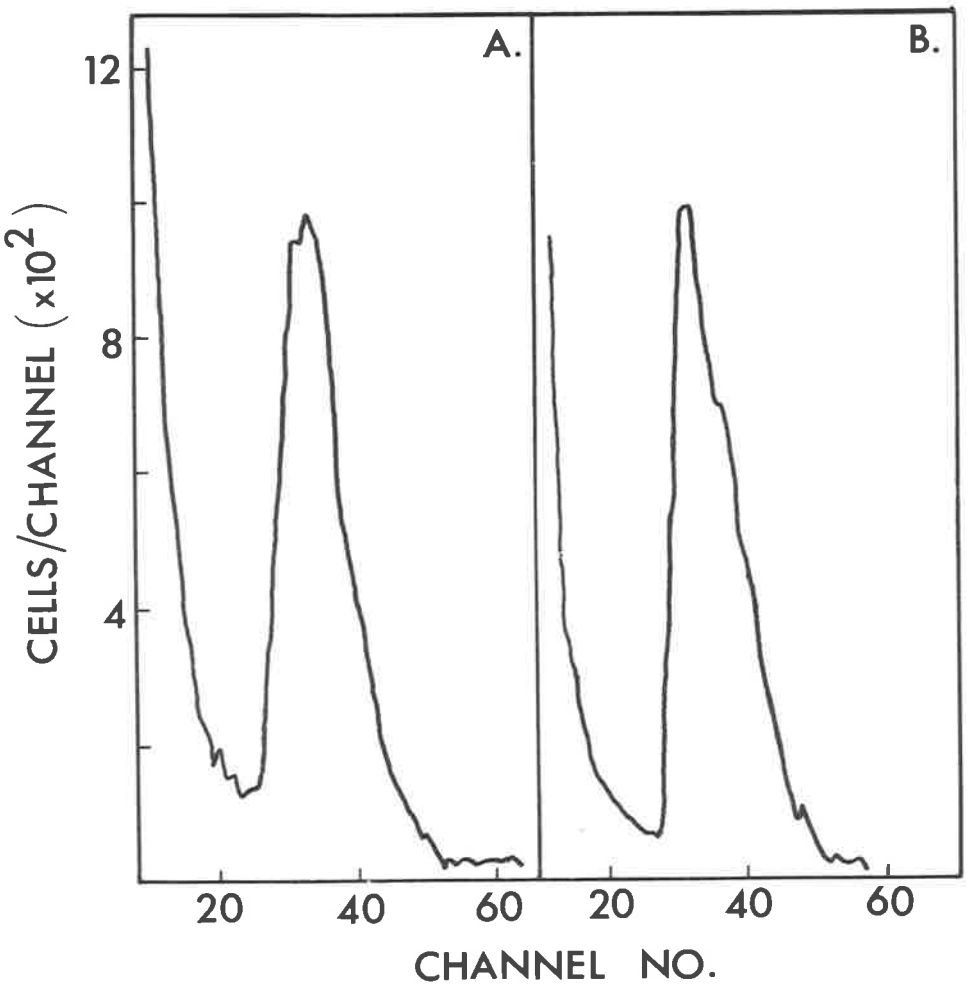
NUCLEAR VOLUME DISTRIBUTIONS

(1) Curve A shows the volume distribution of freshly drawn blood leukocytes.

(2) Curve B shows the volume distribution of lymphocytes from the same subject (separated on a cotton wool column).

Nuclei were analysed within one hour of preparation.

Particle volume per channel was 1.111 μ^3.


A.
B.
12
8
4
CELLS/CHANNEL (x10²)
20
40
60
20
40
60
CHANNEL NO.

on days 2, 3 and 4 of lymphocyte culture, and on days 3 and 4 of blood cell culture are shown in Figure III-6. The proportion of larger cells increased with time in culture, and occurred earlier in lymphocyte cultures. There was little difference between lymphocyte and blood cell cultures on day 4.

(b) Comparison of PHA-stimulated and non-stimulated cultures

Normal cells were processed over a seven day period in two experiments. The results obtained from the cells of one subject are shown in Figure III-7. The curve obtained in PHA-stimulated cultures in this subject on day four developed a shoulder on the downward slope, representing a relative increase in the volume of a partially distinct group of particles. This shoulder was not evident in the cell nuclei of cultures from 40 other subjects, measured by volume analysis on day 4 of culture, although curves did not always tail-off quickly in channels with nuclear counts of less than 500. This shoulder may have resulted from the presence of excessive debris. Examination of processed cells by phase contrast microscopy showed that it was not due to leukocyte clumping or to the presence of cytoplasm around cell nuclei.

The modal volume (μ^3) of cell nuclei from the two experiments is shown in Table III-3.

FIGURE III-6

COMPARISON BETWEEN LYMPHOCYTE AND BLOOD CELL CULTURES

Values on the ordinate ($x10^2$) are plotted logarithmically, and on the abscissa, arithmetically.

All cultures were stimulated with PHA in medium supplemented with FCS.

Left hand curve : Blood cell cultures (processed on days 3 and 4).

Right hand curve : Lymphocyte cultures (processed on days 2, 3 and 4).

Particle volume per channel was 1.111 μ^3.

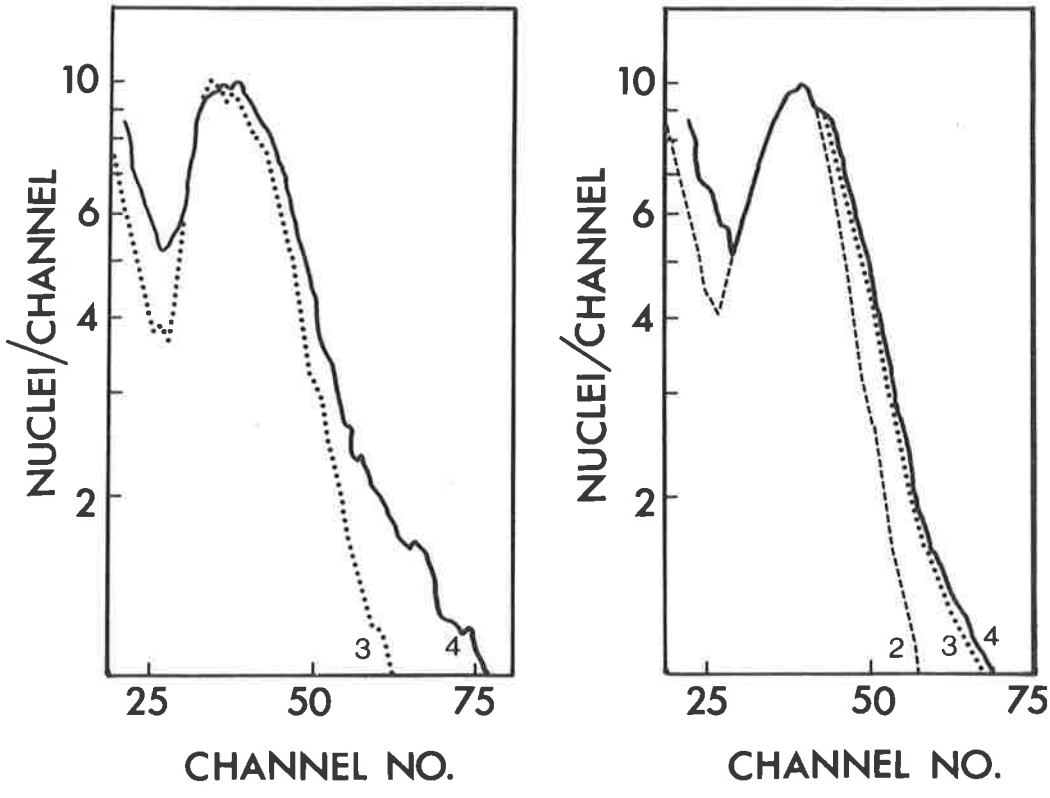

NUCLEI/CHANNEL
10
6
4
2
3
4
25
50
75
CHANNEL NO.
NUCLEI/CHANNEL
10
6
4
2
2
3
4
25
50
75
CHANNEL NO.

FIGURE III-7

TIME COURSE STUDY OF PHA - STIMULATED CHANGES IN NUCLEAR VOLUME

Ordinate: The particle count per channel is plotted logarithmically.

Abscissa: The modal channel is shown. Particle size per channel is 1.111 μ^3.

The dotted line describes the volume distribution curve of PHA-stimulated cultures, and the dashed line, of non-stimulated cultures.

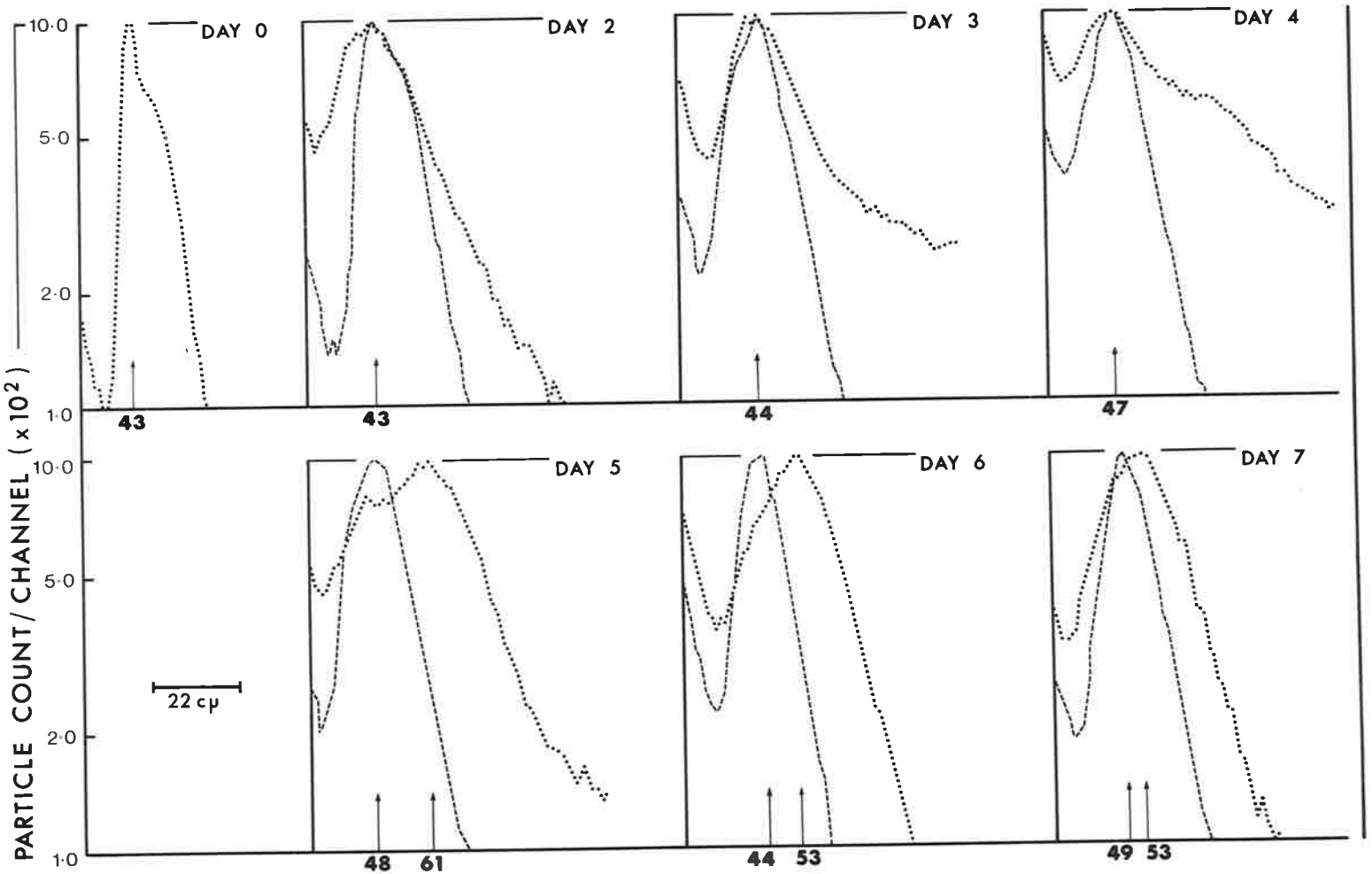

DAY 0
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
DAY 7
10·0
5·0
2·0
1·0
PARTICLE COUNT/CHANNEL (x10²)
22 cμ
43
43
44
47
48
61
44
53
49
53

TABLE III-3

MODAL VOLUME OF CULTURED CELLS

	Day	0	1	2	3	4	5	6	7
1	PHA	43		43	44	47	61	53	53
	Control	43		43	44	47	48	44	49
	*	0	-	0	0	0	13	9	4
2	PHA	41	40	41	41	42	-	46	48
	Control	41	40	-	41	42	-	46	47
	*	0	0	-	0	0	-	0	1

* = difference between PHA-stimulated and control cultures.

The difference in area under the stimulated and control curves (Δ area, see Figure III-4) was measured for cell counts above 500 per channel. This number was selected from data on 40 cultures as the point above which changes in distribution due to the presence of debris were unlikely to occur. The results from experiments 1 and 2 plotted in this way are shown in Figure IV-4. More useful data were obtained in experiment 2 using this criterion (curve A, Figure IV-4) than using the relative increase in the modal volume as an estimate of lymphocyte transformation. The difference in area between stimulated and control cultures was maximal on different days of culture in the two studies.

Further studies using nuclear volume as an estimate of transformation are described in Chapter IV, Part A.

4. HISTOLOGICAL ASSESSMENT OF LYMPHOCYTE TRANSFORMATION

After 96 hours of incubation, cultures were centrifuged at 300g for 15 minutes. The cell button was resuspended in FCS, smeared onto clean glass slides and stained with May-Grunwald-phosphate buffered Jenner-Geimsa stain. Transformation was assessed using the criteria of Yoffey et al (1965). Transformed cells had a large leptochromatic nucleus with one or more nucleoli, and abundant basophilic cytoplasm with or without a perinuclear halo or vacuolation. In large cells the membrane was often irregular, and apparently fragile. The percentage of transformed lymphocytes on the slide was determined by counting 200 intact mononuclear cells. The mean transformation response in 38 control cultures (supplement, FCS), was 67.1%, standard deviation 12.3. Cultures were repeated in two subjects at intervals of two months. The percentages of transformed cells were 67, 53, 66 and 67, 66 respectively.

5. RELATIONSHIP BETWEEN METHODS OF MEASUREMENT OF LYMPHOCYTE TRANSFORMATION

All parameters were normally distributed. Correlation coefficients were calculated for the following pairs and are shown in Table III-4:

(a) Histological transformation and nuclear volume analysis (H.T./N.V.A.)

(b) Histological transformation and ^{3}H-thymidine incorporation (H.T./TdR).

(c) Nuclear volume analysis and ^{3}H-thymidine incorporation (N.V.A./TdR).

TABLE III-4

MEASUREMENT OF TRANSFORMATION - TESTS OF CORRELATION

Test pair	Correlation Coefficient(r)	No.	Significance
H.T./N.V.A.	0.576	26	P < 0.01
H.T./TdR	10^{-5}	38	* N.S.
N.V.A./TdR	10^{-5}	49	N.S.

* Not significant

There was a positive correlation between the results of histological transformation and nuclear volume analysis. Neither parameter correlated with the incorporation of ^{3}H-thymidine into DNA.

6. DISCUSSION

Lymphocyte transformation is commonly measured by the incorporation of ^{3}H- or ^{14}C-thymidine into cell DNA. The radioactive label is found almost exclusively in the DNA (Schellekens and Eijsvoogel, 1968, Ling, 1968). The use of these measurements is based on the assumption that the amount of labelled thymidine incorporated into the DNA of transforming cells is proportional to the rate of DNA synthesis. This is most nearly achieved when the medium external to the cells is flooded with labelled thymidine (Sample and Chretien, 1971) in accordance with the four-factor model of thymidine metabolism (Cleaver, 1967). Such conditions are not

readily maintained if ^{3}H-thymidine is present in the culture for more than four hours (Sample and Chretien, 1971). The results of the current study indicated that flooding conditions were maintained over 4 hours of culture, with ^{3}H concentrations of 2.5 μCi/culture (1.25×10^{-6} molar). These amounts are not likely to cause intra-cellular radiation damage (Cleaver 1967), and the concentration of thymidine was not itself high enough to inhibit DNA synthesis (Sample and Chretien 1971). It has been claimed that short labelling times, e.g. 2 hours, may give meaningless results because of differences in the time of peak activity in different cell cultures, (Ling, 1968). This criticism is less applicable to studies in which the results from large groups of subjects are compared, than to results obtained in individual subjects. Long labelling periods are associated with thymidine breakdown (Ling, 1968). Enzymes present in neutrophil leukocytes also increase thymidine breakdown in culture (Marsh and Perry, 1964; see Discussion, Chapter IV, Part A.) Routine cultures were labelled with ^{3}H-thymidine for four hours in the present study. Measurement of the incorporation of ^{3}H-thymidine into DNA by liquid scintillation methods has been criticised on the grounds that total DNA synthesis is measured without reference to cell morphology (Coulson and Chalmers, 1966, Ling, 1968) or to cell numbers (Chalmers et al, 1967) in the final culture, e.g. depression of ^{3}H-thymidine incorporation may be due to cell death rather than a functional defect in DNA synthesis. Random samples were checked for morphological evidence of disintegration of cells in patient cul-

tures in this study.

Nuclear volume analysis was applied to studies of PHA-induced transformation in canine lymphocytes by Stewart and Ingram (1967); cell debris was digested with the enzyme pronase. This step was found to be unnecessary in human cell cultures in this study, and in the work of Muniz et al (1970). The results reported in the present study have confirmed that there is a significant increase in the nuclear volume of PHA-stimulated lymphocytes. The area under the volume distribution curve was measured by Stewart and Ingram with a planimeter. They compared the curve of stimulated cells with that of small lymphocytes (obtained at the onset of culture). The relative increase in nuclear volume was expressed as a percentage. In contrast to the results of the present study, Muniz et al, (1970) found that the modal volume of unstimulated lymphocytes remained constant; they therefore measured transformation in terms of modal volume. Transformation was most satisfactorily quantitated in the human blood cell cultures of this study by measuring the difference in a defined area under curves of stimulated and control cultures.

Histological estimates of transformation have been expressed as the relative number of transformed cells in three or four day cultures. This percentage is dependent on the original number of lymphocytes in the culture, death and disintegration of non-activated cells, lymphocyte mitoses, and the death of transformed cells or daughter cells (Wilson and Thompson, 1968), factors which also apply to radiochemical estimations. Coulson and Chalmers (1966) emphasized

that the determination of cell counts in a haemocytometer (assuming cell clumps have been dispersed) and the identification of transformed cells, are sources of error in the histological method. The latter can be overcome by the use of autoradiography. Yoffey et al (1965) obtained a good correlation between the percentage of blast cells (which were divided into three types) in three-day cultures, and the percentage of cells in autoradiographs, which incorporated ^{3}H-thymidine. In the present study 67.1% (standard deviation 12.3) of cells in 96 hour blood cultures were transformed, compared with the 32.1% (standard deviation 4.7) obtained by Yoffey in 72 hour lymphocyte-rich cultures. The percentage of transformed cells in blood cultures may have been high because of the inclusion of some cells classified by Yoffey as "transitional lymphocytes." Serial studies in 2 subjects showed that the results were reproducible.

Despite known sources of error in the histological technique of counting transformed cells, there was a good correlation between this parameter and the results of nuclear volume analysis, i.e. the percentage of transformed cells as assessed in this study was an accurate reflection of the extent of lymphocyte transformation. It is often assumed that the morphological assessment of transformation measures the same function as the incorporation of ^{3}H-thymidine into extracts or precipitates of DNA. Loeb et al (1970) found that the maximum percentage of lymphoblasts occurred on the same day of culture as maximal DNA synthesis. In the present study there was no correlation between histological estimates of transformation and

the measurement of DNA synthesis, or nuclear volume analysis and DNA synthesis. This lack of correlation is probably not due to errors in technique. It is known that ^{3}H-thymidine incorporation can be inhibited by 5-fluorodeoxyuridine (Salzman et al, 1966) and by low concentrations of actinomycin (Loeb et al, 1970) without inhibition of blastogenesis, suggesting that the two methods measure different parameters.

Although histological measurement of transformation was satisfactorily applied to blood cell cultures, the technique is subjective and only small numbers of cells were assessed. Nuclear volume analysis and the incorporation of ^{3}H-thymidine into acid-precipitable DNA are objective methods of assessment. Tritiated thymidine studies showed that maximal DNA synthesis occurred uniformly on the fourth day of blood cell culture (see Chapter IV) whereas the relative increase in nuclear volume was maximal on different days of culture in different subjects. It was therefore concluded that the most practicable method for the routine measurement of lymphocyte transformation in blood cell cultures was the incorporation of ^{3}H-thymidine into acid-precipitable DNA.

7. SUMMARY

(a) The concentration of radioactivity used to measure DNA synthesis in cell culture exhibited saturation kinetics. Flooding conditions were obtained with concentrations of 2.5 μCi/culture (when the specific activity was kept constant at 500 mCi/mMol).

(b) The incorporation of ^{3}H-thymidine by PHA-stimulated cells was dependent on the lymphocyte count in the individual subject. The slope of the regression line was variable and always less than unity.

(c) The range of lymphocyte counts in normal blood cultures was small, and had no significant effect on the distribution of results of ^{3}H-thymidine incorporation.

(d) Nuclear volume analysis was readily adapted to measure lymphocyte transformation in blood cell cultures. In two normal subjects the maximum volume of transformed cell-nuclei was reached on different days of culture. Results were most usefully expressed as the difference in a defined area under the curves of stimulated and control cultures.

(e) Histologically, lymphocyte blastogenesis was expressed as the percentage of transformed cells. In some slides there was considerable variation in duplicate counts of 100 cells.

(f) Histological determination of transformation correlated with nuclear volume analysis when measured at 96 hours of culture. Neither parameter correlated with the incorporation of ^{3}H-thymidine into DNA.

CHAPTER IV

PART A

PHYTOHAEMAGGLUTININ-INDUCED LYMPHOCYTE TRANSFORMATION IN BLOOD CELL CULTURES. ESTABLISHMENT OF OPTIMAL CONDITIONS FOR ROUTINE MEASUREMENT

1. INTRODUCTION

The mitogenic properties of PHA were first described in 1959 (Hungerford et al). It has subsequently been established that the peripheral blood cell which is responsive to PHA is the lymphocyte (Ling, 1968), and the extent of this response has been developed as a test of immunological function. Lymphocyte transformation has usually been measured in cultures of blood leukocytes or lymphocytes. Recently, good results have been obtained using unseparated blood components (Junge et al, 1970, Pauly and Sokal, 1972). In this study, the determination of optimal conditions for the use of blood cell cultures in man has been based on the method of Junge et al (1970).

2. METHODS

(a) Technique of Culture

Standard blood cell cultures were established as described in Chapter II. The effect of varying the components of the cultures was studied. In all experiments lymphocyte transformation was measured by the incorporation of ^{3}H-thymidine into DNA. Results were expressed as counts per minute per culture (cpm/culture). The counting efficiency of the spectrometer was 34%. Nuclear volume analysis of cultured cells was performed in some experiments.

(b) Determination of Cell Viability in Culture

(1) Dye exclusion: The ability of intact cells to exclude trypan blue dye was measured by the method of Ling (1968). Cultures were centrifuged at 300g for 10 minutes. The cell button was resuspended in FCS (0.5 ml). Cell suspension (8 drops) was incubated with filtered trypan blue dye (2 drops) for three minutes at 37°C. The percentage of non-stained (viable) cells was determined by light microscopy.

(2) Cell counts: Cultures were processed to disperse cell clumps by removing the cytoplasm from cultured cells (see Chapter III). Cell nuclei were counted in a Coulter counter, Model B, orifice size 50 microns, current setting 1, amplification 0.5, threshold 20-90.

3. RESULTS

(a) Determination of the Time of Maximal Transformation

The culture medium used in this experiment was buffered with sodium bicarbonate at pH 7.3. Cultures were incubated in an atmosphere of 5% carbon dioxide in air.

The results obtained in one of the two normal subjects studied are shown in Figures IV-1 and IV-2. The incorporation of ^{3}H-thymidine was maximal on day 4 in PHA-stimulated cultures and was unchanged in non-stimulated control cultures throughout the culture period. The volume and number of transformed cell nuclei were maximal on day 5. Cell counts in control tubes fell continuously throughout the culture period. There was an

FIGURE IV-1

TIME COURSE STUDY OF LYMPHOCYTE TRANSFORMATION IN BLOOD CELL CULTURES

1. DNA synthesis was measured in triplicate cultures containing heparinized blood (0.2 ml), autologous serum (0.4 ml) and bicarbonate-buffered medium 199 (3.38 ml), maintained in an atmosphere of 5% carbon dioxide in air. Stimulated cultures contained reconstituted PHA (0.02 ml, ■), controls (□) did not. ^{3}H-thymidine (1.0 μCi, spec. activity 500 mCi/mMol) was added at 92 hours.
2. Nuclear volume was measured in PHA-stimulated and non-stimulated cultures. The results were expressed as the difference in area under the two curves.

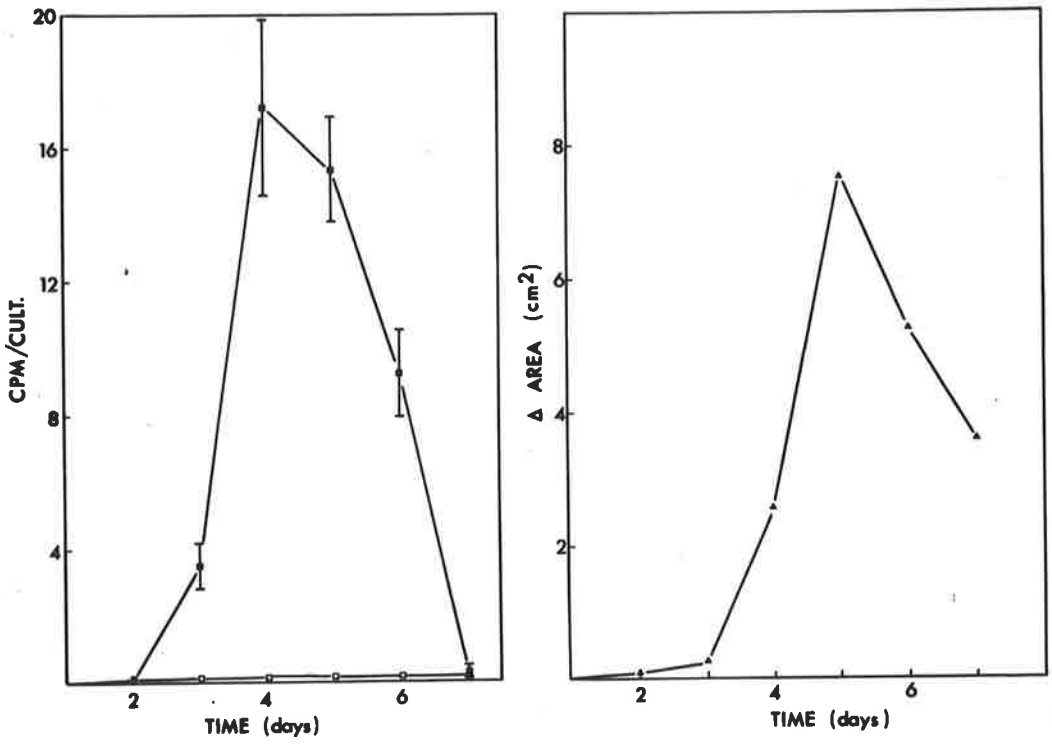
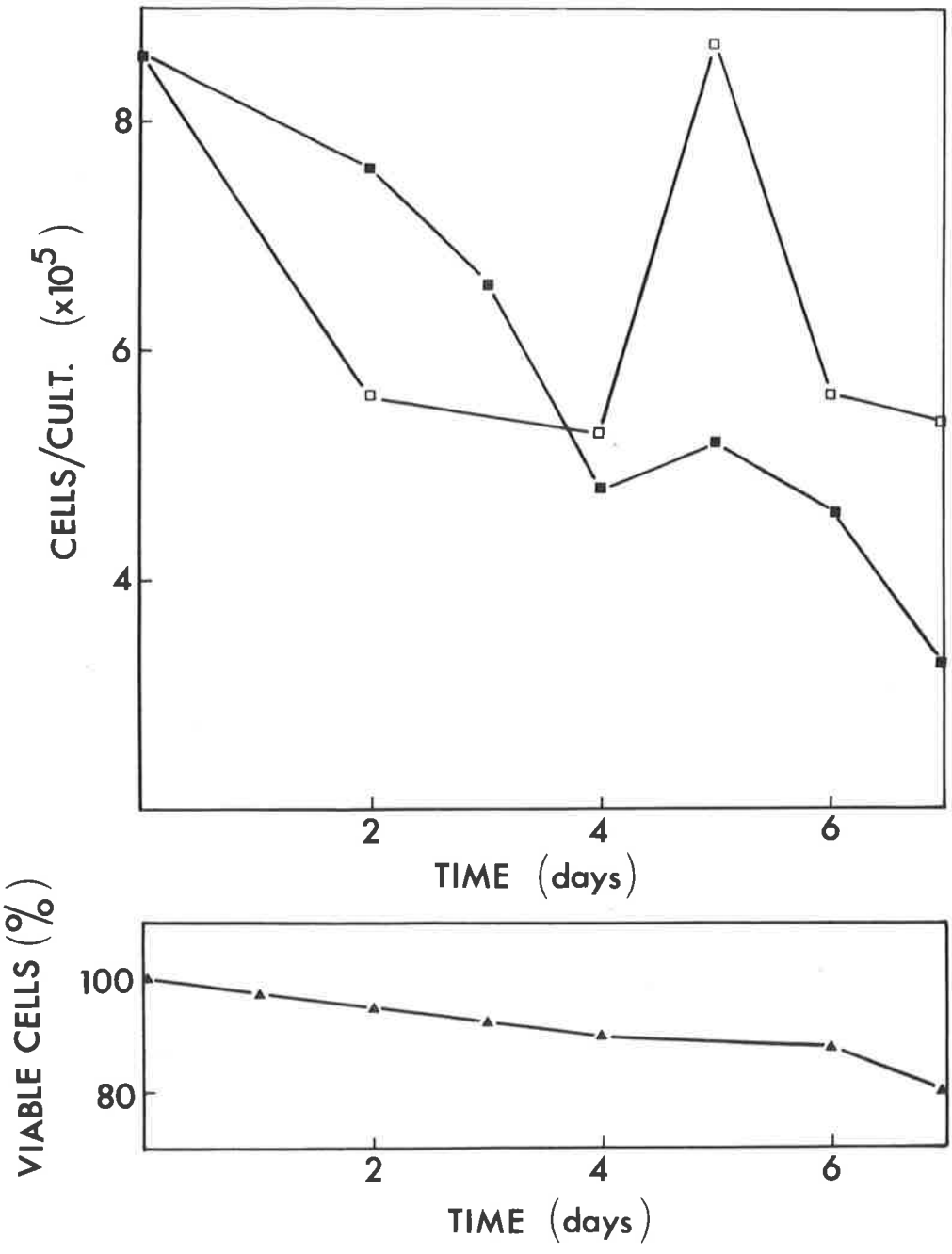

20
16
12
8
4
CPM/CULT.
2
4
6
TIME (days)
8
6
4
2
Δ AREA (cm²)
2
4
6
TIME (days)

FIGURE IV-2

TIME COURSE STUDY OF CELL COUNTS AND VIABILITY IN CULTURE

1. The upper graph shows the number of cell nuclei in PHA-stimulated (□) and non-stimulated (■) cultures, plotted against the duration of the culture.
2. The percentage of intact cells in PHA-stimulated cultures (▲) which excluded trypan blue dye, is plotted against the duration of the culture.


CELLS/CULT. (x10^5)
8
6
4
2
4
6
TIME (days)
VIABLE CELLS (%)
100
80
2
4
6
TIME (days)

initial fall in counts in PHA-stimulated cultures, followed by an increase on day 5, at which time 89% of nucleated cells excluded trypan blue dye.

(b) The Use of HEPES-Buffered Medium 199 in Cell Culture

The effect of pH on lymphocyte transformation in HEPES-buffered medium 199 was studied in two experiments. Representative results of ^{3}H-thymidine incorporation and nuclear volume analysis are shown in Figure IV-3. In the presence of HEPES buffer, pH is temperature dependent (Darzynkiewicz and Jacobson, 1971); the results shown are for the pH at 37°C.

Further studies were carried out at pH 7.5.

(c) Comparison of Lymphocyte Transformation in Medium 199 Buffered with Bicarbonate (pH 7.3) and HEPES (pH 7.5)

Cultures buffered with bicarbonate (HCO_3) were incubated in an atmosphere of 5% carbon dioxide. Five normal subjects were studied. The standard deviations obtained in triplicate cultures were expressed as a percentage of the mean (% error). The results are shown in Table IV-1 (cpm/culture x 10^3). (p.72).

DNA synthesis was increased by an average of 20% in HEPES-buffered medium. The percentage error was not affected by the buffer used.

In subsequent experiments HEPES-buffered medium 199 was used.

FIGURE IV-3

THE EFFECT OF pH ON LYMPHOCYTE TRANSFORMATION IN HEPES-BUFFERED MEDIUM 199

1. Each point shows the mean of triplicate determinations of DNA synthesis.

   ■ PHA-stimulated cultures

   ● Non-stimulated (control) cultures

2. Each point (■) shows the difference in area under the curves of PHA-stimulated and control cultures.

   ▲ Final cell counts (nuclei) per culture.

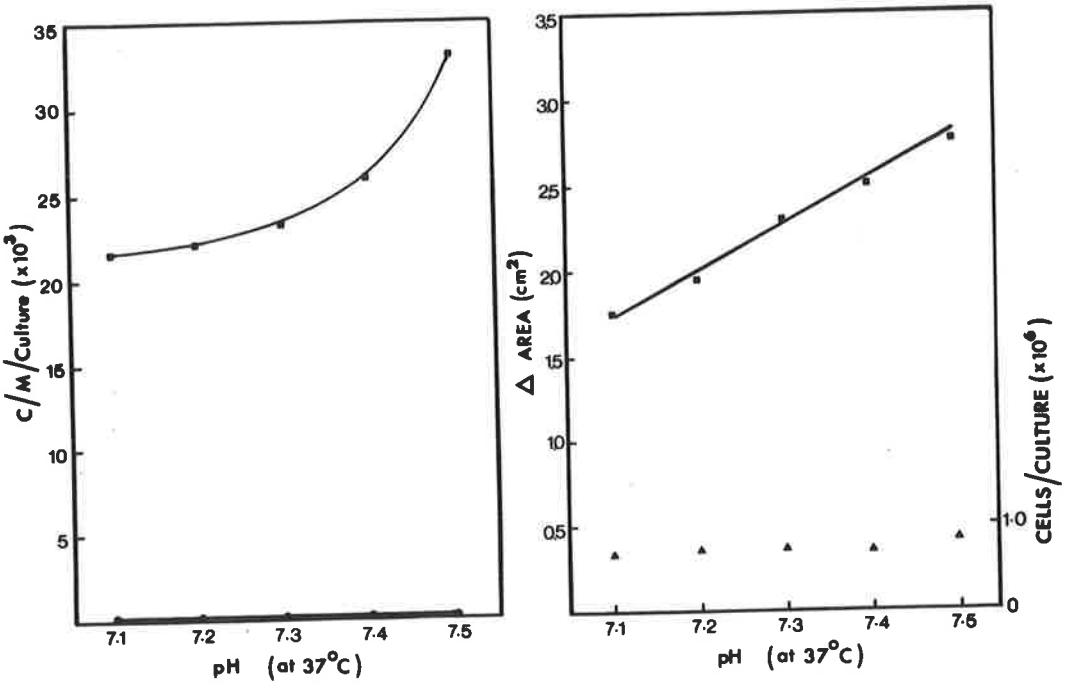

C/M/Culture (x10³)
35
30
25
20
15
10
5
7·1
7·2
7·3
7·4
7·5
pH (at 37°C)
Δ AREA (cm²)
35
30
25
20
15
10
0·5
1·0
0
CELLS/CULTURE (x10⁶)
7·1
7·2
7·3
7·4
7·5
pH (at 37°C)

TABLE IV-1

COMPARISON OF BICARBONATE AND HEPES-BUFFERED MEDIUM

Subject	HCO_3	% Error	HEPES	% Error	$\frac{HEPES}{HCO_3}$
G.S.	17.4	10.4	22.4	3.6	1.3
G.M.	14.2	1.6	15.7	4.4	1.1
J.N.	11.6	4.8	14.8	10.6	1.3
B.B.	16.0	5.6	19.5	1.4	1.2
B.C.	20.2	5.6	20.8	6.7	1.0
Mean	15.9	5.6	18.6	5.3	1.2

(d) Determination of the Time of Maximal Lymphocyte Transformation in HEPES-Buffered Medium 199.

The kinetics of transformation were studied in cultures supplemented with autologous serum (10%, 2 experiments) and FCS (10%, 1 experiment).

The results are shown in Figures IV-4 and IV-5. Nuclear volume data and cell counts were obtained only in cultures supplemented with autologous serum. The percentages of cells which excluded trypan blue dye are shown in Table IV-2.

TABLE IV-2

CELL VIABILITY-DYE EXCLUSION

Day	1	2	3	4	5	6	7
*A	97	95	93	-	-	89	88
*B	98	94	90	87	86	-	-

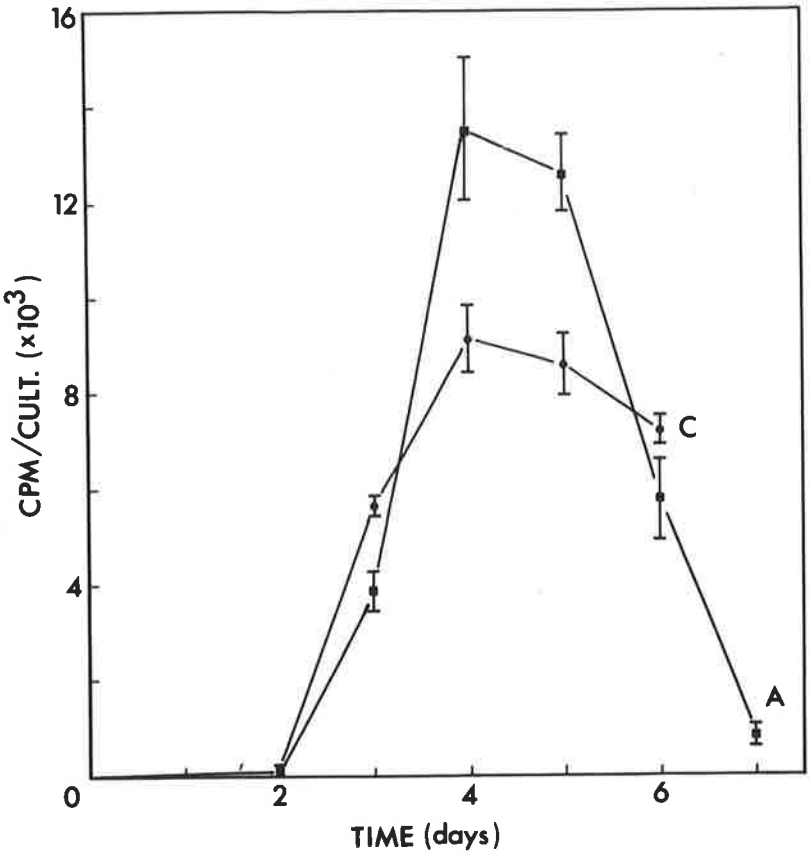
*Subjects A and B

FIGURE IV-4

TIME COURSE STUDY OF LYMPHOCYTE TRANSFORMATION IN HEPES-BUFFERED MEDIUM 199

1. Upper graph: DNA synthesis was measured in triplicate PHA-stimulated cultures in the presence of autologous serum (10% v/v, A) and FCS (10% v/v, C).
2. Lower graph: The relative increase in nuclear volume was measured in PHA-stimulated cultures containing autologous serum (10% v/v, A and B).

The curve of DNA synthesis obtained with cells (supplemented with autologous serum) from subject B was similar in shape to that obtained from subject A.


16
12
8
4
0
CPM/CULT. (x10³)
2
4
6
TIME (days)
C
A

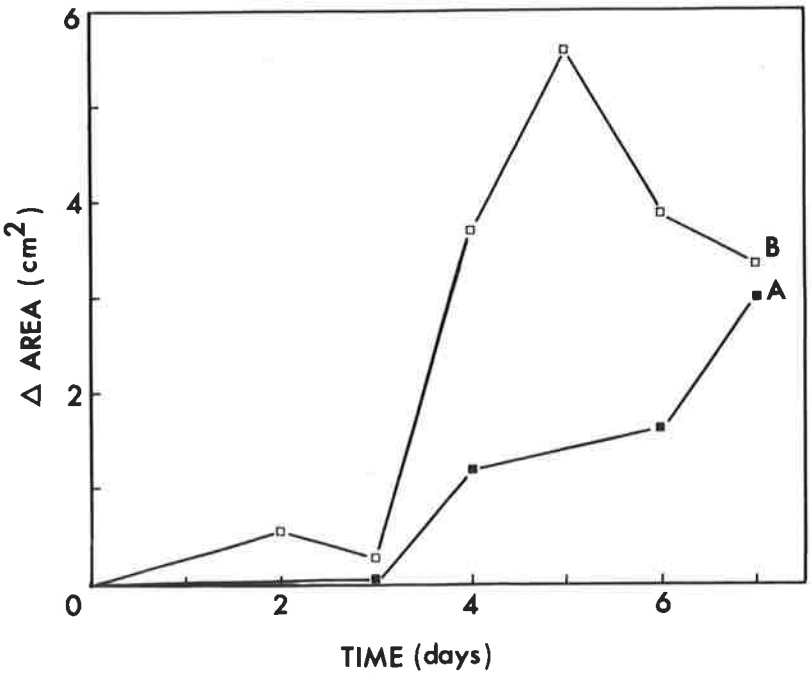

6
4
2
0
Δ AREA (cm²)
2
4
6
TIME (days)
B
A

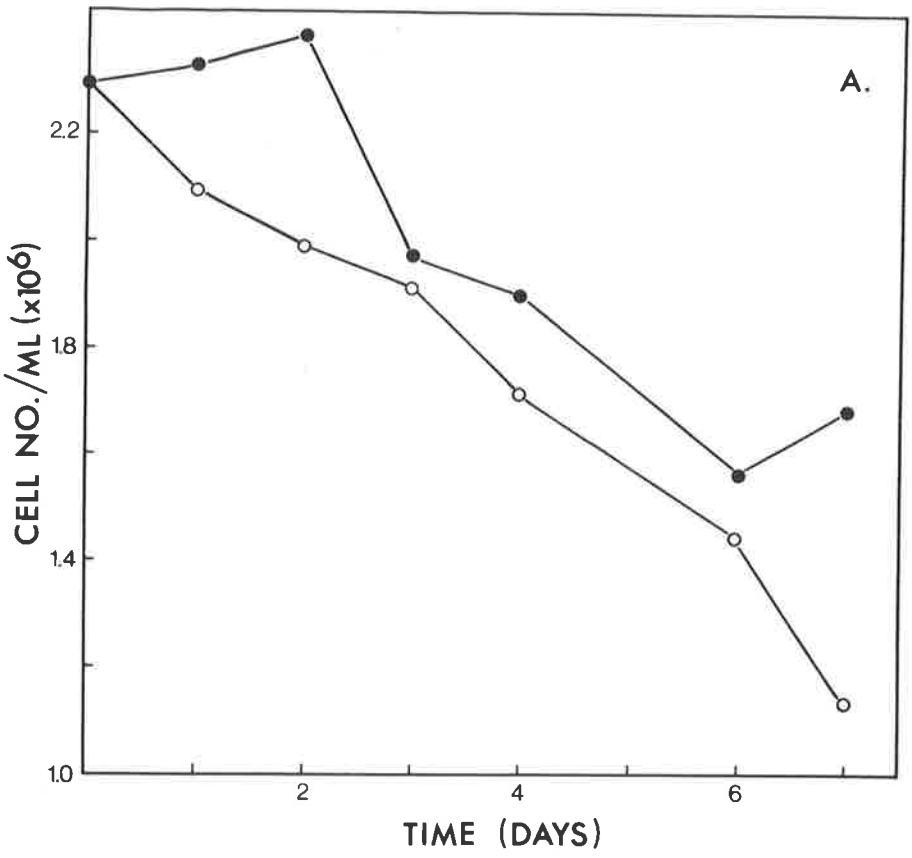
FIGURE IV-5

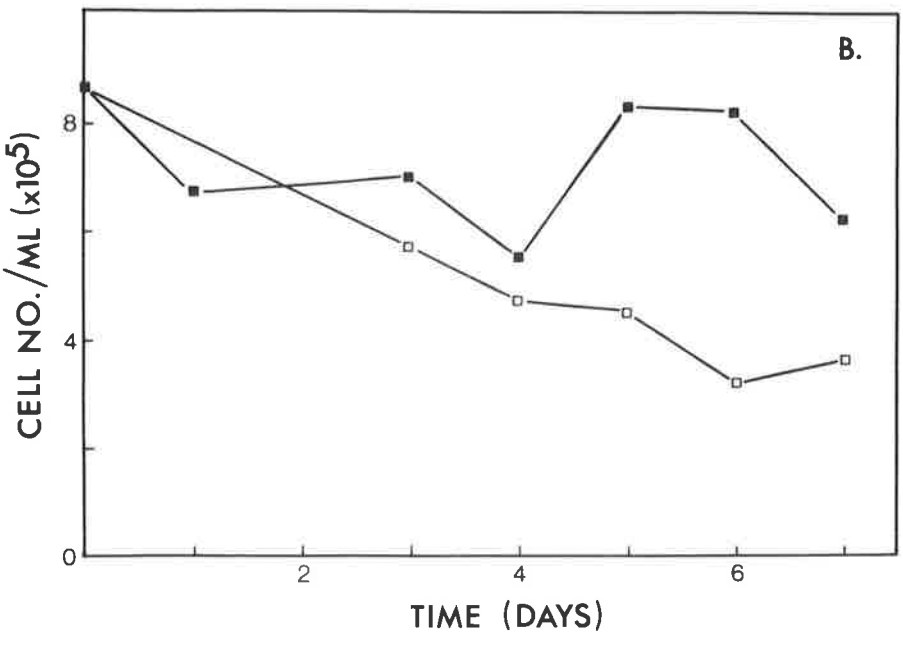
TIME COURSE STUDY OF CELL COUNTS IN BLOOD CELL CULTURES

Determinations were made on single cultures incubated with and without PHA, in subjects A and B.

● ■ PHA-stimulated cultures

○ □ Non-stimulated cultures


A.
2.2
1.8
1.4
1.0
CELL NO./ML (x10⁶)
2
4
6
TIME (DAYS)


B.
8
4
0
CELL NO./ML (x10⁵)
2
4
6
TIME (DAYS)

The incorporation of ^{3}H-thymidine was maximal on the fourth day of culture in each experiment. Nuclear volume was maximal on day 5 in one subject; the greatest difference in cell counts between stimulated and control cultures occurred on day 6 in this subject. In the second experiment these maxima were not determinable during the seven-day culture period.

(e) Comparison of the Effect of Autologous Serum and Autologous Plasma on Lymphocyte Transformation.

Three experiments were performed, using two batches of PHA. ^{3}H-thymidine (1.0 µCi/culture, specific activity 500 mCi/mMol) was added at 92 hours in experiments 1 and 2; ^{3}H-thymidine (2.5 µCi/culture) was added at 92 hours in experiment 3. The results (cpm/culture) are shown in Table IV-3.

TABLE IV-3

COMPARISON BETWEEN AUTOLOGOUS SERUM AND PLASMA

	Serum (10%)	Plasma (10%)	PHA-batch
1.	835±128	643±115	1530
2.	1315±430	1411±340	1530
3.	52690±97	49320±152	3716

There was no significant difference between the results obtained in medium supplemented with autologous serum, and medium supplemented with autologous plasma.

(f) The Effect of Serum Concentration on Lymphocyte Transformation

(1) Autologous serum: The results obtained from two normal donors are shown in Figure IV-6. There was a linear increase in DNA synthesis in the presence of serum concentrations of 5-30%. The slope of each line was greater than unity.

(2) Foetal calf serum: The results shown in Figure IV-7 were obtained in three experiments. The concentration at which DNA synthesis was maximal varied with different batches of FCS. Figure IV-7 also demonstrates that at a fixed concentration of FCS (10% v/v) DNA synthesis varied with the batch used. Batches A and B were used for routine estimations of lymphocyte transformation.

(g) The Effect of Whole Blood Concentration on Lymphocyte Transformation

Cultures were established in medium supplemented with 10% FCS. In three experiments, heparinized blood was washed three times in medium 199 to remove autologous plasma; in three experiments unwashed cells were used.

An increase in the volume of washed or unwashed cells was accompanied by a proportionate increase in DNA synthesis (see Figure IV-8), and in counts of cell nuclei (Figure IV-9). Nuclear volume increased with blood volumes in excess of 0.2 ml/culture (Figure IV-9).

(h) The Effect of PHA Concentration on Lymphocyte Transformation

Figure IV-10 shows the dose-response curve obtained in normal cell cultures using PHA batch 3716. This batch was subsequently

FIGURE IV-6

THE EFFECT OF THE CONCENTRATION OF AUTOLOGOUS SERUM ON LYMPHOCYTE TRANSFORMATION

The results are plotted geometrically.

Each point is the mean of triplicate determinations.

The variation at each point (% error) was less than 10%.

The concentration of autologous serum (% v/v) was calculated without reference to the volume of autologous plasma added in the heparinized blood.

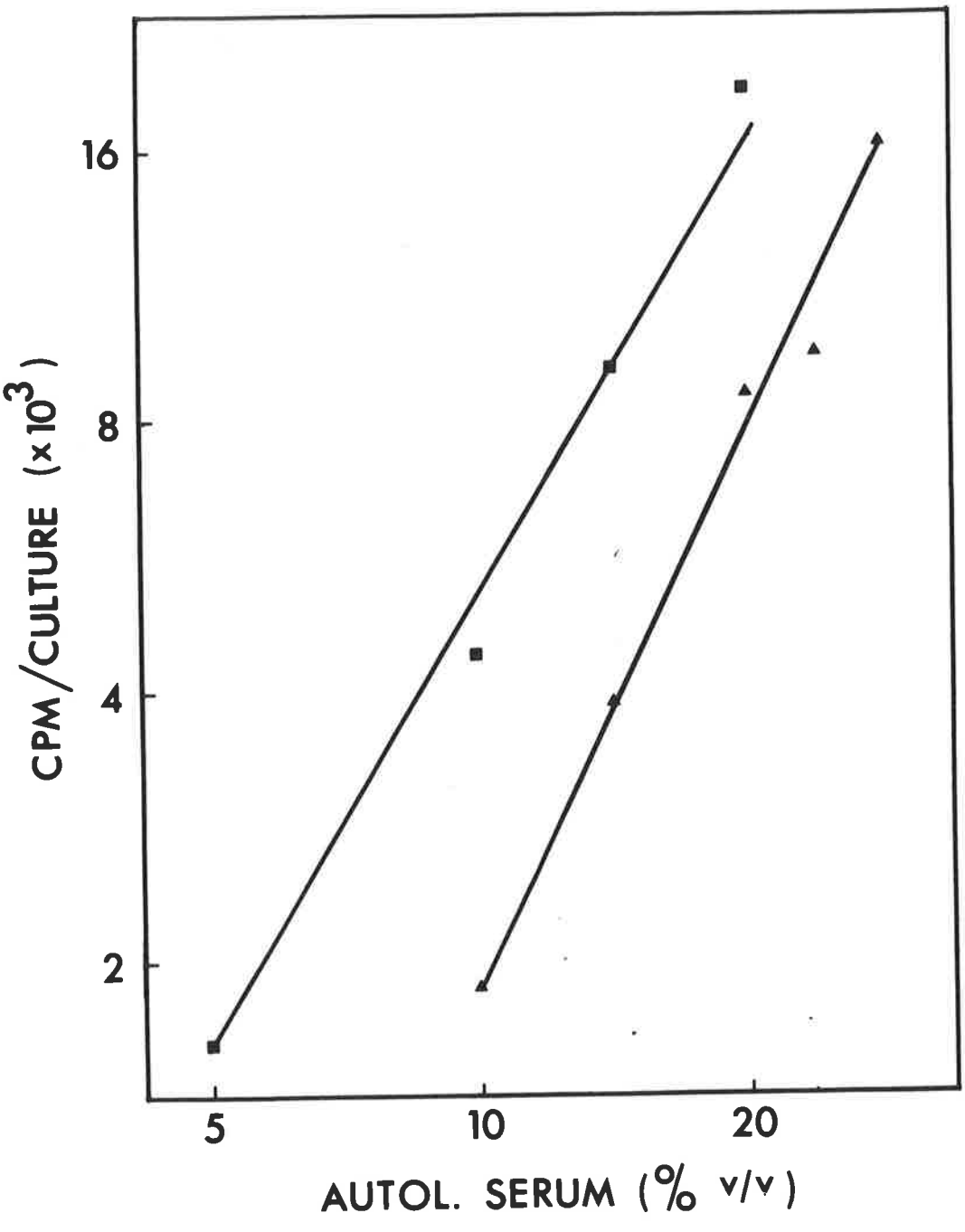

16
8
4
2
CPM/CULTURE (x10^3)
5
10
20
AUTOL. SERUM (% v/v)

FIGURE IV-7

THE EFFECT OF THE CONCENTRATION OF FCS ON LYMPHOCYTE TRANSFORMATION

1. DNA synthesis was compared in PHA-stimulated cultures supplemented with three batches of FCS. Cells from one healthy donor were incubated with FCS batch A, from 2 healthy donors, with FCS batch B, and from 4 healthy donors, with FCS batch C. The mean values are shown for batches B and C.
2. Two batches of FCS (B and D) were used to supplement cultures from a single blood donor. The PHA concentration is expressed as the volume (µl) of reconstituted PHA per ml of culture.

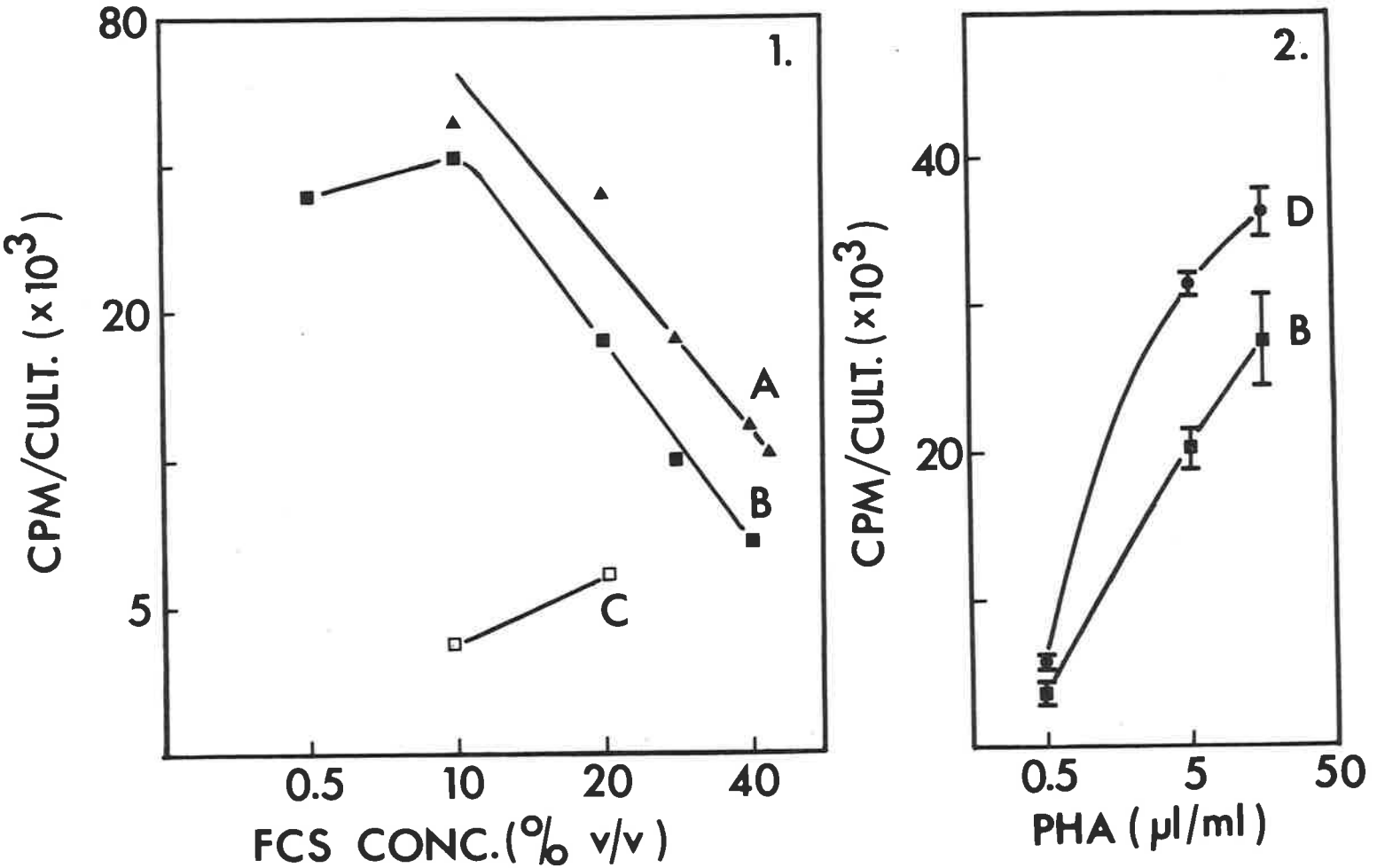

1.
80
20
5
CPM/CULT. (x10³)
A
B
C
0.5
10
20
40
FCS CONC.(% v/v)
2.
40
20
CPM/CULT. (x10³)
D
B
0.5
5
50
PHA (µl/ml)

FIGURE IV-8

THE EFFECT OF BLOOD CELL CONCENTRATION ON LYMPHOCYTE TRANSFORMATION

The results are plotted geometrically, and are derived from 2 representative experiments. The variation in triplicate determinations at each blood concentration was less than 10%.

Abscissa: Values represent the volume of cells (equivalent to the original volume of blood) added to each culture.

Ordinate: DNA synthesis (cpm/culture x 10^3).

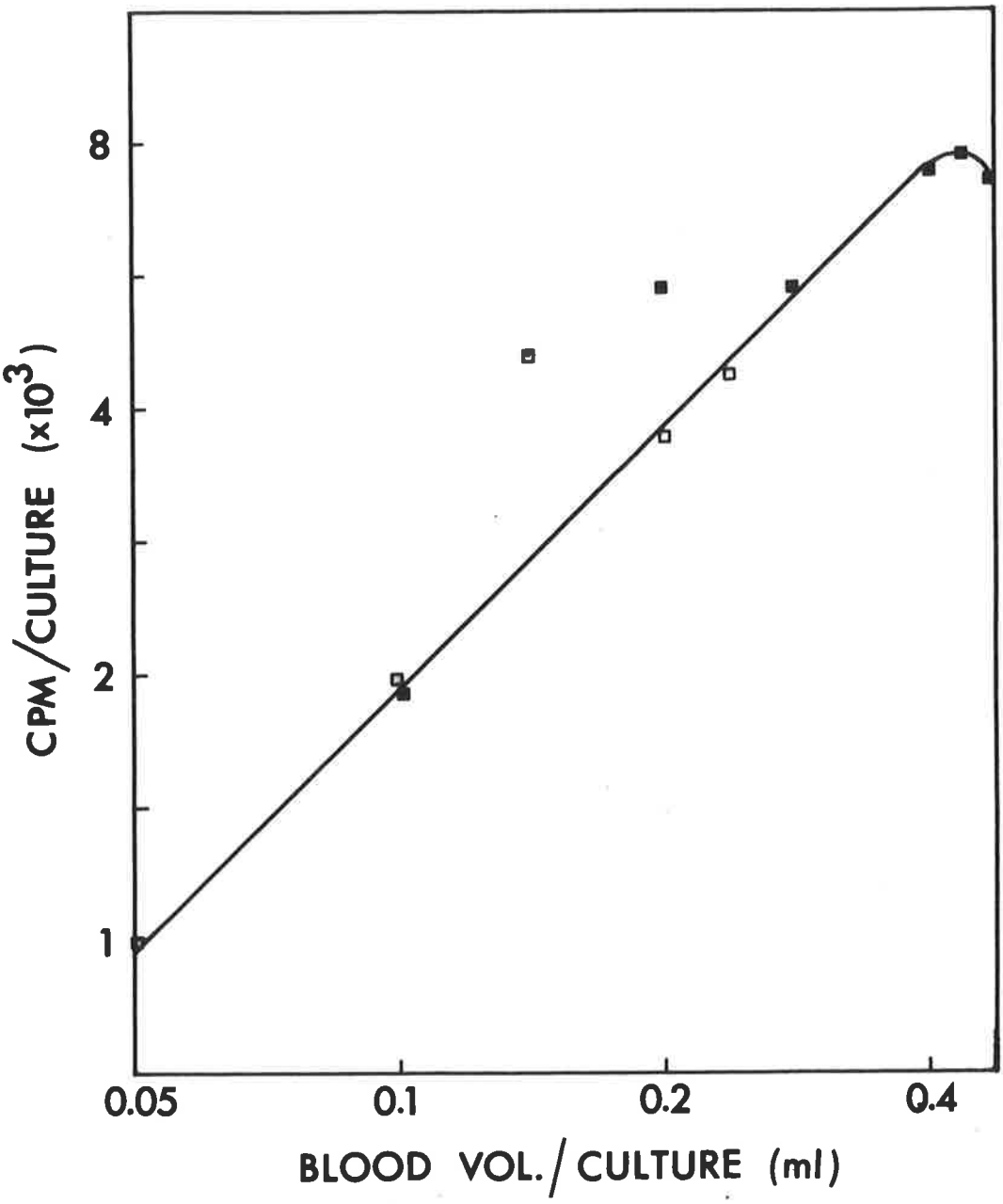

8
4
2
1
CPM/CULTURE (x10^3)
0.05
0.1
0.2
0.4
BLOOD VOL./CULTURE (ml)

FIGURE IV-9

THE EFFECT OF BLOOD CELL CONCENTRATION ON NUCLEAR VOLUME OF TRANSFORMED CELLS AND ON CELL COUNTS

The upper curve (left-hand ordinate) shows the difference in area under the curves of PHA-stimulated and control cultures (■ , washed cells).

The lower curve (right-hand ordinate) shows the relationship between the final cell count in the cultures and the initial blood volume (▲ , washed cells).

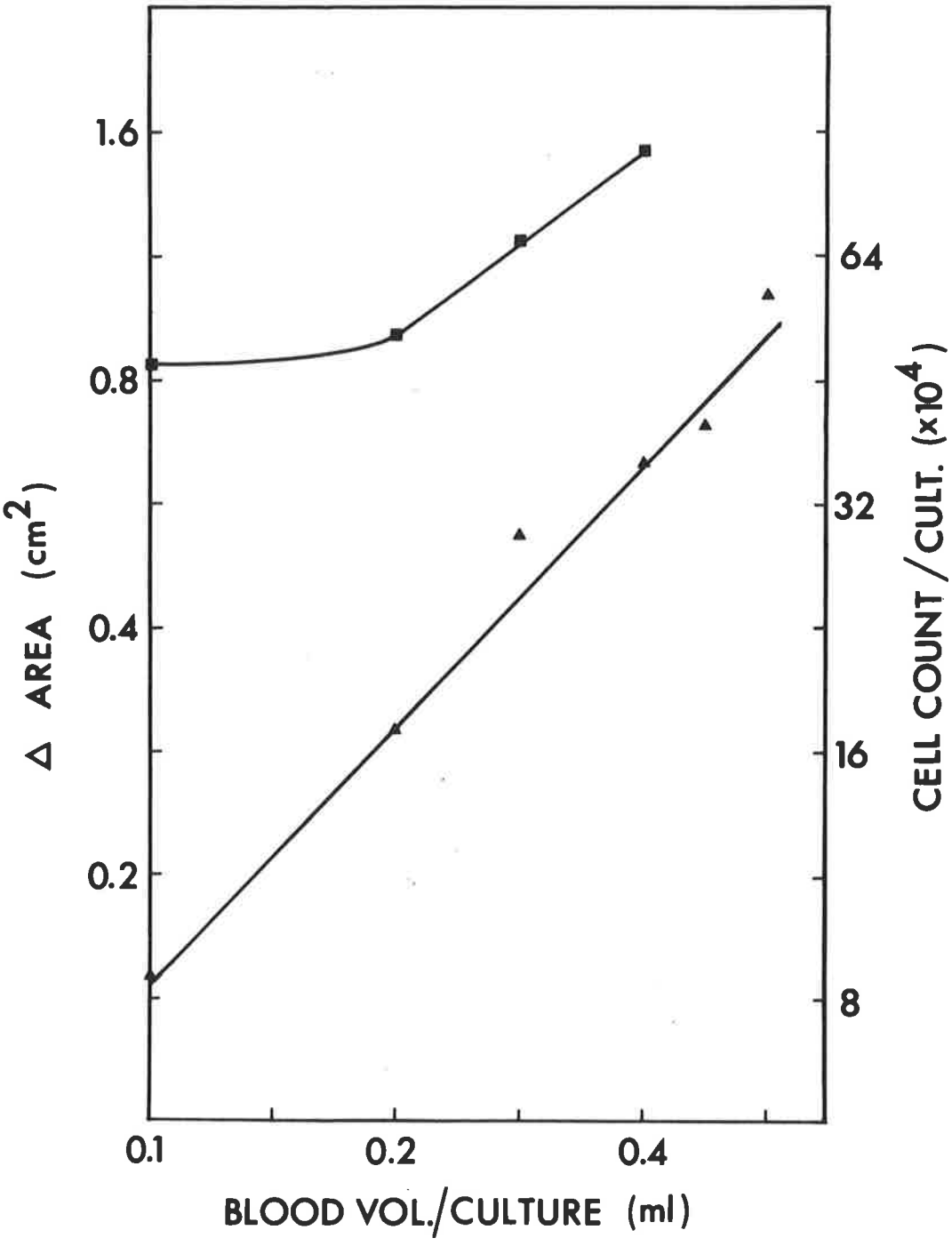

1.6
0.8
0.4
0.2
Δ AREA (cm²)
64
32
16
8
CELL COUNT / CULT. (x10⁴)
0.1
0.2
0.4
BLOOD VOL./CULTURE (ml)

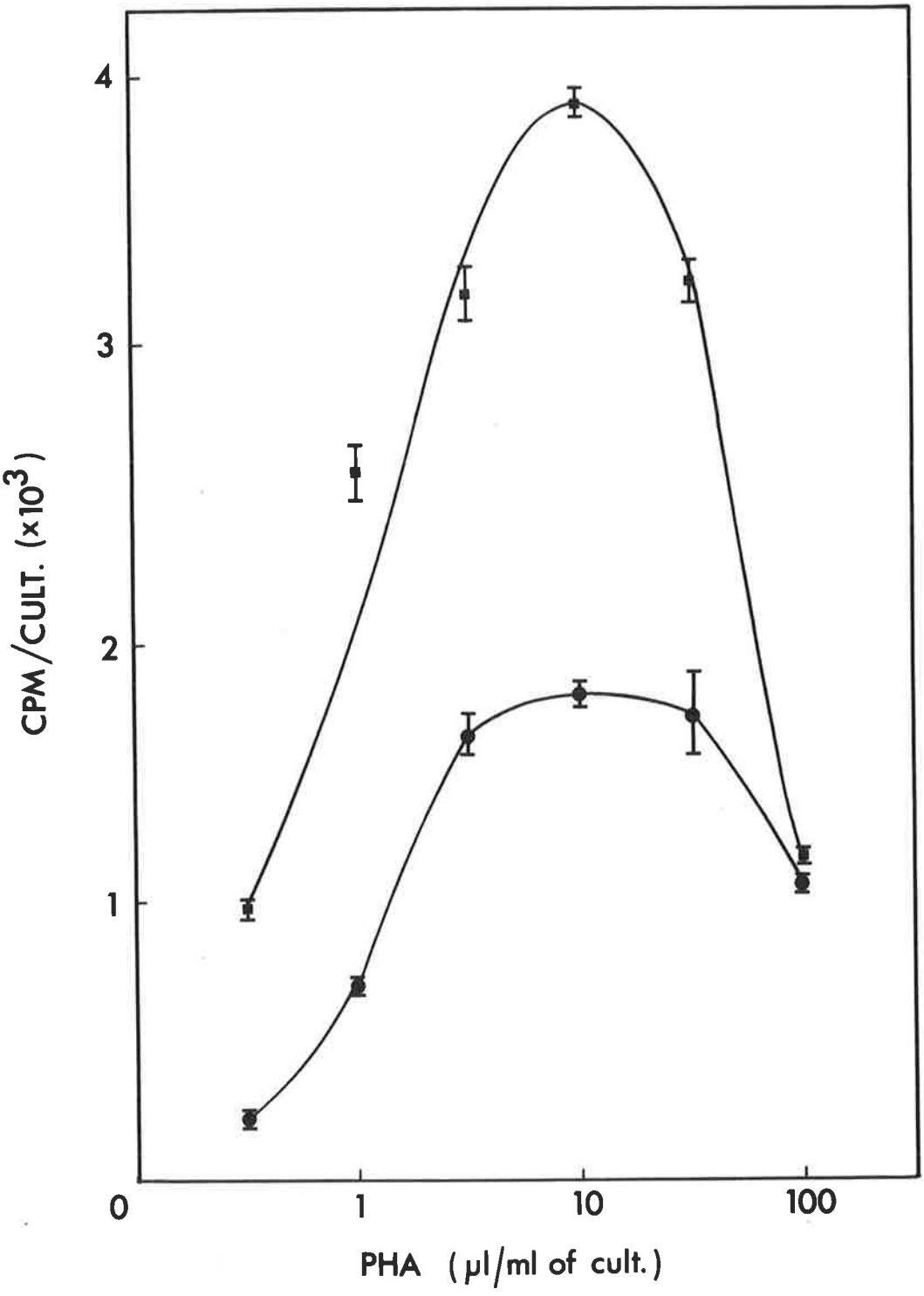
FIGURE IV-10

PHA DOSE-RESPONSE CURVE

Upper curve: Cultures supplemented with autologous serum.

Lower curve: Cultures supplemented with FCS.

The concentration of PHA (batch 3716) is shown as µl of reconstituted PHA/ml of culture.


4
3
2
1
0
CPM/CULT. (x10³)
1
10
100
PHA (µl/ml of cult.)

used in routine studies. The concentration of reconstituted PHA (v/v) at which maximal DNA synthesis occurred varied in different subjects with a given batch of PHA (see Table IV-4) but did not vary in the same subject. However, the extent of individual response to a fixed concentration of PHA (0.02ml/culture) varied with the batch used (see Table IV-5).

TABLE IV-4

PHA - PEAK RESPONSE

Batch	Subject	PHA (µl/ml) -FCS	PHA (µl/ml) -Autol. Serum
3716	M.H.	10.0	10.0
3716	R.S.	50.0	50.0
3716	R.R.	25.0	25.0
3659	T.S.	7.5	-
3659	R.P.	7.5	-

TABLE IV-5

PHA - COMPARISON OF DIFFERENT BATCHES

	PHA - Batch Number			
Subject	3659	3716	5088	Counting Efficiency
T.S.	21.5 ± 0.6	25.3 ± 3.7	-	34%
J.H.	30.7 ± 0.3	40.2 ± 0.8	-	34%
R.R.	-	20.2 ± 1.6	31.4 ± 0.8	38%

4. REPRODUCIBILITY OF THE METHOD

(a) Lymphocyte Transformation in the Presence of FCS

PHA-induced transformation was measured in 13 normal subjects at weekly intervals, for three weeks. The mean values obtained in triplicate cultures from each subject (expressed as dpm/culture x 10^3) are shown in Table IV-6.

TABLE IV-6

STABILITY OF LYMPHOCYTE TRANSFORMATION

Subject	Sex	Week 1	Week 2	Week 3
D.N.	M	45.9	57.7	18.2
J.N.	M	75.6	113.8	69.2
G.H.	M	132.0	126.9	97.1
C.A.	M	120.7	73.8	110.2
S.J.	M	106.2	53.4	84.6
R.W.	M	51.2	40.9	82.9
K.H.	F	49.7	58.8	94.1
G.M.	M	59.9	35.3	27.3
N.W.	M	37.7	84.4	21.0
J.E.	M	63.8	34.4	52.4
G.S.	F	45.0	43.0	37.6
T.S.	F	79.4	58.3	74.3
R.W.	F	53.7	56.5	52.9
Mean$\pm$S.D.		70.8$\pm$29.4	64.3$\pm$27.6	63.2$\pm$29.4

The variances of the three groups were compared using Fisher's F-test. None was significantly different. There was no significant difference between the group means (Student's

t-test) or medians (Wilcoxon's Rank sum test), although considerable variation occurred from week to week in individual subjects.

(b) Lymphocyte Transformation in the Presence of Autologous Serum

PHA-induced transformation was measured in triplicate cultures from 8 normal subjects at weekly intervals, over three weeks. The results, expressed as dpm/culture, are shown in Table IV-7.

TABLE IV-7

STABILITY OF LYMPHOCYTE TRANSFORMATION

Subject	Sex	Week 1	Week 2	Week 3
J.N.	M	73.8	49.7	154.7
R.W.	F	51.7	70.0	50.6
C.A.	M	94.5	99.9	140.9
S.J.	M	86.5	117.6	88.5
R.W.	M	75.0	64.4	117.9
N.W.	M	37.6	84.4	40.6
G.S.	F	10.5	15.1	14.3
T.S.	F	97.8	34.0	27.8
Mean $\pm$ S.D.		65.9 ± 28.5	66.9 ± 31.7	79.4 ± 50.3

Fisher's F-test confirmed that there were no significant differences between the variances of the two groups. The means were not significantly different (Student's t-test), nor were the medians (Wilcoxon's Rank sum test). However, individual responses varied considerably with time.

(c) Variation in the Results of Triplicate Cultures

The relative error obtained in standard (triplicate) cultures from 100 normal subjects was expressed as a percentage of the mean value of each triplicate, and averaged. The mean error was 5.22 percent.

(d) Relationship Between Lymphocyte Transformation in the Presence of Autologous Serum and FCS

Cells from 71 of the normal subjects reported in Chapter II were stimulated in medium 199 supplemented with autologous serum (10%) and in medium supplemented with FCS (10%). DNA synthesis in the presence of FCS was 1.6 times that in autologous serum. There was no simple relationship between the two (i.e. the relationship was not described by a first or second order polynomial equation).

5. COMPARISON OF DNA SYNTHESIS IN PHA-STIMULATED CULTURES OF BLOOD CELLS AND LYMPHOCYTES

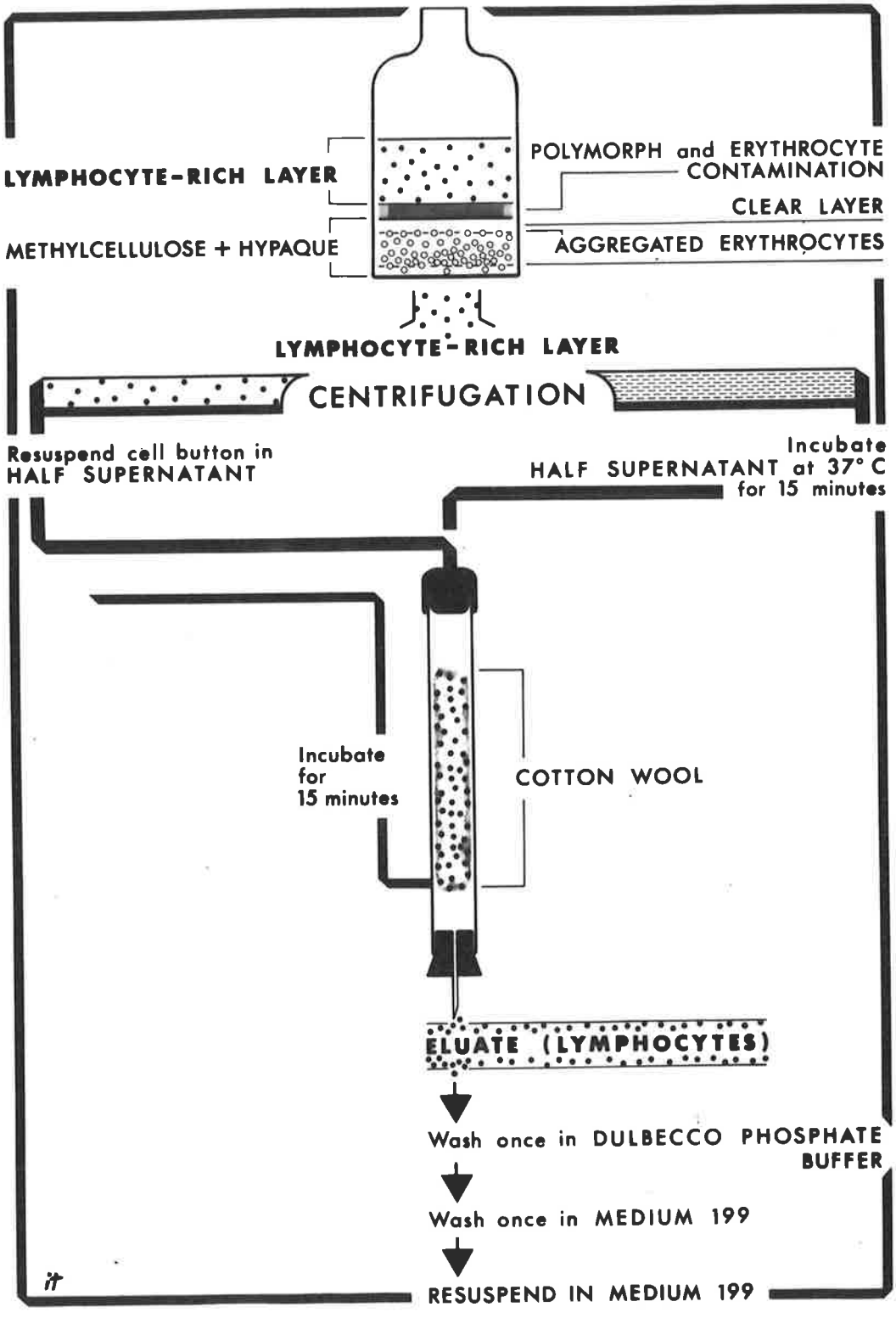
(a) Preparation of Lymphocyte Suspensions

Lymphocytes were purified from peripheral blood using a modification of the methods of Hulliger and Blazkovek (1967) and of Lamvik (1966). The procedure is illustrated in Figure IV-11. Venous blood was anticoagulated with preservative-free heparin (25 units/ml of blood, Weddel Pharmaceuticals Ltd., London) and carefully layered on to methylcellulose-Hypaque solution (see Appendix (viii)) in a sterile bottle. The height of the blood column lay between 1 and 3 cm. The cells were allowed to

FIGURE IV-11

PURIFICATION OF LYMPHOCYTES FROM PERIPHERAL BLOOD

The procedure illustrated is described fully in the text.


LYMPHOCYTE-RICH LAYER
METHYLCELLULOSE + HYPAQUE
POLYMORPH and ERYTHROCYTE CONTAMINATION
CLEAR LAYER
AGGREGATED ERYTHROCYTES
LYMPHOCYTE-RICH LAYER
CENTRIFUGATION
Resuspend cell button in HALF SUPERNATANT
Incubate HALF SUPERNATANT at 37° C for 15 minutes
Incubate for 15 minutes
COTTON WOOL
ELUATE (LYMPHOCYTES)
Wash once in DULBECCO PHOSPHATE BUFFER
Wash once in MEDIUM 199
RESUSPEND IN MEDIUM 199
it

sediment for 90 minutes at room temperature. The lymphocyte-enriched supernatant (approximately two-thirds of the original blood volume) was centrifuged at 300g for 15 minutes, and half of the supernatant (approximately 8 ml) removed for incubation at 37°C. The cell button was resuspended in the remainder of the supernatant, loaded on to a sterile cotton-wool column (Appendix (viii)), and incubated at 37°C for 15 minutes. Non-adherent lymphocytes, contaminated by some erythrocytes and platelets, were eluted with the supernatant plasma, centrifuged at 300g for 15 minutes, washed in Dulbecco phosphate buffer (calcium and magnesium-free, C.S.L., Aust.), then medium 199, and resuspended in medium 199. Cell counts were performed on a haemocytometer. Aliquots of column eluate were also centrifuged at 300g, resuspended in FCS, smeared on to clean glass slides, and stained with May Grunwald - Jenner Giemsa stain.

(b) Recovery of Lymphocytes from Cotton Wool Columns

Examination of smears showed that of the nucleated cells recovered from the columns, more than 98% were mononuclear. The number of lymphocytes recovered in the column eluate (expressed as a percentage of those present in the original blood sample, is shown in Table IV-8. The ratio of contaminating erythrocytes (RBC) to lymphocytes (WBC) is also shown.

TABLE IV-8

RECOVERY OF LYMPHOCYTES FROM COLUMNS

Subject	Leukocyte Recovery	Lymphocyte Recovery	RBC/WBC
C.W.	8	24	3.0
G.M.	13	40	1.3
T.S.	8	27	2.5

(c) Composition of Lymphocyte Cultures

Lymphocyte suspensions were diluted with FCS-medium 199 such that the final suspension contained 2.0 x 10^6 lymphocytes per ml (and 40% v/v FCS). PHA solution (0.02 ml of reconstituted PHA in 2.98 ml of medium 199), 3.0 ml, was mixed with lymphocyte suspension (1.0 ml) in culture tubes. The final culture volume was 4.0 ml; the concentration of FCS, 10% (v/v).

(d) Measurement of Lymphocyte Transformation

^{3}H-thymidine (2.5 µCi, specific activity 500 mCi/mMol) was added to lymphocyte cultures after 68 hours of incubation and to blood cell cultures, after 92 hours. Cultures were processed as described in Chapter II, at 72 and 96 hours respectively. Stimulated and control cultures were processed for counting and volume analysis of cell nuclei.

The mean DNA synthesis obtained in cultures from 4 normal subjects (expressed as cpm/10^6 lymphocytes in the initial culture, x 10^3) is shown in Table IV-9. The standard deviation (% error) is recorded as a percentage of the mean.

TABLE IV-9

DNA SYNTHESIS IN LYMPHOCYTE AND BLOOD CELL CULTURES

Subject	Lymphocyte Cultures		Blood Cell Cultures		*B.C.C./L.C.
	Mean	S.D.	Mean	S.D.	
D.N.	8.0	3.7	14.0	9.4	1.8
M.P.	7.5	16.8	69.3	4.1	9.2
M.H.	11.1	15.7	80.4	2.8	7.2
D.G.	34.3	4.2	51.1	8.3	1.5
Mean	15.2	10.1	53.7	6.2	4.9

*Ratio $\frac{\text{Blood cell cultures (mean)}}{\text{Lymphocyte cultures (mean)}}$

The incorporation of ^{3}H-thymidine into DNA in blood cell cultures was consistently greater than that in lymphocyte cultures, when results were related to the number of lymphocytes initially added to the culture. The ratio of results varied from 1.5 to 9.2, a factor of 6 times.

The results of the morphological estimates of transformation are shown in Tables IV-10 and IV-11.

TABLE IV-10

HISTOLOGICAL ASSESSMENT OF TRANSFORMATION

Subject	Lymphocyte Culture	Blood Culture	B.C.C./L.C.
	% Transformation	% Transformation	
D.N.	43	49	1.1
M.P.	59	67	1.1
M.H.	64	69	1.1
D.G.	?	67	?

TABLE IV-11

NUCLEAR VOLUME ANALYSIS

Subject	Lymphocyte Culture *Area (mm^2)	Blood Culture *Area (mm^2)	$\frac{B.C.C.}{L.C.}$
D.N.	99	65	0.66
M.P.	219	115	0.53
M.H.	119	146	1.23
D.C.	192	50	0.26

*Difference in area of PHA-stimulated and control cultures.

The percentage of transformed cells in blood cell cultures was consistently 1.1 times that in lymphocyte cultures. The ratio of nuclear volume changes in blood cell cultures to that in lymphocyte cultures varied by a factor of 5. Nuclear volume was less in blood cell cultures than in lymphocyte cultures in three subjects, and greater in one.

6. DISCUSSION

Conditions have been established for the routine measurement of PHA-induced lymphocyte transformation in blood cell cultures in man. Transformation has been measured commonly in cultures of blood leukocytes or lymphocytes. Relatively large amounts of blood are required to obtain adequate leukocyte yields, and some methods of cell separation have resulted in detectable impairment of lymphocyte function (Ling, 1968). The whole blood culture technique of Junge et al (1970) was therefore adapted for the measurement of transformation in this study. The components of cell cultures were

selected to obtain an optimal response with a minimum volume of blood and serum.

DNA synthesis was maximal on the fourth day of incubation in cultures containing 0.2 ml of blood and 10% serum in a total volume of 4 ml. In the method of Junge et al cultures of volume 4 ml contained 0.06 ml of blood and 20% serum. DNA synthesis was maximal on the fifth day of incubation. The kinetics of lymphocyte DNA synthesis have been studied in mixed blood cell cultures (mixed lymphocyte reaction, Pauly and Sokal, 1972) and in PHA-stimulated leukocyte cultures (Darzynkiewicz and Jacobson, 1971). Maximal DNA synthesis occurred later in cultures established with fewer cells.

In the present study, HEPES-buffered medium 199 was preferred to bicarbonate-buffered medium 199 in routine studies of lymphocyte transformation because of its technical advantages, in particular, because of its stability in cultures maintained in a non-carbon dioxide-enriched atmosphere (Shipman, 1968). The rate of PHA-induced lymphocyte DNA synthesis in HEPES-buffered medium 199 was dependent on pH, and increased in the range of 7.1 - 7.5, in agreement with the findings of Darzynkiewicz and Jacobson (1971). This increase in DNA synthesis was associated with an increase in the proportion of larger cells and not with a change in the number of cells present at the end of the culture period; it was not noted in non-stimulated (control) cultures. There was no significant difference between the rate of DNA synthesis in PHA-stimulated cultures buffered with HEPES (at pH 7.5) and cultures buffered with bicarbonate (at pH7.3),

confirming the results obtained by Darzynkiewicz and Jacobson (1971) in PHA-stimulated leukocyte cultures with a population density of less than 10^6 cells/ml. These workers found that variation in the results of duplicate cultures were consistently less in medium buffered with HEPES than with bicarbonate, in contrast to the findings of the present study, in which the variation was a constant 5.5% of the mean.

Routine studies were performed in cell cultures supplemented with autologous serum and in cultures supplemented with FCS. There was no significant difference between the growth-supporting effect of autologous serum and plasma in the present study. PHA-induced transformation in lymphocyte rich cultures is usually higher in medium supplemented with autologous serum than with homologous or heterologous serum at the same concentration (Johnson and Russell, 1965, Cooperband et al, 1967). However, in PHA-stimulated cultures from 71 of the normal subjects described in Chapter II, ^{3}H-thymidine incorporation in the presence of 10% FCS was 1.6 times higher than that obtained in the presence of 10% autologous serum. The difference may have been due to the use of heat-inactivated FCS and non-heat-inactivated autologous serum. It has been claimed that serum factors depress PHA-induced transformation in a number of disease states. Transformation is also depressed by certain drugs when present in the serum (Hsu and Leevy, 1971). PHA-induced transformation can be depressed by alpha-globulins present in normal sera. This depression is not due to precipitation of PHA by the serum proteins (Yachnin,

1972). It may be due to binding of the alpha-globulin to receptors for PHA on the cell membrane (Cooperband et al, 1972). High serum concentrations are also known to inhibit PHA-induced transformation in culture. Optimal serum concentrations have been considered to range from 15-20% (Cooperband et al, 1967, Ling, 1968), in leukocyte cultures. The results obtained in the present study showed that maximal DNA synthesis had not been reached at concentrations of autologous serum of up to 30%, but that FCS concentrations of 10-20% were optimal, and dependent on the batch of FCS used. Foetal calf serum can itself stimulate DNA synthesis, but this effect is usually not significant until 6-7 days in lymphocyte-rich cultures (Johnson and Russell, 1965, Froland and Natvig, 1970, Schellekens and Eijsvoogel, 1968).

The rate of DNA synthesis was also dependent on the volume of blood in the culture. A volume of 0.2 ml was required to obtain satisfactory results in patients with low lymphocyte counts, although in normal subjects the incorporation of ^{3}H-thymidine was at least 1000 cpm/culture, and was proportional to the blood volume per culture, within the range 0.05-0.4 ml of blood. Nuclear volume studies showed that the proportion of larger cells increased with blood volumes greater than 0.2 ml/culture. Pauly and Sokal (1972) measured mixed lymphocyte reactions in blood cell cultures supplemented with 20% FCS: they found that the amount of ^{3}H-thymidine incorporated into cell DNA doubled after reducing the blood cell dilution from 1:40 to 1:20. Cell proximity is known to affect the

amount of ^{3}H-thymidine incorporated by transformed lymphocyte cultures; DNA synthesis was markedly decreased in cultures subjected to continuous agitation (Ling, 1968).

The concentration of PHA selected for routine use was approximately half that required to induce maximal DNA synthesis with the batch used. The maximal transformation response depends on the batch and concentration of the stimulating PHA. High concentrations inhibit lymphocyte transformation in both lymphocyte rich (Fitzgerald, 1971), and blood cell cultures (Junge et al, 1970). There is also an inter-dependence between maximal DNA synthesis, PHA concentration and serum concentration (Alford, 1970).

Studies with purified lymphocytes have shown that lymphocyte transformation is affected by the presence in the culture of other blood elements. Yachnin et al, (1972) reported that lymphocyte transformation in response to two mitogenic fractions of PHA was maximal in the presence of autologous platelets in a ratio of 3-10: 1 lymphocyte, and that erythrocytes potentiated the lymphocyte response to the erythro-agglutinating mitogen, PHA-H, in a ratio of 5-10:1. Johnson and Kirkpatrick (1970) found that the maximal response occurred in the presence of erythrocytes in a ratio of 100:1 lymphocyte. Both groups suggested that membrane binding of PHA by these blood elements facilitated lymphocyte transformation. In the present study, platelet-lymphocyte and erythrocyte-lymphocyte ratios were considerably higher than these values. These blood elements were therefore unlikely to contribute significantly to variations in the

results of PHA-induced DNA synthesis in cultures from different subjects. It has been reported that macrophages do not increase DNA synthesis in lymphocyte cultures stimulated with PHA, in contrast to findings in cultures stimulated with specific antigen (Hersh and Harris, 1968). However, other workers have found that PHA-induced transformation is potentiated by glass adherent (phagocytic) cells (Levis and Robbins, 1970). The effect of granulocytes on PHA-stimulated lymphocyte metabolism also depends on their relative number. Cultures containing less than 0.5% granulocytes incorporate the same amount of ^{14}C-thymidine as cultures with a granulocyte-lymphocyte ratio of 2:1 (Schellekens and Eijsvoogel, 1968). Intermediate numbers of granulocytes (2.5 - 25% of white cells) potentiate PHA-induced protein synthesis (Lawton, 1967). In the present study granulocytes constituted at least 50% of the total leukocytes.

Granulocytes and supernatants of homogenized granulocytes contain relatively high concentrations of enzymes responsible for thymidine breakdown (Marsh and Perry, 1964), and their presence may therefore influence the measured DNA synthesis in lymphocytes. These enzymes are also increased in activated lymphocytes (Ling 1968). However, most of the products of thymidine degradation are acid-soluble (Cleaver, 1967) and are removed during cell processing. The remainder are not re-utilised to any extent (Ling, 1968). The problem of thymidine degradation in the cultured cells in present study was overcome by the additional precautions of a short labelling period (4 hours) and the use of relatively low concentrations of

thymidine (1.2×10^{-6} molar).

PHA-stimulated DNA synthesis in blood cell cultures varied considerably in individual subjects over three consecutive weeks, although group means remained stable. Reproducibility in lymphocyte rich cultures from individual subjects is variable (Richter and Naspitz, 1967, McIntyre and Cole, 1969). In blood cell cultures from a single patient, PHA-stimulated DNA synthesis varied from 211-297 times control values over a period of 4 weeks (Junge et al, 1970); the actual values were not stated. Triplicate blood cell cultures from 100 normal subjects varied by 5.2% of the mean in the present study, compared with 13% (Pauly and Sokal, 1972) and 18% (Junge et al, 1970).

The ultimate figure obtained in measurements of lymphocyte transformation is clearly dependent on a number of interacting variables. For routine tests, it would be impossible to optimize individual factors in order to obtain the peak response. Fitzgerald (1971) suggested that more meaningful data could be obtained by constructing PHA dose-response curves for cells from each subject, although the abnormal example quoted by her showed depression of responsiveness at every concentration of PHA. It has been found that dose-response curves from normal and mongoloid subjects do intersect at low concentrations of PHA (approximately one-fifth of the concentration which induces maximal DNA synthesis, (Rigas et al, 1970).) In some cases of infantile sex-linked agammaglobulinaemia, Hosking et al (1971) reported that DNA synthesis in lymphocytes stimulated with PHA

concentrations of one-tenth, or 1-4 times the concentration required to produce maximal DNA synthesis, was within the normal range. In the present study it was technically impossible to establish dose-response curves. Normal and diseased groups were compared on the basis of a single determination of PHA-induced DNA synthesis in the presence of autologous serum, and in the presence of foetal calf serum. The concentration of PHA was approximately half that required to produce maximal DNA synthesis. The results of Rigas et al (1970), Fitzgerald (1971) and Hosking et al (1971) suggest that the concentrations of PHA used in the present study should detect depression of PHA-induced lymphocyte DNA synthesis without the necessity of establishing a dose-response curve.

PART B

DETERMINATION OF CONDITIONS FOR THE MEASUREMENT OF DNA SYNTHESIS IN CIRCULATING LEUKOCYTES

1. METHOD

The basic procedure has been described in Chapter III.

2. THE EFFECT OF SERUM CONCENTRATION ON DNA SYNTHESIS IN CIRCULATING LEUKOCYTES

Autologous serum (0.4 ml or 0.8 ml) was added to triplicate cultures from three normal subjects. Cultures were also established without added serum. The total culture volume was 4.1 ml. The results (expressed as cpm/culture) are shown in Table IV-12.

TABLE IV-12

THE EFFECT OF SERUM CONCENTRATION ON DNA SYNTHESIS

	Serum Content (ml/culture)		
Subject	0	0.4	0.8
T.S.	100 ± 10	105 ± 4	115 ± 5
	110 ± 6	95 ± 4	130 ± 6
M.A.	-	205 ± 14	235 ± 7
R.R.	235 ± 12	260 ± 13	-

These results suggest that there is little difference in DNA synthesis obtained in the presence or absence of added serum. The numbers are too small for meaningful statistical analysis.

3. REPRODUCIBILITY OF DNA SYNTHESIS IN CIRCULATING LEUKOCYTES

Subject	Sex	Week 1	Week 2	Week 3
D.N.	M	297	176	156
J.N.	M	715	462	488
C.A.	M	294	285	171
R.W.	M	324	162	1015
G.S.	F	568	397	362
T.S.	F	494	735	579
R.G.	M	294	185	150
Mean		426	344	417
S.D.		156	191	291

Fisher's F-test confirmed that the variances of the three groups were not significantly different, nor were the medians (Wilcoxon's Rank sum test).

4. DISCUSSION

DNA synthesis measured in blood cell cultures in the absence of added serum was not significantly different from that obtained with 10% serum. Small amounts of plasma (approximately 2.5% v/v) were present in the heparinized blood; this amount is sufficient to ensure reasonable cell survival for periods longer than the four hours used in this study (Ling, 1968). Other workers have incubated whole blood or whole blood-dextran mixtures with ^{3}H-thymidine for periods of up to 1 hour. In whole blood preparations anticoagulated with EDTA, the morphology of the cells changed after 1 hour of incubation (Wood and Frenkel, 1967). Both autoradiographic and liquid scintillation techniques have been used to measure ^{3}H-thymidine incorporation. Rubini et al (1961) studied 48 patients with normal hemograms and found 0.05% cells incorporated DNA label. This percentage remained constant in the individual patient, and did not vary with age. Results obtained in the present study using liquid scintillation methods also suggest that this parameter is stable with time. Bond et al (1958) failed to detect label in myeloid cells or small lymphocytes. Wood and Frenkel (1967) called these DNA-synthesizing cells "atypical lymphocytes" and stated that they were analogous in many respects to the transformed cells of PHA- or antigen-stimulated cell cultures. They reported that the number of these cells was increased in peripheral blood during viral and non-viral illnesses, possibly as a result of rapid production and early release of immature leukocytes in response to antigenic stimulus. Atypical

lymphocytes represented the "irritation cells described by Turk (1898) in the blood of patients suffering from a number of infections". By using separated lymphocyte suspensions, Crowther et al (1969) showed conclusively that at least part of this active cell population is lymphocytic in origin and that these cells appear in human blood after immunization with viral and bacterial antigens. They have been described in drug hypersensitivity (Wood and Frenkel, 1967) and in renal allograft rejection (Page et al, 1971). Recent studies by Sheldon et al (1973) suggested that the atypical mononuclear cells in the blood of patients with infectious mononucleosis were T-cells. Gump and Fekety (1967) injected human volunteers with attenuated tularemia vaccine. The number of DNA-synthesizing cells reached a maximum just after the acute illness; these workers concluded that the active cells represented the immature, dividing cells involved in inflammatory and repair processes.

The number of cells synthesizing DNA is also increased in Hodgkin's disease (Crowther et al, 1969), disseminated lupus erythematosus (Horwitz et al, 1970) and other acute inflammatory conditions. Horwitz et al (1970) concluded that these cells may represent mononuclear-cell precursors released into the blood from the marrow, in response to an inflammatory stimulus, and/or, immature lymphocytes stimulated to divide by specific antigen, and released from the lymphoid tissues to the blood. In the present study it has been assumed that the rate of DNA synthesis in these cells is a measure of immune activity. Such measurement has been included as a

test of T-cell function, although it has not yet been proven that the assumption is justified.

SUMMARY OF RESULTS

PART A

(a) In PHA-stimulated blood cell cultures, the incorporation of ^{3}H-thymidine into acid-precipitable DNA was maximal on the fourth day of incubation. Nuclear volume changes and cell counts were maximal after five or more days of culture. More than 89% of intact cells excluded trypan blue dye after six days of culture.

(b) For routine use, HEPES was a technically more satisfactory buffer than bicarbonate, and gave comparable results when used at pH 7.5 (at 37°C).

(c) DNA synthesis in PHA-stimulated cultures was similar in medium supplemented with autologous plasma to that in medium supplemented with autologous serum.

(d) There was a linear relationship between DNA synthesis and the concentration of autologous serum. PHA-induced DNA synthesis was reduced in cultures containing FCS concentrations of 20% or more, in comparison with cultures containing 10% FCS, when two different batches of FCS were used. However, DNA synthesis in medium supplemented with a third batch of FCS was greater in the presence of 20% serum than 10% serum. The response of normal lymphocytes to a fixed concentration of PHA in medium supplemented with FCS (10% v/v) also varied with the batch of FCS used.

(e) PHA-induced DNA synthesis (in medium supplemented with 10%

serum) was proportional to the volume of blood added to each culture over the range 0.05–0.4 ml of blood.

(f) DNA synthesis varied with both the batch and concentration of PHA in culture.

(g) The composition of blood cell cultures adopted for the routine measurement of lymphocyte transformation was as follows:

heparinized blood (0.2 ml), serum (0.4 ml), reconstituted PHA (0.02 ml) and medium 199 (3.38 ml).

(h) Lymphocyte transformation (DNA synthesis) varied considerably in normal subjects tested over three consecutive weeks. Group means remained stable.

(i) Lymphocyte transformation was compared in blood cell cultures and lymphocyte cultures. The incorporation of ^{3}H-thymidine was consistently higher in blood cell cultures, but by a variable amount (1.5–9.2 times). The percentage of transformed cells in blood cell cultures was consistently 10% higher than in lymphocyte cultures. The relative increase in nuclear volume was less in blood cell cultures than in lymphocyte cultures in three subjects, and greater in the fourth (the ratio of results in the two types of culture varied by a factor of 5).

PART B

(a) DNA synthesis in circulating leukocytes was independent of the presence of added serum.

(b) Mean DNA synthesis, tested at weekly intervals for 3 weeks in a group of 7 healthy subjects, remained stable.

CHAPTER V

IMMUNOLOGICAL FUNCTION IN HUMAN DISEASE STATES

PART A

1. INTRODUCTION

The occurrence of Hodgkin's disease and other lymphomas has been documented in a significant number of patients treated with the anticonvulsant drug, phenytoin sodium (diphenylhydantoin), and other hydantoins (Hyman and Sommers, 1966, Anthony, 1970, Brown, 1971). The association has not been established in patients treated with other types of anticonvulsant.

Immunodeficiency is known to occur in lymphoma (Brown et al, 1967, Sutherland et al, 1971, Aisenberg, 1972), and may itself predispose to the development of neoplasia of both the lymphoid and epithelial tissues (Good, 1972).

A preliminary study was carried out to determine the immunological status of patients treated with phenytoin sodium.

2. PRELIMINARY SURVEY OF PATIENTS TREATED WITH PHENYTOIN

(a) Patients

(1) Forty-nine permanent residents of Strathmont Centre for the Intellectually Retarded had been receiving phenytoin sodium for more than five years, and four patients had been taking it for at least two years, in doses of 100–400 mg/day. Twenty-five patients were male, twenty-eight were female. Their mean age was 21.6 years, with a range of 8–55 years. At the time of study, 21 patients were receiving phenytoin as their only anticonvulsant. Ten were also taking thioridazine.

(2) They were compared with 45 age and sex-matched, non-epileptic, control subjects (21 males and 24 females, mean age 22.9 years, range 8 - 55 years). Twenty control subjects were taking thioridazine.

(3) Fifteen epileptic patients (7 males and 8 females) were receiving anticonvulsant therapy with phenobarbitone or primidone. Their mean age was 21.7 years, with a range of 8 - 55 years.

(4) All patients were ingesting a standard diet, and multi-vitamin capsules (which did not contain folic acid).

(b) Methods

(1) Patients were examined for lymph node enlargement and hepatosplenomegaly. Patients with clinical evidence of infection were excluded from the series.

(2) Immunological function tests were performed as described in Chapter II, with the following exceptions -

(i) Serum complement was not measured.

(ii) PHA-induced lymphocyte transformation was estimated by the microscopic examination of stained smears prepared from purified lymphocyte cultures, which had been incubated with PHA for 72 hours in medium 199 supplemented with 20% autologous serum. The number of transformed cells was expressed as a percentage of the lymphocytes present at 72 hours. (The method for the measurement of DNA synthesis by the incorporation of ^{3}H-thymidine was developed concurrently with this study).

(iii) Serum IgA concentrations was measured only in subjects over the age of 14 years.

(3) Serum phenytoin concentrations were determined by Mr. C. S. Crisp, of the S. Aust. Department of Chemistry, using the technique of Svensmark and Kristensen (1963).

(4) Serum folate concentrations were measured by Dr. R. J. Kimber, of the Institute of Medical and Veterinary Science, using a modification of the method of Baker et al, (1959).

(5) Statistical analyses of the results are shown in Appendix (ix).

(c) Results

(1) Physical examination and blood examination: There was no clinical evidence of lymphadenopathy or hepatosplenomegaly in any patient. Leukocyte counts were within normal limits (4,000 - 12,000 cells/μl) in all but one epileptic patient, in whom the count was 13,000. Eleven of the 43 patients treated with phenytoin had an absolute eosinophilia compared with 5 of 37 non-epileptic controls. All of these subjects came from two villas at the Centre.

(2) Immunoglobulin concentrations: Mean values and standard deviations (mg/100ml) are shown in Table V-1.

TABLE V-1

SERUM IMMUNOGLOBULIN CONCENTRATIONS

Group	No.	IgG	IgA	IgM
Control	45	1604±518	227±110 (38)	183±77
Phenytoin	51	∅1069±294	203±113 (41)	193±80
Barbiturate	15	1610±396	+283±94 (11)	+264±119

( ) Number of IgA measurements (patients over 14 years).

∅ Mean significantly low, $P < 0.001$.

\+ Mean significantly high, $P < 0.05$.

(3) Antibody responses: The results are shown in Table V-2.

All control subjects developed specific antibody 2 weeks after immunization with S. typhi. There was no response in 4 subjects treated with phenytoin. These differences were not significant (Fisher's exact test).

TABLE V-2

ANTIBODY RESPONSES

Group	Responders	Non-responders	Significance
S. typhi			
Control	45	0	
Phenytoin	49	4	P=0.08
Barbiturate	15	0	*N.S.
Tet. toxoid			
Control	45	2	
Phenytoin	53	0	N.S.
Barbiturate	15	0	N.S.

*Not significant.

(4) Antinuclear antibody: Antinuclear antibody (fluorescent type B, i.e. rim or speckled pattern of nuclear fluorescence) was present in the sera of two of fifty-three patients treated with phenytoin, and none of the patients treated with carbamazepine. Neither value was abnormal.

(5) Delayed hypersensitivity reactions: These results are shown in Table V-3.

TABLE V-3

REACTIVITY TO AT LEAST ONE INTRADERMAL ANTIGEN

Group	One or More DHS Reactions	No DHS Reactions	Significance
Control	44	1	
Phenytoin	38	14	*P=0.0005
Barbiturate	14	1	*P=0.3814
			**P=0.0768

*Compared with control, and **phenytoin-treated groups using Fisher's exact test.

Initially, sixteen of the phenytoin-treated patients (30%) failed to react to at least one of the test antigens. Two developed positive reactions on repetition of the tests - one to candida and mumps antigens, the other to streptococcal and mumps antigens. The proportion of non-reactors after re-testing (26.5%) was significantly greater than the proportion of non-reactors in the barbiturate-treated group, one of whom failed to react, and in the control group, one of whom failed to react.

The area and mean diameter of cutaneous induration were

measured for positive reactions to each antigen. There were no significant differences between the three groups. Attempts were made to sensitize twelve of the fourteen non-reactive patients in the phenytoin-treated group to DNCB. They were unsuccessful in ten cases. All of these patients developed an inflammatory reaction to the non-specific irritant, croton oil.

(6) Circulating lymphocyte counts: The mean value and standard deviation of the lymphocyte counts in each group (expressed as the number of cells per ul of blood) are shown in Table V-4.

TABLE V-4

CIRCULATING LYMPHOCYTE COUNTS

Group	No.	Lymphocyte Count	Significance
Control	51	2098 ± 868	
Phenytoin	38	2100 ± 629	*N.S.
Barbiturate	10	1606 ± 715	*N.S.
			**P < 0.05

*Compared with control and **phenytoin-treated patients using Fisher's exact test.

There were no significant differences between the two groups of epileptic patients and the control group. However, lymphocyte counts were significantly lower in the barbiturate-treated patients than in the phenytoin-treated group.

(7) Lymphocyte transformation: A transformation response of 50% or more occurred in all control subjects. This was there-

fore considered as normal. The results obtained in the phenytoin-treated and control groups are shown in Table V-5. This parameter was not measured in the barbiturate-treated patients.

TABLE V-5

LYMPHOCYTE TRANSFORMATION (Tx)

Group	Normal Tx.	Low Tx.	*Significance
Control	45	0	
Phenytoin	41	11	P=0.0006

*Compared by Fisher's exact test

The value was less than 50% in a significant proportion of phenytoin-treated patients (21%).

(8) Total patients with immunological defects: The number of patients with normal immune responsiveness, and the number with at least one abnormality, is shown in Table V-6.

TABLE V-6

TOTAL IMMUNOLOGICAL RESPONSIVENESS

Group	Normal (No.)	Abnormal (No.)	Significance
Control	42	3	
Phenytoin	31	21	*P=0.000079
Barbiturate	13	2	*P=0.2729
			**P=0.0380

*Compared with control, and **phenytoin-treated groups, using Fisher's exact test.

The number of abnormalities was significantly high in the phenytoin-treated patients; at least one parameter was abnormal

in 40% of patients, compared with 7% of controls, and 13% of barbiturate-treated patients.

(9) Relationship between the presence of immunological abnormalities and serum phenytoin concentration: The serum concentration of phenytoin was measured in 34 patients, 35.3% of whom manifested at least one abnormality of immunological function. Attempts were made to relate serum phenytoin concentration to the presence of abnormalities, see Table V-7.

TABLE V-7

THE EFFECT OF SERUM PHENYTOIN CONCENTRATION ON IMMUNOLOGICAL FUNCTION

	*Phenytoin Concentration	Normal Response	Abnormal Response	Total
1.	0.0-0.99	15	7	22
2.	1.0-1.99	5	4	9
3.	2.0-2.99	2	1	3
	Total	22	12	34

*mg/100ml.

The number of patients in group 3 (with serum phenytoin concentrations greater than 2.0 mg/100ml) was too small for meaningful comparison with groups 1 and 2. Patients in the first group were therefore compared with those in the combined second and third groups, using Fisher's exact test. There was no significant difference between the two (P=0.246).

The relationship between individual parameters of immunological function and serum phenytoin concentrations were also studied.

There was a weak negative correlation between serum phenytoin concentration and IgG concentration, and serum phenytoin concentration and circulating lymphocyte counts, see Table V-8.

TABLE V-8

CORRELATIONS BETWEEN PARAMETERS OF IMMUNOLOGICAL FUNCTION AND SERUM PHENYTOIN CONCENTRATION

Parameter	No.	*r	Significance
IgG	34	-0.42	$P < 0.05$
Lymphocyte count	30	-0.40	$P < 0.05$

*Correlation coefficient, r.

There was no correlation between serum phenytoin concentrations and other parameters of immunological function.

(10) Relationship between phenytoin dosage or serum concentration and the type of immunological defect: The relationship between phenytoin dosage (mg/day) and the number of patients with no immunodeficiency, defects of cellular or humoral immune function, or both, is shown in Table V-9. (p. 104).

For daily doses of 100-300 mg of phenytoin, the proportion of patients with normal responses remained approximately the same; increased dosage within this range was associated with a relatively higher proportion of humoral defects, and a lower proportion of cellular defects.

The relationship between serum phenytoin concentration and the type of immunological defect is shown in Table V-10 (p.104).

TABLE V-9

RELATIONSHIP BETWEEN DOSAGE AND TYPE OF ABNORMALITY

Dose	Normal	*Humoral defect	*Cellular defect	**Both
100	5 (31)	2 (13)	6 (37)	3 (19)
200	6 (31)	4 (21)	4 (21)	5 (27)
300	3 (37)	4 (50)	1 (13)	0 (0)
400	0 (0)	1 (33)	0 (0)	2 (67)

*Humoral defect only, cellular defect only, ** humoral and cellular defect. The percentage of patients in each category, for each dosage of phenytoin (mg/day) is shown in parentheses).

TABLE V-10

RELATIONSHIP BETWEEN SERUM PHENYTOIN CONCENTRATION AND THE TYPE OF IMMUNOLOGICAL DEFECT

*Serum level	Normal	Humoral defect	Cellular defect	Both
0.0-0.99	7 (37)	1 (5)	6 (32)	5 (26)
1.0-1.99	1 (11)	5 (56)	0 (0)	3 (33)
2.0-2.99	0 (0)	1 (33)	1 (33)	1 (33)

*mg/100ml.

(11) Serum folate concentrations in phenytoin-treated patients: Serum folate concentrations were measured in 37 patients treated with phenytoin; they were compared with 42 age- and sex-matched controls, and 8 patients treated with barbiturate. The mean values are shown in Table V-11.

TABLE V-11

SERUM FOLATE CONCENTRATIONS

Group	No.	Folate (µg/L)	Significance
Control	42	5.63±2.99	
Phenytoin	37	3.61±2.33	*P < 0.001
Barbiturate	8	2.69±1.47	*P < 0.01

*The values in the phenytoin-treated and barbiturate-treated patients were compared with those in the control group by Wilcoxon's Rank sum test.

Values in the phenytoin and barbiturate groups were significantly lower than those of the control subjects.

The relationship between serum phenytoin concentration and serum folate (in 31 patients in whom both parameters were measured) is shown in Figure V-1. Significantly lower folate concentrations were found in patients with serum phenytoin concentrations above 0.5 mg/100ml. Attempts were made to establish a relationship between serum folate concentrations and the presence of immunological abnormalities (see Table V-12).

TABLE V-12

THE EFFECT OF SERUM FOLATE CONCENTRATION ON IMMUNOLOGICAL PARAMETERS

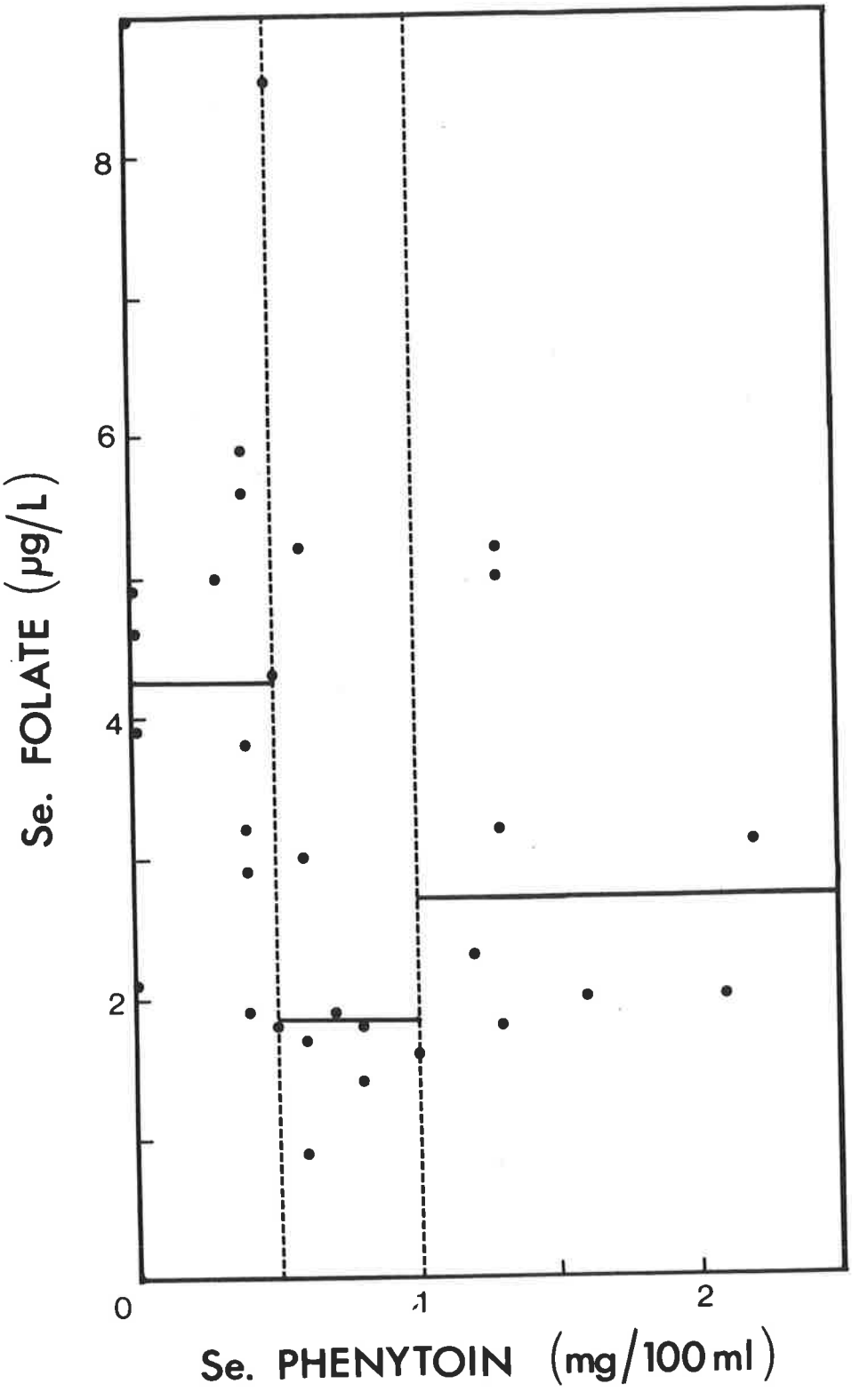
*Folate Concentration	Normal Responses	Abnormal Responses
1.0-1.95	4	7
2.0-3.95	5	7
4.0- or more	10	4

*µg/L

FIGURE V-1

THE RELATIONSHIP BETWEEN SERUM PHENYTOIN AND SERUM FOLATE CONCENTRATIONS

Horizontal bars represent median values for the ranges of serum phenytoin concentrations enclosed by the vertical lines.


Se. FOLATE (µg/L)
8
6
4
2
0
1
2
Se. PHENYTOIN (mg/100 ml)

Patients with folate concentrations less than 4.0 µg/L were compared with those with concentrations greater than 4.0 µg/ml, using Fisher's exact test. The two groups were not significantly different (P=0.462).

(d) Summary

(1) The following abnormalities of immunological function were found in phenytoin-treated patients

- deficiency of circulating IgG
- depression of capacity to manifest DHS reactions.

At least one of these abnormalities was found in 40.4% of phenytoin-treated patients, compared with 6% of controls, and 13% of barbiturate-treated epileptics. The differences were significant (P=0.00079 and P=0.0380 respectively). The number of abnormalities in the barbiturate-treated group was not significantly greater than normal (P=0.2729). The data also indicated that PHA-induced lymphocyte transformation was frequently depressed in patients taking phenytoin, and in some cases, this was the only abnormality.

(2) Cellular immunological defects tended to be more common in patients treated with low doses of phenytoin (100 mg/day) in contrast to humoral immune deficiencies, which were more common in patients on higher doeses (300 mg/day).

(3) The presence of immunodeficiency did not correlate with serum phenytoin concentration. However, the serum concentration of IgG and the circulating lymphocyte count correlated weakly

with the serum phenytoin concentration ($P < 0.05$).

(4) Serum folate concentrations were significantly depressed in the phenytoin-treated and barbiturate-treated groups ($P < 0.001$ and $P < 0.01$, respectively). Folate concentrations were negatively correlated with serum phenytoin concentrations, being significantly lower in patients with phenytoin concentrations greater than 0.5 mg/100ml than in patients with levels less than 0.5 mg/100ml ($P=0.05$). They did not correlate with the presence of immunological abnormalities in the phenytoin-treated group.

PART B

DEFINITIVE STUDY OF IMMUNOLOGICAL FUNCTION IN PATIENTS TREATED WITH PHENYTOIN SODIUM; COMPARISON WITH PATIENTS TREATED WITH CARBAMAZEPINE

1. INTRODUCTION

It having been established that therapy with phenytoin, but not barbiturate, was associated with partial immunosuppression in a significant proportion of patients from a closed community, a more detailed investigation of immunological function was made in two groups of general hospital patients, one treated with phenytoin, and one treated with another anticonvulsant drug, carbamazepine.

2. IMMUNOLOGICAL FUNCTION IN PATIENTS TREATED WITH ANTICONVULSANTS

(a) Patients

(1) Sixty-three general hospital patients (35 males and 28 females) had been ingesting phenytoin regularly for at least one month, most commonly in a dose of 300 mg/day. At the time of study, phenytoin was the only anticonvulsant taken by 37 of

these patients. The mean age of the group was 35.0 years, with a range of 14–70 years.

(2) They were compared with 87 non-epileptic control subjects (48 males and 39 females, mean age 34.2 years, range 14–78 years).

(3) A number of immunological parameters was studied in 11 patients (9 males and 2 females) before the commencement of, and during, therapy with phenytoin.

(4) Fifteen epileptic patients (7 males and 8 females) were taking carbamazepine (Tegretol, Geigy) as their only anticonvulsant. Their mean age was 26.3 years, with a range of 16–56 years.

(5) More than 90% of the epileptic patients were attending the medical or neurological out-patient clinics of The Queen Elizabeth Hospital. The remainder were inpatients, usually admitted for investigation of their epilepsy. The population of control subjects was described in Chapter II.

(b) Methods

(1) Patients were examined for lymph node enlargement, hepatosplenomegaly, and for evidence of infection. Their case records were reviewed for a history of recurrent, severe infection. Patients with current clinical evidence of infection were excluded from the study.

(2) The full range of tests of immunological function was performed, with the exception that patients who failed to manifest DHS reactions were not retested.

(3) Serum phenytoin concentrations were determined by Mr. C.S.

Crisp, of the South Australian Department of Chemistry, using a gas-chromatographic technique (Dill et al, 1971).

(4) Serum folate concentrations were determined as before (see Chapter V, Part A).

(5) Statistical analyses of the results are shown in Appendix (x).

(c) Results

(1) Clinical examination: There was no evidence of enlargement of lymph nodes, liver or spleen in any patient. The prevalence of recurrent, severe infection was not increased in patients taking phenytoin. Circulating leukocyte counts were normal, except in one epileptic patient, in whom the count was 12,600 cells/µl of blood.

(2) Immunoglobulin concentrations and complement: The values obtained in the control, phenytoin-treated, and carbamazepine-treated patients (mg/100ml), their means and standard deviations, are shown in Figures V-2 and V-3. The results of the comparative tests (outlined in Appendix (x)) are shown in Table V-13.

TABLE V-13

IMMUNOGLOBULIN CONCENTRATIONS AND COMPLEMENT

Groups Compared	IgG	IgA	IgM	C3
Control-phenytoin	*N.S.	P < 0.001	P < 0.001	N.S.
Control-carbamazepine	N.S.	P < 0.01	P = 0.001	N.S.
Phenytoin-carbamazepine	N.S.	N.S.	N.S.	N.S.

*Differences not significant

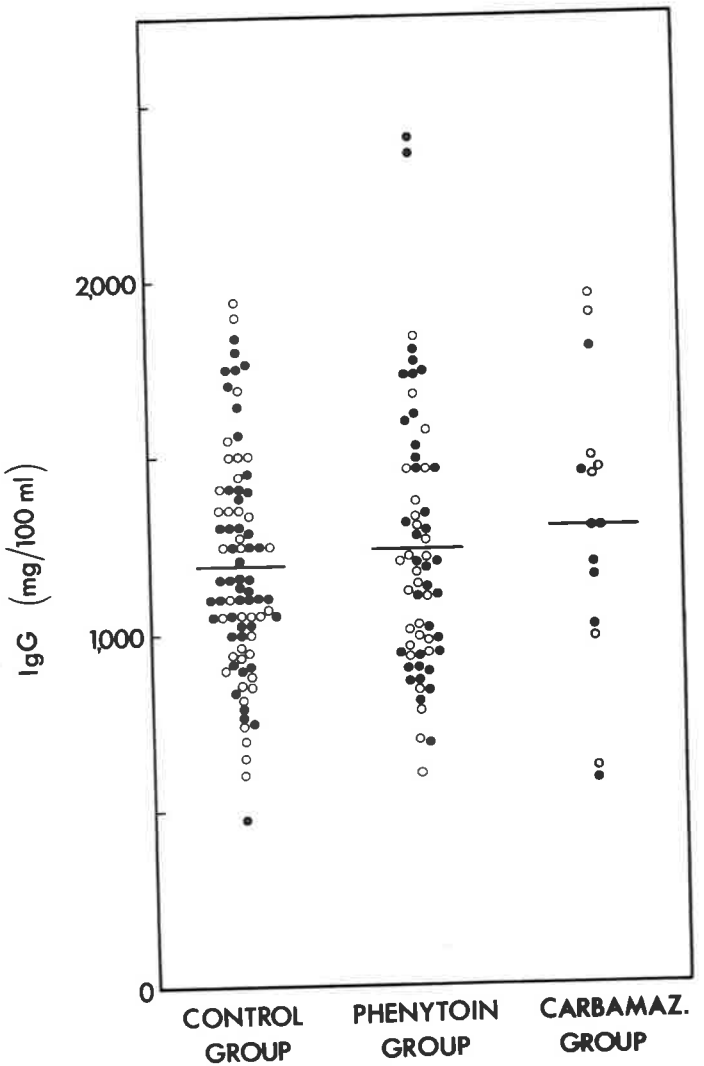
The IgA concentration was decreased in the phenytoin-treated and carbamazepine-treated patients in comparison with

FIGURE V-2

SERUM CONCENTRATIONS OF IgG AND IgA

Values in male subjects are indicated by closed circles, and in female subjects, by open circles.

Horizontal bars show mean values for each group.


IgG (mg/100 ml)
2,000
1,000
0
CONTROL GROUP
PHENYTOIN GROUP
CARBAMAZ. GROUP

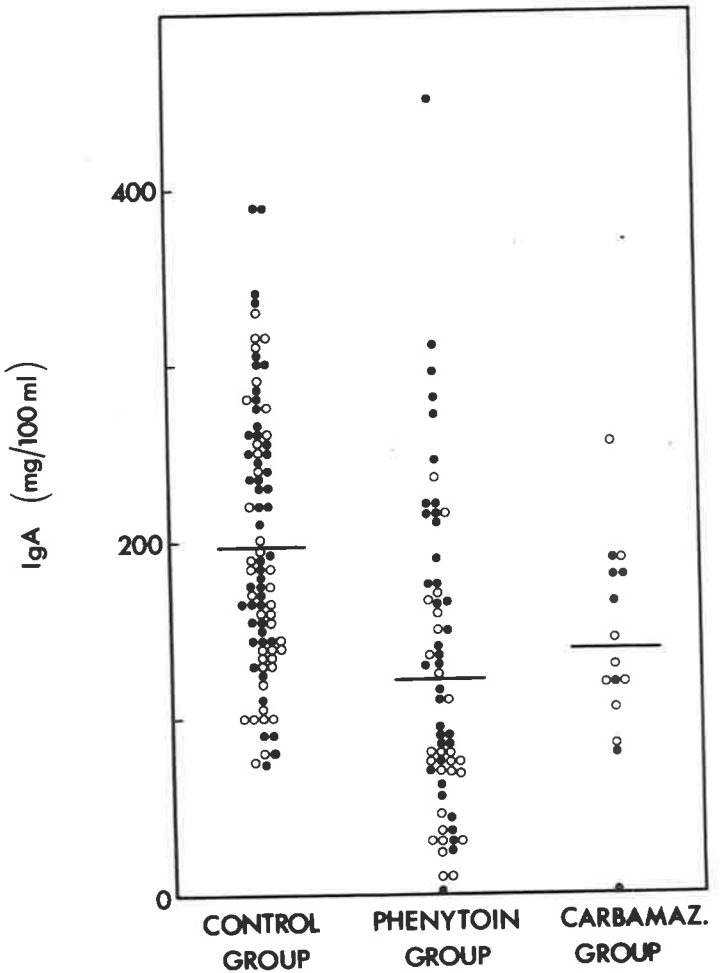
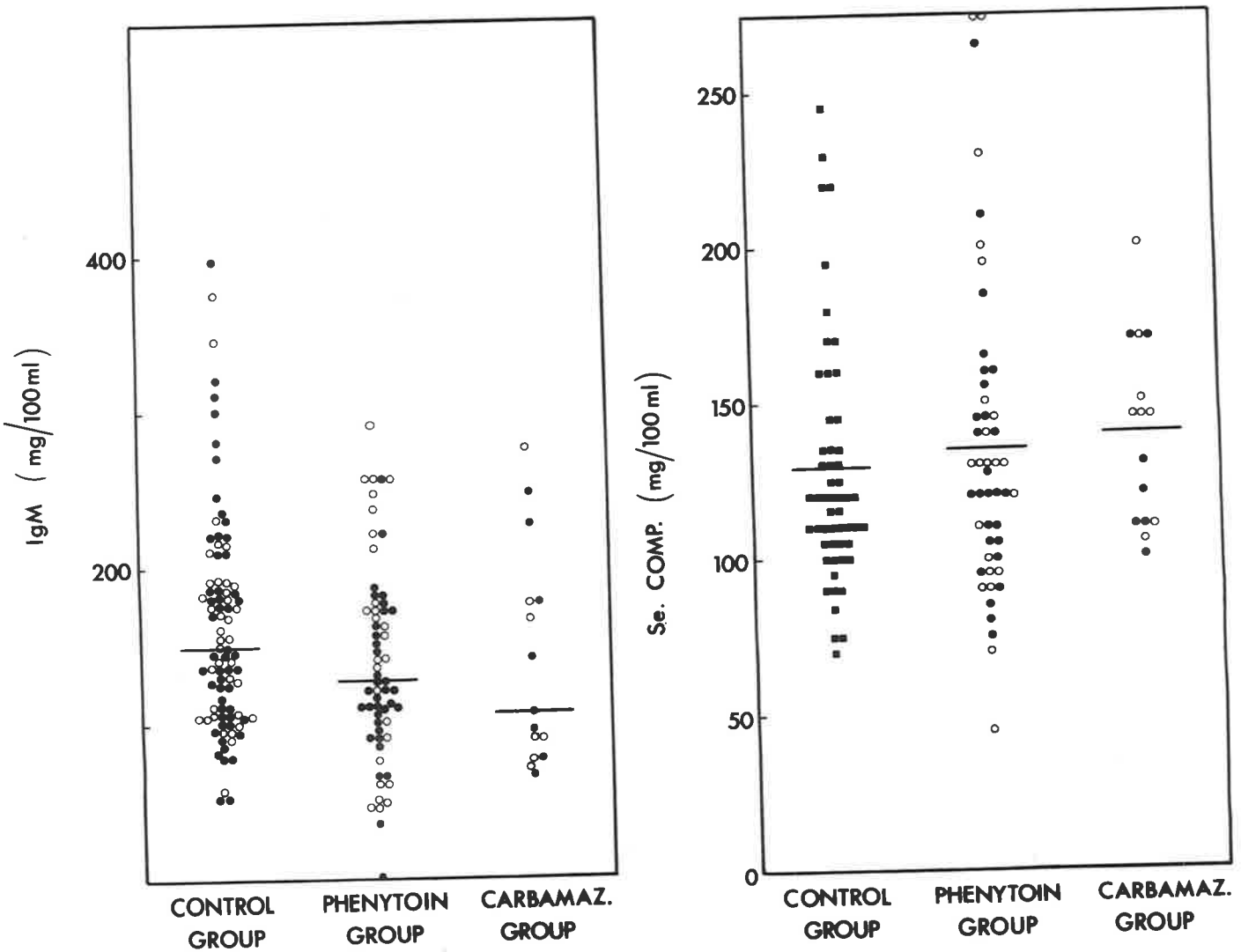

IgA (mg/100 ml)
400
200
0
CONTROL GROUP
PHENYTOIN GROUP
CARBAMAZ. GROUP

FIGURE V-3

SERUM CONCENTRATIONS OF IgG AND COMPLEMENT

Values in male subjects are indicated by closed circles and in female subjects, by open circles.
Values of serum complement concentrations in the control group (shown as squares) are not distinguished by sex.
Horizontal bars represent medians for IgM (log-normally distributed) and means for serum complement (normally distributed).


IgM (mg/100ml)
400
200
CONTROL GROUP
PHENYTOIN GROUP
CARBAMAZ. GROUP
Se. COMP. (mg/100ml)
250
200
150
100
50
0
CONTROL GROUP
PHENYTOIN GROUP
CARBAMAZ. GROUP

the control group. IgA was not detected in the serum of one patient taking carbamazepine; phenytoin therapy had been stopped in this patient because of an hypersensitivity reaction to it, one year before this study. The concentration of IgA was within the normal range in all other patients treated with carbamazepine, although the mean level was still significantly less than that of the non-epileptic control group.

IgM concentrations were significantly low in the phenytoin-treated patients, and in patients taking carbamazepine. The values of the two groups of epileptic patients were not significantly different from each other. The mean concentration of the C3 component of complement was normal in the phenytoin- and carbamazepine-treated patients, see Table V-14.

TABLE V-14

SERUM COMPLEMENT CONCENTRATION (C3)

Group	No.	Complement	Significance
Control	54	128±38	
Phenytoin	49	135±54	*N.S.
Carbamazepine	15	139±29	*N.S.
			**N.S.
Phenytoin (males)	28	132±41	
Phenytoin (females)	21	143±68	N.S.
Phenytoin (alone)	28	136±60	
‡Phenytoin (+ other a/c)	21	137±47	N.S.

Table V-14 (cont.)

ᵘPatients ingesting phenytoin in addition to other anticonvulsants. *Values compared with control, and **phenytoin-treated groups, using either Student's t-test or the parametric test for groups of unequal variance.

The immunoglobulin concentrations of patients within the phenytoin-treated group were tested to determine whether there were differences between males and females, or between patients taking phenytoin alone and patients taking it in combination with other anticonvulsants (a/c). These results are shown in Table V-15.

TABLE V-15

COMPARISON OF IMMUNOGLOBULIN CONCENTRATIONS IN PHENYTOIN SUBGROUPS

Group	No.	IgG	No.	IgA	No.	IgM
Control (male)	48	1210±297	48	212±76	51	163±74
Phenytoin (male)	36	1287±412	37	*150±95	36	ᵘ126±48
Control (female)	37	1161±326	39	180±75	38	161±63
Phenytoin (female)	27	1150±288	26	**90±61	26	151±76
Phenytoin	1160±338(20/17)		125±80(19/17)		139±60(19/17)	
Several a/c	1324±392(16/10)		109±72(18/9)		132±66(17/9)	

*P < 0.005 **P < 0.001 ᵘP=0.0012

The number of males/females is shown in parentheses.

IgA was significantly lower in females taking phenytoin than in males (P < 0.01). However, the means were significantly

low in both sexes in comparison with the respective control groups (they were depressed by 29% in males, and 50% in females). IgM concentrations were significantly low in males treated with phenytoin, but not in females. There were no significant differences in the results of patients on phenytoin alone or phenytoin in combination with other anticonvulsants.

(3) Antinuclear antibody: This was present in the sera of 3% of patients treated with phenytoin, and none of the patients taking carbamazepine. Neither result was abnormal.

(4) Antibody responses: The numbers of patients who developed specific antibody two weeks after immunization with S. typhi are shown in Table V-16.

Specific antibody was not detected in the sera of a significant number of patients taking phenytoin. Five of the six patients with absent responses were treated with phenytoin alone, 4 were males and 2 were females.

TABLE V-16

ANTIBODY RESPONSES TO SALMONELLA TYPHI

Group	Responders (No.)	Non-responders (No.)	Significance #F.E.T.
Control	136	0	
Phenytoin	36	6	*P=0.00013
Carbamazepine	11	1	*P=0.0811 **P=0.3555
Pre-phenytoin	11	0	
Post-phenytoin	10	1	N.S.

#Fisher's exact test *Compared with control group, ** and phenytoin-treated group.

The numbers of patients who developed specific antibody two weeks after immunization with Tetanus toxoid are shown in Table V-17.

A significant number of patients in the phenytoin-treated and carbamazepine-treated groups failed to make detectable antibody. The results of the phenytoin- and carbamazepine-treated groups were not significantly different from each other. Five of the 10 phenytoin-treated patients with absent antibody responses were treated with phenytoin alone, 4 were males, and 6 were females.

TABLE V-17

ANTIBODY RESPONSES TO TETANUS TOXOID

Group	Responders (no.)	Non-responders (no.)	Significance
Control	137	1	
Phenytoin	33	10	*P < 0.00001
Carbamazepine	10	2	*P= 0.0167
			**P= 0.2885

*Compared with control, and **phenytoin-treated groups by Fisher's exact test.

(5) Delayed hypersensitivity reactions: The number of patients reacting to at least one antigen (reactors) and the number failing to react to any antigen (non-reactors) are shown in Table V-18.

TABLE V-18

REACTIVITY TO AT LEAST ONE INTRADERMAL ANTIGEN

Group	Reactors	Non-reactors	Significance
Control	93	1	
Phenytoin	46	3	*P=0.1037
Carbamazepine	14	1	*P=0.240
			**P=0.435

*Compared with control, and **phenytoin-treated groups by Fisher's exact test.

There were no significant differences between control, phenytoin-treated and carbamazepine-treated groups.

The total numbers of positive and negative reactions in each group is shown in Table V-19.

TABLE V-19

DELAYED HYPERSENSITIVITY REACTIONS

Group	Total tests	Reactions (no.)	Reactions (% positive)	Significance
Control	293	220	75	
Phenytoin	139	85	61	*P < 0.005
Carbamazepine	45	26	58	*P < 0.025
				**P = 0.128
Pre-phenytoin	16	16	100	
Post-phenytoin	10	6	63	P=0.0088

*Compared with controls by Chi-square test with Yates' correction, and with **phenytoin-treated patients by Fisher's exact test.

The total number of positive reactions was significantly decreased in both groups of epileptic patients in comparison with the control group. They were not significantly different from one another. Depressed reactions were found in both the male and female phenytoin-treated patients in comparison with their respective controls but not with each other (P=0.0641), Table V-20.

TABLE V-20

DELAYED HYPERSENSITIVITY REACTIONS IN PHENYTOIN SUBGROUPS

Group	Total tests	Reactions (no.)	Reactions (% positive)	Significance
Control (male)	165	122	74	
Phenytoin (male)	87	46	53	P=0.00043
Control (female)	128	98	77	
Phenytoin (female)	62	39	63	P=0.0204
Phenytoin	92	57	62	
Several a/conv.	57	28	49	P=0.042

There were significantly more negative reactions in the group of patients taking more than one anticonvulsant, (P=0.042, Fisher's exact test), than in patients on phenytoin alone (Table V-20.)

(6) Lymphocyte counts: The mean values and standard deviations (cells/µl of blood) for the respective groups are shown in Table V-21.

TABLE V-21

CIRCULATING LYMPHOCYTE COUNTS

Group	No.	Lymphocyte Ct.	Significance
Control	83	1926±430	
Phenytoin	49	1909±522	*N.S.
Carbamazepine	13	1504±486	*P < 0.005
			**P < 0.02

*Compared with control and **phenytoin-treated patients, (Student's t-test).

Lymphocyte counts were depressed only in the carbamazepine-treated patients. Within the phenytoin-treated group, counts were normal in both the male and female patients, and in patients taking phenytoin alone or in combination with other anticonvulsants, see Table V-22. (p.117).

(7) Circulating leukocyte DNA synthesis: The mean values and standard deviations of the three groups (expressed as $\log_{10}$ dpm/culture) are shown in Table V-23. The distribution of results (dpm/culture) is shown in Figure V-4. (For Table V-23, see p.117).

There was a significant depression of DNA synthesis in the female patients treated with phenytoin (Wilcoxon's Rank sum test, $P < 0.05$) but not in the male patients. The results of the patients on phenytoin alone (14 males and 10 females) were no different from those of the patients on several anticonvulsants (9 males and 6 females, Wilcoxon's Rank sum test).

FIGURE V-4

DNA SYNTHESIS IN CIRCULATING LEUKOCYTES

Closed circles represent male subjects, open circles represent female subjects. Medians for each group are shown by horizontal bars.

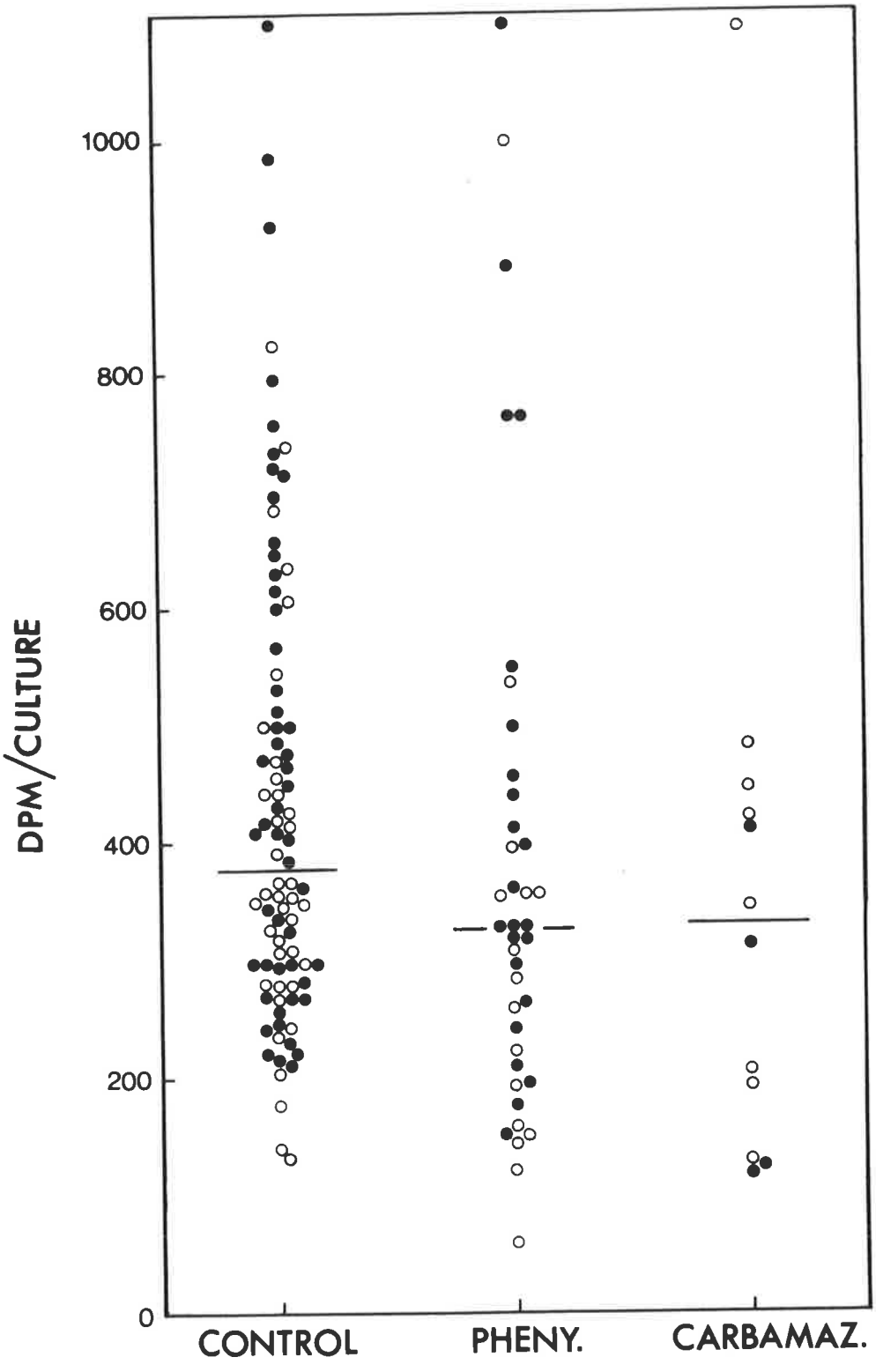

DPM/CULTURE
1000
800
600
400
200
0
CONTROL
PHENY.
CARBAMAZ.

TABLE V-22

LYMPHOCYTE COUNTS IN PHENYTOIN SUBGROUPS

Group	No.	Lymphocyte Ct.	Significance
Control (male)	41	1887±407	
Phenytoin (male)	28	2000±577	N.S.
Control (female)	42	1964±449	
Phenytoin (female)	21	1783±407	N.S.
Phenytoin	28	2010±529	
Several a/c	21	1760±473	N.S.

TABLE V-23

DNA SYNTHESIS IN CIRCULATING LEUKOCYTES

Group	No.	DNA Synthesis	Significance
Control	90	2.5922±0.1949	
Phenytoin	39	2.4939±0.2638	*P < 0.025
Carbamazepine	12	2.4842±0.2931	*N.S.
			**N.S.

*Compared with control, and **phenytoin-treated patients.

(8) PHA-induced DNA synthesis: PHA-induced lymphocyte transformation (DNA synthesis) was measured in medium supplemented with autologous serum (10%) and in medium supplemented with FCS (10%). Mean DNA synthesis (dpm/culture x 10^3) in the presence of autologous serum is shown in Table V-24; the distribution of results is shown in Figure V-5.

FIGURE V-5

PHA-INDUCED DNA SYNTHESIS IN AUTOLOGOUS SERUM AND FCS

Closed circles represent male subjects, open circles represent female subjects.

Means are shown by horizontal bars.

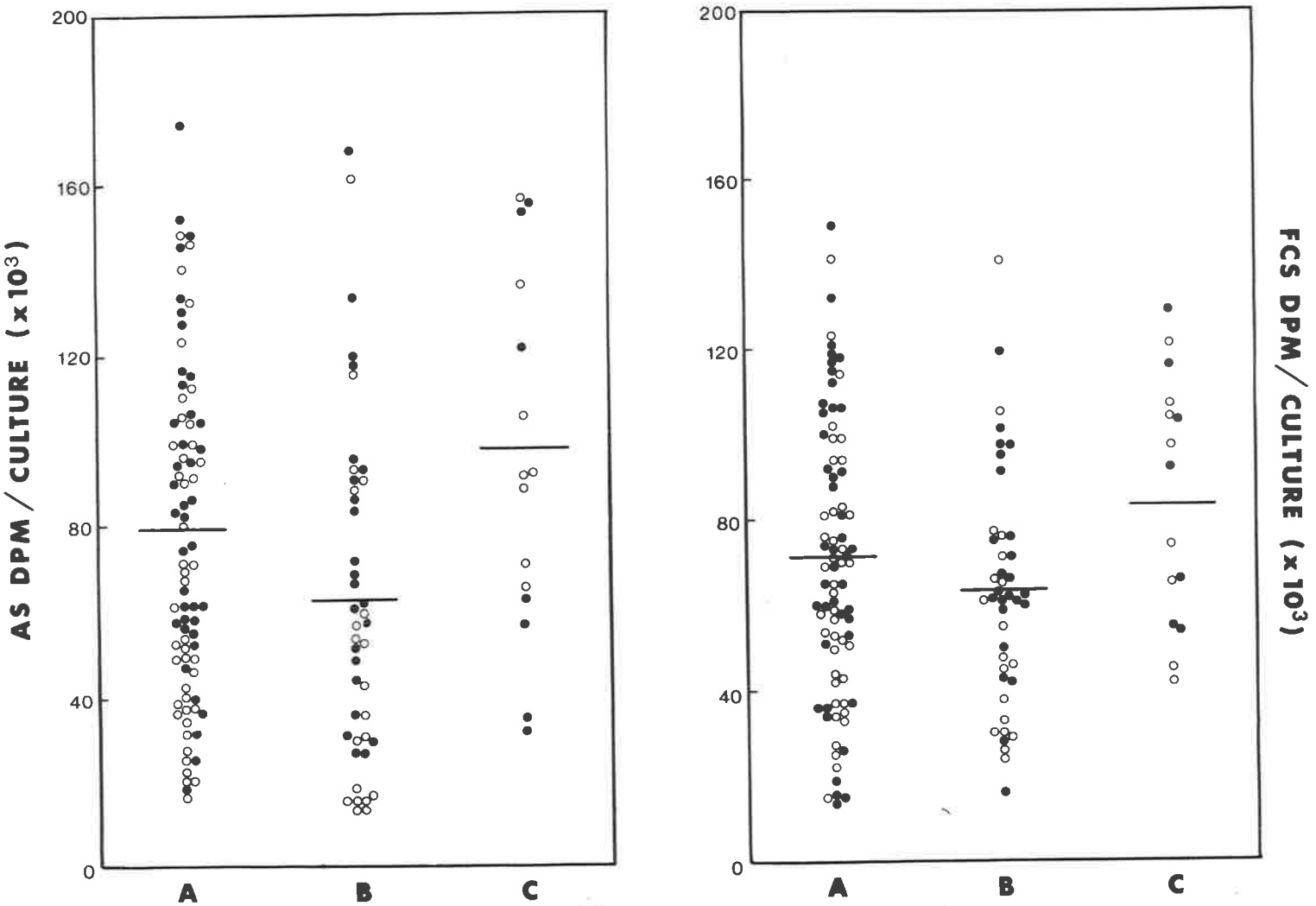

AS DPM / CULTURE (x10³)
200
160
120
80
40
0
A
B
C
FCS DPM / CULTURE (x10³)
200
160
120
80
40
0
A
B
C

TABLE V-24

PHA-INDUCED DNA SYNTHESIS (AUTOLOGOUS SERUM)

Group	No.	DNA Synthesis	Significance
Control	83	78.5±39.0	
Phenytoin	43	62.0±39.6	*P < 0.05
Carbamazepine	15	95.7±41.5	* N.S.
			**P< 0.01

*Compared with control, and **phenytoin-treated patients.

DNA synthesis was depressed in phenytoin-treated patients in comparison with the control, and carbamazepine-treated groups. The mean of the carbamazepine-treated group was not significantly different from normal. Within the phenytoin-treated group, the depression of DNA synthesis was confined to the female patients. There was no significant difference in the results of patients taking phenytoin alone (13 males and 11 females) and patients taking it with other anticonvulsants (11 males and 8 females), see Table V-25.

TABLE V-25

PHA-INDUCED DNA SYNTHESIS IN PHENYTOIN SUBGROUPS

Group	No.	DNA Synthesis	Significance
Control (male)	41	85.6±36.6	
Phenytoin (male)	24	75.7±39.7	N.S.
Control (female)	42	68.4±37.5	
Phenytoin (female)	19	44.7±30.9	P < 0.02
Phenytoin	24	60.6±38.5	
Several a/c	19	63.8±40.0	N.S.

Mean DNA synthesis in the presence of FCS (dpm/culture x 10^3) is shown in Table V-26; the distribution of results is shown in Figure V-5.

TABLE V-26

PHA-INDUCED DNA SYNTHESIS (FCS)

Group	No.	DNA Synthesis	Significance
Control	84	70.7 ± 31.2	
Phenytoin	43	63.1 ± 26.4	*N.S.
Carbamazepine	15	84.7 ± 28.3	*N.S.
			**P < 0.01

*Compared with control, and **phenytoin-treated groups.

DNA synthesis in the phenytoin-treated and carbamazepine-treated patients was not significantly different from that in the control group; values in the phenytoin-treated patients were, however, lower than those of the patients taking carbamazepine. Within the phenytoin-treated group, the results were independent of the sex of the patient and the use of anticonvulsant drugs in addition to phenytoin, see Table V-27.

TABLE V-27

PHA-INDUCED DNA SYNTHESIS (FCS, PHENYTOIN SUBGROUPS)

Group	No.	DNA Synthesis	Significance
Phenytoin (males)	24	67.6 ± 23.5	
Phenytoin (females)	19	55.9 ± 29.1	*N.S.
Phenytoin	24	62.9 ± 24.7	
Several a/conv.	19	61.5 ± 29.7	N.S.

*N.S. = Not significant

(9) Extent of immunological abnormality in phenytoin-treated and carbamazepine-treated patients: One or more immunodeficiencies were found in 30 of 50 phenytoin-treated patients, 7 of 15 carbamazepine-treated patients, and 7 of 87 control subjects, using the following parameters - serum levels of IgG, IgA, IgM and complement, antibody responses to S. typhi and Tetanus toxoid, DHS reactivity, PHA-induced DNA synthesis in autologous serum and foetal calf serum, and DNA synthesis in circulating leukocytes. Differences in the immunological status of males and females treated with phenytoin are shown in Table V-28.

TABLE V-28

IMMUNOLOGICAL STATUS OF THE PHENYTOIN GROUP

Group	Normal (No.)	Abnormal (No.)	Significance
Males	14	16	
Females	6	14	P=0.1196
Total	20	30	

Sixty percent of phenytoin-treated patients (70% of females and 53% of males) manifested at least one abnormality of immunological function. The differences between males and females were not statistically significant (Fisher's exact test). Similarly, there were no significant differences between the numbers of males and females with depression of humoral immunological function or depression of cellular immunological function. All tests were made in 39 hospital patients (Table V-29).

TABLE V-29

IMMUNOLOGICAL ABNORMALITIES IN PHENYTOIN-TREATED PATIENTS

Parameter	Normal (No.)	Abnormal (No.)	Abnormal (%)
IgG	39	0	0
IgA	29	10	26
IgM	38	1	3
C3 component of complement	39	0	0
S. typhi antibody response	33	6	15
Tetanus toxoid antibody response	29	10	26
ANF	39	0	0
DHS reactions	37	2	5
PHA-induced DNA synthesis by lymphocytes in autologous serum	32	7	18
PHA-induced DNA synthesis by lymphocytes in foetal calf serum	38	1	3
Circulating lymphocyte DNA synthesis	36	3	8

Deficiencies of IgA and Tetanus antibody responses were especially common. No particular pattern was evident in the immunosuppressed population, although there were positive correlations between PHA-induced DNA synthesis in autologous serum and in FCS ($P < 0.001$) and between PHA-induced DNA synthesis

in FCS and DNA synthesis in circulating leukocytes ($P < 0.01$). Analysis did not suggest that immunological deficiencies were confined to a definite subgroup of the patient sample. Cellular and humoral deficiencies were present together in 4 of the 22 patients with immunological abnormalities (18%).

(10) Serum phenytoin concentration: Mean values in males and females treated with phenytoin are shown in Table V-30.

TABLE V-30

SERUM PHENYTOIN CONCENTRATION

Group	No.	Serum phenytoin (mg/100ml)	Significance
Males	34	0.85 ± 0.72	
Females	20	0.84 ± 0.77	N.S.

There was no significant difference between the results in males and females, (Wilcoxon's Rank sum test). The serum phenytoin concentration of females with at least one immunological defect was also compared with that of the corresponding males. Again the differences were not significant (Wilcoxon's Rank sum test).

(11) Relationships between serum phenytoin concentration and parameters of immunological function: Patients were divided into three groups on the basis of their serum phenytoin concentration, see Table V-31. (p.123).

The numbers of patients in whom all immunological parameters were normal were compared with those in whom there were one or more abnormalities.

TABLE V-31

CORRELATION BETWEEN SERUM PHENYTOIN CONCENTRATION AND DEFECTS OF IMMUNOLOGICAL FUNCTION

Phenytoin (mg/100ml)		Normal	Abnormal
1.	0.00–0.99	16	17
2.	1.00–1.99	6	3
3.	2.00+	1	3
	Total	25	23

Groups 1 and 2 were compared with group 3, and group 1 was compared with groups 2 and 3, by Fisher's exact test. In each case, the differences were not significant.

Attempts were also made to correlate serum phenytoin concentration with individual parameters of immunological function.

(a) Parameters with continuous distributions: The correlation coefficient, r, was calculated for each normally distributed parameter, and is shown in Table V-32. (p.124.)

There was a negative correlation between serum phenytoin levels and circulating lymphocyte counts, see Figure V-6.

FIGURE V-6

RELATIONSHIP BETWEEN SERUM PHENYTOIN AND

CIRCULATING LYMPHOCYTE COUNT

The linear relationship is given by the equation

$$y = 2340 - 37\,x$$

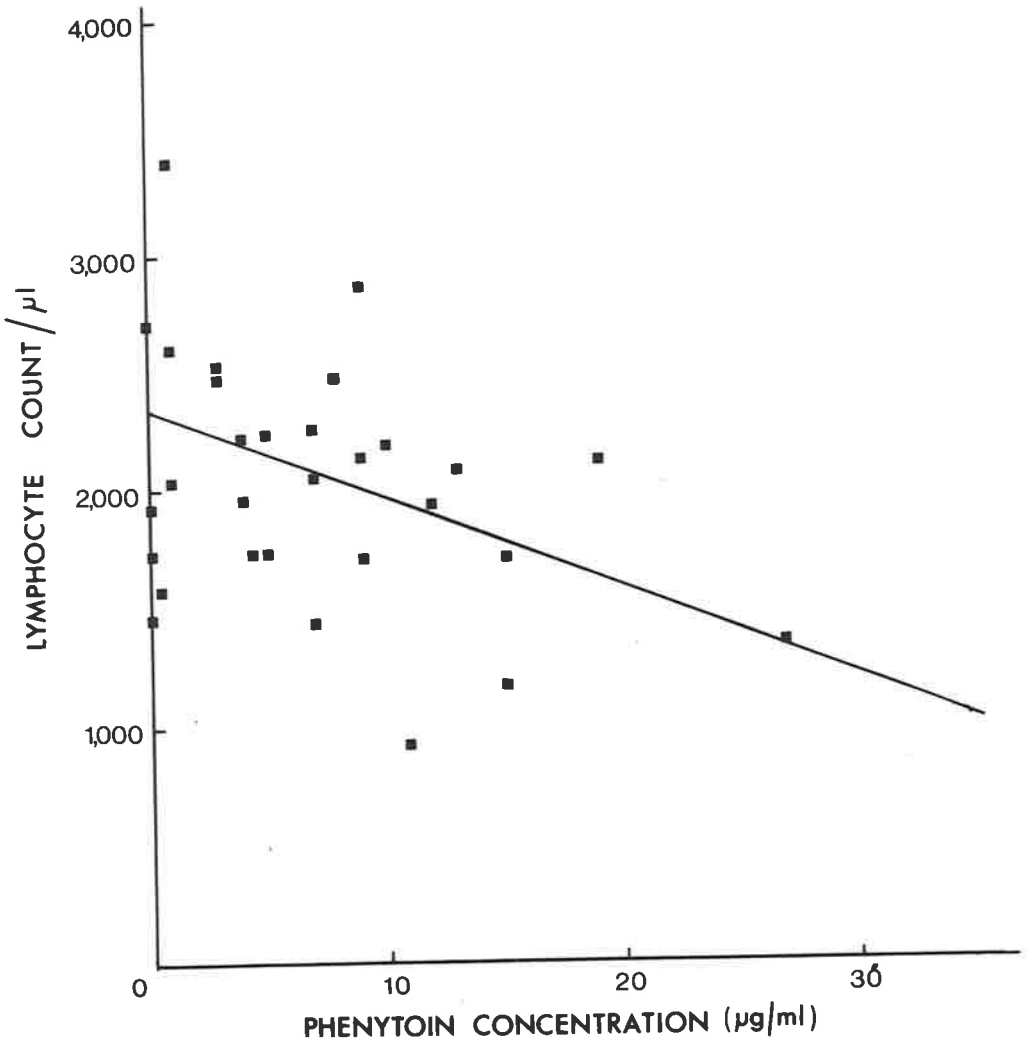

4,000
3,000
2,000
1,000
0
LYMPHOCYTE COUNT /μl
10
20
30
PHENYTOIN CONCENTRATION (μg/ml)

TABLE V-32

CORRELATIONS BETWEEN SERUM PHENYTOIN CONCENTRATION AND PARAMETERS OF IMMUNOLOGICAL FUNCTION

Parameter	No.	r	Significance
IgG	34	-0.094	N.S.
IgA	33	-0.204	N.S.
IgM	33	-0.094	N.S.
Complement	27	-0.134	N.S.
Lymphocyte count	32	-0.356	$P < 0.05$
Transformation (FCS)	29	-0.150	N.S.
Transformation (AS)*	29	-0.119	N.S.
Leukocyte DNA	36	-0.162	N.S.

*Autologous serum

(b) Non-continuous distributions:

(i) Delayed hypersensitivity reactions: Patients were divided into three groups on the basis of serum phenytoin concentrations. The total numbers of positive and negative reactions were compared using 2 x 2 tables, and Fisher's exact test, see Table V-33. (p.125.)

Groups 2 and 3 combined did not differ significantly from group 1.

(ii) Antibody responses: The relationships between serum phenytoin concentrations and antibody responses were not determined because of the small number of non-responders in each range of phenytoin concentrations.

TABLE V-33

THE RELATIONSHIP BETWEEN SERUM PHENYTOIN CONCENTRATION AND DELAYED HYPERSENSITIVITY REACTIONS

Phenytoin (mg/100ml)	Total tests	Reactions (no.)	Reactions (% positive)	Significance
0.00-0.99	90	53	59	
1.00-1.99	27	14	52	*N.S.
2.00+	12	6	50	*N.S.

*Compared with Group 1.

(12) Relationship between phenytoin dosage and the presence of immunological defects: The relationship between phenytoin dosage and the presence of immunological defects is shown in Table V-34. Thirty-nine of the fifty patients were taking phenytoin in a dose of 300 mg/day; of these patients there were no abnormalities identified in 43%. Humoral defects alone were present in 43%, cellular defects alone, in 8%, and combined humoral and cellular defects, in 6%.

TABLE V-34

RELATIONSHIP BETWEEN PHENYTOIN DOSAGE AND THE PRESENCE OF IMMUNOLOGICAL ABNORMALITIES

Dosage (mg/day)	Normal (No.)	Abnormal (No.)	Significance
200	2	3	
300	17	22	
400	1	5	*P=0.1794

*Compared with combined groups 1 and 2 by Fisher's exact test. The differences were not significant.

(13) Relationship between the duration of phenytoin therapy and the presence of immunological defects: Comparisons were made between the responses of patients treated with phenytoin for more than 12 months, and less than 12 months, for more than, and less than 6 months, and for more than, and less than 3 months. The results of the latter two comparisons are shown in Table V-35.

The duration of phenytoin therapy (Rx) did not correlate with the presence of immunological defects.

TABLE V-35

RELATIONSHIP BETWEEN THE DURATION OF PHENYTOIN THERAPY AND THE PRESENCE OF IMMUNOLOGICAL ABNORMALITIES

Duration Rx. (months)	Normal (no.)	Abnormal (no.)	Significance
Less than 6	9	10	
More than 6	11	20	*P=0.1661
Less than 3	8	7	
More than 3	12	23	*P=0.1013

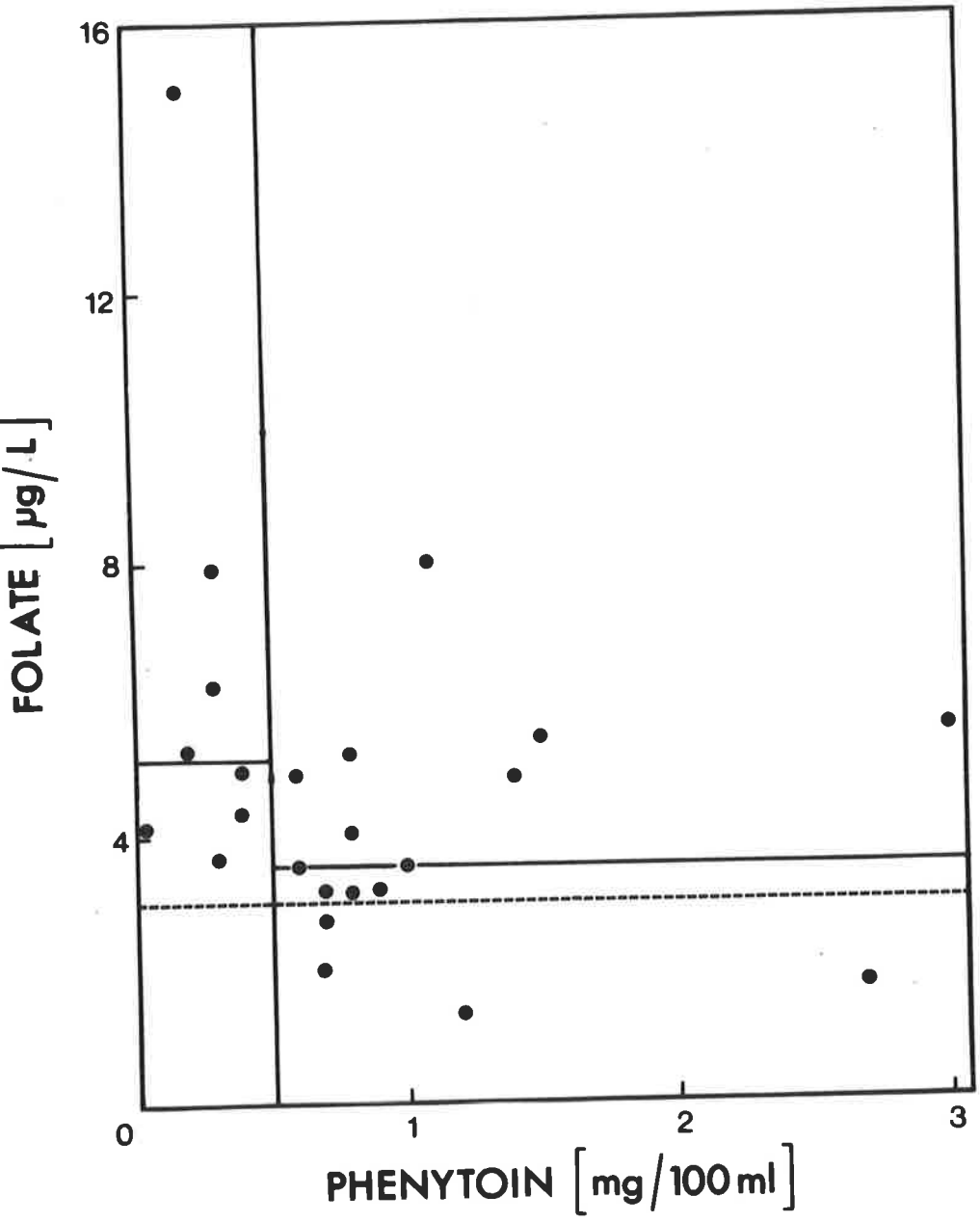
*Compared by Fisher's exact test.

(14) Serum folate concentration: Serum folate concentrations were measured in 24 of the phenytoin-treated patients, and are shown in Figure V-7. Levels were depressed (less than 3.0 µg/L) in 4 patients. The results of patients with serum phenytoin concentrations less than 0.5 mg/100ml were significantly higher than those of patients with phenytoin levels greater than 0.5 mg/100ml ($P < 0.05$, Wilcoxon's Rank sum test). The immuno-

FIGURE V-7

RELATIONSHIP BETWEEN SERUM PHENYTOIN AND SERUM FOLATE CONCENTRATIONS

Horizontal bars represent median values for the ranges of serum phenytoin enclosed by the vertical lines.


16
12
8
4
0
1
2
3
FOLATE [µg/L]
PHENYTOIN [mg/100 ml]

logical responsiveness of patients with folate concentrations less than 3.0 µg/L was compared with that of patients with folate concentrations greater than 3.0 µg/L, see Table V-36.

TABLE V-36

RELATIONSHIP BETWEEN SERUM FOLATE AND IMMUNOLOGICAL DEFECTS

Folate (µg/L)	Normal (no.)	Abnormal (No.)	Significance
Less than 3.0	1	3	
3.0 +	10	10	P=0.2961

The differences were not significant (Fisher's exact test).

(15) Measurement of immunological function before and after the commencement of therapy with phenytoin: The results obtained from 11 patients tested before and at least two months after the commencement of phenytoin therapy are summarized in Appendix (x).

Immunoglobulin concentrations (Figure V-8) and lymphocyte counts were compared in 10 patients by Wilcoxon's test for paired samples. There was a significant depression of IgA after the commencement of therapy ($P < 0.01$). IgG, IgM and lymphocyte counts were unaltered. One patient, who developed a primary antibody response to S. typhi immediately before taking phenytoin, did not make antibody on rechallenge 2 months later (i.e. at rechallenge no antibody was detected in the pre- and post-immunization sera.) This depression of responsiveness was not statistically significant.

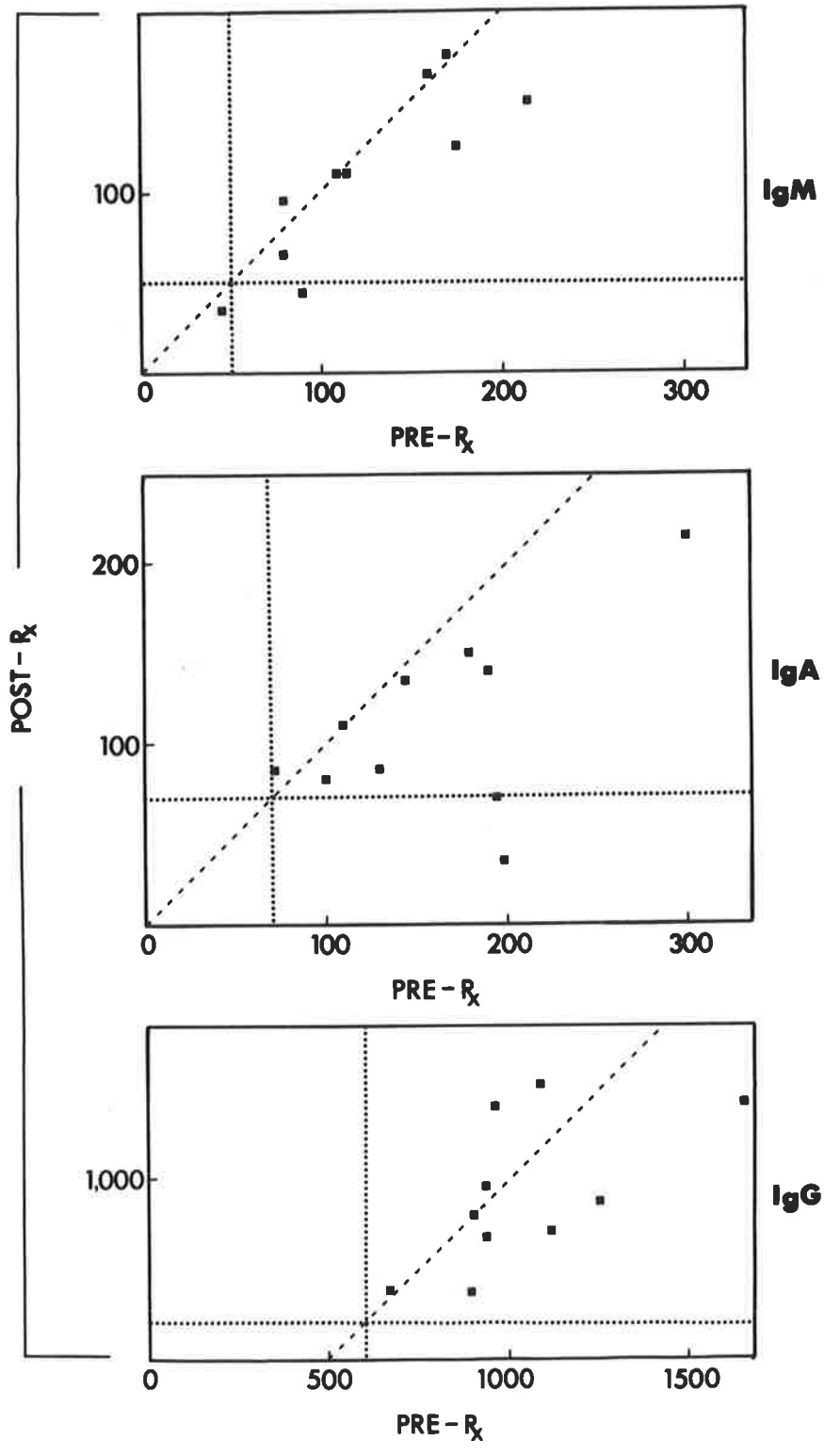
FIGURE V-8

THE EFFECT OF PHENYTOIN ON SERUM IMMUNOGLOBULIN LEVELS

Dotted lines represent the lower limits of normal ranges established from the control population described in Chapter II.

Dashed lines represent the hypothetical line obtained if serum Ig's remained constant before and during phenytoin therapy.

Serum Ig concentrations were measured in mg/100 ml.


POST - Rx
100
0
100
200
300
PRE - Rx
IgM
200
100
0
100
200
300
PRE - Rx
IgA
1,000
0
500
1000
1500
PRE - Rx
IgG

The total number of positive DHS reactions obtained in 10 untreated patients was significantly higher than the number of reactions to the same antigens, measured after the commencement of therapy (P=0.0088). One patient, who reacted to candida and mumps antigens immediately before taking phenytoin, did not react 12 months later.

3. DISCUSSION

These data indicate that depression of cellular and/or humoral immunity is common in patients treated with phenytoin sodium, and that these defects develop after the commencement of therapy. At least one abnormality was found in 60% of patients (70% of female and 53% of male patients) in a series of hospital-treated epileptics, and in 40% of a group of intellectually retarded (I.R.), institutionalized patients. Immunological function was also measured in 2 small groups of patients, one treated with the anticonvulsant carbamazepine, and one with phenobarbitone or primidone (which is partly metabolized to barbiturate (Gallagher and Baumel, 1972), as lymphoma has not been reported in association with these drugs. Immunological defects were found in some carbamazepine-treated patients but in lower frequency than in the phenytoin-treated group. Therapy with barbiturates was not associated with immunodeficiency.

Phenytoin-treated patients failed to manifest DHS reactions to common antigens, and to make antibody to S. typhi and Tetanus toxoid. Serum levels of IgG, IgA, and IgM, DNA synthesis in circulating leukocytes, and PHA-induced transformation in autologous

serum were also low. There were differences in the pattern of immunological deficiencies in different categories of patients. Serum concentrations of IgG were low in the I.R. patients but not in the hospital group. However, Tetanus antibody production was not deficient in the I.R. patients (this is a predominantly IgG response in the study population, (Forbes, 1971)). This was a frequent abnormality in the hospital patients. IgA deficiency was also more frequent in the hospital patients (whose mean daily dosage of phenytoin was higher than that of the I.R. group). This abnormality suggests the possibility of a local effect of phenytoin on the lymphoid tissue of the gastrointestinal tract, which is a major site of synthesis of IgA (Hobbs, 1971). Delayed hypersensitivity reactivity was depressed to a greater extent in the I.R. patients than in the hospital patients taking phenytoin.

Phenytoin appeared to accentuate trends in sex differences in several immunological parameters. IgA levels were depressed to a greater extent in females than in males in the hospital series; IgM was low only in males. These are accentuations of reported sex differences (Buckley and Dorsey, 1971). Depression of PHA-induced DNA synthesis, and the rate of DNA synthesis in circulating leukocytes was observed only in females, accentuating trends found in the control series of the present study. The reason for the differences in the manifestations of phenytoin-induced immunosuppression in institutionalized I.R. patients and general hospital-treated patients is not clear. The underlying epilepsy was more severe in the I.R. patients.

It has been suggested that epilepsy may itself be associated with immunodeficiency. Van Rootselaar and Westendorp Boerma (1968) studied the immunoglobulin concentrations of 21 institutionalized, epileptic children. They reported that the mean IgA concentration was significantly depressed in 10 patients with inborn epilepsy, but not in 11 patients with acquired (post-traumatic) epilepsy. However, details of therapy were not given. These conclusions were not supported by the present study, in which immunological defects were shown to develop after the commencement of treatment. There were also differences in the daily dosage of phenytoin and the duration of therapy in the I.R. patients and the general hospital patients treated with phenytoin. Analysis of the results obtained in the hospital patients showed that the presence of abnormalities was independent of serum concentration of phenytoin, the duration of therapy, the sex of the subject, and the use of additional anticonvulsants, although the type of abnormality (i.e. cellular or humoral defect) did vary with the sex of the patient, and in the case of DHS reactivity, with the use of anticonvulsants in addition to phenytoin. The reason for the latter result is not clear. Analysis of the results obtained in the I.R. patients suggested that the type of abnormality was related to dosage, and to serum concentration of the drug. Patients treated with low doses of phenytoin (100 mg/day) were more likely to manifest deficiencies of cell-mediated immune responses than patients treated with doses of 300 mg/day. The latter patients had a higher proportion of humoral defects. This trend was not seen in the general hospital patients, most of whom

were on a dose of 300 mg/day, although the percentage of patients with humoral defects was similar to that of the I.R. patients taking the same dosage. Studies in animals treated with 6-mercaptopurine have shown that low doses of drug can cause selective depression of DHS reactions (Borel and Schwartz, 1964), and that higher doses can depress antibody responses in different immunoglobulin classes (Schwartz, 1965, 1967). It is likely that differences in the dosage of phenytoin contributed to the differences in the types of immunological defects identified in the I.R. and hospital-treated patients.

Nutrition may also be an important factor in determining the type of phenytoin-induced immunodeficiency. Malnutrition severely affects immunological capacity, particularly cell-mediated immune responses (Smythe et al, 1971). Serum folate concentrations may be low in patients receiving anticonvulsant therapy (Klipstein, 1964) especially if the dietary intake of folate is low, as is often the case in institutionalized patients (Ibbotson et al, 1967). It was shown in the present study, however, that immunological deficiency was not related directly to serum folate concentrations; these were low in the I.R. patients receiving either phenytoin or barbiturates (immunological function being normal in the barbiturate-treated group). However, folic acid is required as a coenzyme for nucleic acid synthesis and phenytoin could interfere with its action in the pathway of DNA biosynthesis. This possibility is considered in Chapter VI.

Independent studies by two groups have recently confirmed that phenytoin therapy is associated with partial immunodeficiency. Grob and Herold (1972) studied 20 patients who had received phenytoin

for at least three years. Lymph nodes of two patients were clinically enlarged at the time of study. They found a deficiency of circulating IgA, failure to develop antibody to polyvalent influenza virus, and depression of DHS reactivity. They also reported a significant depression of the C3 component of complement, and an increased incidence of antinuclear factor in the sera of these patients, results which were not confirmed in the present study. Sensitivity to phenytoin (50 µg/ml) was not detected (by patch-testing) in any patients. IgG levels were found to be low in 22 phenytoin-treated patients in whom IgG, IgA and IgM were measured (MacKinney and Booker, 1972). These workers also found that lymphocyte counts were low in a group of 66 patients, particularly when the serum concentration was greater than 2.0 mg/100 ml. The finding of a negative correlation between serum phenytoin concentration and lymphocyte count in the present study confirms these data.

PHA-induced DNA synthesis was depressed in cultures containing autologous serum but not foetal calf serum. DNA synthesis in circulating leukocytes was also depressed. These results are consistent with the presence of an inhibitory factor in the serum of phenytoin-treated patients. This factor is probably phenytoin itself; it is known to enter cell nuclei (Kemp and Woodbury, 1971) and may thereby affect DNA synthesis directly. The depression of DNA synthesis was observed in the presence of total phenytoin concentrations of not greater than 2 µg/ml, and usually less than 1 µg/ml. Serum concentrations of phenytoin were below the toxic level of

2.0 mg/100 ml (20µg/ml) in all but 7 patients in the combined I.R. and hospital series. Of these 7 patients, immunological defects were present in 5. MacKinney and Booker found that lymphocyte counts were depressed in patients with serum phenytoin concentrations greater than 2.0 mg/100 ml. It seems likely that high concentrations of phenytoin are immunosuppressive in most subjects. The relationship between phenytoin-induced immunosuppression and the development of lymphoma is considered in Chapter VII.

4. SUMMARY

(a) Depression of cellular and humoral immune responses was found in 40% of institutionalized I.R. patients, in 60% of general hospital patients treated with phenytoin, and in 47% of hospital patients treated with carbamazepine. Responses were normal in I.R. patients treated with phenobarbitone or primidone.

(b) IgG, DHS reactions to common antigens and PHA-induced lymphocyte transformation (blastogenesis) were depressed in the I.R. patients. IgA, IgM, antibody responses to S. typhi and to Tetanus toxoid, DHS reactions, PHA-induced DNA synthesis, and DNA synthesis in circulating leukocytes were depressed in the hospital patients. Depression of IgM was confined to males; depression of PHA-induced DNA synthesis and DNA synthesis in circulating leukocytes were present only in females in the hospital series. Except for DHS reactivity, which was more depressed in patients on several anticonvulsants, immunological parameters were significantly depressed in patients on phenytoin

alone, and in patients on phenytoin plus other anticonvulsants.

(c) Antinuclear antibody was not detected in the sera of a significant number of phenytoin-treated patients.

(d) Depression of IgA, DHS reactivity, and probably antibody responsiveness to S. typhi, was shown to develop after the commencement of phenytoin therapy.

(e) The presence of immunological defects was independent on the dosage of drug, its serum concentration, the duration of therapy, and the sex of the subject, although the type of defect (cellular or humoral) varied with the sex of the subject, and there was a negative correlation between serum phenytoin concentration and lymphocyte counts. Defects of cell-mediated immune function were more common in patients taking relatively low doses of phenytoin.

(f) Phenytoin therapy was associated with a general depression of IgA in hospital patients. Depression of IgM, antibody responses, PHA-induced DNA synthesis and DNA synthesis in circulating leukocytes occurred in separate subgroups of the patient sample.

(g) There was no correlation between serum folate levels and the presence of immunological defects. Folate concentrations were low in I.R. patients treated with phenytoin, or barbiturate, but not in general hospital patients.

CHAPTER VI

IN VITRO STUDIES OF PHENYTOIN AND OTHER ANTICONVULSANTS

1. INTRODUCTION

In 1962, McIntyre and Ebaugh incubated PHA-stimulated leukocytes from three patients with different concentrations of phenytoin, and demonstrated that the drug caused depression of the incorporation of ^{32}P into DNA. Ling (1968) reported that phenytoin did not inhibit DNA synthesis in leukocyte cultures stimulated with Staphylococcal filtrate. In the present study, PHA-induced DNA synthesis was depressed in blood cell cultures from phenytoin-treated patients in the presence of autologous serum but not foetal calf serum, (see Chapter V). The effect of phenytoin on nucleic acid and on protein synthesis was therefore studied in vitro.

2. THE EFFECT OF PHENYTOIN ON DNA SYNTHESIS

(a) Method

(1) Cell cultures: Blood cell cultures were prepared in triplicate as described in Chapter II. Phenytoin for intravenous use (Parke Davis, Michigan) 250 mg, was dissolved in a sterile solution (5 ml) of propylene glycol (40%), ethyl alcohol (10%) and distilled water (50%), adjusted to pH 12 with sodium hydroxide, and diluted to the appropriate strength. Defined concentrations of drug in a volume of 0.01 ml were added to triplicate culture tubes and mixed immediately with reconstituted PHA (0.02 ml) in medium 199 (2.98 ml). Control cultures contained drug solvent (0.01 ml). Blood suspension (0.2 ml

in FCS, 0.4 ml, and medium 199, 0.4 ml) was added separately. Cultures were resuspended daily during incubation at 37°C. Phenytoin dose-response curves were also constructed for non-PHA-stimulated cultures. Cultures were processed at the times specified in the text, as described in Chapter II. ^{3}H-thymidine or ^{3}H-deoxyuridine (2.5 µCi, Specific activity 500 mCi/mMol), was added in a volume of 0.01 ml, 4 hours before the termination of cultures. Cultures were also prepared for the microscopic examination of stained smears.

(2) Cell counts: Ninety-six hour cultures were processed for the counting of cell nuclei (Appendix (vi)). The cytoplasm was stripped from the cultured cells by vigorous shaking with filtered counting fluid (18 ml) to which cetrimide solution (2 ml) had been freshly added. Nuclei were counted in a Coulter counter, Model A, current setting 7, threshold 15, orifice diameter 100 microns.

(3) Lymphocyte viability: This was estimated by the trypan blue dye exclusion technique described in Chapter III. The percentage of cells capable of excluding dye was determined by the microscopic examination of wet preparations from lymphocyte cultures after 72 hours of incubation. Purified lymphocyte cultures were studied because of the difficulty in obtaining an accurate measurement of viability with the small number of lymphocytes present in wet preparations of whole blood cultures.

(4) Phenytoin assay: Phenytoin was extracted from cell cultures by the method of Clarke (1969). Cultures were extracted with three successive amounts (5 ml) of solvent ether. The pooled extract was treated twice with sodium bicarbonate (5% w/v), three times with sodium hydroxide (1N), and combined with ether in a separating funnel. This preparation was acidified with hydrochloric acid (0.2 N) and two further volumes of ether were used to extract residual phenytoin from the aqueous phase. The ether extracts were evaporated to dryness, and dissolved in exactly 4 ml of anhydrous methanol. The absorption at 257 nM was read in an ultraviolet spectrophotometer.

(5) Stability of phenytoin in culture: Evidence of the stability of phenytoin was obtained by determination of the ultraviolet absorption pattern of drug extracted from duplicate PHA-stimulated cultures incubated for 1 and 96 hours with drug concentrations of 5, 10, 20, 40, and 62.5 µg/ml.

(6) Solubility of phenytoin in culture: Phenytoin (10 µg/ml and 62.5 µg/ml) was incubated with HEPES-buffered medium 199 for 96 hours at 37°C. At 0 and 96 hours, the mixture was centrifuged at 1500g for 30 minutes. Control mixtures were resuspended without centrifugation. Phenytoin was extracted from aliquots of supernatant (1 ml) and resuspended incubation mixture (1 ml), and assayed as above.

(b) Results

(1) Preliminary study: The effect of the phenytoin solvent (0.01 ml) on PHA-induced DNA synthesis was determined in blood

cell cultures from 4 healthy subjects (see Table VI-1).

TABLE VI-1

THE EFFECT OF PHENYTOIN SOLVENT ON LYMPHOCYTE DNA SYNTHESIS

DNA Synthesis (Control)	DNA synthesis (Solvent)
79.7	64.0
94.4	87.0
15.9	16.0
119.6	136.4
Mean *77.4 ± 38.3	75.9 ± 43.3

* dpm/culture

Phenytoin solvent did not affect PHA-induced DNA synthesis.

(2) Stability and solubility of phenytoin: Absorption of ultraviolet light at a wavelength of 257 nM was constant in cell cultures incubated with PHA and phenytoin for 1 and 96 hours, at all concentrations of drug. There was no change in the shape of the absorption curve, i.e. phenytoin remained stable in culture. Similarly, there was no difference between the results of centrifuged and non-centrifuged incubation mixtures of phenytoin and medium 199 measured at 0 and 96 hours, i.e. phenytoin remained in solution.

(3) Lymphocyte viability: The percentage of intact cells which excluded trypan blue dye is shown in Table VI-2 (p.139). Two counts of 100 cells were made on wet preparations from each culture.

TABLE VI-2

LYMPHOCYTE VIABILITY IN THE PRESENCE OF PHENYTOIN

Drug concentration (μg/ml)	Subject 1	Subject 2
0	93	91
10	94	96
20	93	94
40	94	93
62.5	93	93
125	96	94

Phenytoin was not cytopathic at concentrations of up to 125 μg/ml.

(4) The effect of phenytoin on cultured lymphocyte DNA synthesis and cell counts: (Figure VI-1) There was a concentration-dependent decrease in the incorporation of ^{3}H-thymidine and ^{3}H-deoxyuridine into DNA, which was significant at pharmacological concentrations of phenytoin (10-20 μg/ml, Buchthal and Svensmark, 1971).

At phenytoin concentrations above 40 μg/ml, there was a significantly greater depression of DNA synthesis in PHA-stimulated than in non-stimulated cultures.

Phenytoin concentrations above 40 μg/ml were associated with a depression of cell counts in PHA-stimulated cultures. Counts were compared in PHA-stimulated and non-stimulated cultures from 3 healthy subjects. Although there was a greater fall in the cell counts of PHA-stimulated cultures at phenytoin concentrations above 40 μg/ml, the difference was not significant (Student's t-test).

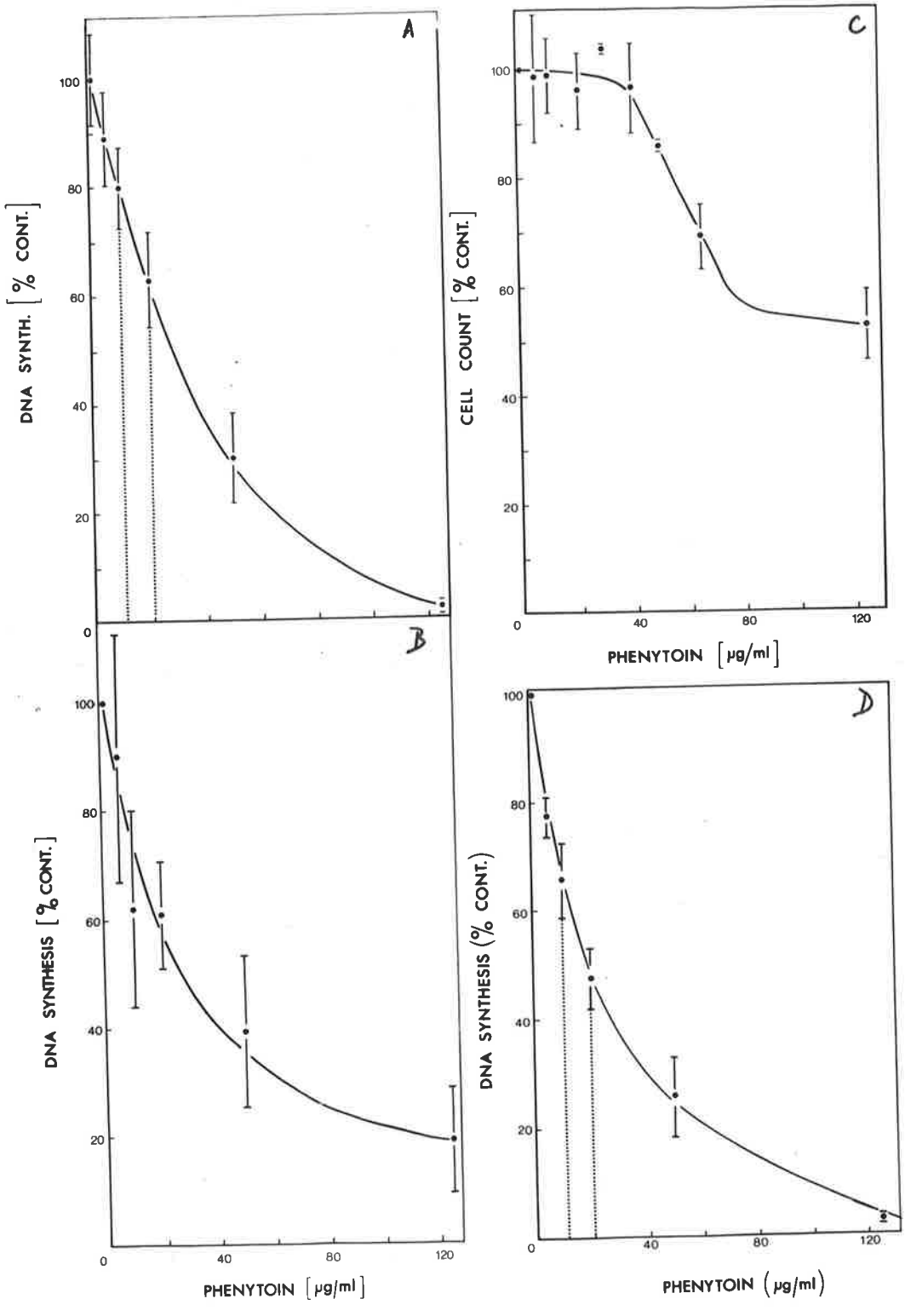
FIGURE VI-1

THE EFFECT OF PHENYTOIN ON CULTURED LYMPHOCYTE DNA SYNTHESIS AND CELL COUNTS

DNA synthesis and cell counts were expressed as a percentage of control values for each concentration of phenytoin. The incorporation of ^{3}H-thymidine into DNA was measured in PHA-stimulated cultures from 5 healthy adults and 2 patients treated with phenytoin (without evidence of side-effects of phenytoin therapy) (curve A), and in non-stimulated cultures from 3 healthy subjects and 6 patients taking phenytoin (curve B). The incorporation of ^{3}H-deoxyuridine into DNA was measured in PHA-stimulated cultures from 2 healthy adults, and one patient treated with phenytoin (curve D).

Cell counts were measured in PHA-stimulated cultures from the 5 healthy subjects and one patient treated with phenytoin used to obtain curve A (curve C).

Dotted lines represent the limits of the recommended therapeutic range of serum phenytoin concentrations.


A
B
C
D
DNA SYNTH. [% CONT.]
DNA SYNTHESIS [% CONT.]
CELL COUNT [% CONT.]
DNA SYNTHESIS (% CONT.)
PHENYTOIN [μg/ml]
PHENYTOIN (μg/ml)
0
20
40
60
80
100
120

(5) The effect of phenytoin on lymphocyte blastogenesis:

(Figure VI-2) There was a concentration-dependent decrease in PHA-stimulated lymphocyte blastogenesis at phenytoin concentrations above 30 µg/ml.

(6) Case study - phenytoin overdosage:

History: Mr. R.G., a 28 year old cleaner with a past history of schizophrenia, unpredictable behaviour, and numerous drug overdosages, presented to hospital after taking a 'handful' of phenytoin capsules. For three months prior to admission he had been treated with diazepam (8 mg/24 hrs) and phenytoin (300 mg/24 hrs). His serum phenytoin concentration on admission was 56 µg/ml.

Investigation: Serum samples were obtained at 0, 3, 24 hours, and 20 days after admission. Phenytoin levels were measured by the technique of Svensmark and Kristensen (1963). Serum was stored at -20°C until day 20, when blood was obtained from the patient for culture. Sera were tested for their ability to inhibit DNA synthesis in 96 hour cultures of PHA-stimulated blood cells. Phenytoin levels ranged from 0.6-18.4 µg/ml in cultures containing 10% or 30% serum. Increments of commercially available phenytoin in the same concentration range (5-20 µg/ml), or phenytoin solvent, were added to cultures of the patient's cells, containing PHA and 30% FCS. DNA synthesis was measured by the incorporation of ^{3}H-thymidine into acid-precipitable DNA.

Results: (Figure VI-3) There was a similar, concentration-dependent decrease in DNA synthesis in cultures containing

FIGURE VI-2

THE EFFECT OF PHENYTOIN ON LYMPHOCYTE BLASTOGENESIS

The percentage of transformed lymphocytes was determined by the microscopic examination of stained smears. The results of 3 counts, each of 100 lymphocytes, were averaged. This value was obtained for each phenytoin concentration and expressed as a percentage of that of the control culture. Figure VI-2 shows the mean results obtained from 5 healthy adults.

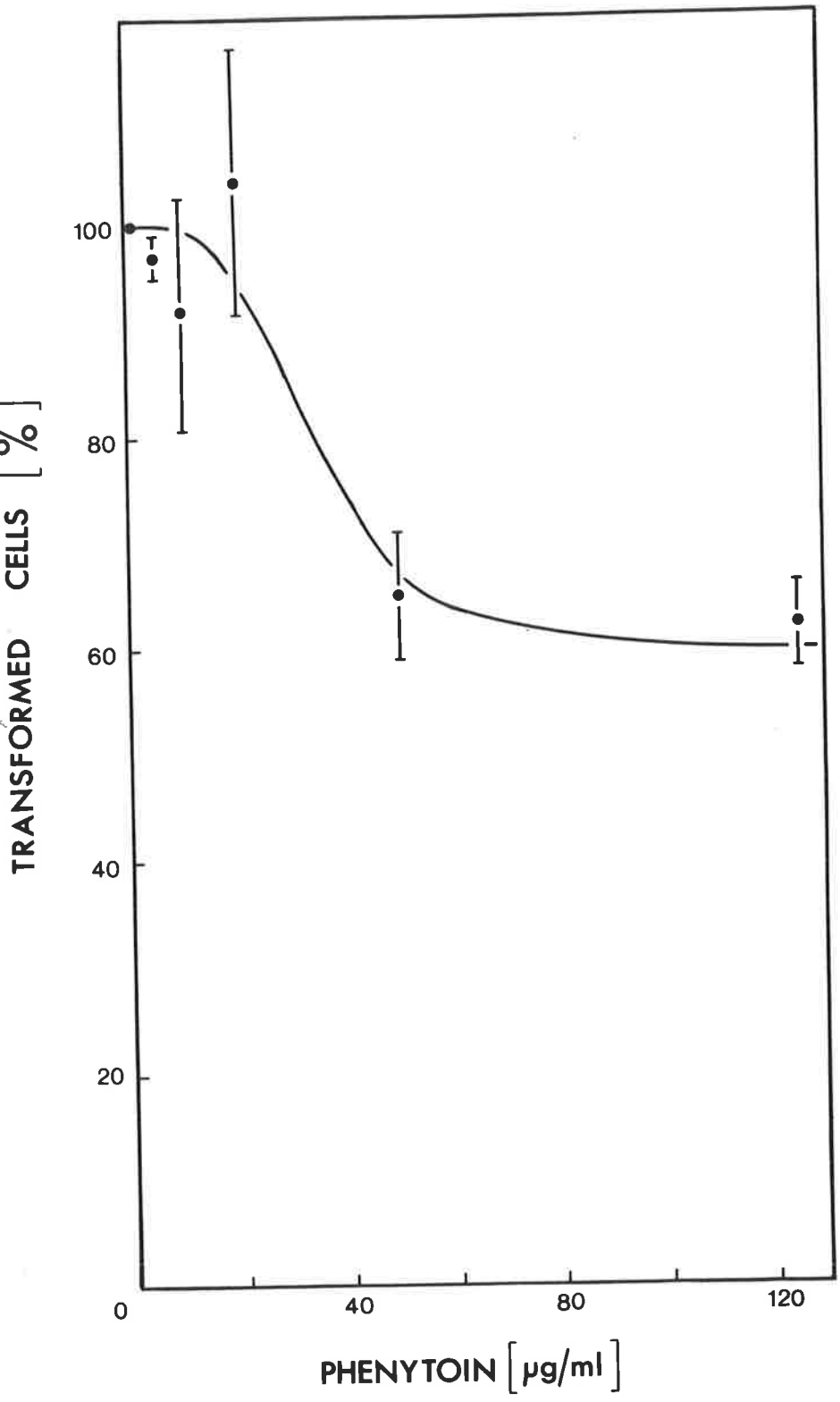
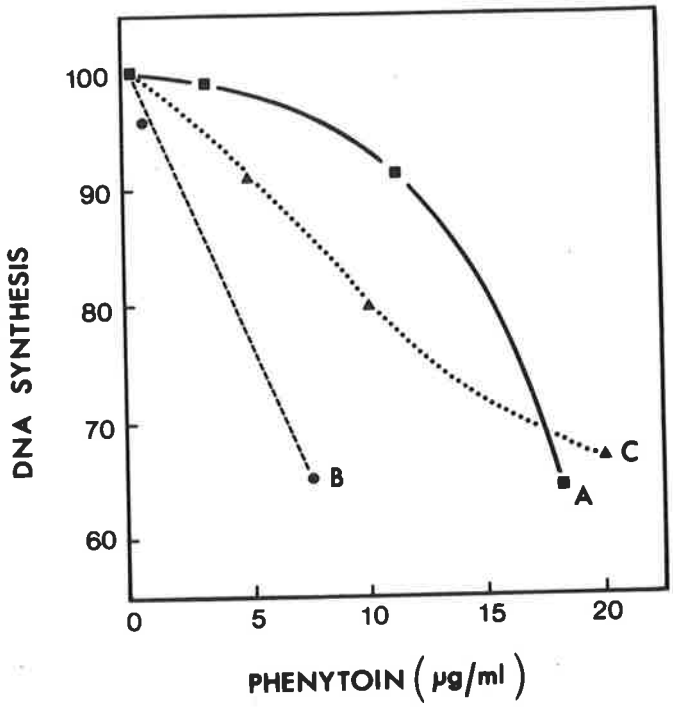

TRANSFORMED CELLS [%]
100
80
60
40
20
0
40
80
120
PHENYTOIN [µg/ml]

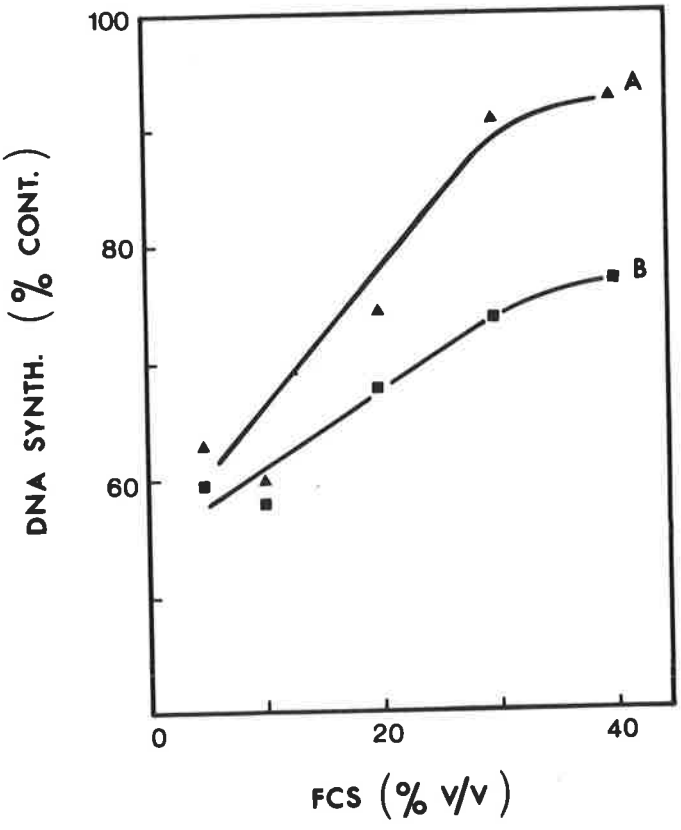
FIGURE VI-3

DEPRESSION OF DNA SYNTHESIS BY PHENYTOIN IN THE SERUM OF A PATIENT AFTER OVERDOSAGE

1. Cultures contained 30% autologous serum (curve A), 30% FCS, to which phenytoin had been added (curve B), or 10% autologous serum (curve C).

2. The effect of serum concentration on the depression of PHA-induced DNA synthesis by phenytoin was studied by incubating blood cells from 2 healthy adults with PHA and phenytoin (20 µg/ml) or phenytoin solvent, in the presence of increasing concentrations of FCS. DNA synthesis at each serum concentration was expressed as a percentage of that in control cultures.


DNA SYNTHESIS
100
90
80
70
60
0
5
10
15
20
A
B
C
PHENYTOIN (µg/ml)


DNA SYNTH. (% CONT.)
100
80
60
0
20
40
A
B
FCS (% v/v)

phenytoin from the two sources. Comparison of the results of cultures containing 10% autologous serum with those containing 30% autologous serum showed that at a given phenytoin concentration there was a significantly greater depression of DNA synthesis in the cultures with less serum.

(7) The effect of serum concentration on depression of DNA synthesis by phenytoin: (Figure VI-3) There was an inverse relationship between phenytoin-induced depression of DNA synthesis and serum concentration.

(8) Kinetic studies of the depression of DNA synthesis by phenytoin in PHA-stimulated cultures:

Method: Two experiments were performed -

(a) Phenytoin (50 µg/ml) 0.01 ml, or phenytoin solvent, 0.01 ml, was added to triplicate cultures at the beginning of the culture period, and PHA was added at specified times throughout.

(b) PHA was added at the beginning of the culture period and phenytoin (50 µg/ml) 0.01 ml, or solvent, 0.01 ml, was added during the culture.

^{3}H-thymidine (2.5 µCi) was added to all cultures at 92 hours of incubation; cultures were processed at 96 hours. Each experiment was repeated once. Cells from two healthy adults were used in the study.

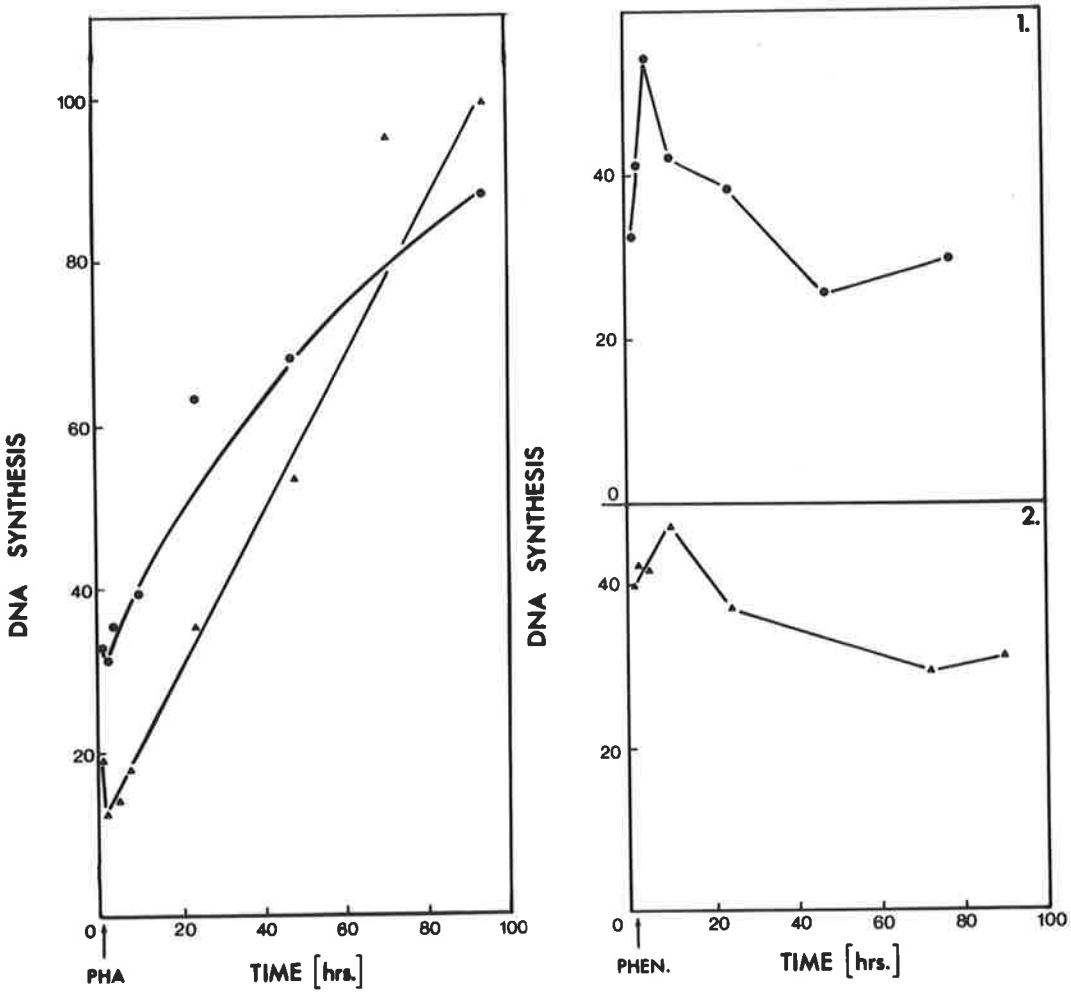
Results: (Figure VI-4) In cultures pre-incubated with phenytoin, significant depression of DNA synthesis occurred when

FIGURE VI-4

KINETIC STUDIES OF THE EFFECT OF PHENYTOIN ON PHA-STIMULATED LYMPHOCYTE DNA SYNTHESIS

1. Figures 1 and 2: The effect of pre-incubation with phenytoin on depression of PHA-stimulated DNA synthesis. PHA was added at the times indicated on the graph by closed circles or triangles.

2. Figure 3: PHA was added at the onset of culture. Phenytoin was added at the times specified on the graph by closed circles or triangles.


DNA SYNTHESIS
100
80
60
40
20
0
PHA
20
40
60
80
100
TIME [hrs.]
DNA SYNTHESIS
1.
40
20
0
2.
40
20
0
PHEN.
20
40
60
80
100
TIME [hrs.]

PHA was added at any time during the culture period. In cultures containing PHA throughout the incubation period, maximal depression of DNA synthesis occurred when phenytoin was added within 4–8 hours of the initiation of culture. Depression of DNA synthesis was not significant when phenytoin was added at 90 hours.

3. THE EFFECT OF PHENYTOIN ON RNA SYNTHESIS

(a) Method

RNA synthesis was measured in lymphocyte cultures of greater than 98% purity, as recommended by Cline (1966). Lymphocytes were separated from heparinized blood by sedimentation over methylcellulose-Hypaque solution, followed by passage through a cotton wool column (see Chapter III). Triplicate cultures containing 1.5×10^6 lymphocytes were incubated for 24 hours with PHA (0.02 ml), FCS (0.2 ml), phenytoin or phenytoin solvent (0.01 ml), and medium 199, such that the culture volume was 2.0 ml. ^{3}H-uridine (3.2 µCi, specific activity 6.4 Ci/mMol) 0.1 ml, was added to cultures at 20 hours. At 24 hours, cultures were cooled rapidly to 0°C in an ice-chip bath. RNA was extracted by the Schmidt-Thannhauser technique as modified by Fleck and Munro (1962).

Cold perchloric acid (PCA, 2.1N) 1 ml, was added to each culture, and allowed to stand for 15 minutes at 4°C. The precipitate was centrifuged at 1500g in a refrigerated centrifuge for 15 minutes, and washed twice with cold PCA (0.7N, 3 ml).

The supernatant was carefully removed, and the precipitate digested with potassium hydroxide (0.3 M, 4 ml) at 37°C for 1 hour. Samples were chilled, and the alkaline mixture neutralized with cold PCA (7 N, 0.115 ml), acidified with cold PCA (1 N, 4 ml) and centrifuged at 1500g for 30 minutes at 4°C. The supernatant (RNA extract) was transferred to collecting tubes, the precipitate washed twice more with cold PCA (0.5 N, 2 ml) and the washings added to the RNA extract. RNA extract (0.2 ml) was then shaken with toluene-ethanol based scintillation fluid (see Appendix (iv)), 15 ml, in glass vials. Radioactivity was measured by cycling the vials three times through a Packard liquid scintillation spectrometer. Samples were corrected for quenching using an automatic external standard.

(b) Results (Figure VI-5)

There was a concentration-dependent decrease in the incorporation of ^{3}H-uridine into RNA, but this was not significant at pharmacological concentrations of phenytoin.

4. THE EFFECT OF PHENYTOIN ON PROTEIN SYNTHESIS

(a) Method

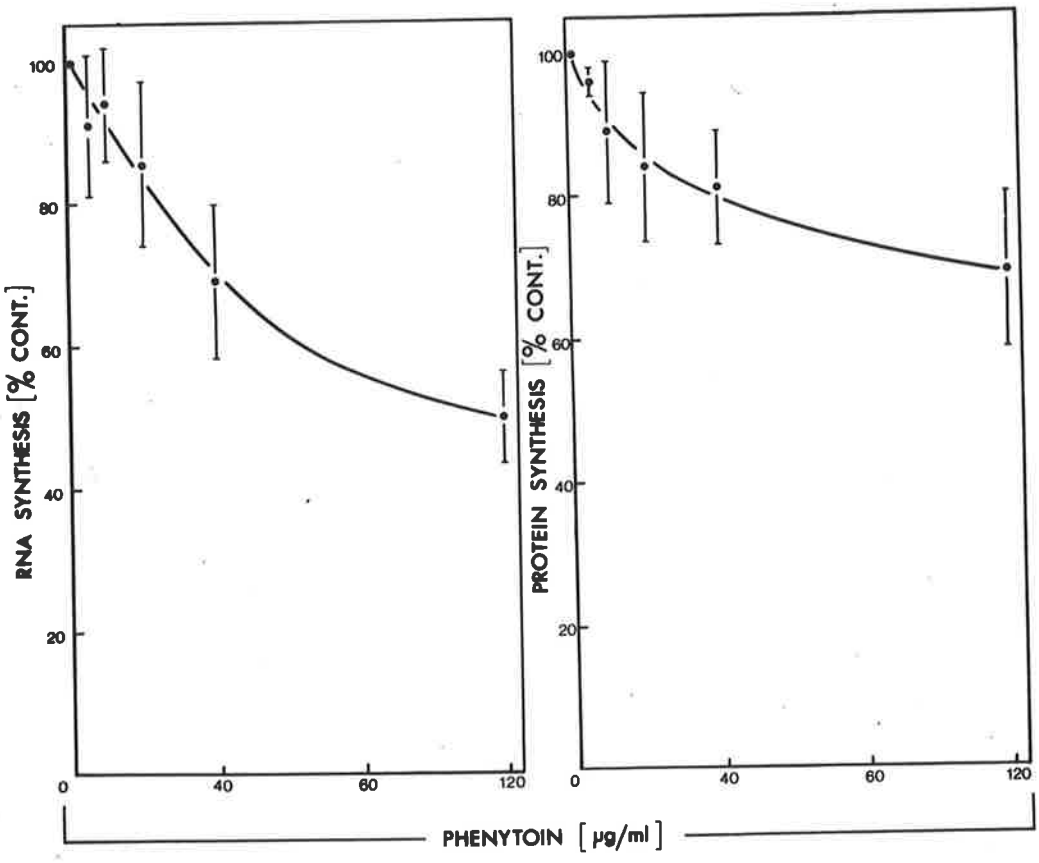
Lymphocytes of greater than 95% purity were obtained by centrifugation through a gradient of Ficoll-Hypaque (see Appendix (xii)) using the method of Froland and Natvig (1970). This method was used in preference to the cotton wool separation technique because the cell yield was higher. Heparinized blood was diluted with Dulbecco's phosphate buffer (calcium and

FIGURE VI-5

THE EFFECT OF PHA ON RNA AND PROTEIN SYNTHESIS

RNA synthesis was measured using lymphocytes from 6 healthy adults.

Protein synthesis was measured in lymphocytes from 5 healthy adults.


RNA SYNTHESIS [% CONT.]
PROTEIN SYNTHESIS [% CONT.]
100
80
60
40
20
0
40
60
120
PHENYTOIN [μg/ml]

magnesium-free, C.S.L., Aust.) 1 part blood: 2 parts buffer. The blood suspension (27 ml) was carefully layered on to the Ficoll-Hypaque solution (11.5 ml) in a sterile pyrex test-tube (internal diameter, 24 mm), and centrifuged with a centrifugal force of 400g (applied to the interface of the blood suspension - Ficoll-Hypaque solution) for 40 minutes at room temperature. The lymphocyte-containing fraction (which formed a white layer at the interface) was carefully removed by Pasteur pipette, and the cell suspension washed twice in Dulbecco's phosphate buffer, and once in HEPES-buffered medium 199. Cell counts were determined by counting two chambers of cells in a haemocytometer.

The lymphocyte suspension was then diluted with medium 199 and FCS and cultures prepared as described for RNA synthesis, except that each 2.0 ml culture contained 2.0×10^6 cells, and ^{14}C-leucine (1.0μCi, specific activity 344 mCi/mMol), was added at the commencement of culture. Cultures were incubated at 37°C for 24 hours. The cells were disrupted by freezing and thawing twice, and washed into plastic centrifuge tubes with ion-free distilled water (2.0 ml). Protein was precipitated with cold trichloroacetic acid (10%, 4 ml) overnight. The precipitate was washed three times in trichloroacetic acid (5%) by centrifugation at 1500g. The supernatant was carefully removed after the last wash, the protein dissolved in Soluene (Packard Instrument Co.), 1.0 ml, and transferred to glass vials with toluene-based scintillation fluid (see Appendix (iv)), 10 ml.

Radioactivity was counted in a Packard counter; results were corrected for quenching with the automatic external standard.

(b) Results

Figure VI-5 shows that there was a slight fall in protein synthesis at high concentrations of phenytoin.

5. THE EFFECT OF OTHER ANTICONVULSANTS ON DNA SYNTHESIS

Carbamazepine, phenobarbitone and diazepam were tested for their ability to depress DNA synthesis in PHA-stimulated cell cultures.

(a) Method

(1) Preparation of drug solutions: Carbamazepine pure substance (Geigy, Switzerland), was dissolved in absolute ethanol-propylene glycol (50% v/v). Phenobarbitone for parenteral use (Farmer-Hill, Aust.) and diazepam pure substance (Roche, Switzerland) were dissolved in propylene glycol (40%), ethanol (10%), and distilled water (50%). All solutions were sterilized by millipore filtration.

(2) Preparation of cultures: Defined concentrations of drug in a volume of 0.01 ml were added to triplicate culture tubes and mixed immediately with reconstituted PHA (0.02 ml) in medium 199 (2.98 ml). Control cultures contained drug solvent (0.01 ml). Cultures were resuspended daily during incubation at 37°C. At 96 hours, they were processed to determine the incorporation of ^{3}H-thymidine into acid-precipitable DNA as described previously. Cultures containing carbamazepine and phenobarbitone were also processed for the counting of cell nuclei.

(3) Lymphocyte viability: Lymphocyte viability in the presence of carbamazepine was estimated in 72 hour lymphocyte cultures by the trypan blue dye exclusion technique (vide supra).

(4) Carbamazepine assay: The concentration of carbamazepine in the culture fluid was determined from its ultraviolet absorption at 257 nM after extraction by the method of Curry (1969). Culture suspension (1 volume) was mixed with ion-free distilled water (3 volumes) and concentrated hydrochloric acid (4 volumes), and heated over a boiling water bath for 1 hour. The mixture was cooled, alkalinized with sodium hydroxide (10 N), extracted with ether, washed with distilled water, and extracted into hydrochloric acid (5 N, exactly 10 ml). The absorption was then measured in the ultraviolet absorption spectrophotometer.

(5) Stability and solubility of carbamazepine: The procedure used to determine these factors in the presence of phenytoin was used for carbamazepine, at the same concentrations of drug (vide supra).

(b) Results

The effect of carbamazepine on PHA-induced DNA synthesis and lymphocyte counts is shown in Figure VI-6, and on lymphocyte viability, in Table VI-3. (p.147.)

Carbamazepine caused a concentration-dependent decrease in DNA synthesis, which was significant at levels of 20 µg/ml ($P < 0.01$), recommended pharmacological concentrations being below 10 µg/ml (Kutt and Louis, 1972, Cereghino et al, 1973).

FIGURE VI-6

THE EFFECT OF CARBAMAZEPINE ON CULTURED LYMPHOCYTE DNA SYNTHESIS AND CELL COUNTS

DNA synthesis and cell counts were determined in cultures from 4 healthy adults and 1 patient treated with carbamazepine.

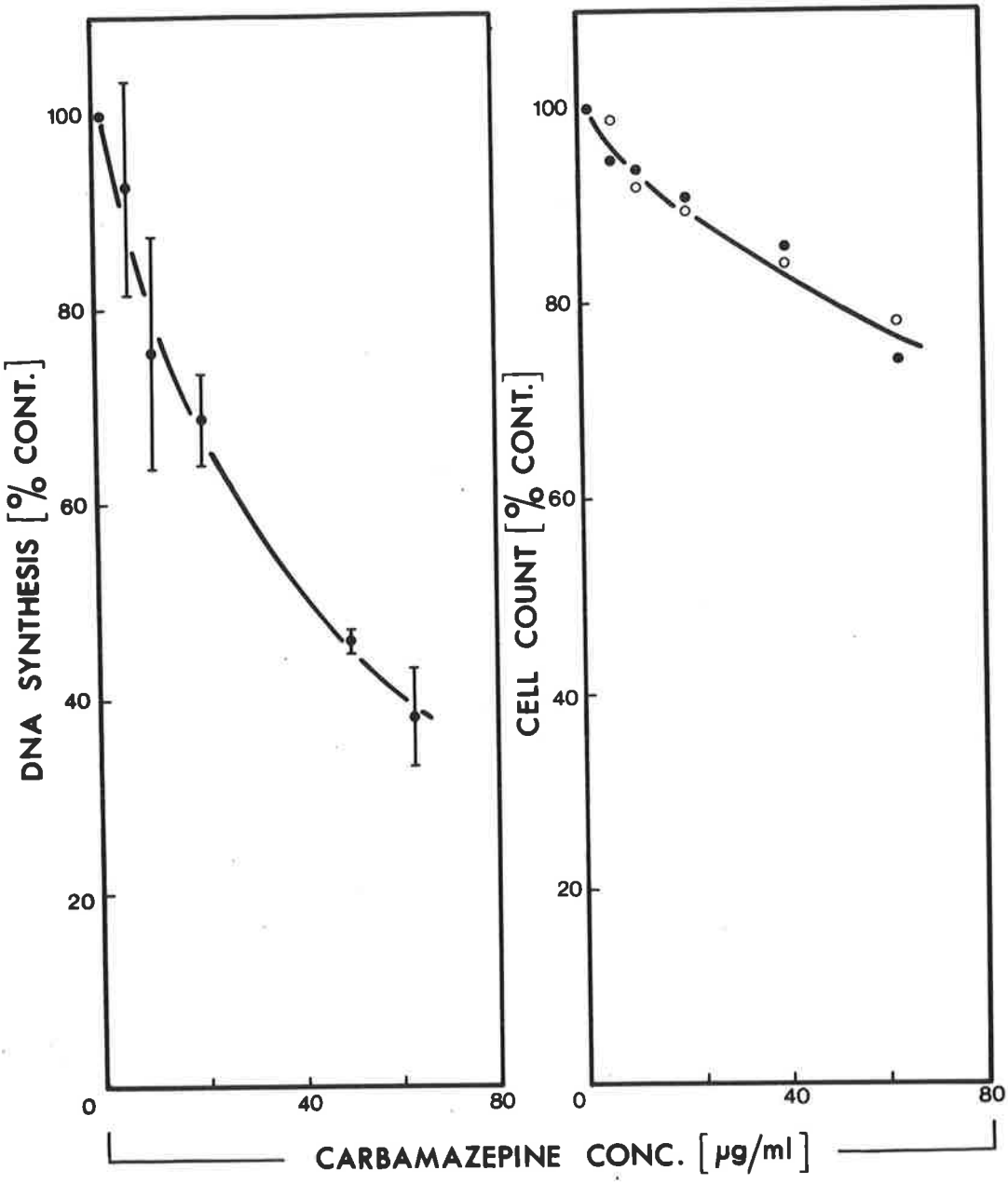

DNA SYNTHESIS [% CONT.]
CELL COUNT [% CONT.]
100
80
60
40
20
0
40
80
CARBAMAZEPINE CONC. [μg/ml]

TABLE VI-3

LYMPHOCYTE VIABILITY IN THE PRESENCE OF CARBAMAZEPINE

Drug concentration (µg/ml)	Subject 1	Subject 2
0	*92	73
10	89	82
20	90	80
40	90	83
62.5	90	81

* Percentage of intact, viable cells.

Serum concentrations in vivo did reach 38 µg/ml in one patient studied; PHA-induced DNA synthesis in the presence of autologous serum was within normal limits in this patient, although DNA synthesis in circulating leukocytes was significantly low. Carbamazepine did not significantly affect cell counts or viability in culture; it remained stable and in solution throughout the incubation period.

Concentrations of phenobarbitone achieved in vivo are similar to those of phenytoin (Buchthal and Svensmark, 1971). Pharmacological concentrations of phenobarbitone did not significantly depress DNA synthesis or cell counts in culture (Figure VI-7).

Similarly, pharmacological concentrations of diazepam (Clarke, 1969) failed to depress lymphocyte DNA synthesis in vitro (Figure VI-8).

FIGURE VI-7

THE EFFECT OF PHENOBARBITONE ON CULTURED LYMPHOCYTE DNA SYNTHESIS AND CELL COUNTS

DNA synthesis (expressed as a percentage of control values) was measured in cultures from 3 healthy subjects.

Cell counts were determined in cultures from 2 of these subjects.

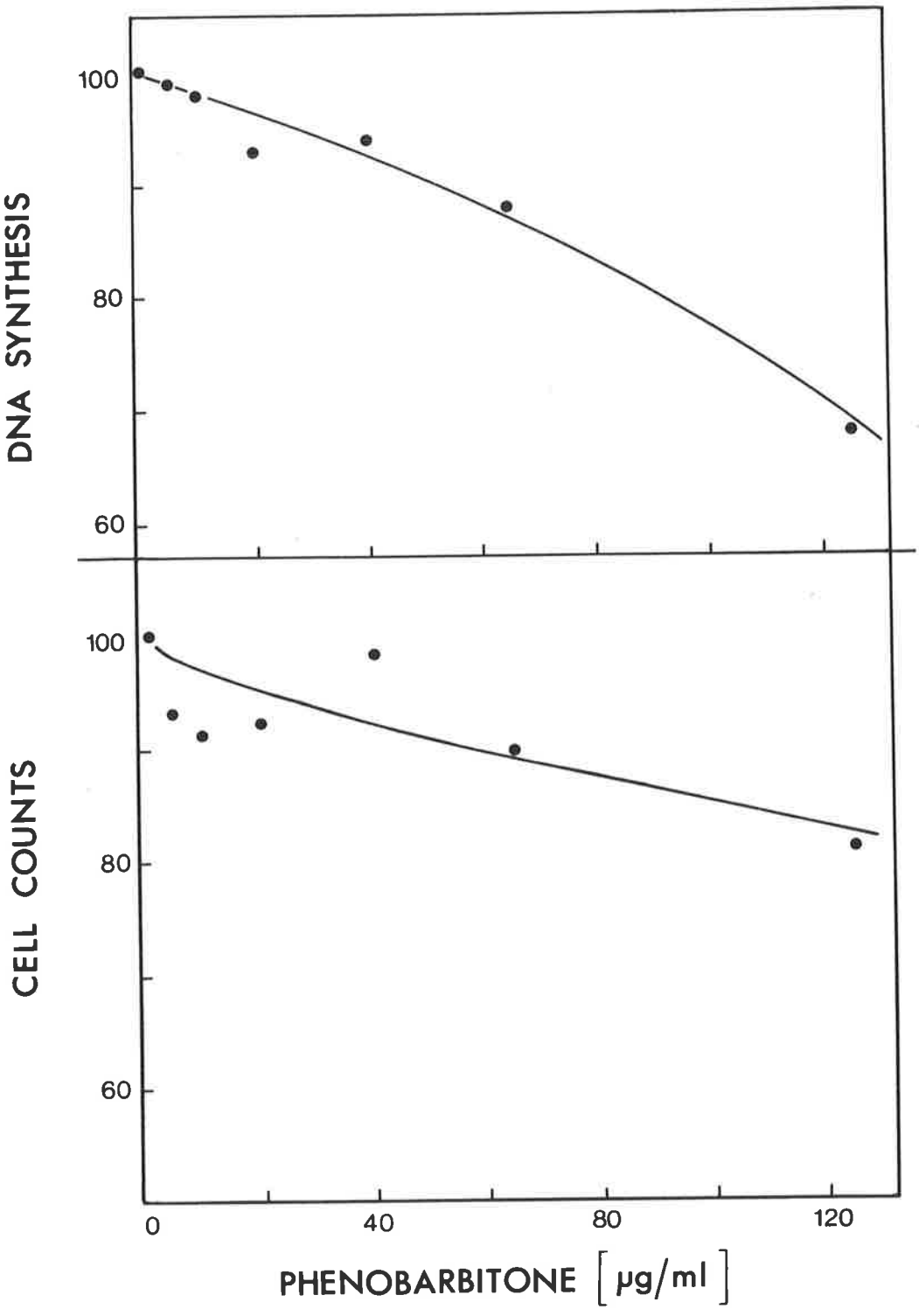
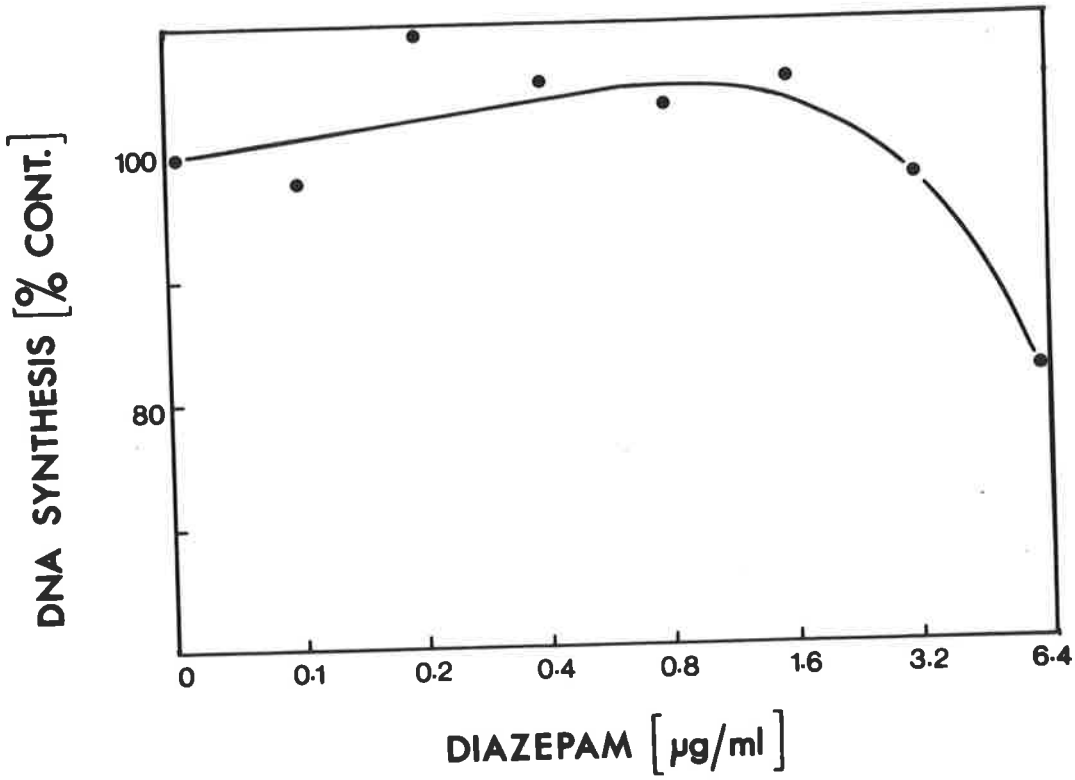

DNA SYNTHESIS
100
80
60
CELL COUNTS
100
80
60
0
40
80
120
PHENOBARBITONE [µg/ml]

FIGURE VI-8

THE EFFECT OF DIAZEPAM ON LYMPHOCYTE DNA SYNTHESIS

DNA synthesis was measured in cultures from 3 healthy adults.


DNA SYNTHESIS [% CONT.]
100
80
0
0·1
0·2
0·4
0·8
1·6
3·2
6·4
DIAZEPAM [µg/ml]

6. DISCUSSION

The effect of phenytoin on DNA synthesis was studied in 96 hour blood cell cultures. Comparable results were obtained in 72 hour lymphocyte cultures in one experiment. The use of blood cell cultures was technically more satisfactory for measurement of DNA synthesis because of two factors - (a) relatively small volumes of blood were required, and (b) preliminary manipulation cells was avoided. RNA synthesis was measured in lymphocyte cultures in which the mononuclear cell content was greater than 98%, as ribonucleases (released by the disruption of lysosomal membranes found particularly in granulocytes) have been shown to cause degradation of RNA during the preliminary part of the extraction procedure (Cline 1966). Protein synthesis was also measured in lymphocyte-rich cultures, to avoid contamination with proteins from erythrocytes, and other leukocytes, and to avoid excessive colour quenching by haeme pigment.

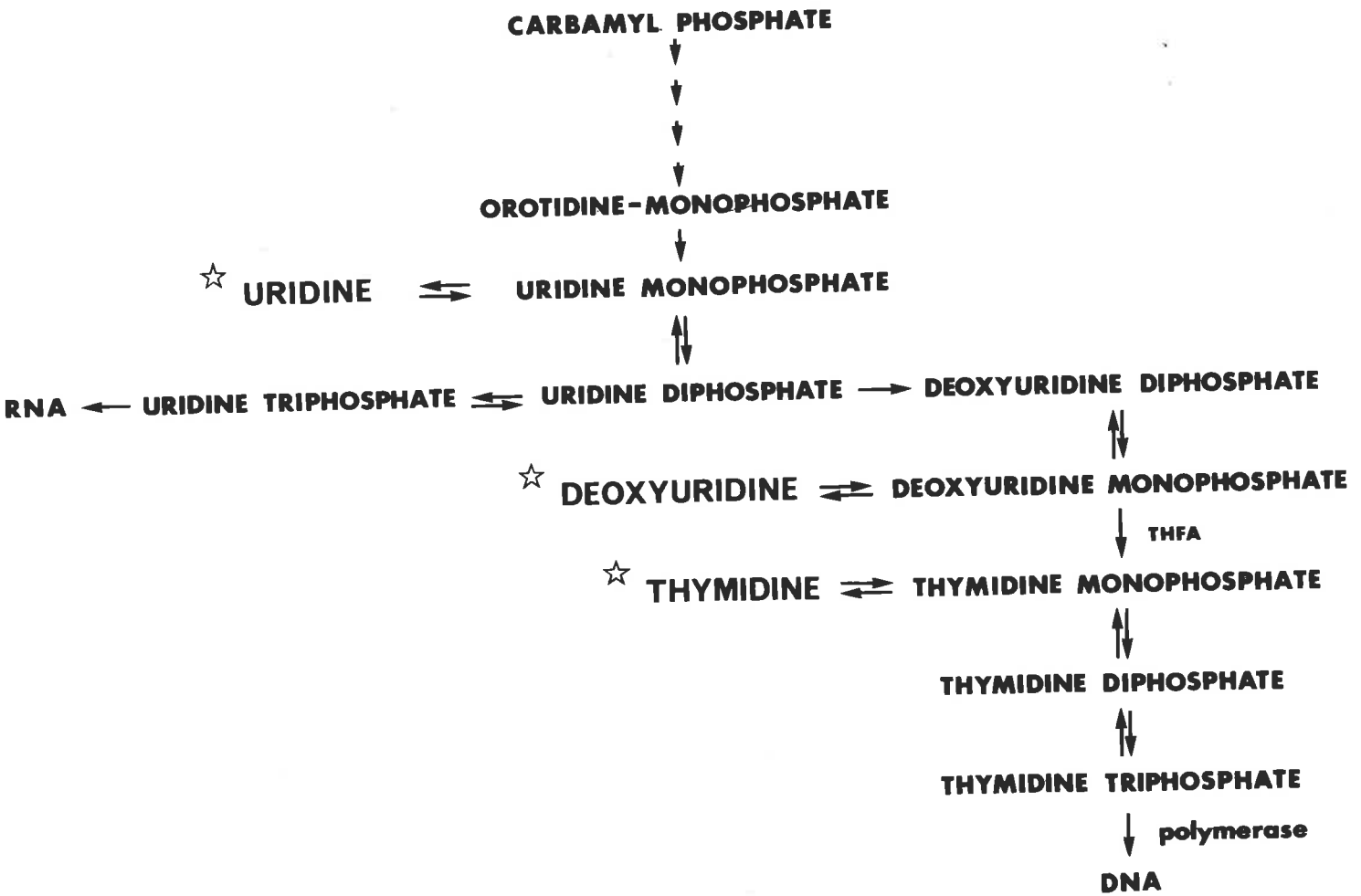
Pathways of nucleic acid synthesis involving the precursor substances used in this study, and the sites of entry of these precursors (Cleaver, 1967) are shown in Figure VI-9.

The results of the present study have shown that phenytoin inhibits DNA synthesis in lymphocytes from normal persons and patients treated with phenytoin. Phenytoin added to cultures in vitro and phenytoin present in serum samples from a patient who had taken an overdose of phenytoin caused a similar depression of DNA synthesis. These findings confirm that the depression of PHA-induced lymphocyte DNA synthesis observed in the population of phenytoin-treated

FIGURE VI-9

THE THYMIDINE BIOSYNTHETIC PATHWAY

☆ Labelled precursors used in the measurement of RNA and DNA synthesis in this study.


CARBAMYL PHOSPHATE
OROTIDINE-MONOPHOSPHATE
☆ URIDINE
URIDINE MONOPHOSPHATE
RNA
URIDINE TRIPHOSPHATE
URIDINE DIPHOSPHATE
DEOXYURIDINE DIPHOSPHATE
☆ DEOXYURIDINE
DEOXYURIDINE MONOPHOSPHATE
THFA
☆ THYMIDINE
THYMIDINE MONOPHOSPHATE
THYMIDINE DIPHOSPHATE
THYMIDINE TRIPHOSPHATE
polymerase
DNA

patients described in Chapter V was due to the presence of phenytoin in the serum, and that lymphocyte DNA synthesis was normal in the absence of drug. Depression of DNA synthesis in vitro was significant at pharmacological concentrations of phenytoin (10–20 μg/ml), and even high concentrations (125 μg/ml) were not cytocidal. The depression of DNA synthesis by phenytoin in vitro varied inversely with the serum concentration, suggesting that only the unbound form was effective. In the presence of high serum concentrations of phenytoin, the depression of PHA-induced DNA synthesis was small. However, PHA-induced DNA synthesis in the presence of sera from patients taking phenytoin was depressed in the presence of total concentrations of phenytoin of (usually) less than 1 μg/ml (Chapter V). Despite the effect of protein binding, depression of lymphocyte DNA synthesis by phenytoin is probably significant in vivo. Pharmacological concentrations of phenytoin did not cause a significant depression of cell counts, lymphocyte blastogenesis, RNA synthesis, or protein synthesis. High concentrations of phenytoin caused depression of all these parameters. RNA and protein synthesis were measured in 24 hour cultures; DNA synthesis was measured in 96 hour cultures. It is possible that the timing of the RNA and protein determinations was such that sensitivity to phenytoin was not detected. However, the results of independent studies have recently been reported by MacKinney and Vyas (1972). They measured DNA, RNA and protein synthesis (using the same precursors) in 72 hour leukocyte cultures, and found that DNA synthesis was selectively depressed at pharmaco-

logical concentrations of phenytoin. They also reported that this depression of DNA synthesis was not due to the diversion of labelled thymidine from the pathway of DNA synthesis, by demonstrating that the same proportion of radioactivity was present in perchloric acid-extractable DNA in control and phenytoin incubated cultures, and that this did not change over 24 hours of incubation with phenytoin.

The depression of nucleic acid and protein synthesis by high concentrations of phenytoin is consistent with the report that toxic concentrations of phenytoin induce chromosomal defects in white cells in vitro (Muniz et al, 1969), and raises the possibility that phenytoin is teratogenic. It is known to cause foetal abnormalities in mice (Harbison and Becker, 1969), but reports of studies in man have been conflicting. It is possible that toxic concentrations of phenytoin in pregnant women do cause an increased incidence of foetal abnormalities (Loughnan et al, 1973).

Results of studies on the effect of phenytoin on nucleic acid synthesis in human lymphocyte and bone-marrow cultures have been conflicting. McIntyre and Ebaugh (1962) found that phenytoin inhibited the incorporation of ^{32}P into the DNA of PHA-stimulated lymphocytes from three patients, one of whom was recovering from phenytoin-induced aplastic anaemia. Ling (1968) reported that non-toxic concentrations of phenytoin (up to 140 µg/ml) caused very little depression of DNA synthesis in lymphocytes stimulated with Staphylococcal filtrate. Yunis et al (1967) studied a patient with phenytoin-induced erythroid aplasia. Nucleic acid synthesis was

measured by gas-flow scintillation spectrophotometry in 4 hour bone-marrow cultures. Phenytoin (20 µg/ml) caused a 25-30% fall in the incorporation of labelled formate into DNA and DNA-thymine, and labelled glycine, adenine and orotic acid into the DNA of erythrocyte precursors; it did not affect the incorporation of labelled deoxy-uridine or thymidine into DNA, glycine into haeme, or lysine into protein. However, phenytoin had no effect on any of these parameters in 10 normal subjects. Holland and Mauer (1965) reported that phenytoin (20 µg/ml) stimulated DNA synthesis and blastogenesis in a child who developed an hypersensitivity reaction to the drug. In another study, phenytoin appeared to increase the incorporation of orotic acid into the RNA precursors uridylic acid and cytidylic acid, in the rat liver (Woodbury and Kemp, 1971).

The early steps in the synthesis of thymidylic acid and uridylic acid include a common pathway (Figure VI-9). Selective depression of DNA synthesis by pharmacological concentrations of phenytoin must be due to inhibition at a point distal to the synthesis of uridine diphosphate, since the incorporation of ^{3}H-deoxyuridine and ^{3}H-thymidine into DNA, but not ^{3}H-uridine into RNA, were depressed in the present study. The conversion of deoxyuridine monophosphate to thymidine monophosphate requires tetra-hydro folic acid. It has been suggested that phenytoin might interfere with the biosynthesis of coenzyme forms of folic acid or their functions (Hawkins and Meynell, 1954) although such an effect has not yet been proven. The drug does not inhibit dihydro-folate (DHFA) reductase, N^{5}, N^{10} -

methylene tetra-hydro-folic acid (THFA) dehydrogenase, or THFA formylase, which catalyse some of the steps of folic acid metabolism (Hamfelt and Wilmanns, 1965). In the present study, phenytoin inhibited the incorporation of both ^{3}H-deoxyuridine and ^{3}H-thymidine into DNA; it is therefore unlikely that phenytoin blocks the conversion of deoxyuridine monophosphate to thymidine monophosphate. In addition, phenytoin concentrations of 10-150 µg/ml have been found not to influence the uptake of ^{3}H-pteroylglutamic acid by PHA-stimulated human lymphocytes (Das and Hoffbrand, 1970).

Kinetic studies suggested that phenytoin affected the early stages of induction or onset of DNA synthesis, causing maximal depression of PHA-induced lymphocyte DNA synthesis when added in the first eight hours of culture. A number of events are known to occur within 60 minutes of the addition of PHA to cell cultures, including a significant increase in the uptake of ^{45}calcium (Allwood et al, 1971, Whitney and Sutherland, 1972), and in the uptake of ^{42}potassium (Quastel et al, 1970). Calcium transport may be linked to sodium transport in some systems (Blaustein and Weissman, 1970). It is known that phenytoin causes depletion of intracellular sodium in excitable tissues, either by stimulating the sodium pump (Woodbury and Kemp, 1971), or by limiting the increase in sodium permeability which occurs during electrical stimulation (Pincus, 1972), although the effects of the drug on sodium transport in non-excitable tissue, e.g. liver, are not so clear-cut (Woodbury and Kemp, 1971). In experiments with isolated jejunum, phenytoin caused an increase in

sodium transport from the lumen, accompanied by a fall in intracellular sodium and a rise in potassium (van Rees et al, 1969). The role of ionic changes in lymphocytes, in the mechanism of phenytoin-induced depression of DNA synthesis, has not been investigated in this study.

Another mechanism by which phenytoin might affect metabolism is via the energy processes of the cell. Markkanen and Peltola (1971) studied the effect of phenytoin on transketolase activity (an enzyme in the pentose-phosphate shunt pathway of aerobic glycolysis) in human leukocytes. Plasma from 5 patients taking phenytoin did inhibit transketolase activity, but the drug was ineffective when added to leukocytes in vitro.

Phenytoin may enter the cell nucleus and inhibit DNA synthesis directly. Kemp and Woodbury (1971) studied the subcellular distribution of phenytoin in the cerebral cortex of rats after injection into the cisterna magna; they found that the drug rapidly entered the nuclear fraction, where it was firmly bound at 30 minutes, possibly to DNA and/or RNA. Later, the drug was found mainly in the microsomal fraction, 70% of it in association with the rough endoplasmic reticulum (the site of ribosomal RNA). In vitro studies showed that, by mixing solutions of RNA and phenytoin at concentrations of phenytoin three times those of the RNA solution, hypochromicity was induced in the RNA molecule, i.e. the drug was bound to RNA. These workers also found that the accumulation of phenytoin in the nuclear fraction and microsomes was blocked by actinomycin (which

inhibits RNA synthesis), and that that in the microsomes was blocked by puromycin (which inhibits the assembly of protein on ribosomes). They suggested that phenytoin was incorporated into, or bound to messenger RNA, and that it might affect protein synthesis via the ribosomal system (Woodbury and Kemp, 1971). In the present study, RNA and protein synthesis in human lymphocytes did not appear to be affected primarily by phenytoin. These differences may be due in part to the type of tissue, and the species studied. The finding of the present study, that the incorporation of ^{3}H-deoxyuridine and ^{3}H-thymidine into DNA were depressed by phenytoin, is consistent with an effect of the drug on DNA polymerase. Inhibition of RNA synthesis by high concentrations of phenytoin is consistent with the hypothesis that RNA polymerase is less sensitive to the inhibitory actions of phenytoin than DNA polymerase. Studies of lymphocytes from phenytoin-treated patients indicated that PHA-induced DNA synthesis was significantly depressed in the presence of autologous serum, but not foetal calf serum, and that the immunosuppression observed in phenytoin-treated patients arose after the commencement of therapy (Chapter V). It can be concluded from these results that depression of DNA synthesis by phenytoin is a major mechanism by which the drug causes immunosuppression.

This conclusion is also supported by the observations that depression of DNA synthesis by carbamazepine in vitro is significantly less than that by phenytoin, in parallel with the lesser immunosuppressive effect observed in vivo, and that phenobarbitone,

which is not immunosuppressive in vivo, causes insignificant depression of DNA synthesis in vitro.

7. SUMMARY

(a) The effect of anticonvulsant drugs on nucleic acid and protein synthesis was studied in cultures of haemopoietic cells.

(b) Pharmacological concentrations of phenytoin caused a significant depression of DNA synthesis in PHA-stimulated and non-stimulated cultures without depression of cell counts, lymphocyte blastogenesis, RNA, or protein synthesis.

(c) High concentrations of phenytoin caused depression of cell counts, lymphocyte blastogenesis, RNA and protein synthesis, but to a much lesser extent than DNA synthesis. Phenytoin was not cytocidal at concentrations of up to 125 μg/ml.

(d) The extent of depression of DNA synthesis by a given concentration of phenytoin varied inversely with the concentration of serum in the culture.

(e) Depression of DNA synthesis by phenytoin was maximal in PHA-stimulated cultures when phenytoin was added within 4-8 hours of the addition of PHA, and was not significantly affected by pre-incubation with phenytoin.

(f) High concentrations of carbamazepine caused a significant depression of DNA synthesis, which was greater than the depression of cell counts. Phenobarbitone and diazepam did not depress DNA synthesis significantly.

CHAPTER VII

IMMUNOLOGICAL FUNCTION IN PATIENTS WITH LYMPHOMA

1. INTRODUCTION

Immunological function is often depressed in patients with untreated Hodgkin's disease or other lymphoma, especially in the more advanced stages of the disease (Brown et al, 1967, Libansky, 1969, Harris and Bagai, 1972, Kaplan, 1972). It has been claimed that patients with early Hodgkin's disease (Stage I) however, are not immunosuppressed (Young et al, 1972, Kaplan, 1972), a finding which is unexpected in view of the considerable circumstantial evidence that immunodepression may be important in the aetiology of lymphoid tissue malignancy (Good, 1972). Phenytoin therapy has been associated with the development of lymphoma (Hyman and Sommers, 1966). Patients with recently diagnosed (untreated) lymphoma were tested for the presence of immunological abnormality, and compared with the group of patients treated with phenytoin.

2. METHOD

(a) Patients

Immunological function was measured in 38 patients (20 males, 18 females; mean age 49.6 years, range 15–79) with untreated lymphoma. Eighteen of these patients had documented Hodgkin's disease (Stage I, 8 patients, Stage II, 5 patients, Stage III, 3 patients, Stage IV, 2 patients), classified by the Ann Arbor modification of the Rye method, 20 had other lymphomas (lymphosarcoma, 13 patients, reticulum cell sarcoma, 2 patients,

and giant follicular lymphoma, 5 patients). Staging of patients with Hodgkin's disease was based on clinical examination, chest X-ray, and bone marrow biopsy. Lymphangiography was performed in all patients except those with Stage IV disease. Where possible, staging laparotomy was undertaken in probable Stage I or II patients. The immunological responses of these patients were compared with those of 38 age and sex-matched control subjects, derived randomly from the control population described in Chapter II.

(b) Immunological Assessment

Immunological function was measured as described in Chapter II, with the following exceptions:

(1) Sera were not tested for the presence of antinuclear factor.

(2) It was not possible to re-test patients with absent DHS reactions before therapy was commenced.

(3) Lymphocyte counts were not computed.

(4) DNA synthesis in circulating leukocytes and PHA-stimulated lymphocytes was not measured. Lymphocyte transformation was assessed by the microscopic examination of stained smears from PHA-stimulated lymphocyte cultures in a number of patients (who were studied before the establishment of the technique for measurement of the incorporation of ^{3}H-thymidine into DNA).

3. RESULTS

(a) Immunoglobulins and Complement

Immunoglobulin concentrations (means and standard deviations, mg/100ml) in the control, lymphoma and lymphoma sub-groups, are shown in Table VII-1. Serum complement concentrations in the control and lymphoma groups are also shown.

TABLE VII-1

IMMUNOGLOBULINS AND COMPLEMENT - SERUM CONCENTRATIONS

Group	No.	IgG	IgA	IgM	C3
Control	38	1127±295	234±93	141±54	**128±38
Lymphoma	38	1142±496	247±168	144±94	+148±60
*H.D.	18	1125±428	248±183	150±95	-
L.S./R.S.	15	1174±617	255±167	143±104	-
G.F.L.	5	1108±245	224±102	128±46	-

*Hodgkin's disease, lymphosarcoma-reticulum cell sarcoma, giant follicular lymphoma.

**Number of subjects 54, +No. = 19.

There were no significant differences between the IgG and IgA concentrations (Student's t-test), or the IgM and complement concentrations (Wilcoxon's Rank sum test) of the control and lymphoma groups. The immunoglobulin concentrations in the lymphoma sub-groups were not significantly different from each other (Wilcoxon's Rank sum test).

(b) Antibody Responses

(1) Salmonella typhi: The results are shown in Table VII-2. (p.159).

The term "responders" refers to the patients who developed measurable serum antibody to S. typhi flagellar H antigen using the technique described in Chapter II. Non-responders did not have detectable antibody in their serum two weeks after immunization.

TABLE VII-2

THE RESPONSE OF PATIENTS WITH LYMPHOMA TO S. TYPHI

Group	Responders	Non-responders	Significance
Control	38	0	
Lymphoma	25	7	*P = 0.0028
H.D.	15	1	
L.S./R.S.	7	4	*N.S.
G.F.L.	3	2	-

*Compared using Fisher's exact test. N.S. = not significant.

A significantly high proportion of patients with lymphoma (29%) failed to develop antibody to S. typhi. This was due to depression of antibody responses in all the lymphoma sub-groups (the Hodgkin's disease and lymphosarcoma-reticulum cell sarcoma groups were not significantly different from each other).

(2) Tetanus toxoid: The results are shown in Table VII-3.(p.160)

A significantly high proportion of patients with lymphoma failed to develop antibody to Tetanus toxoid (41%). Antibody responses were depressed in the lymphoma sub-groups.

TABLE VII-3

THE RESPONSE OF PATIENTS WITH LYMPHOMA TO TET. TOXOID

Group	Responders	Non-responders	Significance
Control	38	0	
Lymphoma	19	13	*P < 0.0001
H.D.	9	7	
L.S./R.S.	8	4	*N.S.
G.F.L.	2	2	-

*Compared by Fisher's exact test. N.S. = not significant.

(c) Delayed Hypersensitivity Reactions

DHS reactivity was regarded as normal if reactions to any of candida, streptoococcal antigen, and mumps, were positive. The numbers of patients with normal reactivity (reactors) are compared with controls in Table VII-4.(p.161)

There was a significant depression of reactivity in the lymphoma group; the results in patients with Hodgkin's disease, and those with lymphosarcoma-reticulum cell sarcoma were both significantly less than controls ($P < 0.005$, $P < 0.004$ respectively), but not from each other ($P = 0.255$).

(d) Lymphocyte Transformation

The percentage of transformed cells was considered to be normal if greater than 50%. The results obtained in cultures from 40 control subjects and 27 patients with lymphoma are shown in Table VII-5 (p.161).

TABLE VII-4

DHS REACTIVITY IN PATIENTS WITH LYMPHOMA

Group	Reactors	Non-reactors	Significance
Control	37	1	
Lymphoma	26	11	*P = 0.0074
H.D.	12	5	
L.S./R.S.	9	4	*N.S.
G.F.L.	4	1	

*Compared by Fisher's exact test. N.S. = not significant.

TABLE VII-5

LYMPHOCYTE TRANSFORMATION IN PATIENTS WITH LYMPHOMA

Group	Normal (No.)	Abnormal (No.)	Significance
Control	40	0	
Lymphoma	20	6	*P = 0.0025

*Compared by Fisher's exact test.

4. COMPARISON OF IMMUNOLOGICAL ABNORMALITIES IN PATIENTS WITH DIFFERENT STAGES OF HODGKIN'S DISEASE

Humoral immunological function, (serum concentrations of IgG, IgA, IgM, and the C3 component of complement, and antibody responses to S. typhi and to Tetanus toxoid), and cellular immunological function (DHS reactivity to intradermal antigens, and PHA-induced lymphocyte blastogenesis) were depressed, alone or in

combination, in patients with all stages of Hodgkin's disease (Table VII-6).

TABLE VII-6

RELATIONSHIP BETWEEN IMMUNOLOGICAL ABNORMALITY AND STAGE IN PATIENTS WITH HODGKIN'S DISEASE

Stage	Normal	Hum. defect	Cell. defect	Both defective
*I	4	1	1	1
II	1	2	2	0
III	0	2	1	0
IV	0	0	0	2

*Antibody responses not tested for in 1 patient.

The number of patients in each group was too small for statistical analysis. However, 3 of 8 patients with Stage I disease showed abnormalities of humoral or cellular immune function, or both, and all patients with Stage III and IV disease were abnormal. Two of the Stage I patients with immunodeficiency had been classified by full investigation, including lymphangiography and laparotomy, one patient had not undergone laparotomy.

5. COMPARISON OF IMMUNOLOGICAL ABNORMALITIES IN PATIENTS WITH LYMPHOMA AND PATIENTS TAKING PHENYTOIN

Immunological defects common to both groups were:

(a) Failure to develop antibody to S. typhi.

(b) Failure to develop antibody to Tetanus toxoid.

(c) Depression of DHS reactivity. This was more marked in the

lymphoma patients; it was only evident in the phenytoin-treated group when the total number of positive reactions was compared with those of the control subjects.

(d) Depression of PHA-induced lymphocyte DNA synthesis was observed in the phenytoin-treated patients in the presence of autologous serum. Although PHA-induced lymphocyte blastogenesis is not a directly comparable parameter (see Chapter III) this was depressed in the patients with lymphoma.

Immunological abnormalities confined to the phenytoin-treated patients were:

(a) Deficiency of circulating IgA.

(b) Deficiency of circulating IgM.

6. CASE STUDY - THE ACTION OF PHENYTOIN AS AN HAPTEN

(a) Method

A 26 year old male (F.K.) had taken phenytoin sodium, 300 mg/day, for 3 years, when he noticed a painless left cervical lump in January, 1972. Two rubbery cervical masses were palpable in April, 1972. Biopsy of one gland showed classical Reed-Sternberg cells, moderate numbers of abnormal reticulum cells, eosinophils and occasional plasma cells, between abnormal follicles. The abnormal follicular pattern replaced the normal lymph node architecture. Further investigations included bone marrow aspiration biopsy and lymphangiography. These failed to demonstrate disease elsewhere. The haemoglobin, leukocyte count, and differential leukocyte count were normal. The

patient was treated with radiotherapy (4500 R) to a thoracic mantle field. Ten months after the radiotherapy, laparotomy and splenectomy were carried out. Biopsies of spleen, an abdominal lymph node, and liver, were negative. No histological abnormality referable to phenytoin therapy (Anthony, 1970, Rausing and Trell, 1971) was seen. Immunological function tests were performed prior to surgery. Blood cells from the patient were incubated with phenytoin in vitro to determine whether the drug stimulated DNA synthesis (thereby suggesting that phenytoin was antigenic in vivo). Triplicate cultures were incubated with defined concentrations of phenytoin as described in Chapter VI for 4 and 5 days. Controls contained drug solvent. Cultures from 6 patients receiving phenytoin (with no enlargement of lymph nodes, liver or spleen), 2 patients with Hodgkin's disease treated by radiotherapy, and 3 apparently healthy subjects were established as controls.

(b) Results

There was evidence of humoral and cellular immunological deficiency in the test patient (see Table VII-7), (p.165). DNA synthesis was stimulated by phenytoin in cultures of the patient's lymphocytes but not in the cultures of any control subject. The difference between the two curves was greater in cultures processed on day 5. The difference was three-fold or more for phenytoin concentrations of approximately 15-100 ug/ml (see Figure VII-1). The patient's cells gave the same response on two occasions.

FIGURE VII-1

STIMULATION OF LYMPHOCYTE DNA SYNTHESIS BY PHENYTOIN

Dotted line : Dose-response curves for patient F.K.

Solid line : Mean dose-response curves obtained from 11 control subjects.

Left hand graph represents cultures processed at 96 hours; and right hand graph, at 120 hours.

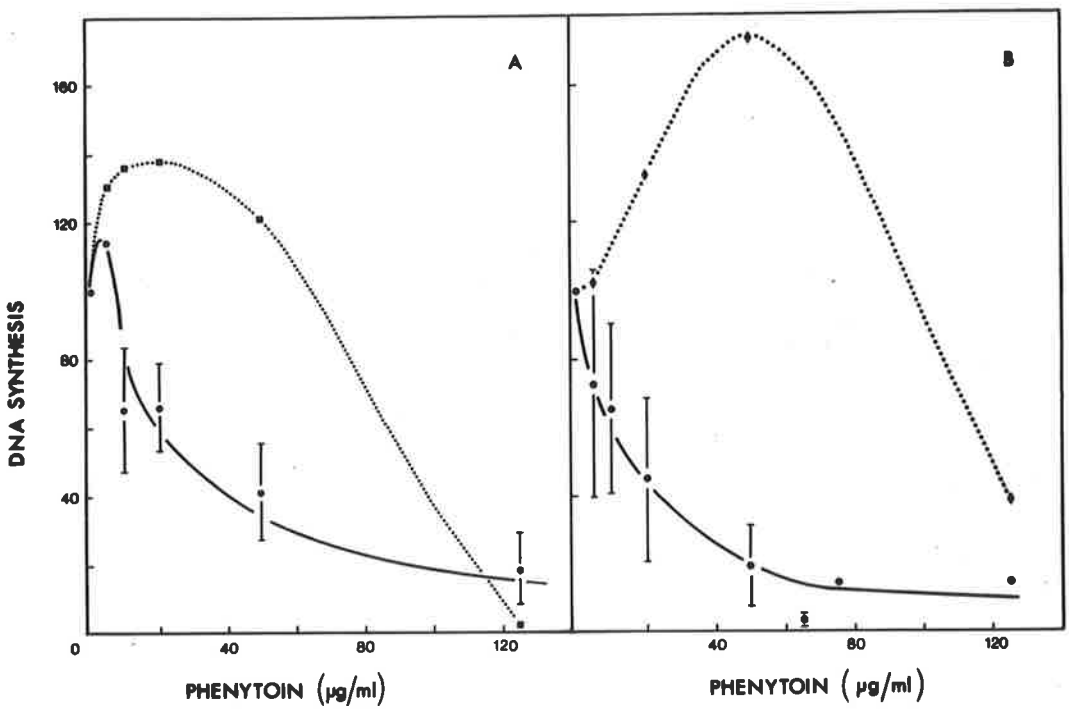

A
B
DNA SYNTHESIS
160
120
80
40
0
40
80
120
PHENYTOIN (µg/ml)
PHENYTOIN (µg/ml)

TABLE VII-7

IMMUNOLOGICAL FUNCTION TESTS IN PATIENT F.K.

	Parameter	Result	Comment
1.	IgG	950 mg/100 ml	Normal
	IgA	110 mg/100 ml	Normal
	IgM	80 mg/100 ml	Normal
	C3	164 mg/100 ml	Normal
2.	S. typhi	Positive ($\geq$:640)	Normal
3.	Tet. toxoid	Negative	Depressed
4.	DHS - Cand.	Negative	
	Strep.	Negative	Depressed
	Mumps	Negative	
5.	PHA-induced DNA synthesis		
	- *AS	600 dpm/culture	Low
	- FCS	3180 dpm/culture	Low
6.	DNA synthesis in circ. leukocytes	460 dpm/culture	Normal
7.	Lymphocyte count	450 cells µl.	Low

*Autologous Serum

7. DISCUSSION

The aetiology and pathogenesis of Hodgkin's disease and the lymphomas remain obscure. Many factors are undoubtedly involved. For example, ionizing radiation, genetic constitution, chemicals such as methylcholanthrene and dimethyl benzanthracene, and viruses are known to be important in the development of lymphoma in rodents

(Kaplan, 1972, Aisenberg, 1973). Age, sex, nutritional status and hormonal balance have been shown to affect susceptibility to the development of leukaemia and lymphoma in certain mouse strains, and simple caloric restriction has been shown to inhibit the development of murine leukaemia (Kaplan, 1972). Immunological factors are important in host resistance against carcinogen-induced tumours in animals (Stutman, 1970). Many workers have postulated that malignant cells survive because of defects in the mechanisms of immunological surveillance (Thomas, 1959, Burnet, 1959, 1967, 1971, Fudenberg, 1971, Krueger et al, 1971, Schwartz, 1972, Laroye, 1973).

Studies in man have been limited. Evidence for the role of viruses in the aetiology of Hodgkin's disease is not convincing. Epidemiological evidence has been obtained in a study of an epidemic in the United States (Vianna et al, 1971). It has recently been reported that a significantly high number of patients with Hodgkin's disease have the HL-A5 histocompatibility group. This is interesting in view of the linkage between the major histocompatibility system (H-2) and susceptibility to viral leukaemogenesis in the mouse (Lilly et al, 1964). Falk and Osoba (1971) reported that there was a significantly frequent association of histocompatibility antigens A1 and A8 with the mixed cellularity and lymphocytic predominance forms of the disease. Patients with nodular sclerosis had an increased frequency of the A5 antigen.

Eisinger et al (1971) cultured lymph, lymph node, spleen, skin, and lung tumour cells from 8 patients with Hodgkin's disease.

The cultured cells appeared to release an RNA-containing agent into the supernatant. Some of the cultured cells underwent blastoid transformation; these free-floating cells released DNA and RNA-containing material. It was suggested that the DNA-containing material represented a Herpes virus, although the relationship of these findings to the pathogenesis of Hodgkin's disease is obscure.

The association between human lymphoid tissue malignancy and therapy with the anticonvulsant drug, phenytoin sodium, has provided another approach to the study of these malignancies in man. It has been known for many years that lymphadenopathy may occur during therapy with hydantoin drugs, especially Nirvanol (5-ethyl, 5-phenyl hydantoin), used originally in the treatment of Sydenham's chorea, (Saltzstein and Ackerman, 1959). More recently, Gams et al (1968) have defined 4 categories of hydantoin-associated lymphadenopathy. The first, most common type, is a benign, pleomorphic hyperplasia with preservation of lymph node architecture, regressing on withdrawal of phenytoin, the second, a reticulum cell hyperplasia resembling malignant lymphoma, but again regressing on drug withdrawal. The third category is histologically indistinguishable from the second, but the lymphoma recurs after cessation of therapy and causes death of the patient. The fourth category is indistinguishable from malignant lymphoma. Seven cases in group 4 were reported by Hyman and Sommers (1966), four with Reed-Sternberg cells characteristic of Hodgkin's disease. At least 9 further cases of non-Hodgkin's lymphoma have been reported (Rausing and Trell, 1971), and 6 of Hodgkin's

disease (Clinicopathologic Conference, 1962, Anthony, 1970, Brown, 1971, Rausing and Trell, 1971, Charlton and Lunsford, 1971). A further case of Hodgkin's disease associated with phenytoin therapy is reported in this Thesis.

Phenytoin has been shown to increase the incidence of lymphomas in mice, in doses comparable to those used in the treatment of epilepsy in man (Kruger and Harris, 1972). Hypersensitivity to phenytoin and evidence of drug-induced immunosuppression were found in affected mice. The lymph nodes of these mice showed areas of distortion and partial atrophy, and areas of hyperplasia containing pyroninophilic reticulum cells, immature plasma cells and secondary follicles (Kruger, 1970). Lymphomas were either of a different histological type, or occurred significantly earlier, than those usually developing in the mouse strains used (Kruger and Harris, 1972). These workers proposed that the combination of chronic antigenic stimulation and partial immunosuppression in phenytoin-treated animals led to the development of lymphoma. The presence or absence of viruses in the lesions was not commented upon. Evidence to support the hypothesis was obtained in BALB/c mice subject to continuous antigenic stimulation with non-oncogenic viruses, Freund's adjuvant, or HeLa cells, and immunosuppression with azathioprine. Malignant lymphoblastic lymphomas developed in 20 percent of BALB/c mice treated with azathioprine and antigen as early as three months later, but not in mice treated with antigen or azathioprine alone. The lymph nodes showed changes of antigen-induced proliferation and

partial atrophy. Foci of atypical reticulum cells were present in 30% of atrophic nodes (Krueger et al, 1971). Virus particles were not looked for in the lesions. These findings, then, are also consistent with the hypothesis of Schwartz, (1972) viz., that the immune response, poorly controlled in the presence of immunosuppression, causes the activation of latent oncogenic viruses. In the present study, immunological defects were found in a large proportion of epileptic patients treated with phenytoin (Chapter V). With the exception of IgA and IgM concentrations, similar types of abnormality were identified in patients with lymphoma.

The deficiencies of cellular and humoral immune responses in the patient (F.K.) who developed Hodgkin's disease three years after the commencement of phenytoin therapy, were not directly comparable with those of the phenytoin-treated and lymphoma patients because of the radiation therapy given him 10 months before this study. However, the lymphocytes of this patient were stimulated to undergo increased DNA synthesis in the presence of phenytoin in vitro, in contrast to the cells of 2 patients with Hodgkin's disease treated previously by radiotherapy, 6 patients taking phenytoin (without clinical evidence of lymphadenopathy), and 3 healthy subjects. Stimulation of lymphocyte DNA synthesis by phenytoin has previously been reported in a case of hypersensitivity to phenytoin (Holland and Mauer, 1965), and is similar to the anamnestic response of lymphocytes to specific antigen in vitro (Pearmain et al, 1963, Ling, 1968). This stimulation of lymphocytes by phenytoin

in the presence of the general hyporeactivity found in the patient studied, is highly significant. Even though the circulating lymphocyte count of this patient was very low (450/µl), DNA synthesis in his blood leukocytes was within the normal range, suggesting that the lymphocytes were constantly being stimulated by phenytoin to undergo DNA synthesis in vivo. A high rate of spontaneous DNA synthesis has recently been demonstrated in lymphocytes from phenytoin-sensitive subjects who have had rashes, fever, or a lupus-like syndrome (MacKinney and Booker, 1972). However, incubation of these cells with phenytoin in vitro was associated with a depression of DNA synthesis, in contrast to the results obtained in patient F.K. It is possible that the combination of lymphocyte sensitivity to phenytoin, and immunosuppression, preceded the development of lymphoma in this patient, in accordance with the hypothesis of Kruger and Harris (1972).

The fact that phenytoin usually suppresses DNA synthesis by lymphocytes in vitro (this study, MacKinney and Booker, 1972) suggests that the drug does not usually act as an hapten in man. The ability to do so is probably genetically determined.

The role of immunosuppression and antigenic stimulation in the aetiology and pathogenesis of lymphomas not known to be associated with any drug or chemical agent requires further study. The prevalence of malignancies of both the lymphoid and epithelial tissues is increased in the mixed primary immunodeficiency syndromes (i.e. syndromes with both T-cell and B-cell defects, Good, 1972) and in immunosuppressed recipients of renal allografts (Starzl

et al, 1971). Malignancy has not been reported in patients with the DiGeorge syndrome, a T-cell deficiency, but B-cell defects are associated with an increased incidence of leukaemia (Good, 1972). In the present study, mixed T-cell and B-cell defects were not common in patients treated with phenytoin (they were present in 4 of the 39 patients in whom all parameters of immunological function were measured), and lymphomas were not present.

Few data are available on the immunological status of patients with early, untreated Hodgkin's disease. Brown et al, (1967) studied primary antibody responses to Tularaemia vaccine, DHS to intradermal antigens and DNCB, lymphocyte counts, and PHA-induced lymphocyte blastogenesis in 50 patients with untreated Hodgkin's disease. All parameters were normal in patients with Stage I disease. However, Sutherland et al (1971) reported that PHA-induced lymphocyte blastogenesis was depressed in a study of 12 untreated patients with Hodgkin's disease; this depression included patients with Stage I disease. Data obtained from 8 patients with untreated Stage I disease using the standard procedures described in the present study, demonstrated abnormalities of both cellular and humoral immunological function. It is likely that subtle derangements of immune capacity will be revealed in early lymphomas by more sophisticated tests (Kaplan, 1972).

The development of lymphoma in patients treated with phenytoin should be a valuable tool in the study of lymphoma in man. More data is required on the immunological status of epileptic

patients, before and after the commencement of therapy with phenytoin, and on the presence of drug hypersensitivity, as manifest by the in vitro induction of DNA synthesis in lymphocytes cultured with phenytoin. Such studies should also permit prediction of the probability that a treated patient will develop lymphoma.

8. SUMMARY

Defects in antibody responses, DHS reactivity, and lymphocyte blastogenesis were found in patients with Hodgkin's disease and other lymphomas. Immunological defects were identified in patients with all stages of Hodgkin's disease. Similar abnormalities were found in patients with lymphoma and patients treated with phenytoin, with the exception of serum IgA and IgM concentrations, which were depressed only in the phenytoin-treated group.

Immunological function was depressed in a patient who developed Hodgkin's disease three years after the commencement of phenytoin therapy; he had received radiotherapy 10 months before study. In vitro, phenytoin stimulated the lymphocytes of this patient to undergo a significant increase in DNA synthesis in comparison with control cells.

APPENDIX (i)

MAINTENANCE OF GLASSWARE AND STERILIZATION PROCEDURE

1. Cleaning of Glassware

Siliconized glassware was

(a) Rinsed in tap water and soaked in a solution of cleaning compound 4-65 (Applied Chemicals Ltd. Aust.)

(b) Washed in the detergent, rinsed four times in tap water, and once in distilled water.

(c) Boiled in distilled water containing Calgon (0.1%, Allbright and Wilson, Aust., Ltd.) and sodium metasilicate (0.09%, Anax, Drug Houses of Aust., Ltd.) for 20 minutes, and rinsed once in distilled water.

(d) Boiled in distilled water for 20 minutes.

(e) Rinsed twice in distilled water, and dried.

Non-siliconized glassware was cleaned with the omission of steps (c) and (d).

2. Sterilization Procedure

(a) Cotton wool plugs were inserted into the neck of pipettes before packaging.

(b) Bottles, disposable culture tubes (caps loosened) and pipettes were sterilized by autoclaving at 120°C for 20-30 mins. They were then dried under vacuum.

APPENDIX (ii)

COMPOSITION OF MEDIUM 199 FOR TISSUE CULTURE

MEDIUM 199 CONCENTRATE, CONTAINED IN EACH 100 ml:

Sodium chloride	8.0 gm	Guanine	0.3 mg
Potassium chloride	0.4 gm	Xanthine	0.3 mg
Magnesium sulphate	0.2 gm	Hypoxanthine	0.3 mg
Disodium phosphate	0.06 gm	Thymine	0.3 mg
Monopotassium phosphate	0.06 gm	Uracil	0.3 mg
Calcium chloride	0.14 gm	Pyridoxal	0.025 mg
Ferric nitrate	0.1 mg	Pyridoxine	0.025 mg
Glucose	1.0 gm	Niacin	0.025 mg
Adenine sulphate	0.01 gm	Niacinamide	0.025 mg
l-Arginine	0.07 gm	p-Amino benzoic acid	0.05 mg
l-Histidine	0.02 gm	Inositol	0.05 mg
l-Lysine	0.07 gm	Riboflavin	0.01 mg
dl-Tryptophane	0.02 gm	Thiamin	0.01 mg
l-Tyrosine	0.04 gm	Calcium pantothenate	0.01 mg
l-Cystine	0.02 gm	Choline chloride	0.5 mg
dl-phenylalanine	0.05 gm	Biotin	0.01 mg
dl-Methionine	0.03 gm	Folic acid	0.01 mg
dl-Serine	0.05 gm	Calciferol	0.1 mg
dl-Threonine	0.06 gm	Cholesterol	0.2 mg
dl-Leucine	0.12 gm	Tween 80	0.015 ml
dl-Isoleucine	0.04 gm	Alpha tocopherol	0.01 mg

APPENDIX (ii)-Cont.

dl-Valine	0.05 gm	Menadione	0.01 mg
dl-Glutamic acid	0.15 gm	Ribose	0.5 mg
dl-Aspartic acid	0.06 gm	Desoxyribose	0.5 mg
dl-Alpha alanine	0.05 gm	Adenylic acid	0.2 mg
l-Proline	0.04 gm	Polymixin B sulphate	2,000 U
l-Hydroxyproline	0.01 gm	Neomycin sulphate	1,000 U
Glycine	0.05 gm	Phenyl red	0.02 gm
Glutamine	0.1 gm	Ethyl alcohol	0.2 ml
Sodium acetate	0.05 gm		

SOLUTION DGP FOR MEDIUM 199 CONTAINED IN EACH ml:

l-Cysteine	0.1 mg	Adenosine triphosphate	10.0 mg
Glutathione	0.05 mg	Tween 80	0.005 ml
Ascorbic acid	0.05 mg	Ethyl alcohol	0.01 ml
Vitamin A	0.1 mg		

(Reference: C.S.L. Handbook, 1968).

APPENDIX (iii)

PREPARATION OF HEPES - BUFFERED MEDIUM 199 FOR TISSUE CULTURE

Each litre of solution contained

N-2- hydroxyethylpiperazine-N-2 ethanesulphonic acid (HEPES, A grade - Calbiochem, California)	2.38 gm
Medium 199 concentrate	100 ml
DGP solution	1 ml
Deionized distilled water	approx. 899 ml

The pH was adjusted to 7.7 at 20°C using sodium hydroxide (6N). The medium was sterilized by passage through a millipore membrane filter (pore size 0.22 microns) and stored at 4°C for not more than 10 days before use.

(References: C.S.L. Handbook 1968, Shipman, 1969, Darzynkiewicz and Jacobson, 1971).

APPENDIX (iv)

COMPOSITION OF SCINTILLATION FLUIDS

(a) **Scintillation fluid used in the determination of DNA synthesis and protein synthesis**

1.	1,4 Di-(2-(4- methyl-5-phenyloxazolyl)- benzene (Dimethyl-POPOP, Koch-Light Laboratories Colnbrook, Bucks, England)	0.3 gm
2.	2,5 - Diphenyloxazole (PPO, Koch-Light Laboratories)	4.0 gm
3.	Toluene (AnalaR, BDH Chemicals Ltd., England).	1000 ml

(b) **Scintillation fluid used in the determination of RNA synthesis**

1.	Dimethyl - POPOP	0.3 gm
2.	PPO	5.0 gm
3.	Absolute ethanol (AnalaR, BDH., England)	250 ml
4.	Toluene	750 ml

APPENDIX (v)

STATISTICAL ANALYSES OF CONTROL DATA

1. Immunoglobulin concentrations

(a) IgG:

Fisher's coefficients:

Skewness: $t_1 = 1.054$ ($P < 0.30$)

Kurtosis: $t_2 = -1.405$ ($P < 0.20$)

Conclusion:

Gaussian distribution.

Comparison between males and females:

Student's t-test: $P < 0.50$

Conclusion: The differences are not significant.

Range of IgG concentrations:

(i) Gaussian statistics: 550–1800 mg/100 ml.

(ii) Percentile method: 600–1900 mg/100 ml.

(b) IgA:

Fisher's coefficients:

Skewness: $t_1 = 0.969$ ($P < 0.40$)

Kurtosis: $t_2 = -0.248$ ($P < 0.02$)

Conclusion:

Gaussian distribution.

Comparison between males and females:

Student's t-test: $P < 0.10$

Conclusion: The differences are not significant.

1(b) Cont.

Range of IgA concentrations:

(i) Gaussian statistics: 45–350 mg/100 ml.

(ii) Percentile method: 70–380 mg/100 ml.

(c) IgM:

Fisher's coefficients:

Skewness: $t_1 = 1.774$ ($P < 0.10$)

Kurtosis: $t_2 = -3.541$ ($P < 0.001$)

Conclusion:

The distribution is symmetrical, but not Gaussian.

After conversion of IgM concentrations to logarithms (base 10):

Skewness: $t_1 = -0.400$ ($P < 0.80$)

Kurtosis: $t_2 = -1.262$ ($P < 0.30$)

Conclusion:

Distribution Gaussian (log-normal)

Comparison between males and females:

Wilcoxon's Rank sum test: $P < 0.05$.

Conclusion: The differences are not significant.

Range of IgM concentrations:

(i) Gaussian statistics: 50–350 mg/100 ml

(ii) Percentile method: 50–370 mg/100 ml

APPENDIX (v)-Cont.

2. Complement concentrations

Fisher's coefficients:

Skewness: $t_1 = 1.418$ ($P < 0.20$)

Kurtosis: $t_2 = -1.998$ ($P < 0.05$)

Conclusion:

Gaussian distribution.

The exact distribution of males and females in this group was not known. They could not therefore be compared.

Range of serum complement concentrations:

(i) Gaussian statistics: 50-200 mg/100 ml

(ii) Percentile method: 70-225 mg/100 ml

3. Lymphocyte Counts

Fisher's coefficients:

Skewness: $t_1 = 0.117$ (*$P < 0.95$)

Kurtosis: $t_2 = 0.537$ (*$P < 0.60$)

Conclusion:

Gaussian distribution.

Comparison between males and females

Student's t-test: $P < 0.50$

Conclusion: Thed ifferences are not significant.

*P = Probability. Differences are significant when -

$P < 0.01$ for Fisher's coefficients (Snedecor, 1959).

$P < 0.05$ for Student's t-test.

Range of lymphocyte counts$^{\phi}$

(i) Gaussian statistics: 1000 - 2800 (lymphocytes/µl of blood)

(ii) Percentile method: 900 - 2900 (lymphocytes/µl of blood)

$^{\phi}$ The accepted cut-off points in the control range of values were those determined by the percentile method.

4. PHA-Induced Lymphocyte Transformation

(a) DNA synthesis in the presence of autologous serum:

Fisher's coefficients:

Skewness: $t_1 = 1.37$ ($P < 0.20$)

Kurtosis: $t_2 = 1.80$ ($P < 0.10$)

Conclusion:

Gaussian distribution.

Comparison between males and females:

Student's t-test: $P < 0.10$

Conclusion: The differences are not significant.

Effect of age on lymphocyte transformation:

Wilcoxon's Rank sum test: The differences are not significant ($P > 0.05$).

Range of PHA-induced DNA synthesis (autologous serum):

(i) Gaussian statistics: 0.5 - 156.0 (dpm/culture x 10^3)

(ii) Percentile method: 19.0 - 152.0 (dpm/culture x 10^3)

4
(b) DNA synthesis in the presence of FCS:

Fisher's coefficients:

Skewness: $t_1 = 0.95$ ($P < 0.40$)

Kurtosis: $t_2 = 1.80$ ($P < 0.10$)

Conclusion:

Gaussian distribution.

Comparison between males and females:

Student's t-test: $P < 0.50$

Conclusion: The differences are not significant

Effect of age on DNA synthesis:

Wilcoxon's Rank sum test: Intergroup differences are not significant.

Range in control subjects:

(i) Gaussian statistics: 10–130 (dpm/culture x 10^3)

(ii) Percentile method: 15–130 (dpm/culture x 10^3)

5. DNA Synthesis in Circulating Leukocytes

Fisher's coefficients:

Skewness: $t_1 = 3.8$ ($P < 0.001$)

Kurtosis: $t_2 = 1.56$ ($P < 0.20$)

Conclusion:

The distribution is skewed.

After conversion to logarithms (base 10),

Skewness: $t_1 = 1.03$ ($P < 0.40$)

Kurtosis: $t_2 = 1.71$ ($P < 0.10$)

Conclusion:

Gaussian distribution (i.e. log-normal).

APPENDIX (v)-Cont.

5. (Cont)

Means and standard deviations:

Mean ($\log_{10}$dpm/culture) = 2.5922

Standard deviation = 0.1949

Geometric mean = 391

Comparison between males and females:

Wilcoxon's Rank sum test: $P < 0.90$

Conclusion: The differences are not significant.

Range of leukocyte DNA synthesis:

(i) Gaussian statistics: 160 - 960 (dpm/culture)

(ii) Percentile method: 155 - 965 (dpm/culture)

APPENDIX (vi)

COUNTING FLUID FOR DETERMINATION OF CELL COUNTS, AND NUCLEAR VOLUME ANALYSIS

Solution A:

Each litre contained

(a)	Formaldehyde	10 ml
(b)	Glacial acetic acid	5 ml
(c)	Sodium chloride 0.9%, ('Venosol', Farmer-Hill Pty. Ltd., Aust.)	985 ml

Dust and other particles were removed by filtration through a millipore filter (pore size 0.22 microns).

Solution B:

Each millilitre contained

(a)	Cetrimide	50 mg
(b)	Sodium chloride, 0.9%	1 ml

To avoid the formation of air bubbles, particles were removed by gravity filtration through a Whatman No. 42 filter paper.

Solutions were always freshly prepared. Aliquots of Solution B were added to aliquots of Solution A immediately before cell processing (see text).

APPENDIX (vii)

USE OF COULTER COUNTER AND MULTI-CHANNEL ANALYSER

1. Determination of Coulter Counter Settings for use in the Counting of Cultured Cells and for Nuclear Volume Analysis

Nuclear volume analyses were performed on a Coulter counter, Model B, coupled to a 128 channel analyser (Nuclear Data).

(a) Orifice diameter - 50 microns.

In accordance with the recommendations of Walker and Hutka (1971) the diameter of the particles to be measured was less than 30% of the orifice diameter.

(b) Current setting = 1

Amplification setting = 2

Satisfactory pulse heights were obtained on the oscilloscope at these settings.

(c) Threshold settings - 20 - 90.

The lower threshold was determined by plotting the cell count against the lower threshold setting. There was a plateau of cell counts at lower threshold settings of 20-30. The lower threshold was therefore set at 20.

(d) Coincidence correction was applied where necessary.

2. Nuclear Volume Analysis - Correlation Between Coulter Counter and Multi-Channel Analyser

The modal volume of the processed cells was determined on the Coulter Counter by measuring the number of cells in successive pairs of settings. The peak channel obtained from the Coulter Counter was

2. (cont.)

consistently only one less than that obtained on the multi-channel analyser.

3. Routine Standardisation of Multi-Channel Analyser

Polystyrene latex particles (diameter 5.02 microns, Dow Chemical Co., Indianapolis) were analysed at current setting 1, and amplification settings of 1 and 2; 10^4 particles were counted in the modal channel. Under these conditions, the width of the curve measured at half of its height in the modal channel doubled as the amplification was doubled. Results were also corrected for modal channel drift using the formula -

$$a = y - 2x$$

where a = distance of the distribution curve (no. of channels) from the origin.

x = modal channel recorded at an amplification setting of 1.

y = modal channel at an amplification of 2.

The value of 'a' was then added to the value of 'x' to obtained the actual peak channel.

APPENDIX (viii)

SEPARATION OF LYMPHOCYTES FROM WHOLE BLOOD

1. Preparation of Methyl-cellulose - Hypaque Solution

Methyl-cellulose (British Drug Houses Ltd., England) was prepared as a 2% solution in deionized distilled water. Hypaque (sodium 3, 5 - diacetamido - 2, 4, 6 - triiodobenzoate, 50% solution, Winthrop Laboratories, Sydney, Australia) was diluted to a 37% solution with distilled water. The methyl-cellulose and Hypaque solutions were mixed in a ratio of 16:10, and sterilized in an autoclave at 120°C for 20 minutes.

2. Preparation of Cotton Wool Column

Glass columns (Pyrex, England) measured 18 cm in length, with an internal diameter of 1.2 cm. Each was packed moderately firmly with cotton wool in its middle 10 cm. The inlet was covered by a rubber stopper, for aseptic introduction of a hypodermic needle. The outlet contained a rubber bung pierced by a 20 gauge hypodermic needle. Both ends of the column were packaged before autoclaving at 120°C for 20 mins. Columns were vacuum dried.

APPENDIX (ix)

RESULTS OF PRELIMINARY STUDY - STATISTICAL ANALYSES

Immunoglobulin concentrations

Immunoglobulins were measured in 45 non-epileptic control patients, 50 patients treated with phenytoin, and 15 epileptic patients treated with barbiturate. These numbers were not large enough to determine the distribution of results, which were therefore assumed to be Gaussian. Variances were compared by Fisher's F-test.

1. IgG

Comparison between control and phenytoin-treated patients:

F test: $F = 3.104$

Conclusion: The variances are significantly different.

Comparison of means:

(variances unequal) $P < 0.001$

Comparison between control and barbiturate-treated patients:

F-test $F = 1.711$

Conclusion: The variances are the same.

Student's t-test: $t = 0.041$

$P > 0.05$

Conclusion: The differences are not significant.

Comparison between phenytoin and barbiturate-treated patients:

F test: $F = 0.551$

Conclusion: The variances are the same.

Student's t-test: $P < 0.001$

APPENDIX (ix)-(Cont.)

2. IgA

Comparison between control and phenytoin-treated patients:

F test: F = 1.055

Conclusion: The variances are the same.

Student's t-test: t = 0.9497

Conclusion: The differences are not significant

Comparison between control and barbiturate-treated patients:

F test: F = 1.369

Conclusion: The variances are the same.

Student's t-test: t = 1.5860

P < 0.20

Conclusion: The differences are not significant.

Comparison between phenytoin and barbiturate-treated patients:

F test: F = 1.445

Conclusion: The variances are the same.

Student's t-test: t = 2.2019

P < 0.05

Conclusion: The differences are significant.

3. IgM

Comparison between control and phenytoin-treated patients:

F test: F = 1.079

Conclusion: Variances are the same.

Student's t-test: t = 0.6181

P > 0.05

Conclusion: The differences are not significant.

3 (cont)

Comparison between controls and barbiturate-treated patients:

Wilcoxon's non-parametric Rank sum test: $P < 0.05$

Conclusion: Values significantly high in barbiturate-treated group.

Comparison between phenytoin and barbiturate-treated patients:

Wilcoxon's Rank sum test: $P < 0.05$

Conclusion: Values significantly high in barbiturate-treated group.

Lymphocyte Counts

Comparison between control and phenytoin-treated patients:

F test: $F = 1.9043$

Conclusion: The variances are not significantly different.

Student's t-test: $t = 0.0120$

$P > 0.05$

Conclusion: The differences are not significant.

Comparison between control and barbiturate-treated patients:

F test: $F = 1.9412$

Conclusion: The variances are the same.

Student's t-test: $t = 1.9230$

$P > 0.05$

Conclusion: The differences are not significant.

APPENDIX (ix)-(Cont.)

Lymphocyte Counts (cont.)

Comparison between phenytoin and barbiturate-treated patients:

F test: $F = 0.774$

Conclusion: Variances are the same.

Student's t-test: $t = 2.149$

$P < 0.05$

Conclusion: Means are significantly different.

APPENDIX (x)

RESULTS OF DEFINITIVE STUDY OF PHENYTOIN-TREATED PATIENTS - STATISTICAL ANALYSES

Immunoglobulin Concentration

1. IgG

The distribution of results in the control group (85 subjects) was Gaussian (see Chapter II). The distribution in the phenytoin-treated group (61 patients) was tested by calculation of Fisher's coefficients:

Skewness: $t_1 = 1.485$ ($P < 0.20$)
Kurtosis: $t_2 = -2.429$ ($P < 0.02$)
Conclusion: Gaussian distribution

The sample values in the carbamazepine-treated group (15 patients) were assumed to be part of a normally distributed population.

The variances of the respective groups were compared by Fisher's F-test. The results have been tabulated below.

Group	F-test (F=)	Significance
Control-phenytoin		N.S.
Control-carbamazepine	0.623	N.S.
Phenytoin-carbamazepine	0.882	N.S.
Control-phenytoin (males)	1.552	N.S.
Control-phenytoin (females)	1.238	N.S.
phenytoin alone - multiple a/c	0.743	N.S.

When the variances of the two groups were not significantly different, the means were compared using Student's t-test; where unequal, means were compared by the method described in Documentia Geigy (1962 p.172).

2. IgA

The distribution of results in the control group (87 patients) was Gaussian. Fisher's coefficients were calculated for the 62 values in the phenytoin-treated group:

Skewness:	$t_1 = 1.548$, $P < 0.20$
Kurtosis:	$t_2 = -1.262$, $P < 0.20$
Conclusion:	Gaussian distribution

The sample values in the carbamazepine-treated group (15) were assumed to form part of a normally distributed population.

The variances of the respective groups were compared by Fisher's F-test. They have been tabulated below.

Group	F value	Significance
Control-phenytoin	0.975	N.S.
Control-carbamazepine	1.763	N.S.
Phenytoin-carbamazepine	1.809	N.S.
Control-phenytoin (males)	1.563	N.S.
Control-phenytoin (females)	1.512	N.S.
Phenytoin alone - multiple a/c	1.235	N.S.

Means were compared by Student's t-test. The results are given in the text (Chapter v).

3. IgM

The distribution of results in the control group (89 subjects) was log-normal. Fisher's coefficients were calculated for the 62 values in the phenytoin-treated group:

Skewness: $t_1 = 1.2579$ ($P < 0.20$)
Kurtosis: $t_2 = -0.5402$ ($P < 0.60$)
Conclusion: Gaussian distribution.

i.e. the distribution of results in the two groups was different. Values were therefore compared using Wilcoxon's non-parametric Rank sum test. The results are given in the text.

4. Complement Concentration

The results in the control group (54 subjects) were normally distributed (see Chapter II). The sample values in the phenytoin-treated group (49 patients), and the carbamazepine-treated group (15 patients) were assumed to be normally distributed.

The variances of the respective groups were compared using Fisher's F test. These results have been tabulated below.

COMPLEMENT CONCENTRATION

Group	F-test (F=)	Significance
Control-phenytoin	2.019	*S
Control-carbamazepine	1.882	**N.S.
Phenytoin-carbamazepine	3.719	S
Phenytoin (males-females)	2.751	S
Phenytoin (alone - mult. a/c)	1.630	N.S.

* Differences significant (at 5% level)

** Differences not significant.

Groups in which the variances were not significantly different were compared using Student's t-test; groups with different variances were compared by the method described in Documentia Geigy (1962, p.172). The results are given in the text.

5. Lymphocyte Counts

The distribution of results in the control group (83 subjects) was Gaussian. It was symmetrical in the phenytoin-treated group (49 patients, Pearson's coefficient of skewness), and was assumed to be Gaussian.

The variances of the respective groups were compared by Fisher's F-test; the results are tabulated below.

LYMPHOCYTE COUNT

Group	F-test	Significance
Control-phenytoin	0.679	N.S.
Control-carbamazepine	0.783	N.S.
Phenytoin-carbamazepine	1.154	N.S.
Control-phenytoin (males)	2.010	S.
Control-phenytoin (females)	1.217	N.S.
Phenytoin (alone) - multiple a/c	1.251	N.S.

The male control group was compared with the male phenytoin-treated group using the test for normal populations with unequal variance. Other means were compared by Student's t-test. Results are given in the text.

APPENDIX (x)-(Cont.)

6. Circulating Leukocyte DNA Synthesis

The distribution of results in the control group (90 subjects) was log-normal. The sample values in the phenytoin-treated group (39 patients) and in the carbamazepine-treated group (12 patients) were assumed to be log-normally distributed. Calculations were based on results converted to $\log_{10}$ values.

The variance of the respective groups were compared by Fisher's F-test, and have been tabulated below.

CIRCULATING LEUKOCYTE DNA SYNTHESIS

Group	F-test (F=)	Significance
Control-phenytoin	0.546	N.S.
Control-carbamazepine	0.442	N.S.
Phenytoin-carbamazepine	0.810	N.S.

The means of the respective groups were compared by Student's t-test, (for results, see text). The results of patients on phenytoin alone were compared with those of patients on additional anticonvulsants using Wilcoxon's Rank sum test. Similarly, results of male and female patients on phenytoin were compared with their respective controls by Wilcoxon's Rank sum test. Results are given in the text.

7. PHA-induced DNA Synthesis - FCS

The results in the control sample (84 subjects) were normally distributed (see Chapter II); they were assumed to be so in the phenytoin-treated group (44 patients) and the carbamazepine-treated group (15 patients).

APPENDIX (x)-(Cont.)

Variances were compared by Fisher's F-test, and are tabulated below.

PHA-INDUCED DNA SYNTHESIS (FCS)

Group	F test (F=)	Significance
Control-phenytoin	1.397	N.S.
Control-carbamazepine	1.220	N.S.
Phenytoin-carbamazepine	0.875	N.S.
Phenytoin (males-females)	1.563	N.S.
Phenytoin (alone) - multiple a/c	1.404	N.S.

The means of these groups were compared by Student's t-test, (see text).

8. PHA-induced DNA Synthesis (autologous serum)

The values in the control sample (83 subjects) were normally distributed; they were assumed to be so in the phenytoin-treated group (42 patients) and in the carbamazepine-treated group (15 patients).

The variances of the respective groups were compared using Fisher's F-test, and are tabulated below.

PHA-INDUCED DNA SYNTHESIS - AUTOLOGOUS SERUM

Group	F	Significance
Control-phenytoin	0.970	N.S.
Control-carbamazepine	0.885	N.S.
Phenytoin-carbamazepine	0.913	N.S.
Control-phenytoin (males)	0.965	N.S.
Control-phenytoin (females)	1.473	N.S.
Phenytoin (alone)-multiple a/c	0.923	N.S.

The means of the respective groups were compared using Student's t-test; results are given in the text.

APPENDIX (xi)

IMMUNOLOGICAL FUNCTION BEFORE AND DURING THERAPY WITH PHENYTOIN

Patient	Sex	Age	Time lag (months)	IgG		IgA		IgM		S. typhi		DHS Reactions		Lympho Count	
				Pre Rx	Post Rx	Pre Rx	Post Rx	Pre Rx	Post Rx	Pre Rx	Post Rx	Pre Rx	Post Rx	Pre Rx	Post Rx
1	M	49	15	675	690	198	35	46	35	N.D.	N.D.	N.D.	N.D.	1932	1740
2	F	45	6	930	840	100	80	90	45	N.D.	N.D.	N.D.	N.D.	1672	1728
3	M	41	3	1250	940	300	215	110	110	≥640	320	3	3	3152	2520
4	M	16	5	930	980	145	135	175	125	≥640	320	2	2	2449	2142
5	M	20	3	900	900	110	110	80	95	80	0	3	2	2296	2706
6	M	46	12	1641	1210	73	85	170	175	≥640	320	2	0	1840	2828
7	M	33	9	792	695	190	140	78	65	≥640	160	1	1	1218	1682
8	F	26	2	1080	1260	195	70	160	165	≥640	≥640	1	0	896	946
9	M	56	3	960	1200	180	150	115	110	≥640	≥640	1	1	1092	1120
10	M	30	3	1115	860	130	85	215	150	N.D.	N.D.	1	0	1650	1560
11	M	33	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	≥640	40	2	1	N.D.	N.D.

Pre Rx = Before immunization
Post Rx = After immunization
N.D. = None done.

APPENDIX (xii)

PREPARATION OF FICOLL-HYPAQUE GRADIENT

Ficoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden)

Hypaque Sodium (sodium 3, 5 - diacetamido - 2,4,6 - triiodobenzoate, 50% solution, Winthrop Labs, Sydney, Aust.)

1. Ficoll, 9 gm, was dissolved in distilled water, 100 ml.
2. Hypaque sodium 20 ml, was diluted with distilled water, 9.5 ml.
3. Ficoll solution, 24 parts, was mixed with Hypaque sodium, 10 parts, to give a mixture of density 1.077.
4. The solution was sterilized by autoclaving.

BIBLIOGRAPHY

AISENBERG, A.C. Manifestations of immunologic unresponsiveness in Hodgkin's disease. (1966) Cancer Res. 26, 1152.

AISENBERG, A.C. Lymphocyte function in malignant lymphoma. (1972) Front. Radiation Ther. Onc. 7, 65.

AISENBERG, A.C. Malignant lymphoma. (1973) New Eng. J. Med. 288, 883.

ALARCON-SEGOVIA, D., FISHBEIN, E., REYES, P.A., DIES, H., SHWADSKY, S. Antinuclear antibodies in patients on anticonvulsant therapy. (1972) Clin. Exp. Immunol. 12, 39.

ALEPA, F.P., ZVAIFLER, N.J., SLIWINSKI, A.J. Immunologic effects of cyclophosphamide treatment in rheumatoid arthritis. (1970) Arth. Rheumatism 13, 754.

ALFORD, R.H. The effects of autologous plasma on human lymphocyte transformation. (1970) P.S.E.B.M. 133, 1443.

ALLANSMITH, M., McCLELLAN, B., BUTTERWORTH, M. Stability of human immunoglobulin levels. (1967) P.S.E.B.M. 125, 404.

ALLISON, A.C. Tumour development following immunosuppression. (1970) Proc. roy. Soc. Med. 63, 1077.

ALLWOOD, G., ASHERSON, G.L., DAVEY, M.J., GOODFORD, P.J. The early uptake of radioactive calcium by human lymphocytes treated with phytohaemagglutinin. (1971) Immunology 21, 509.

AMMANN, A.J. Diseases of cellular immunity. U.C.L.A. Conference (1972) Ann. Int. Med. 77, 101.

ANTHONY, J.J. Malignant lymphoma associated with hydantoin drugs. (1970) Arch. Neurol. 22, 450.

ARNASON, B.G., JANKOVIC, B.D., WAKSMAN, B.H. Effect of thymectomy on "delayed" hypersensitive reactions. (1962) Nature 194, 99.

ASOFSKY, R., and CANTOR, H. Cited by Miller, J.F.A.P. et al, in (1971) Cell Immunol. 2, 469.

AUGUST, C.S., ROSEN, F.S., FILLER, R.M., JANEWAY, C.A., MARKOWSKI, B., KAY, H.E.M. Implantation of a foetal thymus, restoring immunological competence in a patient with thymic aplasia (DiGeorge's Syndrome). (1968) Lancet II, 1210.

BAILEY, N.J.T. Statistical methods in biology. 4th Ed. (1968). English Universities Press Ltd., London.

BAKER, H., HERBERT, V., FRANK, O., PASHER, I., HUTNER, S.H., WASSERMAN, L.R., SOBOTKA, H. A microbiologic method for detecting folic acid deficiency in man. (1959) Clin. Chem. 5, 275.

BANKHURST, A.D., WARNER, N.L., SPRENT, J. Surface immunoglobulins on thymus and thymus-derived lymphoid cells. (1971) J. Exp. Med. 134, 1005.

BELLANTI, J.A., and SCHLEGEL, R.J. The diagnosis of immune deficiency diseases. (1971) Ped. Clin. Nth. America 18, 49.

BERENBAUM, M.C. Immunosuppressive agents: The design of selective therapeutic schedules. (1969) Antibiotica et Chemotherapia 15, 155.

BIANCO, C., PATRICK, R, NUSSENZWEIG, V. A population of lymphocytes bearing a membrane receptor for antigen-antibody-complement complexes. (1970) J. Exp. Med. 132, 702.

BIENENSTOCK, J., and DOLEZEL, J. Peyers Patches: Lack of specific antibody-containing cells after oral and parenteral immunization. (1971) J. Immunol. 106, 938.

BIENENSTOCK, J., and PEREY, D.Y.E. Immune mechanisms of mucosal resistance. (1972) Med. Clin. Nth. Amer., 56, 391.

BJORNBERG, A., and HOLST, R. Generalized lymphadenopathy as a drug reaction to hydantoin. (1967) Acta Neurol. Scandinav. 43, 399.

BLAUSTEIN, M.P., and WEISMANN, W.P. Effect of sodium ions on calcium movements in isolated synoptic terminals. (1970) Proc. Nat. Acad. Sci., 66, 664.

BOND, V.P., CRONKITE, E.P., FLIEDNER, T.M., SCHORK, P. Deoxyribonucleic acid synthesizing cells in peripheral blood of normal human beings. (1958) Science 128, 202.

BOREL, Y., and SCHWARTZ, R. Inhibition of immediate and delayed hypersensitivity in the rabbit by 6-mercaptopurine. (1964) J. Immunol. 92, 754.

BROCK, N., and HOHORST, H.-J. cited by FORBES, I.J. and SMITH, J.L. (1967) Lancet II, 334.

BROWN, J.M. Drug-associated lymphadenopathies with special reference to the Reed-Sternberg Cell. (1971) Med. J. Aust. 1, 375.

BROWN, R.S., HAYNES, H.A., FOLEY, H.T., GODWIN, H.A., BERARD, C.W., CARBONE, P.P. Hodgkin's disease - immunological, clinical and histological features of fifty untreated patients. (1967) Ann. Int. Med. 67, 291.

BRUTON, O.C. Agammaglobulinemia. (1952) Pediatrics 9, 722.

BUCHTHAL, F., and SVENSMARK, O. Serum concentrations of diphenylhydantoin (phenytoin) and phenobarbital and their relation to therapeutic and toxic effects. (1971) Psychiat. Neurol. Neurochir. 74, 117.

BUCKLEY, C.E., and DORSEY, F.C. The effect of aging on human serum immunoglobulin concentrations. (1970a) J. Immunol. 105, 964.

BUCKLEY, C.E., and DORSEY, F.C. A comparison of serum immunoglobulin concentrations in sarcoidosis and tuberculosis. (1970b) Ann. Int. Med. 72, 37.

BUCKLEY, C.E., and DORSEY, F.C. Serum immunoglobulin levels throughout the life-span of healthy man. (1971) Ann. Int. Med. 75, 673.

BURNET, F.M. Immunological aspects of disease. (1967) Lancet I, 1171.

BURNET, F.M. Immunological surveillance in neoplasia. (1971) Transplant. Rev. 7, 3.

BURNET, Sir MacFarlane. Auto-immune disease. I. Modern immunological concepts. (1959) Brit. Med. J. 2, 645.

BUTTERWORTH, M., McCLELLAN, B., ALLANSMITH, M. Influence of sex on immunoglobulin levels. (1967) Nature 214, 1224.

CANTOR, H. cited by Raff, M.C., in "Surface Antigenic Markers of Lymphocytes". (1971) Transplant. Rev. 6, 52.

CEREGHINO, J.J., VAN METER, J.C., BROCK, J.T., PENRY, J.K., SMITH, L.D., WHITE, B.G. Preliminary observations of serum carbamazepine concentration in epileptic patients. (1973) Neurology 23, 357.

CHALMERS, D.G., COOPER, E.H., EVANS, C., TOPPING, N.E. Quantitation of the response of lymphocytes in culture to specific and nonspecific stimulation. (1967) Int. Arch. Allergy 32, 117.

CHARLTON, M.H., and LUNSFORD, D. Le Sostanze di idantoina come possibili cause del linfoma maligno. (1971) Minerva Medica 62, 2185.

CHASE, M.W. Delayed-type hypersensitivity and the immunology of Hodgkin's disease, with a parallel examination of sarcoidosis. (1966) Cancer Res. 26, 1097.

CHILGREN, R.A., MEUWISSEN, H.J., QUIE, P.G., GOOD, R.A., HONG, R. The cellular immune defect in chronic mucocutaneous candidiasis. (1969) Lancet I, 1286.

CLAMAN, H.N., and CHAPERON, E.A. Immunologic complementation between thymus and marrow cells - A model for the two-cell theory of immunocompetence. (1969) Transplant. Rev. 1, 92.

CLARKE, E.G.C. Isolation and identification of drugs in pharmaceuticals, body fluids and post-mortem material. (1969) The Pharmaceutical Press, London, p.496.

CLEAVER, J.E. "Thymidine Metabolism and Cell Kinetics". (1967) North-Holland Publ. Co., Amsterdam. Ed. A. Neuberger and E.L. Tatum. Frontiers of Biology - Vol. 6. North-Holland Research Monographs.

CLEVELAND, W.W., FOGEL, B.J., BROWN, W.T., KAY, H.E.M. Foetal thymic transplant in a case of DiGeorge's Syndrome (1968) Lancet II, 1211.

CLINE, M.J. Isolation and characterization of RNA from human leukocytes (1966) J. Lab. Clin. Med. 68, 33.

CLINICOPATHOLOGIC CONFERENCE. Lymphoma or drug reaction occurring during hydantoin therapy for epilepsy. (1962) Am. J. Med. 32, 286.

COOPER, M.D., LAWTON, A.R., KINCADE, P.W. A two-stage model for development of antibody-producing cells. (1972) Clin. Exp. Immunol. 11, 143.

COOPER, M.D., PEREY, D.Y., McKNEALLY, M.F., GABRIELSEN, A.E., SUTHERLAND, D.E.R., GOOD, R.A. A mammalian equivalent of the avian Bursa of Fabricius. (1966) Lancet I, 1388.

COOPER, M.D., PETERSON, R.D.A., GOOD, R.A. Delineation of the thymic and bursal lymphoid systems in the chicken. (1965) Nature 205, 143.

COOPERBAND, S.R., BADGER, A.M., DAVIS, R.C., SCHMID, K., MANNICK, J.A. The effect of immunoregulatory α globulin (IRA) upon lymphocytes in vitro. (1972) J. Immunol. 109, 154.

COOPERBAND, S.R., GREEN, J.A., KENNEDY, M.A., GRANT, M.M. Dissociation and inhibition of the stimulatory effect of phytohaemagglutinin on protein and DNA synthesis in human lymphocyte cultures. (1967) Nature 214, 1240.

COULSON, A.S., and CHALMERS, D.G. Quantitation of peripheral blood lymphocyte cultures. (1966) Nature 209, 378.

CRADDOCK, C.G., LONGMIRE, R., McMILLAN, R. Lymphocytes and the immune response. (1971) New Eng. J. Med. 285, 324.

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

CURRY, A.S. Poisons detection in human organs. (1969) Publ. C.C. Thomas, Springfield, Illinois, p.93.

CURTIS, J.E., HERSH, E.M., HARRIS, J.E., McBRIDE, C., FREIREICH, E.J. The human primary immune response to keyhole limpet haemocyanin: Interrelationships of delayed hypersensitivity, antibody response and in vitro blast transformation. (1970) Clin. Exp. Immunol. 6, 473.

DAGUILLARD, F. Immunologic significance of in vitro lymphocyte responses. (1972) Med. Clin. Nth. Am. 56, 293.

DARZYNKIEWICZ, Z., and JACOBSON, B. HEPES-buffered medium in lymphocyte cultures. (1971) P.S.E.B.M., 136, 387.

DAS, K.C., and HOFFBRAND, A.V. Studies of folate uptake by phytohaemagglutinin-stimulated lymphocytes. (1970) Brit. J. Haemat. 19, 203.

DAVIES, A.J.S. The thymus and the cellular basis of immunity. (1969) Transplant. Rev. 1, 43.

DI GEORGE, A.M. Discussion of a paper by Cooper, M.D., Peterson, R.D.A., and Good, R.A. (1965) J. Pediatrics 67, 907.

DILL, W.A., CHUCOT, L., CHANG, T., GLAZKO, A.J. Simplified benzophenone procedure for determination of diphenylhydantoin in plasma. (1971) Clin. Chem. 17, 1200.

DOCUMENTIA GEIGY. Scientific tables Sixth Edn., Ed. Konrad Diem, Publ. Geigy Pharmaceuticals, St. Leonards, N.S.W., Aust., (1962).

DRINKER, C.K., and YOFFEY, J.M. Lymphatics, lymph and lymphoid tissue. (1941) Harvard Univ. Press. Cited by Yoffey, J.M., 1964 Ann. Rev. Med. 15, 125

DUBIN, I.N. The poverty of the immunological mechanism in patients with Hodgkin's disease. (1947) Ann. Int. Med. 27, 898.

DUMONDE, D.C. "Lymphokines": Molecular mediators of cellular immune responses in animals and man. (1970) Proc. roy. Soc. Med. 63, 899.

DUMONDE, D.C., and MAINI, R.N. The clinical significance of mediators of cellular immunity. (1971) Clin. Allergy 1, 123.

DWYER, J.M., and MACKAY, I.R. Antigen-binding lymphocytes in human blood. (1970) Lancet I, 164.

EDELMAN, G.M., and GALL, W.E. The antibody problem. (1969) Ann. Rev. Biochem. 38, 415.

EILBER, F.R., and MORTON, D.L. Impaired immunological reactivity and recurrence following cancer surgery. (1970) Cancer, 25, 362.

EISINGER, M., FOX, S.M., DE HARVEN, E., BIEDLER, J.L., SANDERS, F.K. Virus-like agents from patients with Hodgkin's disease. (1971) Nature 233, 104.

FALK, J., and OSOBA, D. HL-A antigens and survival in Hodgkin's disease. (1971) Lancet II, 1118.

FELDMANN, M. Induction of B-cell tolerance by antigen specific T-cell factor. (1973) Nature New Biol. 242, 82.

FELDMANN, M., and BASTEN, A. The relationship between antigenic structure and the requirement for thymus-derived cells in the immune response. (1971) J.E.M. 134, 103.

FITZGERALD, M.G. The establishment of a normal human population dose-response curve for lymphocytes cultured with PHA (phytohaem-agglutinin). (1971) Clin. Exp. Immunol. 8, 421.

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

FORBES, I.J. Measurement of immunological function in clinical medicine. (1971) Aust. N.Z.J. Med. 2, 160.

FORBES, I.J., and SMITH, J.L. Effects of anti-inflammatory drugs on lymphocytes. (1967) Lancet II, 334.

FROLAND, S.S., and NATVIG, J.B. Effect of polyspecific rabbit anti-immunoglobulin antisera on human lymphocytes in vitro. (1970) Int. Arch. Allergy 39, 121.

FROLAND, S., and NATVIG, J.B. Surface-bound immunoglobulin as a marker of B lymphocytes in man. (1971) Nature New Biol. 234, 251.

FUDENBERG, H.H. Genetically determined immune deficiency as the predisposing cause of "autoimmunity" and lymphoid neoplasia. (1971) Am. J. Med. 51, 295.

FUDENBERG, H., GOOD, R.A., GOODMAN, H.C., HITZIG, W. et al. Primary immunodeficiencies (WHO Report: Special article) (1971) Pediatrics 47, 927.

GAJL-PECZALSKA, K.J., BIGGAR, W.D., PARK, B.H., GOOD, R.A. B lymphocytes in DiGeorge Syndrome. (1972) Lancet I, 1344.

GALLAGHER, B.B., and BAUMEL, I.P. Cited in GALLAGHER, B.B., BAUMEL, I.P., MATTSON, R.H. Metabolic disposition of primidone and its metabolites in epileptic subjects after single and repeated administration. (1972). Neurology 22, 1186.

GAMS, R.A., NEAL, J.A., CONRAD, F.G. Hydantoin-induced pseudopseudolymphoma. (1968) Ann. Int. Med. 69, 557.

GATTI, R.A., and GOOD, R.A. Occurrence of malignancy in immunodeficiency diseases. (1971) Cancer 28, 89.

GOLD, E.R., and FUDENBERG, H.H. Chromic chloride: A coupling reagent for passive hemagglutination reactions. (1967) J. Immunol. 99, 859.

GOOD, R.A. Relations between immunity and malignancy. (1972). Proc. Nat. Acad. Sci. U.S.A. 69, 1026.

GOULLET, P.P., and KAUFMANN, H. Etudes sur la response immunitaire selon l'age chez le rat. (1964/5) Gerontologia 10, 76.

GREY, H.M., RABELLINO, E., PIROFSKY, B. Immunoglobulins on the surface of lymphocytes. IV Distribution in hypogammaglobulinemia, cellular immune deficiency, and chronic lymphatic leukemia. (1971) J. Clin. Invest. 50, 2368.

GROB, P.J., and HEROLD, G.E. Immunological abnormalities and hydantoins (1972) Brit. Med. J. 2, 561.

GROVE, D.I., BURSTON, T.O., FORBES, I.J. Immune function in multiple myeloma and macroglobulinaemia. (1973) Unpublished.

GROVE, D.I., FORD, R.M., FORBES, I.J. Immunological function in asthma. II: Antibody responses and cellular immunity (1973) Unpublished.

GROVE, D.I., O'CALLAGHAN, S.J., BURSTON, T.O., FORBES, I.J. Immunological function in dystrophia myotonica. (1973) Brit. Med. J. 3, 81.

GUDAT, F.G., HARRIS, T.N., HARRIS, S., HUMMELER, K. Studies on antibody-producing cells. 1. Ultrastructure of 19S and 7S antibody-producing cells. (1970) J. Exp. Med. 132, 448.

GUMP, D.W., and FEKETY, F.R. (Jr.) The relationship of infection and DNA-synthesizing cells in human blood. (1967) J. Lab. Clin. Med. 69, 428.

HABER, E. Immunochemistry. (1968) Ann. Rev. Biochem. 37, 497.

HAFERKAMP, O., SCHLETTWEIN-GSELL, D., SCHWICK, H.G., STORIKO, K. Serum protein in an ageing population with particular reference to evaluation of immune globulins and antibodies. (1966) Gerontologia 12, 30.

HAMFELT, A., and WILMANNS, W. Inhibition studies on folic acid metabolism with drugs suspected to act on the myeloproliferative system. (1965) Clin. Chim. Acta 12, 144.

HARBISON, R.D., and BECKER, B.A. Relation of dosage and time of administration of diphenylhydantoin to its teratogenic effect in mice. (1969) Teratology 2, 305.

HARRIS, J., and BAGAI, R.C. Immune deficiency states associated with malignant disease in man. (1972) Med. Clin. Nth. Am. 56, 501.

HAWKINS, C.F., and MEYNELL, M.J. Megaloblastic anemia due to phenytoin sodium. (1954) Lancet II, 737.

HELLSTROM, I., HELLSTROM, K.E., PIERCE, G.E., YANG, J.P.S. Cellular and humoral immunity to different types of human neoplasms. (1968) Nature 220, 1352.

HELLSTROM, I., HELLSTROM, K.E., SJOGREN, H.O. Serum mediated inhibition of cellular immunity to methylcholanthrene-induced murine sarcomas. (1970) Cellular Immunol. 1, 18.

HERSH, E.M., CARBONE, P.P., FREIREICH, E.J. Recovery of immune responsiveness after drug suppression in man. (1966). J. Lab. Clin. Med. 67, 566.

HERSH, E.M., and HARRIS, J.E. Macrophage-lymphocyte interaction in the antigen-induced blastogenic response of human peripheral blood leukocytes. (1968) J. Immunol. 100, 1184.

HOBBS, J.R. Immunoglobulins in clinical chemistry. (1971) Adv. Clin. Chem. 14, 219.

HOLLAND, P., and MAUER, A.M. Diphenylhydantoin-induced hypersensitivity reaction. (1965) J. Pediat. 66, 322.

HORWITZ, D.A., STASTNY, P., ZIFF, M. Circulating deoxyribonucleic acid-synthesizing mononuclear leukocytes. I. Increased numbers of proliferating mononuclear leukocytes in inflammatory disease. (1970) J. Lab. Clin. Med. 76, 391.

HOSKING, C.S., FITZGERALD, M.G., SIMONS, M.J. Quantified deficiency of lymphocyte response to phytohaemagglutinin in immune deficiency diseases. (1971) Clin. Exp. Immunol. 9, 467.

HOWARD, J.C. The life-span and recirculation of marrow-derived small lymphocytes from rat thoracic duct. (1972) J. Exp. Med. 135, 185.

HSU, C.C.S., and LEEVY, C.M. Inhibition of PHA-stimulated lymphocyte transformation by plasma from patients with advanced alcoholic cirrhosis. (1971) Clin. Exp. Immunol. 8, 749.

HULLIGER, L., and BLAZKOVEK, A.A. A simple and efficient method of separating peripheral-blood leukocytes for in-vitro studies. (1967) Lancet I, 1304.

HUMPHREY, J.H. Cell-mediated immunity - general perspectives. (1967) Br. Med. Bull. 23, 93.

HUNGERFORD, D.A., DONNELLY, A.J., NOWELL, P.C., BECK, S. The chromosome constitution of a human phenotypic intersex. (1959) Am. J. Hum. Genet. 11, 215.

HYMAN, G.A., and SOMMERS, S.C. The development of Hodgkin's disease and lymphoma during anticonvulsant therapy. (1966) Blood 28, 416.

IBBOTSON, R.N., DILENA, B.A., HORWOOD, J.M. Studies on deficiency and absorption of folates in patients on anticonvulsant drugs. (1967) Aust. Ann. Med. 16, 144.

ISHIZAKA, K. The identification and significance of gamma E in Immunobiology. (1971) Ed. Good, R.A. and Fisher, D.W. p.84.

JACOBS, A., ENTWISTLE, C.C., CAMPBELL, H., WATERS, W.E. A random sample from Wales. IV. Circulating gastric and thyroid antibodies and antinuclear factor. (1969) Brit. J. Haemat. 17, 589.

JOHNSON, R.A., and KIRKPATRICK, C.H. Augmentation of phytohemagglutinin mitogenic activity by RBC membranes. (1970) Fed. Proc. 29, Abst. No. 703.

JOHNSON, M.W., MAIBACH, H.I., SALMON, S.E. Skin reactivity of patients with cancer. Impaired delayed hypersensitivity or faulty inflammatory response? (1971) N.E.J.M. 284, 1255.

JOHNSON, G.J., and RUSSELL, P.S. Reaction of human lymphocytes in culture to components of the medium. (1965) Nature 208, 343.

JUNGE, U., HOEKSTRA, J., WOLFE, L., DEINHARDT, F. Microtechnique for quantitative evaluation of in vitro lymphocyte transformation. (1970) Clin. Exp. Immunol. 7, 431.

KAPLAN, H.S. Hodgkin's Disease. (1972) Harvard Univ. Press, Cambridge, Massachusetts. First Ed.

KEMP, J.W., and WOODBURY, D.M. Subcellular distribution of 4-^{14}C-diphenylhydantoin in rat brain. (1971) J. Pharmacol. Exp. Therap. 177, 342.

KINCADE, P.W., LAWTON, A.R., COOPER, M.D. Restriction of surface immunoglobulin determinants to lymphocytes of the plasma cell line. (1971) J. Immunol. 106, 1421.

KLIPSTEIN, F.A. Subnormal serum folate and macrocytosis due to anticonvulsant drug therapy. (1964) Blood 23, 68.

KRETSCHMER, R., SAY, B., BROWN, D., ROSEN, F.S. Congenital aplasia of the thymus gland (DiGeorge's Syndrome). (1968) New Eng. J. Med. 279, 1295.

KRUEGER, G.R.F., MALMGREN, R.A., BERARD, C.W. Malignant lymphomas and plasmacytosis in mice under prolonged immunosuppression and persistent antigenic stimulation. (1971) Transplantation 11, 138.

KRUGER, G. Effect of Dilantin in mice. 1. Changes in the lymphoreticular tissue after acute exposure. (1970) Virchows Arch. Abt. A Path. Anat. 349, 297.

KRUGER, G.R.F., and HARRIS, D. Is phenytoin carcinogenic? (1972) Lancet I, 323.

KUTT, H., and LOUIS, S. Untoward effects of anticonvulsants. (1972) New Eng. J. Med. 286, 1316.

LAMB, D., PILNEY, F., KELLY, W.D., GOOD, R.A. A comparative study of the incidence of anergy in patients with carcinoma, leukemia, Hodgkin's disease and other lymphomas. (1962) J. Immunol. 89, 555.

LAMVIK, J.O. Separation of lymphocytes from human blood. (1966) Acta Haemat. 35, 294.

LAROYE, G.J. Cancer caused by an inherited selective defect in immunological surveillance. (1973) Lancet I, 641.

LAWRENCE, H.S. Transfer factor. (1969) Adv. Immunol. 11, 195.

LAWTON, J.W.M. M.D. Thesis, (1967) p.99.

LEVIN, R.H., LANDY, M., FREI, E. The effect of 6-mercaptopurine on immune response in man. (1964) N.E.J.M. 271, 16.

LEVIS, W.R., and ROBBINS, J.H. The effect of glass-adherent cells on the blastogenic response of "purified" lymphocytes to PHA. (1970) Exp. Cell Res. 61, 153.

LIBANSKY, J. Study of immunologic reactivity in hemoblastosis. Circulating antibody formation as a response to antigenic stimulus in leukemia, malignant lymphoma, myeloma and myelofibrosis. (1965) Blood 25, 169.

LIBANSKY, J. The investigation of the cellular type of immunity in patients with lymphoproliferative and myeloproliferative diseases. (1969) Int. J. Cancer 4, 288.

LILLY, F., BOYSE, E.A., OLD, L.J. Genetic basis of susceptibility to viral leukaemogenesis. (1964) Lancet II, 1207.

LING, N.R. Lymphocyte stimulation. (1968) North-Holland Publishing Co., Amsterdam.

LISCHNER, H.W., PUNNETT, H.H., DIGEORGE, A.M. Lymphocytes in congenital absence of the thymus. (1967) Nature 214, 580.

LOEB, L.A., EWALD, J.L., AGARWAL, S.S. DNA polymerase and DNA replication during lymphocyte transformation. (1970) Cancer Res. 30, 2514.

LO SPALLUTO, J., MILLER, W. Jr., DORWARD, B., FINK, C.W. The formation of macroglobulin antibodies. I. Studies on adult humans. (1962) J. Clin. Invest. 41, 1415.

LOUGHNAN, P.M., GOLD, H., VANCE, J.C. Phenytoin teratogenicity in man. (1973) Lancet I, 70.

LOUIE, J.S., and GOLDBERG, L.S. Lymphocyte-monocyte defect associated with anergy and recurrent infections. (1972) Clin. Exp. Immunol. 11, 469.

McINTYRE, O.R., and COLE, A.F. Variation in the response of normal lymphocytes to PHA. (1969) Int. Arch. Allergy 35, 105.

McINTYRE, O.R., and EBAUGH, F.G. The effect of phytohemagglutinin on leukocyte cultures as measured by P^{32} incorporation into the DNA, RNA and acid-soluble fractions. (1962) Blood 19, 443.

MacKINNEY, A.A., and BOOKER, H.E. Diphenylhydantoin effects on human lymphocytes in vitro and in vivo. An hypothesis to explain some drug reactions. (1972) Arch. Intern. Med. 129, 988.

MacKINNEY, A.A., and VYAS, R. Diphenylhydantoin-induced inhibition of nucleic acid synthesis in cultured human lymphocytes. (1972) Proc. Soc. Exp. Biol. Med. 141, 89.

MAKELA, O. Analogies between lymphocyte receptors and the resulting humoral antibodies. (1970) Transplant. Rev. 5, 3.

MARKKANEN, T., and PELTOLA, O. Pentose-phosphate pathway of leucocytes. (1971) Acta Haemat. 46, 36.

MARSH, J.C., and PERRY, S. Thymidine catabolism by normal and leukaemic human leukocytes. (1964) J. Clin. Invest. 43, 267.

MAXIMOW, A.A. (1909) Folia Haemat. 8, 125. (Cited by Lawton, J.W.M., 1967).

MERRITT, H.H., and PUTNAM, T.J. Sodium diphenyl hydantoinate in the treatment of convulsive disorders. (1938) J. Amer. Med. Ass. 111, 1068.

MEYER, K.K., WEAVER, D.R. et al. Lymphocyte immune deficiency following irradiation for carcinoma of the breast. (1972) Front. Radiation Ther. Onc. 7, 179.

MILLER, J.F.A.P. Immunity and the thymus. (1963) Lancet I, 43.

MILLER, J.F.A.P., BASTEN, A., SPRENT, J., CHEERS, C. Interaction between lymphocytes in immune responses. (1971) Cellular Immunol. 2, 469.

MOORE, M.A.S., and OWEN, J.J.T. Chromosome marker studies in the development of the haemopoietic system in the chick embryo. (1965) Nature (Lond.) 208, 956.

MOORE, M.A.S., and OWEN, J.J.T. Stem cell migration in developing myeloid and lymphoid systems. (1967) Lancet II, 658.

MULLER-EBERHARD, H.J. Complement. (1969) Ann. Rev. Biochem. 38, 389.

MUNIZ, F.J., HOUSTON, E.W., CRUZ-ABAD, L., RITZMANN, S.E., LEVIN, W.C. Nuclear volume distribution of phytohemagglutinin-stimulated human lymphocytes. (1970) Proc. Soc. Exp. Biol. Med. 135, 334.

MUNIZ, F., HOUSTON, E., SCHNEIDER, R., NUSYOWITZ, M. Chromosomal effects of diphenylhydantoins. (1969) Clin. Res. 17, 28.

NASPITZ, Ch.K., RICHTER, M. The action of phytohemagglutinin in vivo and in vitro, a review. (1968) Prog. Allergy 12, 1.

NATHAN, C.F., KARNOVSKY, M.L., DAVID, J.R. Alterations of macrophage functions by mediators from lymphocytes. (1971) J. Exp. Med. 133, 1356.

OSOBA, D. Thymic function, immunologic deficiency, and autoimmunity. (1972) Med. Clin. Nth. Am. 56, 319.

PAGE, D., POSEN, G., STEWART, T., HARRIS, J. Immunological detection of renal allograft rejection in man. (1971) Transplantation 12, 341.

PARKER, F.Jr., JACKSON, H.Jr., FITZHUGH, G., SPIES, T.D. Studies of diseases of the lymphoid and myeloid tissues. IV. Skin reactions to human and avian tuberculin. (1932) J. Immunol. 22, 277.

PARKER, J.W., and LUKES, R.J. The variability of PHA-induced lymphocyte transformation in repetitive studies. Cited in McIntyre, O.R. and Cole, A.F. (1969) Int. Arch. Allergy 35, 105.

PARROTT, D.M.V., and DE SOUSA, M. Thymus-dependent and thymus-independent populations: Origin, migratory patterns and life span. (1971) Clin. Exp. Immunol. 8, 663.

PAULY, J.L., and SOKAL, J.E. A simplified technique for in vitro studies of lymphocyte reactivity. (1972) P.S.E.B.M. 140, 40.

PEARMAIN, G., LYCETTE, R.R., FITZGERALD, P.H. Tuberculin-induced mitosis in peripheral blood leucocytes. (1963) Lancet I, 637.

PENTYCROSS, C.R. Lymphocyte transformation in young people. (1969) Clin. Exp. Immunol. 5, 213.

PHILLIPS, B., and ROITT, I.M. Evidence for transformation of human B lymphocytes by PHA. (1973) Nature New Biol. 241, 254.

PICK, E., and TURK, J.L. The biological activities of soluble lymphocyte products. (1972) Clin. Exp. Immunol. 10, 1.

PINCUS, J.H. Diphenylhydantoin and ion flux in lobster nerve. (1972) Arch. Neurol. 26, 4.

PISCIOTTA, A.V., WESTRING, D.W., DE PREY, C., WALSH, B. Mitogenic effect of phytohaemagglutinin at different ages. (1967) Nature (Lond.) 215, 193.

PLAYFAIR, J.H.L. Cell co-operation in the immune response. (1971) Clin. Exp. Immunol. 8, 839.

QUASTEL, M.R., DOW, D.S., KAPLAN, J.G. In Proc. Fifth Leukocyte Culture Conference. J.E. Harris Ed. (1970) Academic Press, N.Y. p.97 Cited in 1972 Cell Immunol. 5, 137.

RAFF, M.C., NASE, S., MITCHISON, N.A. Mouse specific bone marrow-derived lymphocyte antigen as a marker for thymus-independent lymphocytes. (1971) Nature 230, 50.

RAPPAPORT, H. Atlas of tumour pathology, Section III - Fascicle 8. Tumors of the hemopoietic system. (1966) Publ. Armed Forces Inst. Pathol., Washington, D.C.

RAPPAPORT, H., WINTER, W.J., HICKS, E.B. Follicular lymphoma. A re-evaluation of its position in the scheme of malignant lymphoma. (1956) Cancer 9, 792.

RAUSING, A., and TRELL, E. Malignant lymphogranulomatosis and anticonvulsant therapy. (1971) Acta med. scand. 189, 131.

REED, A.H., HENRY, R.J., MASON, W.B. Influence of statistical method used on the resulting estimate of normal range. (1971) Clin. Chem. 17, 275.

RHODES, K., SCOTT, A., MARKHAM, R.L., MONK-JONES, M.E. Immunological sex differences. A study of patients with rheumatoid arthritis, their relatives, and controls. (1969) Ann. Rheum. Dis. 28, 104.

RICHTER, M., and ALGOM, D. The heterogeneity of lymphocytes. (1972) Med. Clin. Nth. Amer. 56, 305.

RICHTER, M., and NASPITZ, C.K. The variation in response of human peripheral lymphocytes to phytohemagglutinin in vitro. (1967) Int. Arch. Allergy 32, 288.

RIGAS, D.A., ELASSER, P., and HETCH, F. Impaired in vitro response of circulating lymphocytes to phytohaemagglutinin in Down's syndrome: Dose- and time-response curves and relation to cellular immunity. (1970) Int. Arch Allergy Appl. Immunol. 39, 587.

ROCKLIN, R.E., ROSEN, F.S., DAVID, J.R. In vitro lymphocyte response of patients with immunologic deficiency diseases. (1970) New Eng. J. Med. 282, 1340.

ROSENBERG, S.A., and DAVID, J.R. Inhibition of leukocyte migration: An evaluation of this in vitro assay of delayed hypersensitivity in man to a soluble antigen. (1970) J. Immunol. 105, 1447.

ROSENBERG, E.B., POLMAR, S.H., WHALEN, G.E. Increased circulating IgE in trichinosis. (1971) Ann. Int. Med. 75, 575.

ROWLEY, M.J., BUCHANAN, H., MACKAY, I.R. Reciprocal change with age in antibody to extrinsic and intrinsic antigens. (1968) Lancet II, 24.

ROWLEY, M.J., and MACKAY, I.R. Measurement of antibody-producing capacity in man. 1. The normal response to Flagellin from Salmonella Adelaide. (1969) Clin. Exp. Immunol. 5, 407.

RUBINI, J.R., BOND, V.P., KELLER, S., FLIEDNER, T.M., CRONKITE, E.P., DNA synthesis in circulating blood leukocytes labelled in vitro with ^{3}H-thymidine. (1961) J. Lab. Clin. Med. 58, 751

SALTZSTEIN, S.L., and ACKERMAN, L.V. Lymphadenopathy induced by anticonvulsant drugs and mimicking clinically and pathologically malignant lymphomas. (1959) Cancer, 12, 164.

SALZMAN, N.P., PELLEGRINO, M., FRANCESCHINI, P. Biochemical changes in phytohemagglutinin stimulated human lymphocytes. (1966) Exp. Cell. Res. 44, 73.

SAMPLE, W.F., and CHRETIEN, P.B. Thymidine kinetics in human lymphocyte transformation: determination of optimal labelling conditions. (1971) Clin. Exp. Immunol. 9, 419.

SCHELLEKENS, P.TH.A., and EIJSVOOGEL, V.P. Lymphocyte transformation in vitro. 1. Tissue culture conditions and quantitative measurements. (1968) Clin. Exp. Immunol. 3, 571.

SCHREK, R., RAPPAPORT, H., RUBNITZ, M.E., KWARN, H.C. Cytology and reactions of viable cells from malignant lymphoma. (1969) Cancer, 23, 1061.

SCHWARTZ, R.S. Immunosuppressive drugs. (1965) Prog. Allergy. 9, 246.

SCHWARTZ, R.S. Alteration of immunity by antimetabolites in immunity, cancer, and chemotherapy. (1967) Ed. E. Mihich, Academic Press, N.Y. and Lond.

SCHWARTZ, R.S. Immunoregulation, oncogenic viruses and malignant lymphomas. (1972) Lancet I, 1266.

SEGAL, S., COHEN, I.R., FELDMAN, M. Thymus-derived lymphocytes: humoral and cellular reactions distinguished by hydrocortisone. (1972) Science 175, 1126.

SELL, S. Development of restrictions in the expression of immunoglobulin specificities by lymphoid cells. (1970) Transplant. Rev. 5, 19.

SHANNON, D.C., JOHNSON, G., ROSEN, F.S., AUSTEN, K.F. Cellular reactivity to candida albicans antigen. (1966) New Eng. J. Med. 275, 690.

SHELDON, P.J., PAPAMICHAIL, M., HEMSTED, E.H., HOLBOROW, E.J. Thymic origin of atypical lymphoid cells in infectious mononucleosis. (1973) Lancet I, 1153.

SHIPMAN, C. Evaluation of 4-(2-Hydroxyethyl)-1-piperazineethane-sulphonic acid (HEPES) as a tissue culture buffer. (1969) Proc. Soc. Exp. Biol. Med. 130, 305.

SHORTMAN, K., BRUNNER, K.T., CEROTTINI, J-C. Separation of stages in the development of the "T" cells involved in cell-mediated immunity. (1972) J. Exp. Med. 135, 1375.

SHORTMAN, K., DIENER, E., RUSSELL, P., ARMSTRONG, W.D. The role of nonlymphoid accessory cells in the immune response to different antigens. (1970) J. Exp. Med. 131, 461.

SMITH, J.L. Lymphocyte metabolism. (1968) M.D. Thesis.

SMYTHE, P.M., SCHARLAND, M., BRERETON-STILES, G.C., COODAVIA, H.M., GRACE, H.J., LOENING, W.E.K., MAFOYANE, A., PARENT, M.A., VOS, G.H. Thymolymphatic deficiency and depression of cell-mediated immunity in protein-calorie malnutrition. (1971) Lancet II, 939.

SNEDECOR, G.W. Statistical methods. (1959) Fifth Ed. Iowa State College Press, Ames, Iowa.

SOKAL, J.E., and PRIMIKIRIOS, N. The delayed skin test response in Hodgkin's disease and lymphosarcoma. Effect of disease activity. (1961) Cancer 14, 597.

SOLOMON, A., and McLAUGHLIN, C.L. Immunoglobulin disturbances and their clinical significance. (1973) Med. Clin. Nth. Amer. 57, 499.

STARZL, T.E., PENN, I., PUTNAM, C.W., GROTH, C.G., HALGRIMSON, C.G. Iatrogenic alterations of immunologic surveillance in man and their influence on malignancy. (1971) Transplant. Rev. 7, 112.

STEWART, C.C., and INGRAM, M. A method for counting phytohemagglutinin-stimulated lymphocytes. (1967) Blood, 29, 628.

STUTMAN, O. In "Immune Surveillance" Academic Press, New York, (1970) Eds. Smith, R.T. and Landy, M. p. xi.

SUTER, E., and RAMSEIER, H. Cellular reactions in infection. (1964) Adv. Immunol. 4, 117.

SUTHERLAND, R.M., INCH, W.R., McCREDIE, J.A. Phytohemagglutinin (PHA)-induced transformation of lymphocytes from patients with cancer. (1971) Cancer 27, 574.

SVENSMARK, O., and KRISTENSEN, P. Determination of diphenylhydantoin and phenobarbital in small amounts of serum. (1963) J. Lab. Clin. Med. 61, 501.

SWANSON, M.A., and SCHWARTZ, R.S. Immunosuppressive therapy. The relation between clinical response and immunologic competance. (1967) New Eng. J. Med., 277, 163.

SZENBERG, A., and WARNER, N.L. Immunological function of the thymus and bursa of fabricius. (1962) Nature, 194, 146.

TAKAHASHI, T., OLD, L.J., McINTIRE, K.R., BOYSE, E.A. Immunoglobulin and other surface antigens of cells of the immune system. (1971) J. Exp. Med. 134, 815.

THOMAS, L. Discussion of a paper by Medawar, P.B. Reactions to homologous tissue antigens in relation to hypersensitivity. in Cellular and humoral aspects of the hypersensitive states. Symposium no. 9. N.Y. Acad. Med. p. 530. Ed. H. Sherwood Lawrence. (1959) Publ. Hoeber-Harper.

TOMASI, T.B. The gamma A globulins: first line of defense. (1972) "Immunobiology", First Edn., Ed. R.A. Good and D.W. Fisher; Publ. Sinauer Ass., Inc., Connecticut.

TURK, W. Cited by Crowther, D., Fairley, G.H., Sewell, R.L. (1969) J. Exp. Med. 129, 849.

UHR, J.W. The heterogeneity of the immune response. (1964) Science 145, 457.

UHR, J.W. Intracellular events underlying synthesis and secretion of immunoglobulin. (1970) Cellular Immunol. 1, 228.

Van REES, H., WOODBURY, D.M., NOACH, E.L. Effects of ouabain and diphenylhydantoin on electrolyte and water shifts during intestinal absorption in the rat. Cited in 1971. Psychiat. Neurol. Neurochir. 74, 91.

Van ROOTSELAAR, F.J., and WESTENDORP BOERMA, F. Serum levels of immunoglobulins in mongolism, in epilepsy and in unclassified mental deficiency. (1968) Psychiat. Neurol. Neurochir. 71, 501.

VIANNA, N.J., GREENWALD, P., DAVIES, J.N.P. Extended epidemic of Hodgkin's disease in high school students. (1971) Lancet I, 1209.

WALKER, P.H., and HUTKA, J. Use of the Coulter counter (model B) for particle-size analysis of soils. Division of Soils Technical Paper No. 1. (1971) C.S.I.R.O. Aust.

WATSON, D.W. Immune responses and the gut. (1969) Gastroenterology, 56, 944.

WEISSMAN, I.L. Thymus cell migration. (1967) J. Exp. Med. 126, 291.

WELLER, T.H., and COONS, A.H. Fluorescent antibody studies with agents of varicella and herpes zoster propagated in vitro. (1954) Proc. Soc. Exp. Biol. Med. 86, 789.

WEST, C.D., HONG, R., HOLLAND, N.H. Immunoglobulin levels from the newborn period to adulthood and in immunoglobulin defioiency states. (1962) J. Clin. Invest. 41, 2054.

WHITNEY, R.B., and SUTHERLAND, R.M. Enhanced uptake of calcium by transforming lymphocytes. (1972) Cell. Immunol, 5., 137.

WHITTINGHAM, S., and MACKAY, I.R. Design and functions of a department of clinical immunology. (1971) Clin. Exp. Immunol. 8, 857.

WILSON, J.D., and THOMPSON, A.E.R. Death and division of lymphocytes, neglected factors in assessment of PHA-induced transformation. (1968) Lancet II, 1120.

WINKELSTEIN, A., MIKULLA, J.M., NANKIN, H.R., POLLOCK, B.H., STOLZER, B.L. Mechanisms of immunosuppression. Effects of cyclophosphamide on lymphocytes. (1972) J. Lab. Clin. Med. 80, 506.

WOOD, T.A., and FRENKEL, E.P. The atypical lymphocyte. (1967) Am. J. Med. 42, 923.

WOODBURY, D.M., and KEMP. J.W. Pharmacology and mechanisms of action of diphenylhydantoin. (1971) Psychiat. Neurol. Neurochir. 74, 91.

YACHNIN, S. Inhibition of phytogemagglutinin-indiced lymphocyte transformation by α-globulins, lack of correlation with phytohemagglutinin precipitation by serum proteins. (1972) J. Immunol. 108, 845.

YACHNIN, S., ALLEN, L.W., BARON, J.M., SVENSON, R.H. The potentiation of phytohemagglutinin-induced lymphocyte transformation by cell-cell interaction; a matrix hypothesis. (1972) Cellular Immunol. 3, 569.

YOFFEY, J.M., WINTER, G.C.B., OSMOND, D.G., MEEK, E.S. Morphological studies in the culture of human leucocytes with phytohemagglutinin. (1965) Brit. J. Haemat. II, 488.

YOUNG, R.C., CORDER, M.P., HAYNES, H.A., DeVITA, V.T. Delayed hypersensitivity in Hodgkin's disease. A study of 103 untreated patients. (1972) Am. J. Med. 52, 63.

YUNIS, A.A., ARIMURKA, G.K., LUTCHER, C.L., BLASQUEZ, J., HALLORAN, M. Biochemical lesion in dilantin-induced enythroid aplasia. (1967) Blood, 30, 587.

ZACHARSKI, L.R., ELVEBACK, L.R., LINMAN, J.W. Leukocyte counts in healthy adults. (1971) Am. J. Clin. Path. 56, 148.

ZIEGLER, J.L., COHEN, M.H., MORROW, R.H., KYALWAZI, S.K., CARBONE, P.P. Immunological studies in Burkitt's lymphoma. (1970) Cancer 25, 734.

ZWEIMAN, B., PAPPAGIANIS, D., MAIBACH, H., HILDRETH, E.A. Coccidioidin delayed hypersensitivity: skin test and in vitro lymphocyte reactivities. (1969) J. Immunol. 102, 1284.

ZWEIMAN, B., and SILBERBERG, D.H. In vitro lymphocyte responsiveness of human subjects receiving azathioprine. (1971) Int. Arch. Allergy Appl. Immunol. 41, 428.