LYMPHOCYTE FUNCTION IN PATIENTS WITH CERTAIN NEUROLOGICAL DISEASES

by

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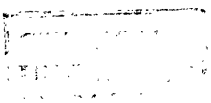
A thesis submitted for the degree of Master of Science in the University of Glasgow

October 1980



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ACKNOWLEDGEMENTS

I am grateful to Professor J. A. Simpson, Professor of Neurology, University of Glasgow, in whose department this work was carried out. I am also grateful to Professor D. R. Newth, Professor of Zoology, University of Glasgow, for supervising this thesis.

Mr. D. G. T. Thomas, consultant neurosurgeon in the Institute of Neurological Sciences, Glasgow (now Senior Lecturer, National Hospital, Queen's Square, London) provided help in the diagnosis of patients with brain tumours, and Dr. M. Gunar, also from the Department of Neurosurgery, assisted in the collecting of blood samples and intradermal injections for testing cutaneous reactivity.

My thanks also to Dr. J. T. Nichols (Clinical Research Department, Pharmaceuticals Division, Imperial Chemical Industries Ltd., Macclesfield), who recommended patients taking β-blocking drugs, and supplied samples of these drugs for in vitro investigations. Drug samples were also supplied by Astra Chemicals Ltd., Watford, and Ciba Laboratories, Horsham.

Dr. R. A. Shakir, Department of Neurology, University of Glasgow, kindly provided blood samples and information on patients with epilepsy, and calculated the serum IgA levels.

I am grateful to Miss Wendy Ogg and the Department of Medical Illustration, Institute of Neurological Sciences, Southern General Hospital, Glasgow for all their help, and to Mrs. J. Henderson for typing this work.

Dr. P. O. Behan, Senior Lecturer in Neurology, University of Glasgow, provided me with much help and guidance, both in the design of the experiments undertaken, and the presentation of these results. I am very grateful for all his encouragement. Dr. W. M. H. Behan also helped in the compilation of this thesis.

I was supported in this work by a grant from the Muscular Dystrophy Group.

SUMMARY

The quantitative evaluation of the reactivity in human phytohaemagglutinin-stimulated lymphocyte cultures was studied using a whole blood technique. Cells were cultured for 22 hours in various concentrations of PHA, and by measuring the amount of incorporation of tritium labelled leucine into the proteins synthesised, a dose response curve could be drawn up for each donor. The patients studied fell into three main categories.

(a) 22 patients with brain tumours. Depressed protein synthesis was detected in many of these when compared to 35 normal healthy controls, and also to 20 patients with various chronic neurological diseases. A 3-day culture technique was developed to test the effect of sera from patients with brain tumours, and other neurological complaints, on normal lymphocytes. No serum blocking factor was found.

(b) Patients undergoing practolol therapy. Impaired T-cell function was seen in all patients whether they manifested adverse reactions to the drug in vivo or not. Normal lymphocytes were cultured for 2 days in the presence of various β-blocking drugs, then their response to PHA was measured. Only propranolol was seen to cause depression of protein synthesis.

(c) 31 patients with epilepsy who were taking various anticonvulsant drugs, including phenytoin. Low responses were seen in all patients on anticonvulsants when compared to 35 normal healthy controls.

ABBREVIATIONS

ATP	Adenosine triphosphate
B-lymphocyte	Non-thymus derived lymphocyte
cAMP	Cyclic adenosine 3', 5'-monophosphate
cGMP	Cyclic guanosine 3', 5'-monophosphate
C3	The third component of complement
Con A	Concanavelin A
DNA	Deoxyribonucleic acid
DNCB	Dinitrochlorobenzene
DNFB	Dinitrofluorobenzene
FCS	Foetal calf serum
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
mRNA	Messenger RNA
P	Probability value
PHA	Phytohaemagglutinin
PPD	Purified protein derivative of tuberculin
PWM	Pokeweed mitogen
RNA	Ribonucleic acid
s.d.	Standard deviation
SK/SD	Streptokinase/Streptodornase
T-lymphocyte	Thymus derived lymphocyte
tRNA	Transfer RNA
$\bar{x}$	Mean value

INTRODUCTION

Knowledge of the workings of the immune system has expanded enormously in recent years. Early workers in this field concentrated on serological reactions, basing their concepts on historical observations that patients who survived certain infectious diseases were protected from subsequent infection. This ability to ward off infections was considered to be a function purely of humoral substances only. Attention was drawn to the possible role of cells in immunity by the great Russian, Eli Metchnikoff, who, having resigned his professorship at Messina in 1882, began to study the behaviour of microscopic marine organisms. From his work, a theory of phagocytosis arose, and it is a reflection of the ignorance at that time, that two different camps of immunology evolved. We thus have the beginning of modern immunology in which we now know that both cellular and humoral factors are involved.

In the late nineteenth century, Robert Koch described a modified reaction to tuberculosis in animals which had previously been exposed to infection. He observed that the inoculation of cultures of bacilli into a tuberculous guinea pig caused the appearance of an inflamed area after 1 to 2 days, whereas little reaction occurred in normal animals similarly injected (Koch, 1890). This Koch phenomenon could be elicited with living, killed, or glycerine extracts of tubercle bacilli. Further studies of the skin reaction taking place demonstrated that it differed from humoral immune reactions in (a) its time of onset, (b) its dermal characteristics, and (c) its

histological components. The term, delayed hypersensitivity, was applied to this skin reaction and it was initially studied with infectious agents such as tuberculosis, hydatid disease, coccidioidomycosis, histoplasmosis and leprosy. In 1928 Dienes demonstrated that a similar reaction could be elicited to proteins other than bacterial.

The characteristic skin changes in a delayed hypersensitivity reaction include oedema with perivenous mononuclear cell infiltration of lymphocytes, macrophages and epithelial cells. A variety of evidence began to accumulate which showed that the lymphocyte was the cell responsible for this reaction. This rested on the facts that lymphocytes are always present in the lesion, and that the reaction may be transferred by living lymphoid cells in syngeneic animals and not by serum. The passive transfer of contact sensitivity by Landsteiner and Chase (1942) and of sensitivity to tuberculin by Chase (1945) demonstrated that viable cells from peritoneal exudates, lymph nodes and spleens could mediate this reaction.

Despite the early attention paid to the cellular factors in immunology, the explosion of knowledge of cellular immune mechanisms only took place in the middle of this century. Our present knowledge of cellular immune function grew out of the ability to study cells in tissue culture.

The central importance of the lymphocyte was established largely by the work of Gowans (1966), who, using a radioactive label to follow their fate <u>in vivo</u>, showed the presence of a pool of recirculating leukocytes which pass from the blood into lymph nodes, thymus, spleen

and bone marrow, by way of the major lymphatic channels, such as the thoracic duct. In general, the lymphocyte is a spherical cell with a large round nucleus and a narrow rim of cytoplasm which appears to be lacking endoplasmic reticulum, and a Golgi apparatus, structures normally associated with protein synthesis, and secretion. In this group of cells, heterogeneous with respect to life-span, origin and function, two main morphological types appear to exist: small lymphocytes with an overall diameter of 7-8 microns, making up 92% of all lymphocytes; and large lymphocytes with a diameter of around 12 microns.

Before 1960, the only known activity of small lymphocytes was motility, and it was generally suggested that the small lymphocytes did not themselves perform physiologically, but that they served as precursors of other cell types. As techniques and experimental skills became more advanced, the small lymphocyte began to emerge as the primary cell involved in the defence of the body, being responsible for recognition of foreign antigens, and for initiating reactions against them. Lymphocytes were shown to be involved in antibody production and in graft rejection, as well as delayed hypersensitivity reactions.

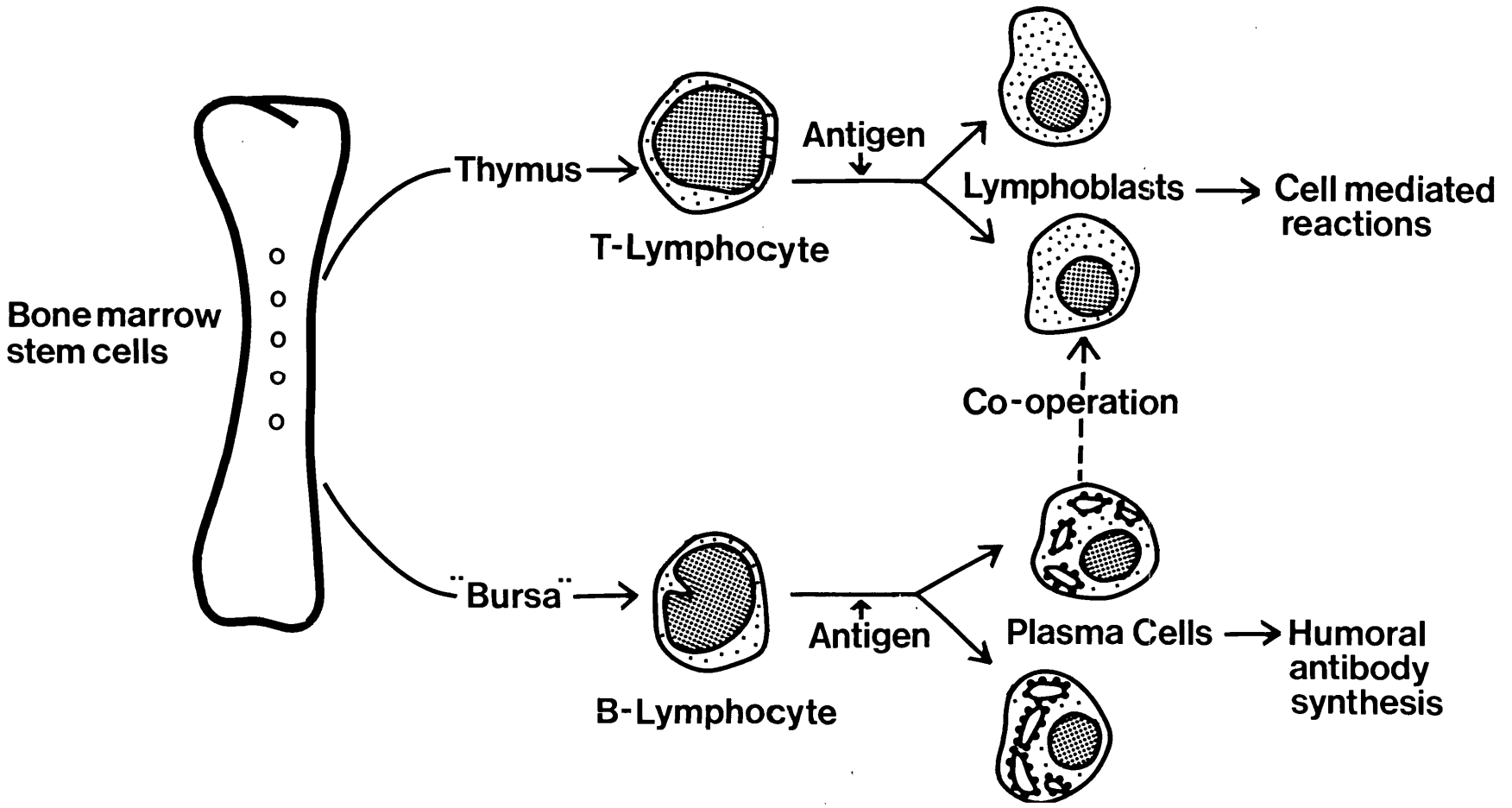
The discovery that the thymus was involved in the development of the immune system (Miller, 1961; Archer, Pierce, Papermaster and Good, 1962), proved to be of central importance. From this came the finding that lymphocytes associated with the thymus were not capable themselves of making antibody, but co-operated with other non-thymus derived lymphocytes to do so (Davies, 1969;

Miller and Mitchell, 1969). These cells were christened T-cells by Roitt, Greaves, Torrigiani, Brostoff and Playfair (1969). The non-thymus derived lymphocytes they called B-cells due to the discovery in chickens of the Bursa of Fabricius, a lymphoid organ which is responsible for the development of immunocompetance in cells destined to make humoral antibody. The equivalent of the bursa in man and other mammals has not yet been clearly defined, although gut-associated lymphoid tissue such as the tonsils, appendix and Peyer's patches have been nominated as possible candidates.

On stimulation by antigen both populations undergo morphological changes (Fig. 1). The B-lymphocytes develop into the plasma cell series, and so are responsible for the production and secretion of immunoglobulins, and for the formation of germinal centres within the primary nodules of lymphoid tissues. T-lymphocytes transform to lymphoblasts which are responsible for all cell mediated immune reactions including graft rejection, delayed hypersensitivity, tuberculin test reactions and defence against viral infections and some bacteria. T-lymphocytes are also known to co-operate with B-cells for the production of antibodies to certain antigens. In this case the T-cell is probably releasing a specific factor which activates B-lymphocytes either directly or through the participation of a third cell, a macrophage.

Various subsets of T-lymphocytes have recently been described: "helper"-cell - a thymus derived cell whose presence is required both <u>in vivo</u> and <u>in vitro</u> for the production of normal levels of antibody by B-lymphocytes

Fig. 1 Proliferation and transformation of lymphocytes on antigenic stimulation.


Bone marrow stem cells
Thymus
T-Lymphocyte
Antigen
Lymphoblasts
Cell mediated reactions
Co-operation
Bursa
B-Lymphocyte
Antigen
Plasma Cells
Humoral antibody synthesis

p. 5 line 11 should read:- ".....e.g. in the mouse, helper cells are phenotypically $Ly1^+$"

to certain antigens known as thymus dependent antigens, and to haptens: "suppressor"-cell - a T-cell capable of regulating the magnitude of antibody production by inhibiting the expression of the various types of B-cells reactive to a particular antigen: "killer"-cell - a T-lymphocyte which in vitro is cytotoxic to cell cultures. As well as being classified according to function, these sub-classes may be identified phenotypically on the basis of antigenic surface markers, e.g. helper cells are phenotypically $Ly1^+$, cytotoxic and suppressor cells are $Ly2^+$ and $Ly3^+$, and immature cells $Ly1^+$, $Ly2^+$ and $Ly3^+$.

Studies in laboratory animals using well defined antigens, and under controlled conditions, have shown that certain diseases involving the immune system can be transferred by living lymphoid cells. Research arising out of studies of these cells in tissue culture have opened up many avenues for the study of possible human immunological disorders. These tissue culture, in vitro, techniques developed from the studies of Rich and Lewis (1932) on the movement of peritoneal macrophages, and on the observations of Hungerford, Donnelly, Nowell and Beck (1959) on the morphological changes that occurred in human peripheral blood lymphocytes on culture with an extract of the red kidney bean, Phaseolus vulgaris.

At the present time there are many techniques for studying the activity of lymphocytes in culture. Most of these are based on the fact that sensitised lymphocytes interact with their corresponding antigen to produce lymphokines (Dumonde, Wolstencroft, Panayi, Matthew, Morley and Howson, 1969), soluble factors which cause a

variety of effects including lymphocyte transformation, inhibition of migration of macrophages and peripheral blood lymphocytes, activation of macrophages, chemotaxis of mononuclear cells, skin reaction, cytotoxicity and inhibition of virus multiplication. In vitro techniques have therefore been developed to give accurate measurement of the lymphokine activity, and thus a measurement of the degree of sensitisation of a population of lymphocytes to a particular antigen.

One technique which has particular advantages is lymphocyte protein synthesis. This technique involves the measurement of protein produced in preparation for mitosis of lymphocytes in contact with either non-specific mitogenic substances, e.g. plant lectins; antigen against which the lymphocyte donor has been previously immunised: or lymphocytes from a genetically foreign individual. Changes in protein synthesis can be detected in lymphocytes within hours of such contact, and measurement of this phenomenon has been used to detect cell mediated immunity (Waithe and Hirschhorn, 1971; Rosenberg and Levy, 1972). By the addition of radioactively labelled amino acids to the culture medium of stimulated cells, it is possible, after a specific time interval, to measure the amount of incorporation into either the proteins or DNA, synthesised by those cells.

Phytohaemagglutinin (PHA), an extract of Phaseolus vulgaris, was the first non-specific mitogen to be demonstrated (Hungerford et al, 1959; Nowell, 1960). Since then, many other lymphocyte mitogens have been found, some of which are extracts from other plants, e.g.

Concanavelin A (Douglas, Kamin and Fudenberg, 1969) and Pokeweed mitogen (PWM) (Farnes, Barker, Brownhill and Fanger, 1964); and some which are bacterial products, e.g. Staphylococcal culture filtrate (Ling, Spicer, James and Williamson, 1964) and Streptolysin S (Hirschhorn, Schreibman, Verbo and Gruskin, 1964). Also included are antibodies against cellular antigens (Grasbeck, Nordman and De La Chapelle, 1964) and against globulins on the cell surface (Adinolfi, Gardner, Gianelli and McGuire, 1967) and various chemicals and ions.

Blastogenesis with non-specific mitogens occurs without prior sensitisation, and equally well in both adult and cord blood (Lindahl-Kiessling and Book, 1964). Unlike the stimulation with antigen, where only a small fraction of the cells respond (Elves, Roath and Israëls, 1963; Pearmain, Lycette and Fitzgerald, 1963), PHA transforms a major proportion of the T-lymphocytes. Additionally, some B-cells are affected, although their response appears to be T-dependent. The picture is emerging that helper T-cells are preferentially stimulated by PHA, and suppressors by Con A (Roitt, 1977). PWM activates both T and B lymphocytes, while lipopolysacharide (in the mouse at least) is a B-cell mitogen. Most of these mitogenic stimulants have a high molecular weight and a relatively complex structure and, as yet, the molecular basis for their mitogenic activity is unknown.

Although the sequence of morphological changes in transformation is well established (Elves, Chapman, Gough and Israëls, 1964; Polliack, Touraine, De Harven, Lampen and Hodden, 1976), the metabolic changes which accompany

and permit morphogenesis are still under investigation.

It has been shown (Powell and Leon, 1970; Allan, Auger and Crumpton, 1971) that the first step essential for activation is the interaction of the carbohydrate binding site of the lectin with the cell surface glycoprotein, or glycolipid receptor. The exact chemical nature of the receptor site is unknown, but work by Toyshima, Fukuda and Osawa (1972) suggests the existence of distinct specific lymphocyte receptors for PHA and Con A. This was confirmed by Faguet (1977) who further suggested that the majority of lymphocyte membrane receptors for PHA and Con A are "spare" or redundant since as little as 3-15% receptor occupancy by the ligand was sufficient for maximal cell response (Novogrodsky, Biniaminov, Ramot and Katchalski, 1972; Faguet, 1976). It also appears that the stimulation of lymphocytes by antigen or lectins usually, and perhaps always, requires that the receptors be crosslinked by multivalent ligands (Greaves and Janossy, 1972; Allan and Crumpton, 1973). Recent studies on the distribution and mobility of lymphocyte surface receptors, and the modulation of receptor movement by lectin reagents, suggests that the surface receptors may have a common anchorage system, consisting of microfilaments and microtubules within the cell (Petris and Raff, 1973; Yahara and Edelman, 1973). It has been proposed (Edelman, Yahara and Wang, 1973) that such a system may also serve the function of regulating and transducing a variety of external signals to the appropriate intracellular messengers. Binding is not itself sufficient to trigger DNA synthesis (Hellström, Dillner, Hammarström and Perlmann, 1976), but causes a

number of biochemical changes.

Within the first minute, and thus preceding other known metabolic alterations in lectin stimulated cells, activation of the enzyme adenylate cyclase, causes a small, but consistent, increase in cyclic adenosine monophosphate (cAMP) with a maximal response obtained after 5-15 minutes (Smith, Steiner and Parker, 1971; Lyle and Parker, 1974; Parker, Sullivan and Wedner, 1974) followed by a decline to control values after 1-2 hours.

This increase leads to a selective activation of one, or a group of protein kinases, under the control of cAMP, and of membrane metabolism and transport, allowing increased uptake of Ca^{++} and other essential nutrients into the cytoplasm (Allwood, Asherman, Davey and Goodford, 1971; Whitney and Sutherland, 1972). Parker (1977) has shown that high concentrations of cAMP (10-100 μM) markedly inhibits the DNA synthetic response to PHA, whereas in low doses, it can stimulate mitogenesis, although the magnitude of change is only 2-3 fold, and much less than the 50-100 fold response seen with PHA or Con A (Hirschhorn, Grossman and Weissman, 1970; Smith, Steiner, Newberry and Parker, 1971).

The exact role of cAMP is poorly understood, but its importance in lymphocyte activation, both as a positive and a negative modulatory agent is clear. Work in several laboratories indicates that cAMP is also involved in the production of lymphokines by stimulated lymphocytes (Kishimoto, Miyake, Nishizawa, Watanabe and Yamamura, 1975). Smith et al (1971) and Hadden, Hadden, Haddox and Goldberg (1972) implicate cGMP as well as cAMP as intracellular

regulators of mammalian cell growth and differentiation. Intracellular levels of cGMP have been shown to increase after human peripheral lymphocytes have been induced to proliferate by Con A or PHA (Hadden et al, 1972), and have been shown to reverse the inhibitory effect of cAMP (Bourne, Lichtenstein, Melmon, Henney, Weinstein and Shearer, 1974). Bourne et al (1974) also suggested that cAMP and cGMP may act in a "push-pull" fashion to regulate lymphocyte functions. The importance, however, of cGMP in early phases of human T-cell activation is controversial (Wedner, Dankner and Parker, 1975) although a good case exists for a modulatory role of cGMP in responses of murine B-lymphocytes to liposaccharides (Watson, 1975; Weinstein, Segal and Melmon, 1975).

During the first hour of the response, PHA and Con A produce a 2-10 fold increase in calcium ion uptake, with the most marked changes occurring during the first few minutes (Parker, 1977; Allwood et al, 1971; Whitney et al, 1972). This change in membrane permeability may be related to the change in cGMP and cAMP levels, but more work is needed to clarify this.

In addition to Ca^{++} and the cyclic nucleotide changes, the possible role of microtubules and microfilaments in the regulation of lymphocyte function must be considered. Most eukaryotic cells contain a rich array of microtubules and microfilaments in close association with the plasma membrane. Morphological studies (Bryan, 1974; Wessels, Spooner, Ash, Bradley, Ludvena, Taylor, Wrenn and Yamada, 1971) suggest that these structures can extend from the cell membrane into

the cytoplasm, and thus either, or both, could be a part of a mechanical transduction system through which perturbations exerted at the lymphocyte surface are conveyed to the interior of the cell.

Which of these early alterations is responsible for transmission of mitogenic signal to the nucleus is not yet clear (Fig. 2).

The amino acid uptake of responding lymphocytes is seen to increase 2-4 fold, 30-60 minutes after contact with PHA or Con A (Parker, 1977). Later RNA (Cooper and Rubin, 1965; Mueller and La Mahieu, 1966) and protein (Bach and Hirschhorn, 1963; Kay and Korner, 1966) synthesis accelerate, and the activities of several amino acids are increased. Protein production takes place mainly in the first two hours (Bach and Hirschhorn, 1963) with not much more by 24 hours. Synthesis of this protein takes place on the ribosomes, which are found bound to the endoplasmic reticulum in the cytoplasm. The ribosomes consist of around 40% protein and 60% RNA, and this RNA, termed messenger or mRNA acts as a template upon which new proteins are built. DNA is required as the primer in the synthesis of mRNA, thus the specific organisation and alignment of amino acids in the polypeptide chain of a protein is a direct reflection of the base sequence in the gene (DNA). Transfer, or tRNA is responsible for carrying a specific amino acid to the site for protein synthesis, and it appears there is at least one specific tRNA for each of the twenty different amino acids (Fig. 3). Energy requirements for DNA and protein synthesis are sustained by glycolysis (Cooper, Barkham and Hale, 1961).

Fig. 2 Some early events in mitogenesis.

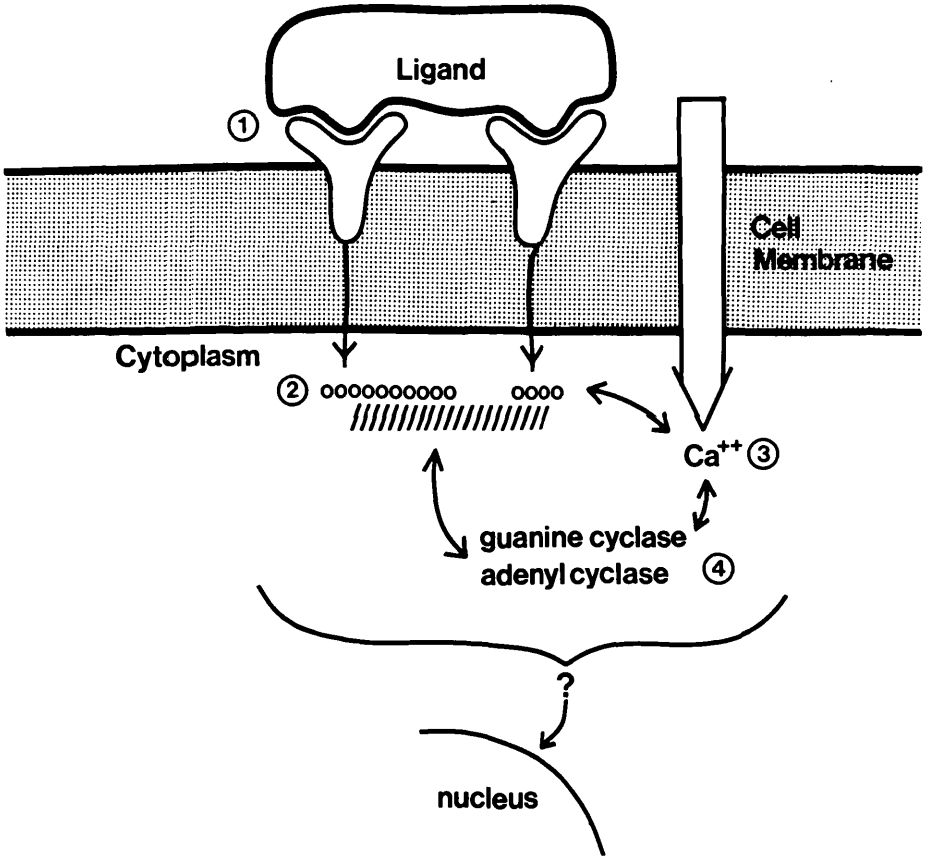
(1) Binding of ligand to cell surface receptors.

(2) Receptors are assumed to interact directly or indirectly with an assembly of sub-membranous proteins including (///////) microfilaments and (oooooo) microtubules, which in turn can be in various states of association and disassociation.

(3) Transport of calcium ions.

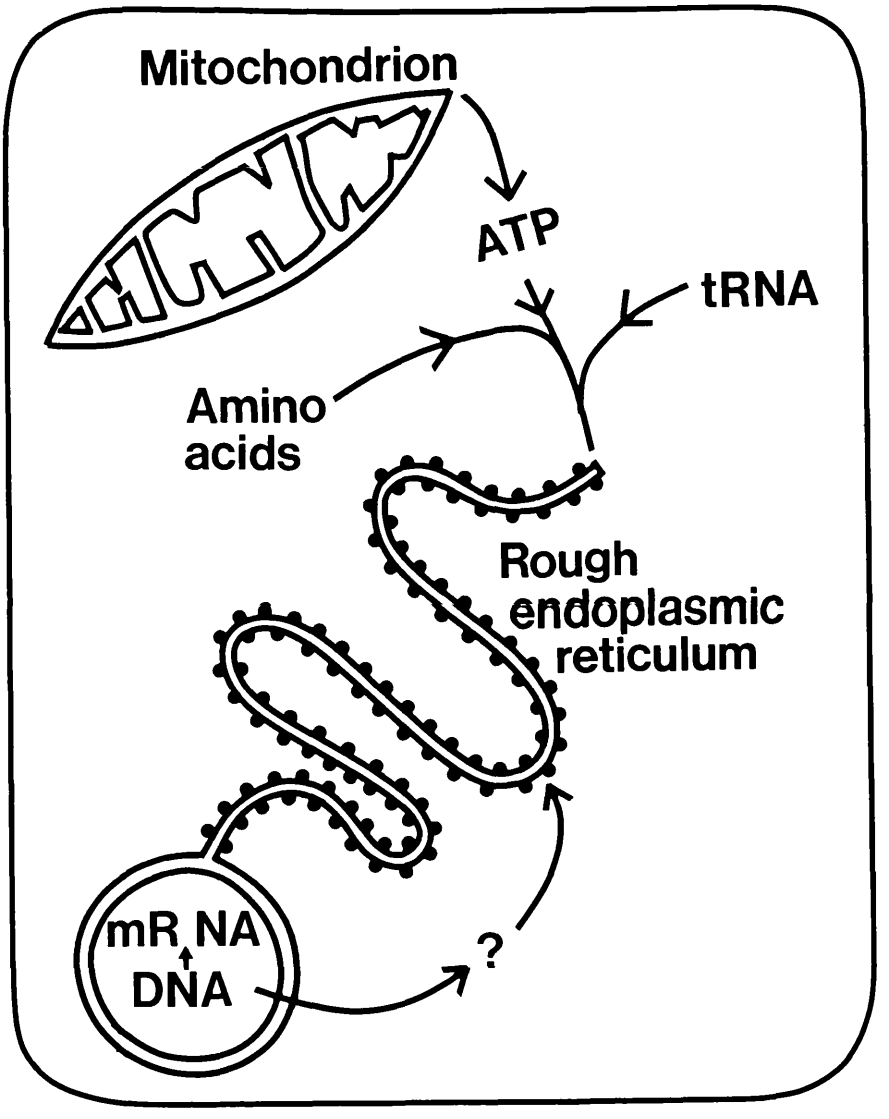
(4) Activation of cyclase systems for cGMP and cAMP.

The proximate surface-to-nuclear signal is not known.



Diagrammatic representation of some early events in mitogenesis.

Fig. 3 Intracellular actions involved in synthesis of protein in an animal cell.



Intracellular actions involved in synthesis of protein in an animal cell

A wide variety of specific proteins are synthesised, among them albumin, transferrin, lgG, lipoprotein and interferon (Lucas, 1971). Also produced are many non-antibody immune effector factors such as lymphotoxin (LT) (Granger and Kolb, 1968), migratory inhibitory factor (MIF) (Bennett and Bloom, 1968) and mitogenic factor (Wolstencroft and Dumonde, 1970). The chemical characterisation of these synthesised proteins was done by Turner and Forbes in 1966. Active DNA synthesis occurs about 48 hours after initiation of the PHA response, and shortly after this the cells enter mitosis (Weinstein, Melmon and Segal, 1976).

Mitosis of sensitised lymphocytes after contact with specific antigen, has been used as an assay for the presence of cellular immunity, and for detecting foreign histocompatability antigens for several years (Bach and Hirschhorn, 1964; Ling, 1968; Oppenheim, Leventhal and Hersh, 1968). The degree of transformation can be measured either by morphological examination or by uptake of radioactive labelled thymidine. Many culture systems have been devised using leukocytes (Hungerford et al, 1959; Nowell, 1960), purified lymphocytes (Cooper and Rubin, 1965; Schellekens and Eijsvoogel, 1968; Polliack et al, 1976) or whole blood (Junge, Hoekstra, Wolfe and Deinhardt, 1970; Pauly, Sokal and Han, 1973; Heitman, 1967; Park and Good, 1972). The use of a whole blood culture technique offers a number of advantages as well as considerable savings in time, cost and volume of blood required. By eliminating the cumbersome and often complex steps required for preparing pure lymphocyte suspensions, one of the major sources of

technical variability and error responsible for poor reproducibility is removed.

The main difference between a culture of whole blood and one of purified lymphocytes is the presence of large numbers of erythrocytes and granulocytes in the culture. Knight and Ling (1969) have shown that the addition of erythrocytes does not alter the response of purified lymphocytes to staphylococcal filtrate, and Junge et al (1970) even found a generally better and prolonged lymphocyte growth rate in comparison to pure lymphocyte cultures. This improved growth rate may be due to the dense red cell button acting as a type of "feeder" system (Pauly et al, 1973) which supports the buffy coat of leukocytes and maintains a desirable in vitro environment for lymphocyte activation. Another explanation proposed is that PHA adsorbed on erythrocyte membranes, is in a particularly favourable steric configuration or local concentration to stimulate lymphocytes, although direct attempts to stimulate lymphocytes with PHA attached to erythrocyte membranes have been successful only at very high PHA or erythrocyte concentrations (Kay, 1971; Johnson, Fink and Ziff, 1972). This would suggest that perhaps the membrane alone is the important feature and that exchange of information between cells is not required.

The ^{14}C-thymidine uptake by purified PHA-stimulated lymphocytes is not significantly altered by the addition of granulocytes (Schellekens and Eijsvoogel, 1968), but responses to antigens are usually higher in lymphocyte cultures containing other white cell elements than in cultures of lymphocytes purified by cotton wool or glass

bead column filtration (McFarland, 1969). The addition of monocytes to purified lymphocyte populations can increase the mitogen-induced proliferation (Oppenheim et al, 1968; Hollister and Jarrett, 1978). The exact role played by macrophages is not yet understood, but evidence suggests a synergism exists between lymphocytes and macrophages in lymphocyte activation by antigens in culture (Seeger and Oppenheim, 1970). There is good cytological evidence (McFarland, Heilman and Moorhead, 1966) that lymphocytes form aggregates around macrophages, particularly in the presence of a stimulant. The lymphocytes attach to the macrophages by means of a uropod, and this contact is often maintained over a prolonged period. It has been observed that the most rapidly growing cells in PHA-stimulated cultures are found within cell aggregates, while a higher proportion of those outside aggregates show fewer signs of activation (Ling and Kay, 1975). Macrophages appear to be superior to monocytes in performing this synergistic role (Hanifin and Cline, 1970).

The effect of autologous serum present in whole blood cultures must be considered. At blood dilutions required for eliciting maximal lymphocyte responses, enough autologous plasma is present to support good lymphocyte viability and reactivity, and avoids the need for unautologous supplements to the medium which may introduce artifactual errors (Ling, 1968). However, a vast array of reports have been published describing non-antigenic specific suppressive factors present in serum (Tomasi, 1977), which may affect the response to mitogens by either competing with sites on the cell surface for

stimulant, or by directly suppressing the cellular response. Many of these substances have been reasonably well characterised chemically (Cooperband, Nimberg, Schmid and Mannick, 1976) but it must be remembered that this information has been derived from *in vitro* systems, and whether data extrapolated from these studies can be applied *in vivo* is questionable.

The response of lymphocytes to PHA may be affected by many factors. Drugs such as quinine (Thong and Ferrante, 1978), phenobarb (Park and Brody, 1971), and aspirin (Pachman, Esterly and Peterson, 1971) all have been shown to suppress DNA synthesis in the presence of PHA, although no decrease in cell viability was found. Pachman shows, however, that the effect on RNA and protein synthesis of stimulated lymphocytes was less pronounced. Age is another important factor. Low responses are obtained with cells of the very young and of the very old, in agreement with the more comprehensive studies in animals (Hagen and Frølund, 1973). Relatively low responses have been found for young people (under 26 years old) compared to older adults (Pentycross, 1969), however decreased PHA responsiveness is most marked in old age, particularly beyond the age of 80 years (Hallgren, Buckley, Gilbertson and Yunis, 1973; Fernandez, McSween and Langley, 1976; Lavanchy and Rosenthal, 1978), where there is a general decline in cellular immune activity. Conditions within the test itself, such as cell number, surface area, duration of culture are also important, and once a technique has been decided it must be strictly adhered to if meaningful comparisons are to be made.

Delayed cutaneous hypersensitivity is considered the best *in vivo* method for assessment of cellular immunity (Rocklin and David, 1976), and therefore it is often useful to correlate results from *in vitro* methods with this *in vivo* test. Kerby (1968) found that the degree of lymphocyte transformation in response to tuberculin antigen correlated with the intensity of the cutaneous reaction to the same antigen, in the same donor. A correlation between cutaneous reactivity and *in vitro* lymphoblast response was also shown by Hinz, Daniel and Baum (1970) but no correlation was seen with antibody production and skin testing. Chaparas, Sheagren, DeMeo and Hedrick (1970) on the other hand could find no association between the skin reaction *in vivo* and lymphoblastic transformation *in vitro*. Since these tests are carried out using various methods, and having differing culture conditions, it may be that each is measuring a slightly different function of lymphocyte response.

The purpose of this thesis is to present the results of applying the *in vitro* technique of lymphocyte protein synthesis to a large number of patients with various neurology-related diseases, and to controls. The diseases were studied because immune function may be involved in the pathogenesis. The evidence which led to the selection of these particular diseases will be discussed later in this work.

METHODS

Patients

The studies reported in this thesis were done on patients suffering from various neurological complaints. These patients were drawn mainly from the neurological and neurosurgical wards, and the outpatient clinics of the Institute of Neurological Sciences in Glasgow. Patients were also studied from the Epilepsy Centre, Quarrier's Homes, Bridge of Weir, Renfrewshire and from the outpatient clinics of the Victoria Infirmary, Glasgow.

Techniques

Blood Sampling

Blood samples were taken by venepuncture from an antecubital vein after cleaning the area with spirit. If lymphocyte protein synthesis was to be measured the syringe used was wetted with preservative-free heparin (1,000 units/ml, Evans Medical) and the blood collected in a sterile plastic universal container. If serum was required the blood was collected in plain glass tubes which were centrifuged when clotted and the serum stored at $-20^{\circ}C$ until required.

Cutaneous Reactivity

Skin testing was done by an intradermal injection of 0.1 ml of antigen into a cleaned area on the volar aspect of the arm. The antigens used were: 5 units of streptokinase/streptodornase (Lederle Laboratories, American Cyanamid Company); 0.5% Candida albicans (Bencard); and 10 units PPD (purified protein derivative of tuberculin,

Evans Medical). The results were examined at 6, 24 and 48 hours, and the product of two maximal diameters measured. The test was considered positive when the indurated area was 1 cm or greater in diameter.

Lymphocyte Protein Synthesis

Peripheral blood lymphocyte protein synthesis was measured by a modification of a whole blood technique (Pauly, Sokal and Han, 1973). Essentially 10-12 mls venous blood was taken as previously described. A lymphocyte count was then done, using an Improved Neubauer Counting Chamber, and the concentration of cells adjusted with RPMI 1640 tissue culture medium (Gibco Europe) containing 50 units of penicillin and 50 µg streptomycin/ml, to give approximately 1 x 10^6 lymphocytes per culture tube. To this cell suspension was added tritiated leucine (Radiochemical Centre, Amersham) specific activity 19 curies/mmol, to give 1 µCi of tritiated leucine per culture. After careful mixing, the cell suspension was pipetted in 3 ml aliquots into 16 x 120 mm glass culture tubes containing 0.1 ml of a given concentration of purified phytohaemagglutinin (Burroughs Wellcome). In general seven dilutions of PHA were used, giving final concentrations of 16.66, 8.33, 2.08, 1.04, 0.26, 0.065 and 0.016 µg/ml. The culture tubes were tightly capped and placed in an upright position in an incubator at 37°C. After 22 hours the tubes were removed and the cells washed in 10 mls normal saline to remove excess tritiated leucine. After an eight minute spin at 350 g, the cell pellet was resuspended and given two washes with cold 3% acetic acid. The cell pellet from the final spin was bleached with one

drop of hydrogen peroxide (30 vols) which was subsequently boiled off by heating at 84°C for 20-30 minutes. 0.6 ml NCS tissue solubiliser (Amersham/Searle Corporation) was then used to dissolve the cell pellet, and 10 mls scintillation fluid added. The scintillation fluid used was a mixture of PPO (91%) and dimethyl POPOP (9%) at a concentration of 4 g/litre in toluene. This solution was then transferred to a scintillation vial and counted in a Packard Tri-carb liquid scintillation counter. Efficiencies of 40-50% were usual. Responses to PHA were expressed as

$$\% \text{ Ratio of } \frac{\text{Counts/min in culture with PHA}}{\text{Counts/min in unstimulated culture}}$$

Lymphocyte Transformation

A similar whole blood technique was used to investigate lymphocyte transformation in terms of tritiated thymidine uptake in response to PHA and various antigens. 1 μCi tritiated thymidine, specific activity 19 Ci/mmol (Radiochemicals, Amersham) was added to each lymphocyte culture 24 hours before harvesting. PHA cultures were harvested as described above, at 3 days to determine tritiated thymidine uptake. The antigen-stimulated cultures were harvested at 6 days.

CHAPTER 1

MEASUREMENT OF THYMUS-DERIVED LYMPHOCYTE FUNCTION IN PATIENTS WITH MALIGNANT BRAIN TUMOUR

Introduction

There is compelling evidence that an increased incidence of malignant disease is found in patients with impaired cell-mediated immunity. This occurs whether the cell-mediated immune deficiency is congenital (Gatti and Good, 1971) or due to prolonged immunosuppressive therapy (Penn, Halgrimson and Starzl, 1971; Liebowitz and Schwartz, 1971). Lymphomas and epithelial tumours have been reported most frequently, and in renal transplant patients in whom delayed hypersensitivity responses have been depressed, an unexpectedly increased number of cerebral lymphomas has occurred (Schneck and Penn, 1971; McCracken, 1973).

In patients with immune deficiency who develop tumours, there is some evidence that restoration of the immunity may abort the tumour (Valentine and Lawrence, 1971). Similarly, in syngeneic animals grafted with malignant tumours, suppression or restoration of their cell-mediated immunity may cause enhancement or regression of the tumour (Balner and Dersjant, 1969; Denlinger, Swenberg, Koestner and Wechster, 1973; Arnstein, Taylor, Nelson-Rees, Huebner and Lennette, 1974).

Lymphocyte function has been shown to be defective in patients with cancer, as measured in vivo by impairment of cutaneous reactivity to common antigens (Solowey and Rapaport, 1965; Hughes and MacKay, 1965), delayed homograft rejection (Brunschwig, Southam and Levin, 1965), impaired ability to become sensitised to dinitrofluoro-

benzene (DNFB) (Levin, McDonough, Miller and Southam, 1964), or to dinitrochlorobenzene (DNCB) (Eilber and Morton, 1970; Catalona, Taylor, Rabson and Chretien, 1972). In vitro tests of peripheral blood lymphocytes from these patients have also revealed a defect in T-cell function, namely, abnormally low responses to phytohaemagglutinin (Gatti, Garrioch and Good, 1970; Whittaker, Rees and Clark, 1971).

It was decided therefore to study a large group of patients with brain tumours, and to compare their responses to PHA to those of patients with other neurological diseases and normal healthy controls. A technique was also set up to test for a serum blocking factor in these patients, as previous workers have suggested that the immune defect found in patients with primary intracranial tumours might be due to a serum factor causing suppression of lymphocyte blast transformation (Brooks, Netsky, Normansell and Horwitz, 1972).

Methods

Patients

In this study the patients with benign or malignant brain tumours were all in good general health, apart from their neurological symptoms. They had no evidence of weight loss, anaemia or electrolyte disturbances. At the time of study, no operations (apart from biopsy) had been undertaken and no treatment had started.

Group A - 28 patients with malignant glioma in whom the diagnosis had been confirmed histologically. There were 16 males and 12 females whose ages ranged from 16 to 71 years, with a mean age of 53.

Group B - 11 patients with disseminated carcinomatosis; 9 males and 2 females, whose ages ranged from 31 to 70 years with a mean age of 63.

Group C - 9 patients with meningioma; 4 males and 5 females aged 30 to 54 with a mean age of 42 years.

Group D - 20 patients with various chronic neurological diseases including cerebrovascular accident (3), intracranial hypertension (3), motor neurone disease (6), myasthenia gravis (6) and multiple sclerosis (2). There were 9 males and 11 females whose ages ranged from 18 to 66 with a mean age of 49 years.

Group E - 21 normal controls consisting of medical and nursing personnel (8), patients with inter-vertebral disc lesions (10) and patients with migraine (3). There were 10 males and 11 females whose ages ranged from 25 to 56 with a mean age of 38 years.

None of the patients studied were receiving steroids or immunosuppressive therapy at the time of the tests.

Cutaneous Reactivity

All subjects in groups A-E were skin tested as described in METHODS. Intradermal injections were performed by Dr. M. Gunar.

Peripheral Lymphocyte Protein Synthesis

Carried out as described in METHODS.

Tests for a serum blocking factor using the assay of peripheral blood lymphocyte protein synthesis

(a) Healthy donor lymphocytes and test serum:

60 mls of venous blood from healthy donors was collected as previously described. The cells obtained from each sample were washed x3 in tissue culture fluid RPMI 1640 and a lymphocyte count was done. The cell suspension was then divided into four aliquots and tissue culture medium added to each to give a final concentration of 1×10^6 lymphocytes per 3 mls.

The medium was supplemented with one of the following:-

(i) 10% autologous serum.

(ii) 10% foetal calf serum.

(iii) 10% serum from patients with glioma.

(iv) 10% serum pooled from patients with chronic neurological diseases.

The cells were then cultured for 48 hours at $37^{\circ}C$. Following this tritiated leucine was added and the material divided into 3 ml aliquots. Protein synthesis on stimulation by various concentrations of PHA was then measured as described over the next 22 hours.

(b) Repeated washing of lymphocytes from patients with glioma to remove putative serum blocking factor:

Lymphocytes from six patients with malignant glioma were taken and the cells from each individual patient were washed carefully x3 in large volumes of RPMI 1640. They were then resuspended in tissue culture fluid supplemented with autologous or foetal calf serum, and protein synthesis was measured over 22 hours as described.

Results

Cutaneous Reactivity

All normal controls (Group E) showed a positive skin response of greater than 1 cm to SK/SD and Candida, and 56% of the controls had a positive response to tuberculin (Table I). These results are in marked contrast to the poor responses in patients with glioma or in those with generalised carcinomatosis. Patients with benign tumours and patients with other neurological diseases showed no significant difference to healthy controls. Significance was calculated using Chi squared distribution.

Lymphocyte Function In Vitro

Figure 4 and Table II show graphically the response to lymphocytes in vitro from all five groups of patients. There is highly significant (as measured by Student's t-test) depression of lymphocyte protein synthesis in patients with glioma and carcinomatosis. Depressed lymphocyte responses to a much lesser extent were found in two other groups, i.e. patients with meningioma, and, as expected (Simpson, Behan and Dick, 1976; Behan, Dick and Durward, 1977), those with immune disorders in the control group. This is also seen in a scatter diagram (Fig. 5) of stimulation ratio of patients and control subjects at 8.33 μg/ml concentration of PHA.

Tests for a Serum Blocking Factor

No evidence of serum blocking factors was found. Cell cultures from healthy donors showed no difference in their responses to varying concentrations of PHA, whether the cells were grown in autologous serum, foetal calf

TABLE I

CUTANEOUS REACTIVITY TO SK/SD, PPD AND CANDIDA

	Clinical State	No. in Group	Percentage showing response greater than 1 cm diameter		
			SK/SD*	PPD**	CANDIDA+
A	Glioma	28	18.5	11.1	18.5
	P value		.001	.005	.001
B	Carcinomatosis	11	18	45.5	36
	P value		.0001	NS	.001
C	Meningioma	9	100	44.9	100
	P value		NS	NS	NS
D	Chronic neurological diseases	20	80	55	80
	P value		NS	NS	NS
E	Normal healthy controls	21	100	56	100

*SK/SD - 5 units/0.1 ml

**PPD - 10 units/0.1 ml

+CANDIDA - 0.5%

Fig. 4 shows graphically the response of lymphocytes *in vitro* from all five groups of patients. There is a highly significant ($P < 0.00001$) depression of lymphocyte protein synthesis in patients with glioma and carcinomatosis.

Patient Group	Mean Tritium uptake in 10^3 cpm*	Standard Deviation in 10^3 cpm*
A	5.73	1.48
B	6.06	0.54
C	6.02	0.91
D	5.76	1.05
E	5.78	2.49

* cpm - counts per minute

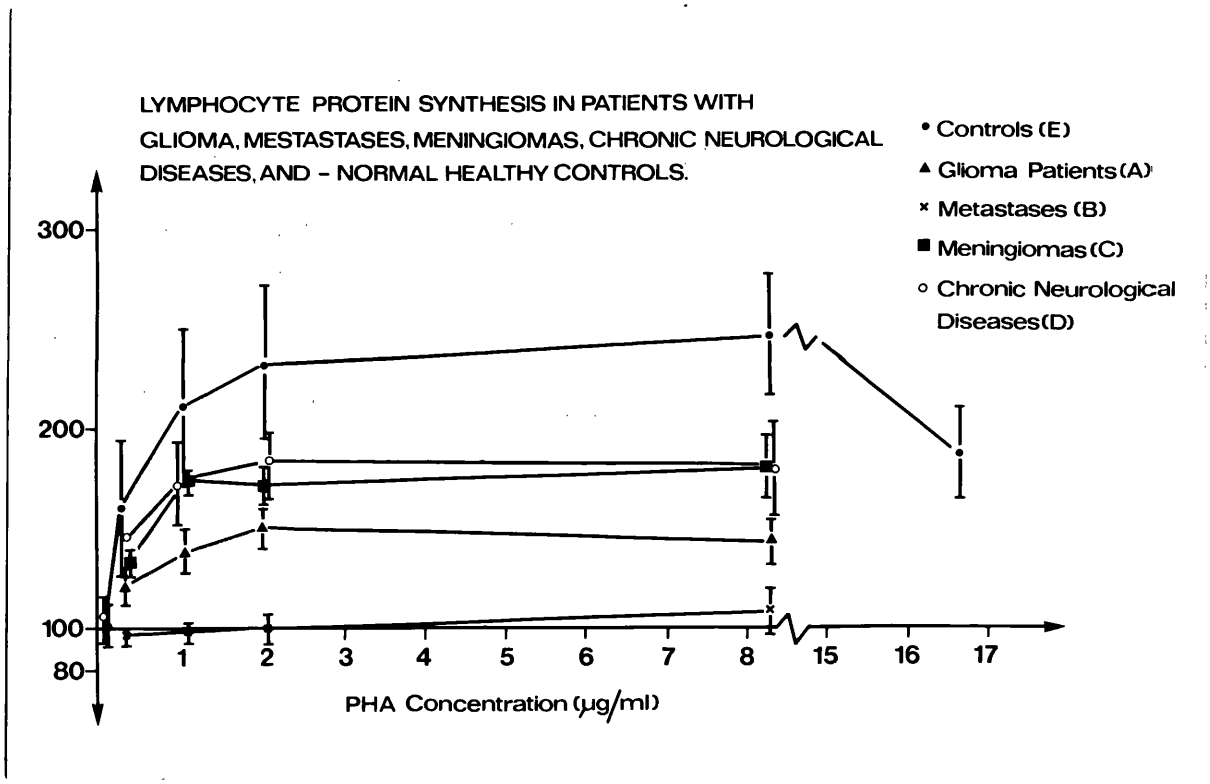

LYMPHOCYTE PROTEIN SYNTHESIS IN PATIENTS WITH GLIOMA, MESTASTASES, MENINGIOMAS, CHRONIC NEUROLOGICAL DISEASES, AND - NORMAL HEALTHY CONTROLS.
• Controls (E)
▲ Glioma Patients (A)
× Metastases (B)
■ Meningiomas (C)
○ Chronic Neurological Diseases (D)
300
200
100
80
1
2
3
4
5
6
7
8
15
16
17
PHA Concentration (µg/ml)

TABLE II

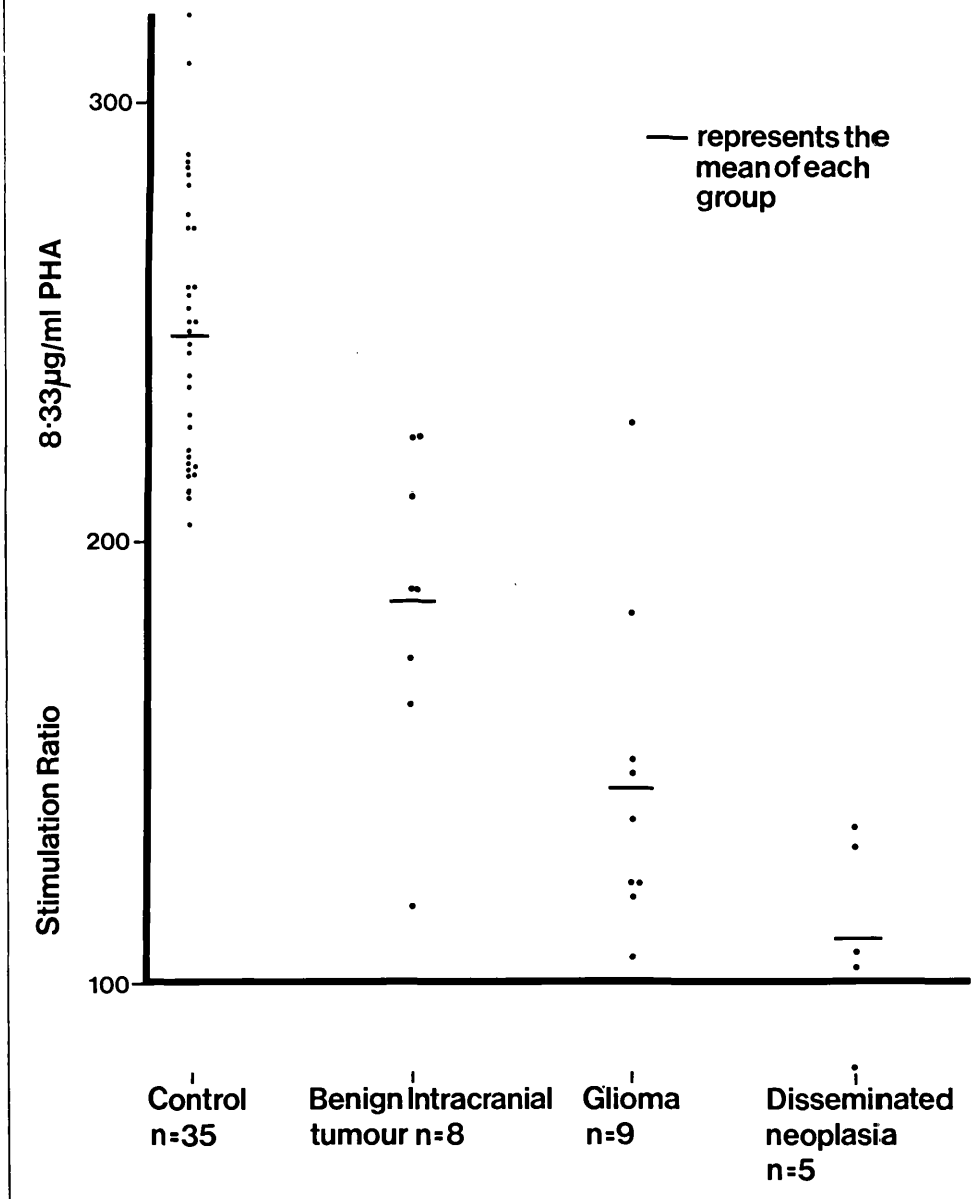
LYMPHOCYTE RESPONSES IN PATIENTS WITH GLIOMA, CARCINOMATOSIS, MENINGIOMAS, CHRONIC NEUROLOGICAL DISEASES, AND IN NORMAL HEALTHY CONTROLS

Patient group		Stimulation indices (S.I.) at varying concentrations of PHA (ug/ml).				
		8.33	2.08	1.04	0.26	0.065
A	Mean	1.43	1.50	1.39	1.20	-
	s.d.	0.12	0.12	0.11	0.09	-
	P.	< 0.00001	<0.00001	<0.00001	<0.00001	-
B	Mean	0.09	1.00	0.98	0.95	-
	s.d.	0.11	0.07	0.05	0.02	-
	P.	<0.00001	<0.00001	<0.00001	<0.00001	-
C	Mean	1.80	1.70	1.71	1.32	-
	s.d.	0.16	0.11	0.09	0.07	-
	P.	<0.00001	<0.00001	<0.00001	<0.00001	-
D	Mean	1.79	1.82	1.72	1.41	1.07
	s.d.	0.24	0.19	0.22	0.18	0.12
	P.	<0.00001	<0.00001	<0.00001	0.006	0.187
E	Mean	2.47	2.33	2.11	1.61	1.03
	s.d.	0.31	0.38	0.40	0.34	0.10

s.d. = standard deviation

P. = value of probability when compared with control group (E).

Fig. 5 Scatter diagram of stimulation ratios of patients and controls to stimulation by PHA at a concentration of 8.33 µg/ml.


300
200
100
8·33μg/ml PHA
Stimulation Ratio
— represents the mean of each group
Control n=35
Benign Intracranial tumour n=8
Glioma n=9
Disseminated neoplasia n=5

serum, serum from patients with other neurological disorders, or serum from patients with glioma (Fig. 6 and Table III). Similarly, lymphocytes from patients with malignant brain tumour revealed the same depression of protein synthesis whether they were washed repeatedly in tissue culture fluid and grown in RPMI to which foetal calf serum had been added, or whether they were washed and grown in the presence of autologous serum (Fig. 7 and Table IV).

Discussion

These studies show that patients with primary malignant intracranial tumours have impaired lymphocyte function, as measured *in vivo* by cutaneous reactivity to recall antigens, and *in vitro* by depressed lymphocyte protein synthesis. At the time of study these patients were otherwise healthy with no evidence of infection, weight loss, haematological or hepatic dysfunction. The significance of the anergy found is unknown and whether or not it contributes to the induction of the tumour or modifies its rate of growth remains to be determined.

There is good evidence from clinical observations and from animal experiments that the immune response of the host may influence tumour growth in other systems of the body (Valentine and Lawrence, 1971). Patients with naturally occurring, congenital or acquired defects of cell-mediated immunity have an increased incidence of tumour (Gatti and Good, 1970) and indeed lymphomas are unexpectedly frequent in the central nervous system of patients receiving immunosuppressive therapy for renal transplantation (Schneck and Penn, 1971). These tumours

TABLE III

CELLULAR RESPONSES OF WASHED HEALTHY LYMPHOCYTES IN SERUM OF PATIENTS WITH GLIOMA AND OTHER DISEASES

	Stimulation indices (S.I.) at varying concentrations of PHA (ug/ml).						
	33.33	8.33	2.08	1.04	0.26	0.065	0.016
Autologous							
n	9	23	23	23	23	23	9
Mean	2.10	2.72	2.83	2.85	2.24	1.75	1.08
s.d.	0.44	0.47	0.66	0.64	0.36	0.44	0.17
P.	-	-	-	-	-	-	-
Foetal calf							
n	7	19	19	19	19	19	7
Mean	1.92	2.15	2.74	2.73	2.14	1.45	1.15
P.	0.39	0.02	0.73	0.67	0.6	0.08	0.42
Pooled serum from patients with chronic neurological diseases							
n	7	7	7	7	7	7	7
Mean	2.11	2.61	2.67	2.47	2.1	1.63	1.13
s.d.	0.3	0.42	0.54	0.61	0.52	0.4	0.27
P.	0.91	0.63	0.61	0.25	0.56	0.58	0.68
Pooled serum from patients with glioma							
n	18	18	18	18	18	18	18
Mean	2.07	2.41	2.46	2.45	1.91	1.43	1.05
s.d.	0.45	0.53	0.4	0.51	0.39	0.26	0.21
P.	0.85	0.14	0.15	0.12	0.04	0.07	0.7

n = number of tests done

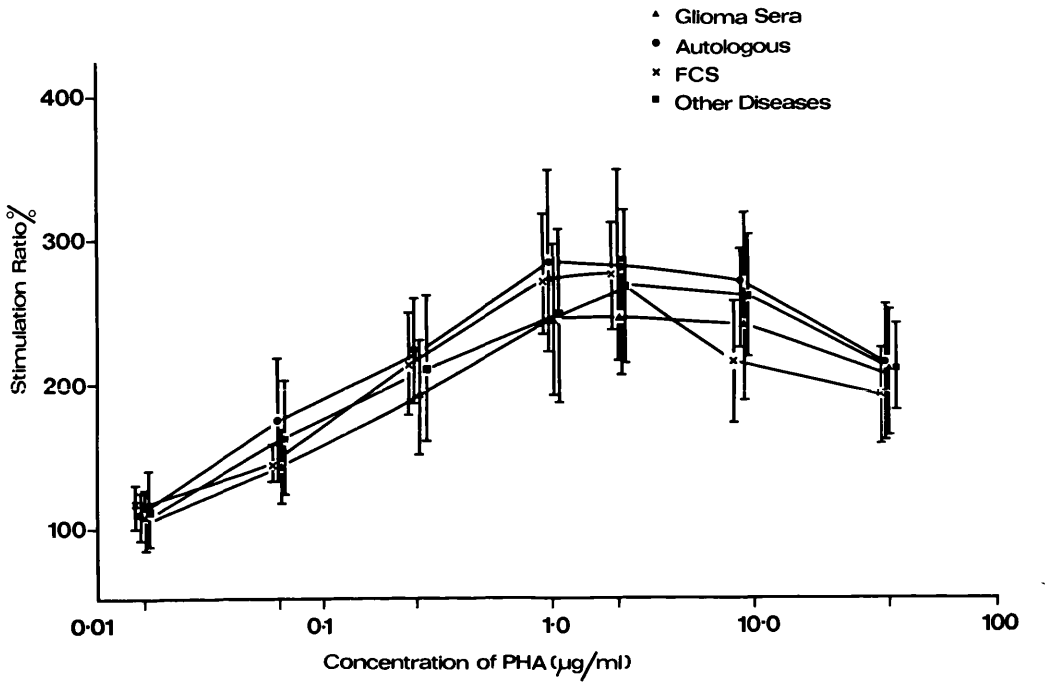
s.d.= standard deviation

P. = value of probability when compared with autologous serum.

Fig. 6 shows cellular responses of washed healthy lymphocytes cultured in sera from patients with glioma and other diseases.

Fig. 7 shows cellular responses of lymphocytes from patients with glioma. These cells were cultured in either foetal calf or autologous serum, following repeated washing.

STIMULATION INDICES OF WASHED HEALTHY LYMPHOCYTES IN SERUM OF PATIENTS WITH GLIOMA & OTHER DISEASES



Cellular Responses in Autologous & Foetal Calf Sera

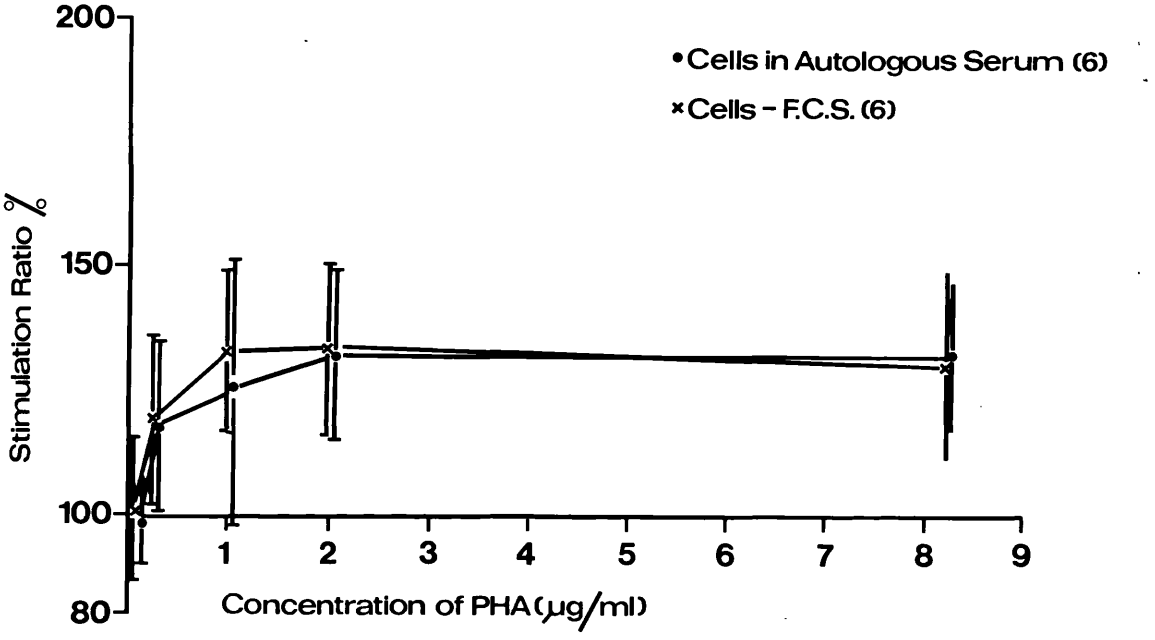


TABLE IV

CELLULAR RESPONSES IN AUTOLOGOUS AND FOETAL CALF SERA

Patient	Serum supplement	Stimulation indices (S.I.) at varying concentrations of PHA (ug/ml).				
		8.33	2.08	1.04	0.26	0.065
1	Autologous	1.23	1.18	1.18	1.05	0.85
	Foetal calf	1.27	1.34	1.34	1.24	1.03
2	Autologous	1.55	1.49	1.47	1.42	1.10
	Foetal calf	1.37	1.62	1.64	1.43	1.22
3	Autologous	1.41	1.46	1.56	1.24	1.09
	Foetal calf	1.09	1.26	1.27	1.16	1.07
4	Autologous	1.16	1.43	1.30	1.27	0.94
	Foetal calf	1.21	1.24	1.22	1.32	1.00
5	Autologous	1.21	1.05	0.85	0.94	0.98
	Foetal calf	1.23	1.12	1.21	1.00	0.96
6	Autologous	1.36	1.33	1.13	1.17	1.00
	Foetal calf	1.65	1.41	1.30	1.00	0.78
	Autologous sera					
	Mean S.I.	1.32	1.32	1.25	1.18	0.99
	S.D.	0.15	0.17	0.26	0.17	0.09
	Foetal calf serum					
	Mean S.I.	1.30	1.33	1.33	1.19	1.01
	s.d.	0.19	0.17	0.16	0.17	0.14
	(P.	0.82	0.88	0.54	0.88	0.77)

s.d. = standard deviation

P. = probability value.

p. 26 line 13 should read:- "responses of lymphocytes from patients with glioma to Concanavelin A ...."

may diminish in size or disappear on withdrawal of immunosuppressive treatment (Wilson, Hager, Hampers, Corson, Merrill and Murray, 1968; Zukosi, Killen, Ginn, Matter, Lucas and Seigler, 1970; Schneck and Penn, 1970).

Several studies have previously suggested that immunocompetence may be altered in patients with glioma, e.g. there is a reduction in the number of circulating lymphocytes and skin reactions to PPD, SK/SD, mumps, candida, and trichophyton antigens are impaired, as is the ability of these patients to become sensitised to DNCB (Brooks et al, 1972; Mahaley, 1975). Young, Sakalas and Kaplan (1976) confirmed the above findings and also noted reduced in vitro responses of patients with glioma to Concanavalin A. They found that the abnormalities were confirmed to patients with astrocytomas of Grade III and IV, in contrast to the results of the previous workers. In a separate study, Mahaley, Brooks, Roszman, Bigner, Dudka and Richardson (1977) confirmed that the skin reactions to foreign antigens were diminished to a much greater extent in patients with the more malignant tumours.

In contrast to these reports of generalised anergy in patients with glioma, the results of other studies suggest that the body may mount an immunological attack on these tumours. For instance, Kumar and Taylor (1973) demonstrated cytotoxic lymphocytic activity against astrocytomas and similar results, with cytotoxic effects to a common antigen from astrocytomas, were reported by Levy, Mahaley and Day (1972). The importance of these two studies lies also in the fact that both groups detected a serum blocking factor in the patients which impaired the ability of

immunocompetent cells to kill tumour cells in vitro. The nature of the factor or factors was not identified.

Brooks et al (1972) demonstrated a serum factor capable of depressing in vitro lymphocyte transformation and stated that the amount of the factor present could be correlated with the degree of anergy. The blastogenic responses of lymphocytes from both patients and normal controls were diminished by the serum factor; and lymphocytes from patients which had shown impaired responses were found to be capable of normal in vitro reactions after repeated washings. We have been unable to confirm these observations in this study. The difference, however, may be more apparent than real, in that we took lymphocyte protein synthesis measured over 22 hours as an indication of lymphocyte function, whereas Brooks et al (1972) measured DNA synthesis over 2 hours in lymphocytes cultured with mitogens or antigens for 4-6 days. Our technique, therefore, may not have been sensitive enough to measure this blocking factor.

Inhibitors of lymphocyte function have been described in the serum of patients with cancers outside the central nervous system (Silk, 1967; Sample, Gertner and Chretien, 1971) and specific blocking or tumour-enhancing activity has also been demonstrated in the serum of both humans and animals with neoplasms (Hellström and Hellström, 1970). Brooks et al (1972) thought that the blocking factor in the serum of patients with glioma corresponded to the immunoglobulin fraction and was IgG but others have disagreed (Young et al, 1976). In a detailed study of patients with solid tissue neoplasms Glasgow, Nimberg,

Menzoian, Saporoschetz, Cooperband, Schmid and Mannick (1974) found a circulating immunosuppressive serum component in the first peak, Fraction 1, after ion-exchange chromatography of the serum. They had previously shown that there was an immunoregulatory fraction in the alpha-globulin of normal human serum (Cooperband, Badger, Davis, Schmid and Mannick, 1972). They concluded that the peptide fraction isolated was different to the blocking factor found in the serum of cancer patients by Hellström and Hellström (1970) or by Baldwin, Price and Robbins (1973).

Similar blocking factors have also been reported in animals (Kamrin, 1959; Mowbray, 1963), and in other conditions, such as in patients undergoing rejection of renal transplants (Riggio, Schwartz, Bull, Stenzel and Rubin, 1969), patients with infectious diseases (Pearmain et al, 1963) or in patients with presumed autoimmune disorders (Jensen, 1968). It is clear, therefore, that the immunosuppressive serum factors detected by different workers may not be identical to one another and controversy certainly exists, not only as to their mode of action, identification and precise nature, but even as to their existence.

We have demonstrated a general anergy in patients with glioma, but there are several reports indicating that patients with glioma may become specifically sensitised to the tumour. These comprise tissue culture studies (Eggers, 1972), skin-testing (Bloom, Carstairs, Crompton and McKissock, 1960; Febvre, Manoury, Constans and Trouillas, 1972; Grace, Perese, Metzgar, Sasabe and

Holdridge, 1961; Trouillas, 1972) and cytotoxicity experiments (Ciembroniewicz and Kolar, 1969; Jagarlamooday, Aust, Tew and McKhann, 1971; Koprowski and Fernandes, 1962; Levy et al, 1972; Mitts and Walker, 1965; Wahlström, Saksela and Troupp, 1973).

In addition, histological evidence may suggest an immune response: lymphocyte infiltration of a wide variety of malignant tumours has been observed (Tsakraklides, Anastassiades and Kersey, 1973; Pratt, Brynes, Vardiman and Coppleson, 1975), and a correlation has been demonstrated between the clinical course of these patients and the immunological responses to the tumour. Indeed, in patients with breast cancer, the degree of lymphocyte infiltration has been stated to give as good an index of prognosis as the histological grade of dedifferentiation (Hamblin, 1968). Some workers have suggested that a similar state of affairs may exist for the degree of lymphocyte infiltration around brain tumours and survival (Ridley and Cavanagh, 1971; di Lorenzo, Palma and Nicole, 1977; Brooks, Markesbery, Gupta and Roszman, 1978). Observation of transplanted tumours in animals has provided further evidence that aggressive lymphocytes may be responsible for tumour regression, and that the rate of elimination of the grafted tumours is related to the degree of the cell-mediated immune response (Scheinberg, Edelman and Levy, 1964; Scheinberg, Levy and Edelman, 1965; Scheinberg and Taylor, 1968).

This study has confirmed that otherwise healthy patients with malignant brain tumours have significant depression of lymphocyte function. There is compelling evidence that a similar type of anergy can influence tumour

growth in other systems and, in regard to the brain, a preliminary report has suggested that therapy with autologous lymphocytes may modify the rate of growth of glioblastoma multiforme (Young, Kaplan and Regelson, 1977). Further study to clarify the factors operative in the disordered immunity shown by patients with glioma is clearly warranted.

CHAPTER 2

THE EFFECT OF β-BLOCKERS ON LYMPHOCYTES IN VITRO

Introduction

Various adverse reactions have been reported in patients undergoing treatment with practolol, a beta-blocking drug used to control angina and hypertension. These include exfoliate dermatitis (Rowland and Stevenson, 1972); eczematous, lichenoid, psoriasiform skin rashes (Felix, Ive and Dahl, 1974); a syndrome resembling systemic lupus erythematosis (Raftery and Denman, 1973); nephrotic syndrome (Farr, Wingate and Shaw, 1975); fibrinous peritonitis (Brown, Baddeley, Read, Davies and McGarry, 1974; Hensen, Rhemrev and Kapteyn, 1975; Windsor, Kurrein and Dyer, 1975); and the oculomucocutaneous syndrome (Wright, 1975).

The discovery in patients with ocular damage of high titres of antinuclear antibodies and antibodies binding to the intercellular region of xenogeneic epithelial tissues (Amos, Brigden and McKerron, 1975) and the demonstration of the deposition of IgG, IgM and C3 at the dermoepidermal junction and circulating antinuclear antibodies in several patients with skin reactions (Felix et al, 1974) suggested that an abnormal immune response might be involved. Further, the possibility of a primary immunological mechanism for the oculomucocutaneous syndrome was suggested when a survey of reports of patients carried out by ICI found that adverse reactions were not related to dosage and, therefore, were unlikely to be pharmacologically or toxicologically induced.

It was decided therefore to measure the protein synthesis, *in vitro*, of peripheral blood lymphocytes of patients taking practolol, and who had developed adverse reactions. The results are compared with those obtained in a group of patients in whom no adverse effects had developed and in a control group of normal healthy individuals, who had never taken practolol. Lymphocyte blast transformation was also measured by culturing cells from patients with adverse symptoms, in the presence of PHA, and the drug itself, and comparing results to those of normal lymphocytes cultured under similar conditions.

In addition, further studies were carried out to test the effect of several beta-blocking drugs on normal peripheral blood lymphocytes *in vitro*.

Methods

Patients

The following groups were studies:-

Group A - 15 patients in whom either cutaneous lesions or a combination of cutaneous and ocular lesions developed whilst on practolol. There were 4 females and 11 males with an average age of 61 years (range 35-75 years). The average dose of practolol was 1200 mg/day (range 200-2400 mg/day) and the average duration of practolol treatment before the first symptoms of the oculomucocutaneous syndrome appeared was 29 months (range 9 to 51 months). At the time of study, 3 patients were still taking practolol, 1 had been off for 6 days, and the remainder had stopped taking practolol for 1 to 36 months. However, the rash persisted in 4 patients and 9 still had ocular symptoms and signs. In the 2 remaining patients the rash had

resolved. Most patients had been transferred to an alternative beta-blocker together with a thiazide diuretic.

Group B - 3 patients, 1 male and 2 females who had sclerosing peritonitis confirmed by laparotomy. Practolol (200-300 mg/day) had been used for 18 to 36 months before the onset of abdominal symptoms. In 2 of the patients ocular lesions were said to have developed as well as the sclerosing peritonitis, and the third had had an exfoliative dermatitis. All 3 patients had stopped taking practolol for up to 18 months before the start of the present study.

Group C - 6 male patients who had been receiving 400 mg/day of practolol for about 2 years for the treatment of ischaemic heart disease, and all were still on practolol at investigation.

Group D - 35 healthy controls, 20 males and 15 females whose mean age was 38 years (range 30-56 years), made up of medical and nursing personnel.

(This study was carried out before the withdrawal of practolol ('Eraldin') tablets from general use.)

Drugs

Drugs tested in vitro were

(a) Practolol (Imperial Chemical Industries Ltd., Alderley Park, Macclesfield).

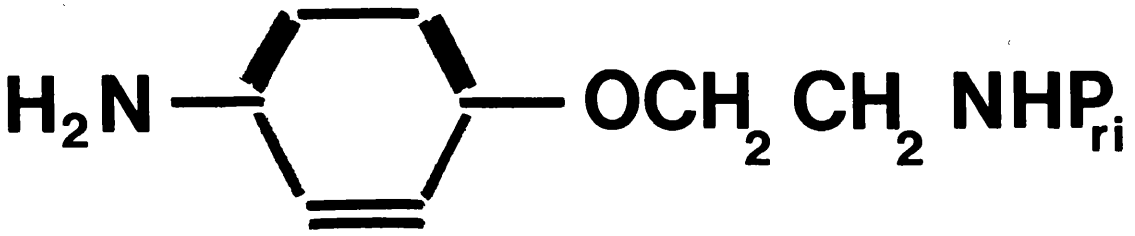
(b) A metabolite of practolol (ICI, Macclesfield) (Fig. 8).

(c) Propranolol (ICI, Macclesfield) (Fig. 9).

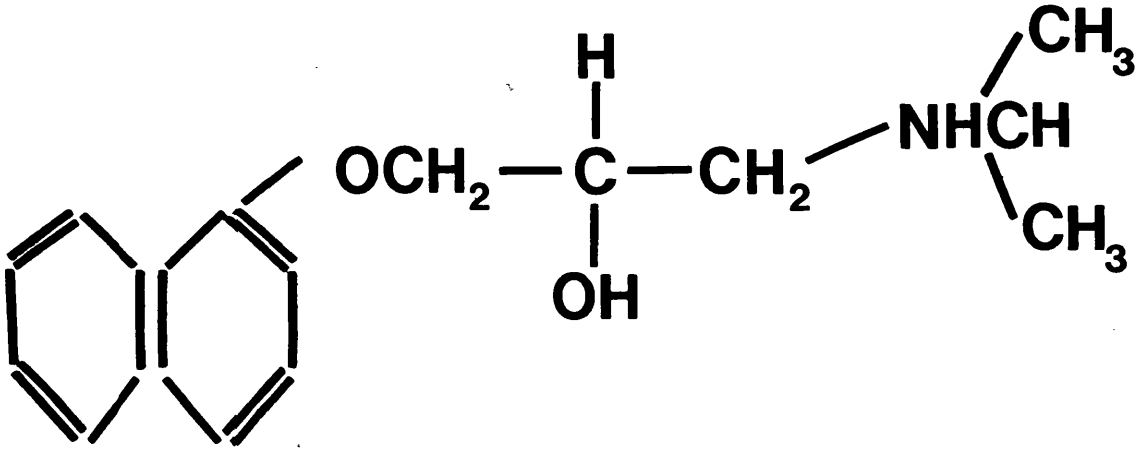
(d) Metoprolol tartrate ('Betaloc' , Astra Chemicals Ltd., Watford).

Fig. 8

Fig. 9



a metabolite of practolol (I.C.I.)



Propranolol (I.C.I)

(e) Oxprenolol hydrochloride ('Trasicor', Ciba Laboratories, Horsham).

Protein Synthesis by Peripheral Blood Lymphocytes

Carried out as described in METHODS.

Lymphocyte Transformation

Carried out as described in METHODS. Lymphocytes were cultured in the presence of PHA at a concentration of 8.7 µg/ml, and also in the presence of practolol, and a metabolite of practolol both at a final concentration of 50 µg/ml. Candida albicans antigen was also used at a concentration of 0.1 ml of the standard solution (Bencard) in each 3 ml culture.

To test the effect of beta-blocking drugs, in vitro, on normal healthy lymphocytes, by measurement of protein synthesis

Venous blood from healthy donors was collected as previously described. A cell count was done, and the cells prepared as for peripheral lymphocyte protein synthesis. Before the addition of the tritiated leucine, however, a measured amount of each of the drugs was added to each bottle containing cells, and these were then incubated at 37°C for 48 hours. Five different drugs were used, each at a final concentration of 10, 50, 100 and 250 µg/ml. Tritiated leucine was then added, the material divided into 3 ml aliquots and protein synthesis on stimulation by two concentrations of PHA (8.33 and 2.04 µg/ml) measured over the next 22 hours, as described previously.

This technique was further modified by extensively washing the cultured cells after 48 hours with tissue culture fluid then comparing the results of their protein synthesis with those of cells cultured in a similar fashion, but not washed before the addition of PHA.

Results

Protein Synthesis in Response to PHA

The overall response of lymphocyte protein synthesis was significantly less in the patients with the oculo-mucocutaneous syndrome (Group A) than in the controls (Group D), as shown in Fig. 10. Similar results (Fig. 11) were obtained in the 3 patients (Group B) with sclerosing peritonitis. The results of lymphocyte testing in patients who had been on practolol for 2 years (Group C) were also abnormal (Fig. 12). At the optimum concentration of PHA there was a significant ($P = < 0.01$) between the response of their lymphocytes and that of control lymphocytes.

Lymphocyte Transformation Tests

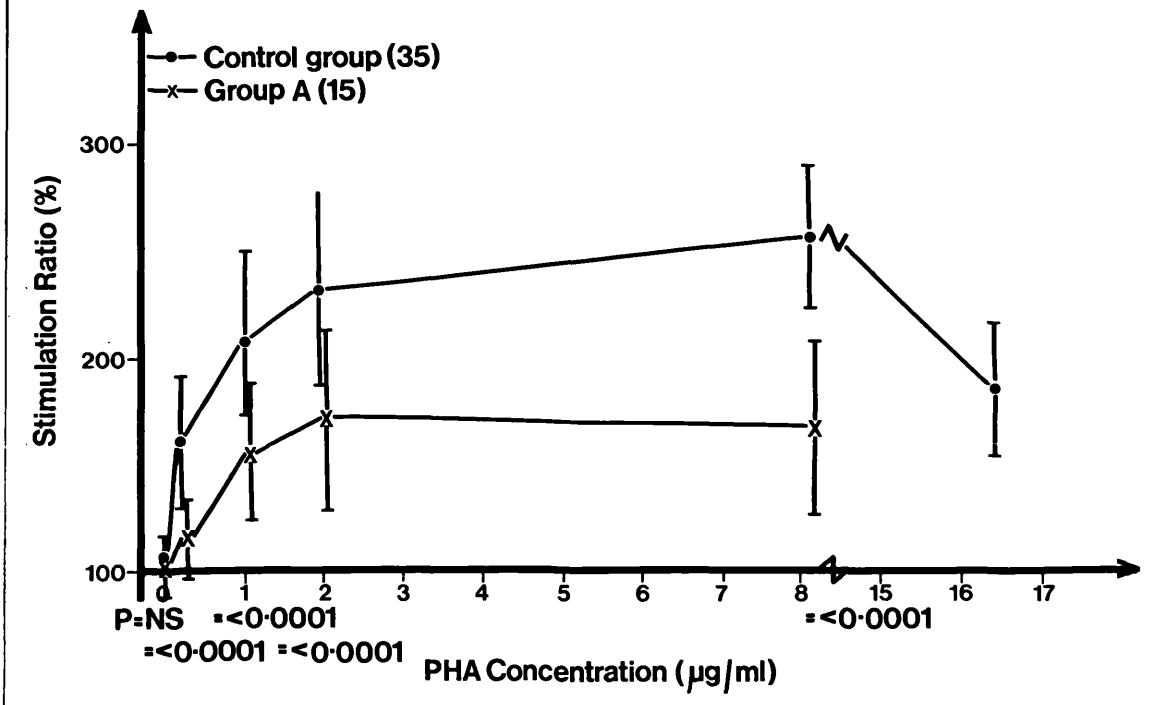
The lymphocyte PHA responses after culture for 3 days, as measured by tritiated thymidine uptake, were depressed in the patients with eye and skin manifestations of the oculomucocutaneous syndrome (Table V). There was no significant response to practolol or one of its possible metabolites in any of the patients or controls.

Effect of Beta-blocking Drugs In Vitro

Incubation of normal healthy lymphocytes with practolol had no effect on the response to PHA at either

Fig. 10 Protein synthesis by PHA-stimulated lymphocytes from patients with the oculomucocutaneous syndrome (Group A) compared with normal controls (Group D).

Fig. 11 Protein synthesis by PHA-stimulated lymphocytes from patients with sclerosing peritonitis (Group B) compared with normal controls (Group D).


Control group (35)
Group A (15)
Stimulation Ratio (%)
300
200
100
0
1
2
3
4
5
6
7
8
15
16
17
P=NS
=<0·0001
=<0·0001
=<0·0001
=<0·0001
PHA Concentration (µg/ml)

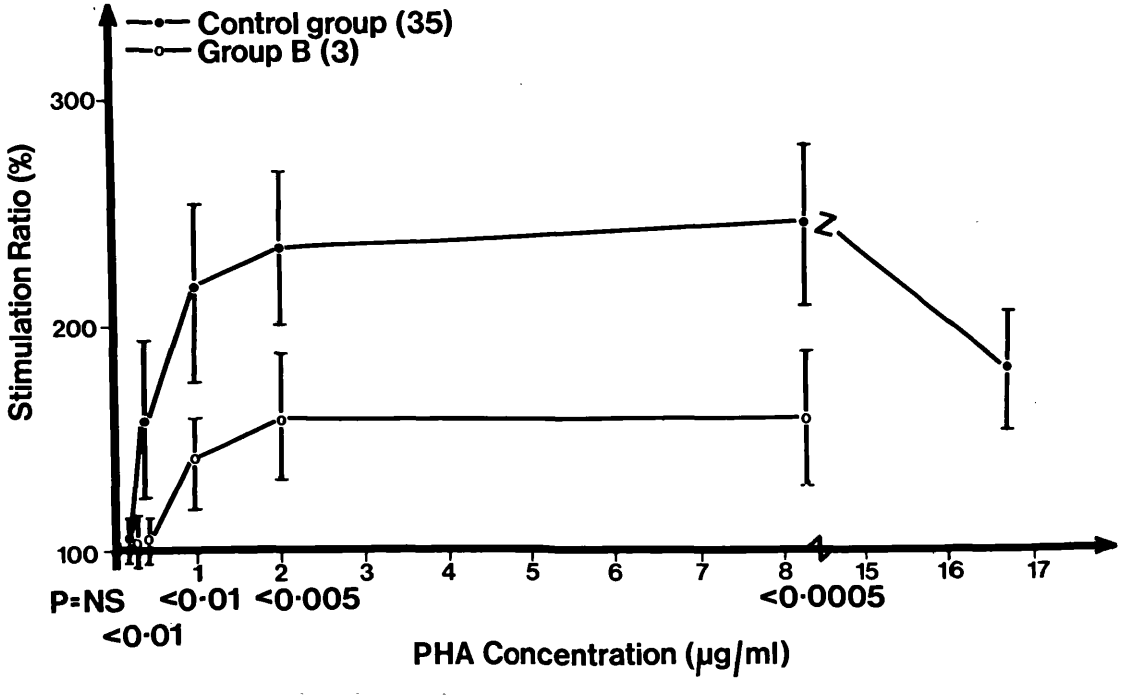

Control group (35)
Group B (3)
Stimulation Ratio (%)
300
200
100
1
2
3
4
5
6
7
8
15
16
17
P=NS
<0·01
<0·005
<0·01
<0·0005
PHA Concentration (µg/ml)

Fig. 12 Protein synthesis by PHA-stimulated lymphocytes from patients on long-term practolol therapy without lesions (Group C) compared with normal controls (Group D).

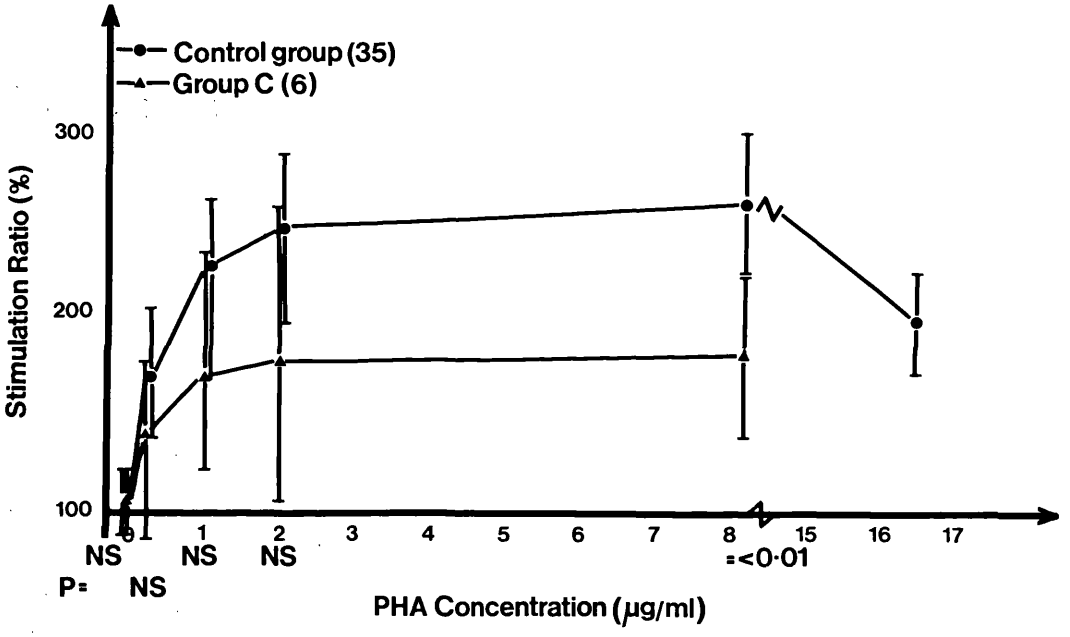

Control group (35)
Group C (6)
Stimulation Ratio (%)
300
200
100
0
1
2
3
4
5
6
7
8
15
16
17
NS
NS
NS
=<0·01
P=
NS
PHA Concentration (µg/ml)

TABLE V

LYMPHOCTYE BLAST TRANSFORMATION IN PATIENTS FROM GROUP A WITH OCULOMUCOCUTANEOUS SYNDROME COMPARED WITH NORMAL HEALTHY CONTROLS.

GROUP		STIMULATION RATIO (%)			
		PHA* (8ug/ml)	PRACTOLOL** (50ug/ml)	METABOLITE** (50ug/ml)	CANDIDA**
Normal Healthy Controls (n = 5)	$\bar{x}$	21.15	1.06	1.26	1.04
	s.d.	± 10.58	0.06	0.17	0.27
GROUP A (n = 15)	$\bar{x}$	9.88	1.05	1.37	0.96
	s.d.	8.59	0.07	0.26	0.15
	P.	0.02	NS	NS	NS

* 3 day culture

** 6 day culture

P calculated using Students t test.

p. 36 line 14 should read:- "....there was no difference between the responses...."

concentration (Fig. 13). Very little effect was also seen after culture with the metabolite or with Betaloc which showed only slight impairment of the lymphocyte response at the higher drug concentrations (Figs. 14 and 15). Trasicor had a more pronounced effect on the reactivity of the PHA stimulated cells (Fig. 16) but the greatest depression of response was seen after cultures with propranolol (Fig. 17) where, at concentrations of 50 µg/ml and over, comparisons to controls were highly significantly different with $P < 0.00001$.

Comparison of washed and unwashed cells was done on 3 sets of cells cultured with four concentrations of Trasicor. In both cultures with PHA at 8.33 and 2.04 µg/ml there is no difference between the responses of washed and unwashed lymphocytes cultured alone, or in the presence of the drug at a concentration of 10 µg/ml. At concentrations of Trasicor of 50, 100 and 250 µg/ml, however, washing of the cells before incubation with PHA increased the response seen (Table VI).

Discussion

Practolol therapy may be associated with various adverse reactions (British Medical Journal, 1975). Although all of the practolol reactors were not still on practolol when they became involved in this investigation, they did, however, continue to have symptoms and signs related to practolol toxicity. A survey of reports on patients with the oculomucocutaneous syndrome carried out at ICI indicates that the reaction is not related to dose, dosage regimen, or total cumulative amount of practolol received. Further, there appeared to be no

Fig. 13 *In vitro* effect of practolol on normal PHA-stimulated lymphocytes.

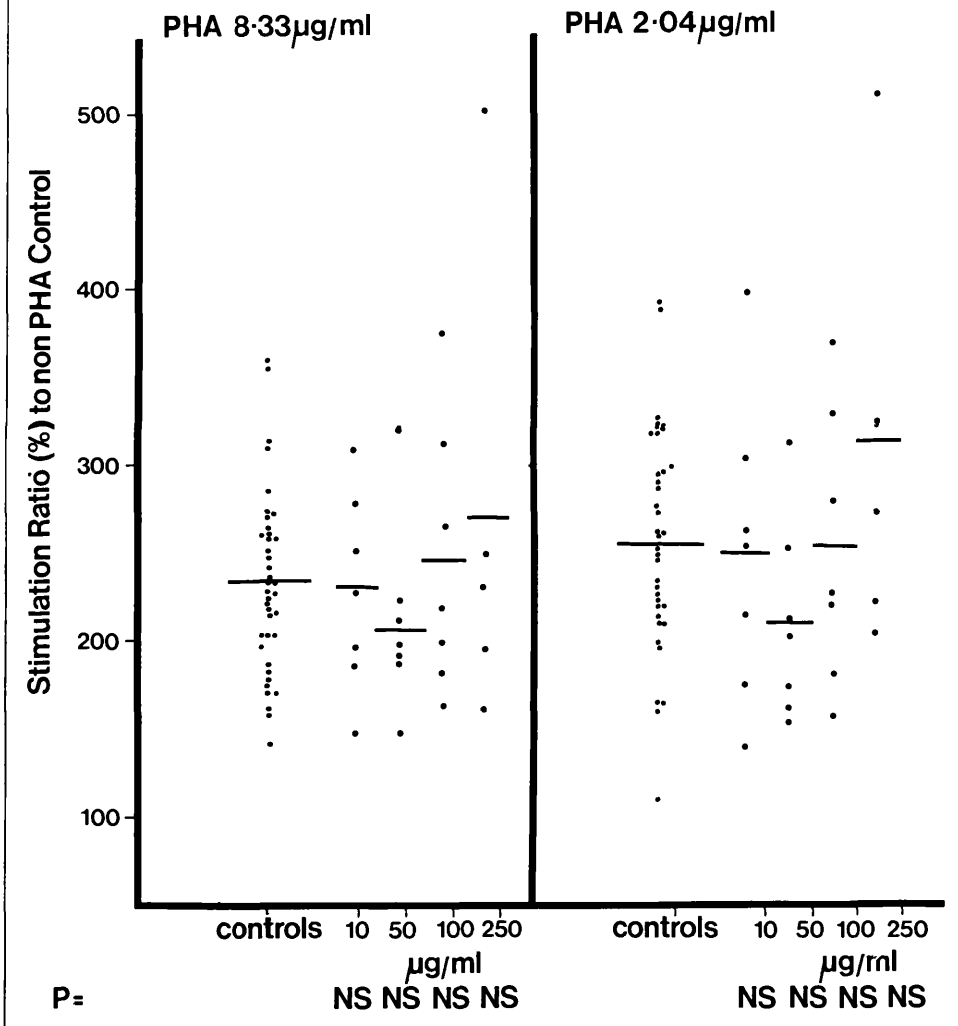

PHA 8·33µg/ml
PHA 2·04µg/ml
Stimulation Ratio (%) to non PHA Control
500
400
300
200
100
controls
10
50
100
250
µg/ml
P=
NS NS NS NS
NS NS NS NS

Fig. 14 Measurement of protein synthesis in normal PHA-stimulated lymphocytes incubated in the presence of a metabolite of practolol.

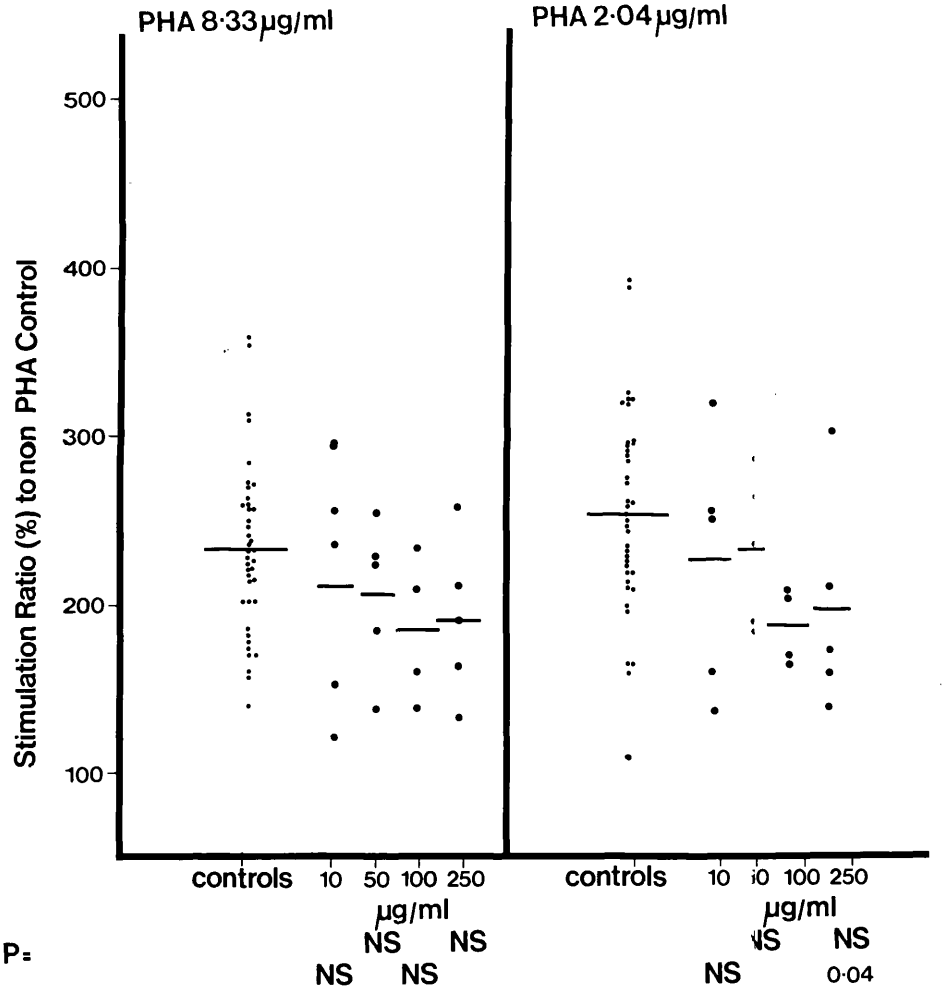

PHA 8·33µg/ml
PHA 2·04µg/ml
Stimulation Ratio (%) to non PHA Control
500
400
300
200
100
controls
10
50
100
250
µg/ml
P=
NS
NS
NS
NS
controls
10
100
250
µg/ml
NS
NS
0·04

Fig. 15 The effect of Betaloc *in vitro* on normal PHA-stimulated lymphocytes.

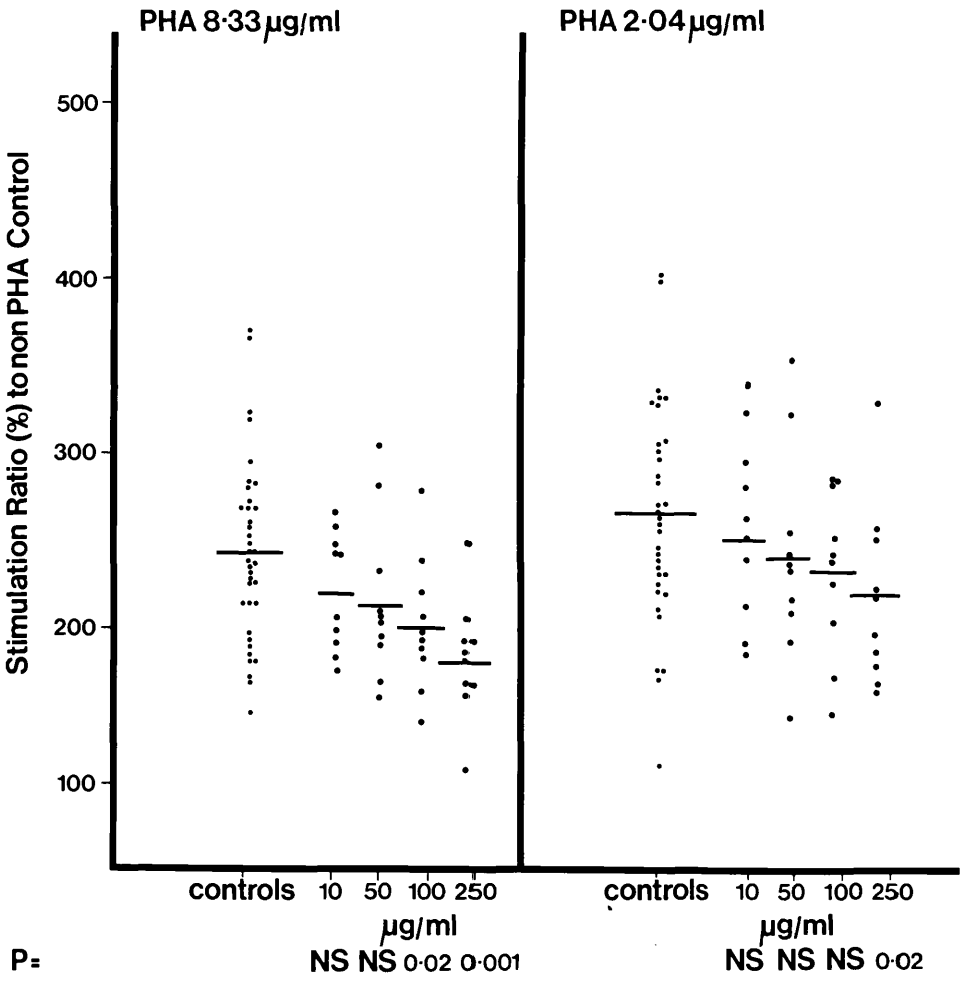

PHA 8·33 µg/ml
PHA 2·04 µg/ml
Stimulation Ratio (%) to non PHA Control
500
400
300
200
100
controls 10 50 100 250
µg/ml
P=
NS NS 0·02 0·001
controls 10 50 100 250
µg/ml
NS NS NS 0·02

Fig. 16 The effect of Trasicor *in vitro* on normal PHA-stimulated lymphocytes.

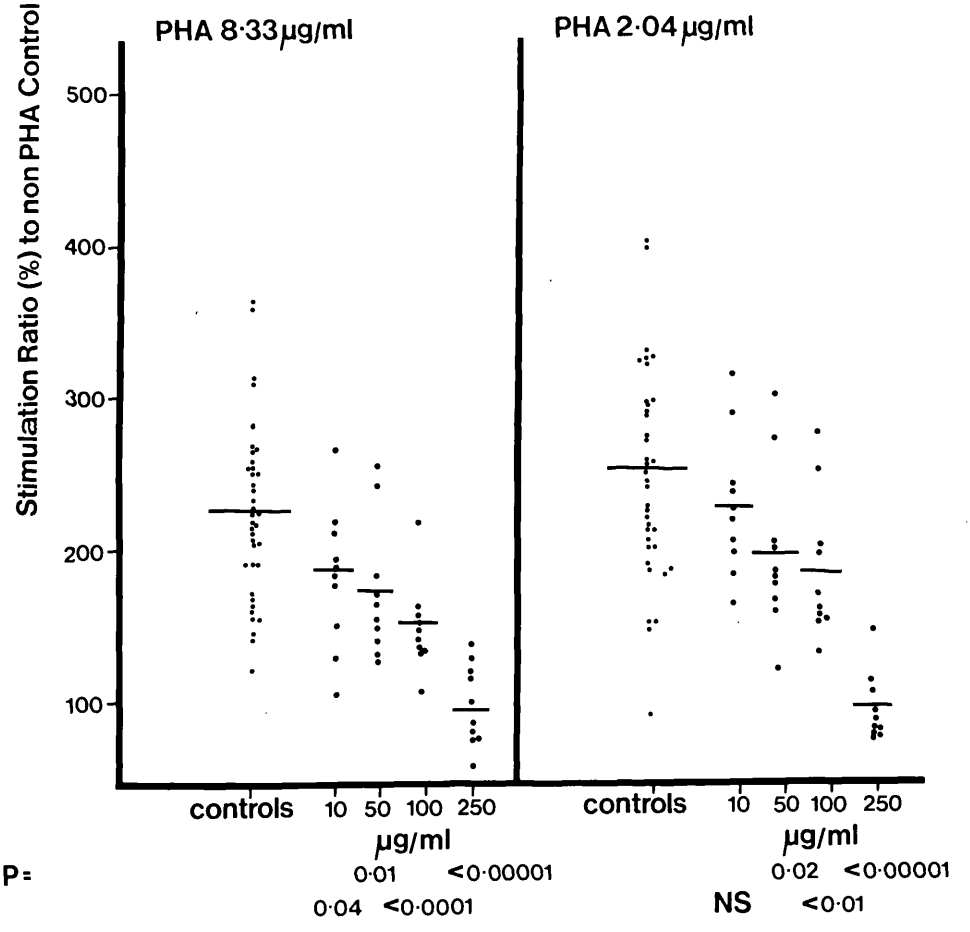

PHA 8·33 µg/ml
PHA 2·04 µg/ml
Stimulation Ratio (%) to non PHA Control
500
400
300
200
100
controls
10
50
100
250
µg/ml
P=
0·01
<0·00001
0·04
<0·0001
0·02
<0·00001
NS
<0·01

Fig. 17 In vitro effect of propranolol on normal PHA-stimulated lymphocytes.

ERRATUM

Fig. 17 P value at PHA concentration of 8.33 µg/ml, drug concentration of 10 µg/ml should read < 0.00001.

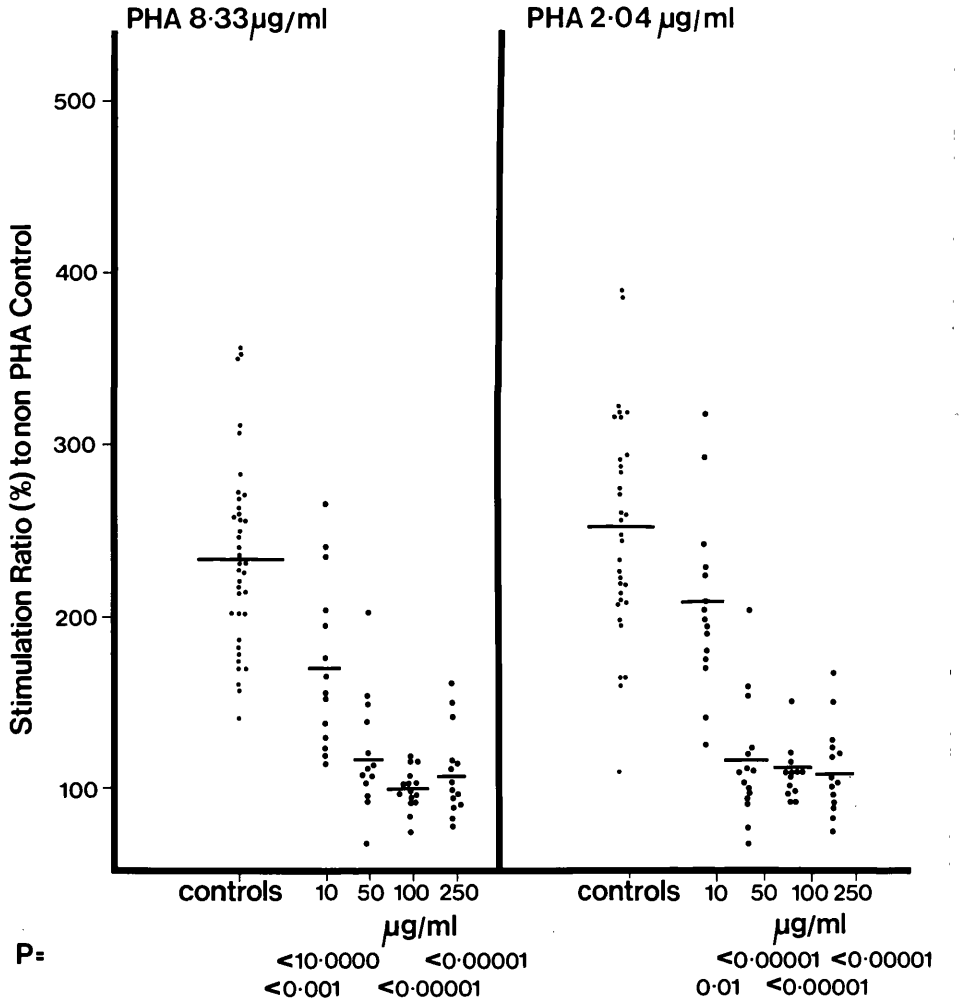

PHA 8·33μg/ml
PHA 2·04 μg/ml
Stimulation Ratio (%) to non PHA Control
500
400
300
200
100
controls
10
50
100
250
μg/ml
P=
<10·0000
<0·00001
<0·001
<0·00001
<0·00001
<0·00001
0·01
<0·00001

TABLE VI

COMPARISON OF PHA STIMULATION RATIOS OF WASHED AND UNWASHED LYMPHOCYTES CULTURED IN THE PRESENCE OF TRASICOR

CONCENTRATION OF TRASICOR		PHA 8.33 ug/ml		PHA 2.04 ug/ml	
		UNWASHED	WASHED	UNWASHED	WASHED
Controls (n = 3)	$\bar{x}$	1.53	1.65	1.53	1.62
	s.d.	0.17	0.29	0.12	0.31
	P.	-	NS	-	NS
10 ug/ml (n = 3)	$\bar{x}$	1.54	1.59	1.57	1.45
	s.d.	0.34	0.25	0.18	0.27
	P.	-	NS	-	NS
50 ug/ml (n = 3)	$\bar{x}$	1.40	1.61	1.45	1.77
	s.d.	0.25	0.27	0.25	0.29
	P.	-	NS	-	NS
100 ug/ml (n = 3)	$\bar{x}$	1.34	1.65	1.37	1.60
	s.d.	0.13	0.45	0.38	0.29
	P.	-	NS	-	NS
250 ug/ml (n = 3)	$\bar{x}$	1.06	1.39	1.06	1.44
	s.d.	0.07	0.14	0.14	0.19
	P.	-	0.02	-	0.04

causal relation with co-administered drugs, and there was no reason to believe that any of the other drugs that the patients were taking at the time of the investigation were likely to affect the immunological parameters being measured.

All of the patients who had reacted to practolol showed depressed cell-mediated immunity as demonstrated by the lowered in vitro responses of their peripheral blood lymphocytes to stimulation by PHA, both in a short term and also a three day culture. There was also evidence, however, of depressed lymphocyte function in those patients who had been on long term practolol therapy without the development of overt toxic reactions to the drug. It is not possible to say whether the depression of lymphocyte function is a precursor of the oculomuco-cutaneous reaction, as this syndrome did not later develop in any of those patients on long term practolol with impaired lymphocyte function.

Neither practolol itself nor one of its potential metabolites affected the transformation of lymphocytes derived from patients with oculomucocutaneous syndrome. This may have been due to the time of testing, since Houwerzijl and de Gast (1977) did sequential studies on two patients with a hypersensitivity reaction to carbamazepine, and found that during the reaction there was an impaired response with PHA and no transformation in the presence of carbamazepine, whereas some months the sensitivity reaction, a higher lymphocyte reactivity to PHA, and transformation with carbamazepine were found.

In a study of the sera from patients on practolol

p. 38 line 3 should read:- ".... and rheumatoid"

with oculomucocutaneous syndrome, Behan, Behan, Zacharias and Nicholls (1976) showed the presence of various auto-antibodies, especially antinuclear antibody, and rhumatoid factor. This may suggest an autoimmune disorder, however patients on long term practolol therapy with no adverse reactions also were found to possess various autoantibodies in their sera.

Certain beta-blockers were shown to depress the reaction of normal lymphocytes to PHA. This was especially true of propranolol and Trasicor. The reaction, however, was not irreversible, as washing the cells before culturing with PHA restored normal responses. In order to explain what is happening in this <u>in vitro</u> situation it is necessary to understand the role of adrenergic receptors.

Communication between neurones and end organs (e.g. muscles, glands) is primarily via transmission of chemical signals across juxtapositioned specialised cell surface regions which together constitute a synapse. Studies using various methods (Daniels and Vogel, 1975) have revealed that the post synaptic membranes are packed with receptors, specific for each of the known neuro-transmitters. Cholinergic synapses are found between post-ganglionic motor nerve fibres and skeletal striated muscle, and the neurotransmitter molecule is mainly acetylcholine. Sympathetic nerve post-ganglionic synapses with smooth muscles and glands are, in contrast, exclusively adrenergic, and transmission is mediated by norepinephrine. Although these transmitters are only produced by cells of 'neural' origin, they can function either locally as adrenergic neurotransmitters, or systemically as adrenergic hormones.

The subdivision of adrenoreceptors into two broad categories α and β, was first suggested by Ahlquist (1948), and is now well established. These two receptors differ in physical or chemical properties in such a way as to distinguish their affinity for various sympathomimetic amines. In general, the effect on α receptors is excitatory, and on β receptors inhibitory, but this is not an absolute rule. Sympathetic receptor cells may have α or β receptors on their surface, or both.

There is much less agreement, however, on the validity of the further subdivision of β-adrenoreceptors into β_1 and β_2 subgroups (Lands, Arnold, McAuliff, Ludufna and Brown, 1967), principally because there seem to be too many receptors which will not fit neatly into either group (Bristow, Sherrod and Green, 1970; Lefkowitz, 1975). Nevertheless it remains a clinically useful concept since it has been possible to develop relatively cardioselective (β_1) adrenoreceptor antagonists such as practolol, and metaprolol, which may be used to treat angina or hypertension, whereas selective β_2 agonists such as salbutamol and terbutaline are being used in the treatment of bronchial asthma since they produce bronchodilation.

While looking for a model system to study adrenergic receptor function and malfunction in relation to disease, it was found that leucocytes possess both α and β adrenoreceptors (Logsdon, Middelton and Coffey, 1972). Assessment of the lymphocyte β-adrenoreceptor was done by Connolly and Greenacre (1977) who categorised it as β_2. They also showed that the lymphocyte βadrenoreceptor is readily blocked by propranolol, but appears to be far less sensitive to the effect of practolol. It was also noted

that practolol is far more polar than propranolol, and in experiments using aqueous media, it will preferentially partition into the medium rather than into the lipid of the cell membrane, and this may hinder access to the receptors. For the lipid soluble propranolol the reverse will be the case, tending to exaggerate the already substantial difference in potency of the two drugs with respect to the β_2 receptor.

It is assumed that neurotransmitters initiate responses on excitable neurones, or non-neural tissue by a purely cell surface action on receptors. This is supported by studies on reconstituted acetylcholine receptors in artificial membranes (Rang, 1973; Axelrod, 1974). Greaves (1975) states that activation of the cell surface β-adrenergic receptor causes the enzyme adenyl cyclase to act as a catalyst in the formation of cyclic AMP from magnesium ATP. The important role that cyclic AMP plays in regulating cell proliferation and growth has already been discussed earlier in this work.

Thus we see there is a direct link between the β-adrenergic receptor on lymphocytes, and the process of protein synthesis by these cells. This would explain why propranolol should have such an adverse effect on normal lymphocytes <u>in vitro</u> when stimulated by PHA.

CHAPTER 3

IN VITRO LYMPHOCYTE FUNCTION IN PATIENTS ON ANTICONVULSANTS

Introduction

Several immunological abnormalities have been described in patients taking phenytoin (di-sodium phenylhydantoin), an anticonvulsant drug widely used in the treatment of epilepsy. These abnormalities have included depressed cellular and humoral immunity, and low serum immunoglobulin A (IgA) (Sorrell, Forbes, Burness and Rischbieth, 1971; Grob and Herold, 1972; Slavin, Fenton, Laundy and Reynolds, 1974). In a detailed study cell-mediated immunity was measured *in vivo* by skin testing with ubiquitous recall antigens, and *in vitro* by measurement of lymphocyte DNA and RNA synthesis, and was found to be depressed. Low IgA levels together with defective antibody production to *Salmonella typhii* and tetanus toxoid were also found (Sorrell and Forbes, 1975). It was subsequently suggested that epilepsy with constitutional characteristics might predispose to low IgA and that low IgA only occurs when hydantoins are given (Fontana, Grob, Sauter and Joller, 1976). It was therefore decided to study a large group of patients on phenytoin and other anticonvulsants to determine their immunological responses, and to see if there was any correlation with their HLA histocompatability status.

Methods

Patients

Thirty one patients suffering from epilepsy were studied, whose ages ranged from 27 to 76 years, with a

mean age of 47 years. These patients were divided into four groups.

(i) 10 patients (7 males and 3 females) who were taking phenytoin as part of their anticonvulsant medication, and were shown to have constantly low serum IgA levels;

(ii) 10 patients (5 males and 5 females) who were taking phenytoin but had consistently normal serum IgA levels;

(iii) 10 patients (4 males and 6 females) who were on anticonvulsant drugs other than phenytoin (including phenobarbitone, primidone, carbamazepine, sodium valproate and ethotoin), and had normal serum IgA levels;

(iv) 1 patient (female) who was on no drugs.

The duration of treatment was greater than 10 years in all patients.

Serum IgA levels had been determined by Dr. R. Shakir, Institute of Neurological Sciences, Glasgow, using radial immunodiffusion, and the Technicon AIP system. The 100% mean normal adult value (MNA) for IgA was 248 mg/dl, and the normal range was 50-175% MNA (Fahey and McKelvey, 1965). Any result, therefore, under 50% MNA was taken to indicate a low serum IgA level.

Peripheral Blood Lymphocyte Protein Synthesis

This was carried out as described in METHODS.

Results

A highly significant depression of protein synthesis was found in all 30 patients taking anticonvulsant drugs, irrespective of their IgA levels, or which drug was being

given. They all showed significant depression when compared to the controls, at PHA concentrations 1, 2 and 8 μg/ml (Figs. 18-20). The dose response curve of the one patient on no drugs was in the range of the normal controls (Fig. 21).

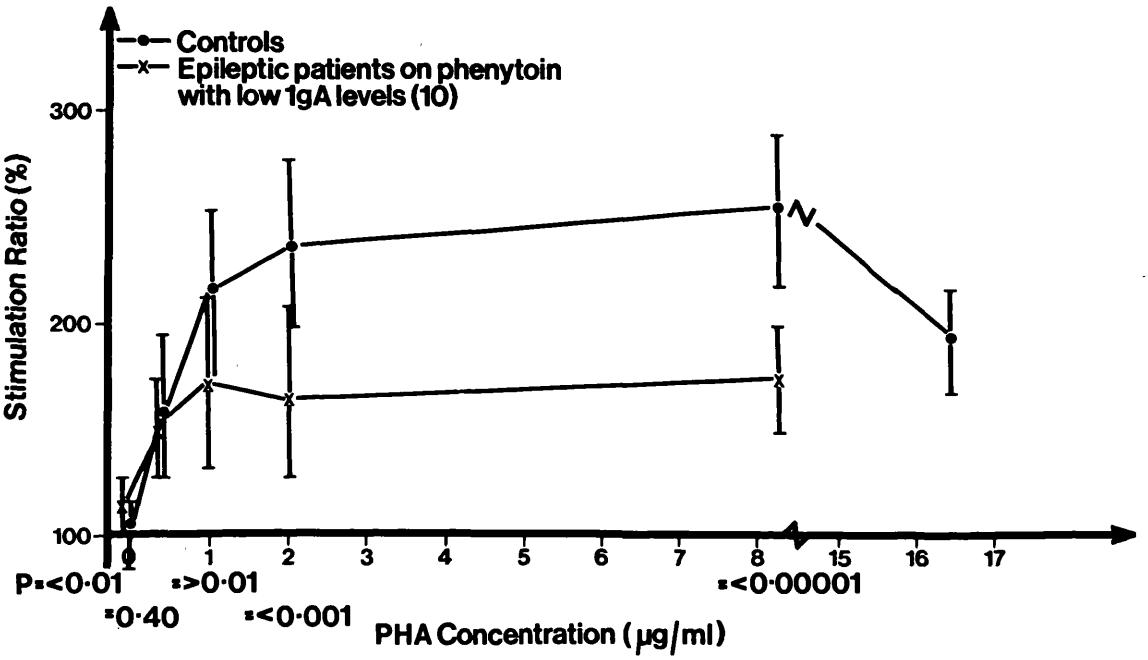
Discussion

There is good evidence that immunological abnormalities are found in patients taking anticonvulsants. Shakir (1977) demonstrated that phenytoin affects the IgA producing plasma cells confirming reports of depressed IgA levels in both serum and saliva of epileptic patients by Aarli (1976a; 1977). Impairment of T-cell function as measured *in vivo* by lack of cutaneous reactivity to ubiquitous recall antigens, and *in vitro* by depressed lymphocyte DNA and RNA synthesis, has also been shown in patients on phenytoin therapy (Sorrell and Forbes, 1975; Masi, Paolucci, Perocco and Franceschi, 1976). The presence of immunological defects, however, was independent of the dosage of drug, its serum concentration, the duration of therapy and the sex of the subject (Sorrell and Forbes, 1975; Shakir, Behan, Dick and Lambie, 1978).

Conspicuous impairment of T-cell function, as measured by lymphocyte protein synthesis was seen in all patients on anticonvulsants examined in this study. The only normal response was seen in one patient whose epilepsy was not yet being treated with drugs. Clearly more patients of this type have to be studied before any conclusions can be drawn but this finding could tie in with work done by Aarli (1976b), in which normal levels of IgA in untreated patients began to fall during the

Fig. 18 Lymphocyte protein synthesis in epileptic patients on phenytoin with low IgA levels.

Fig. 19 Lymphocyte protein synthesis in epileptic patients on phenytoin with normal IgA levels.


Controls
Epileptic patients on phenytoin with low IgA levels (10)
Stimulation Ratio (%)
300
200
100
0
1
2
3
4
5
6
7
8
15
16
17
P=<0·01
=>0·01
=<0·00001
=0·40
=<0·001
PHA Concentration (µg/ml)

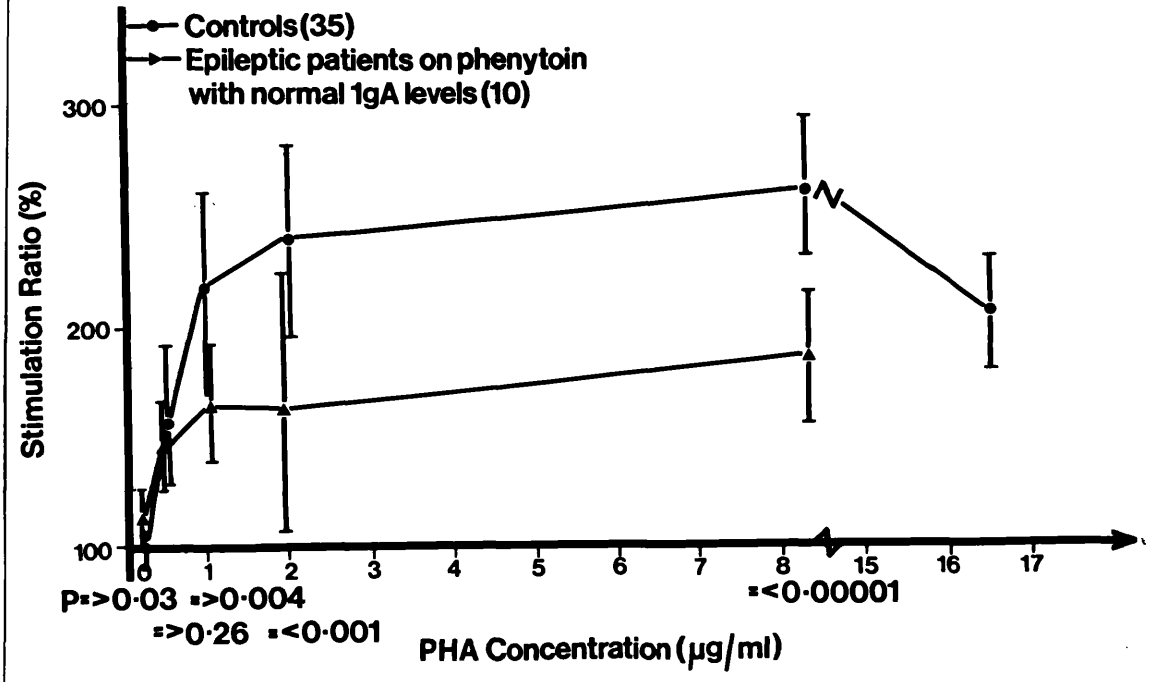
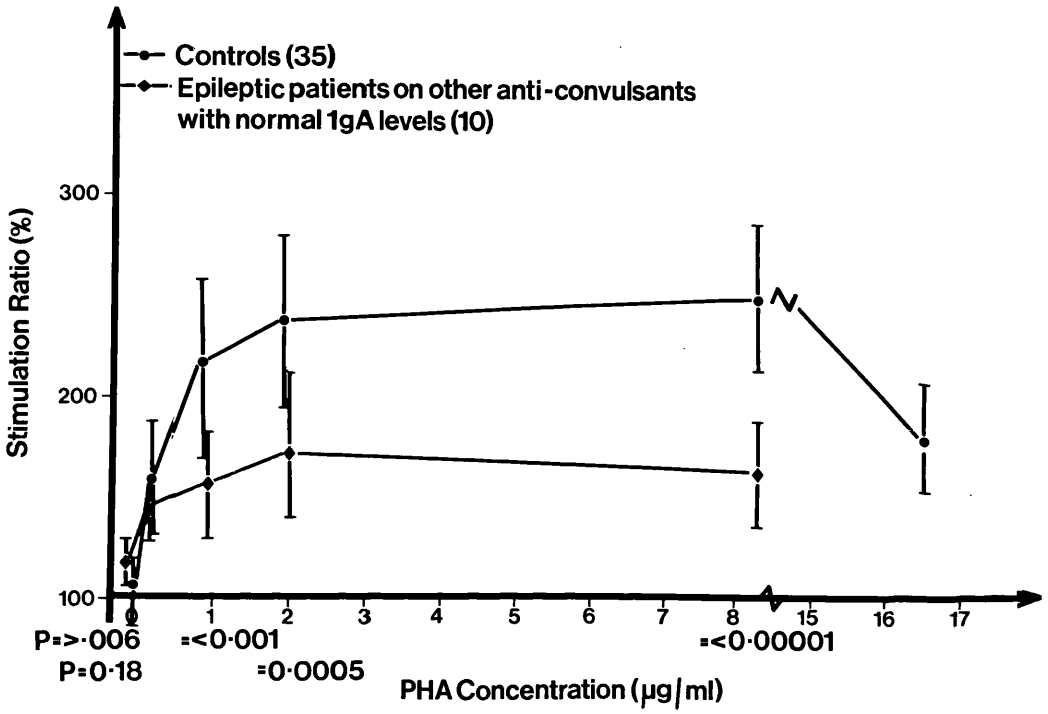
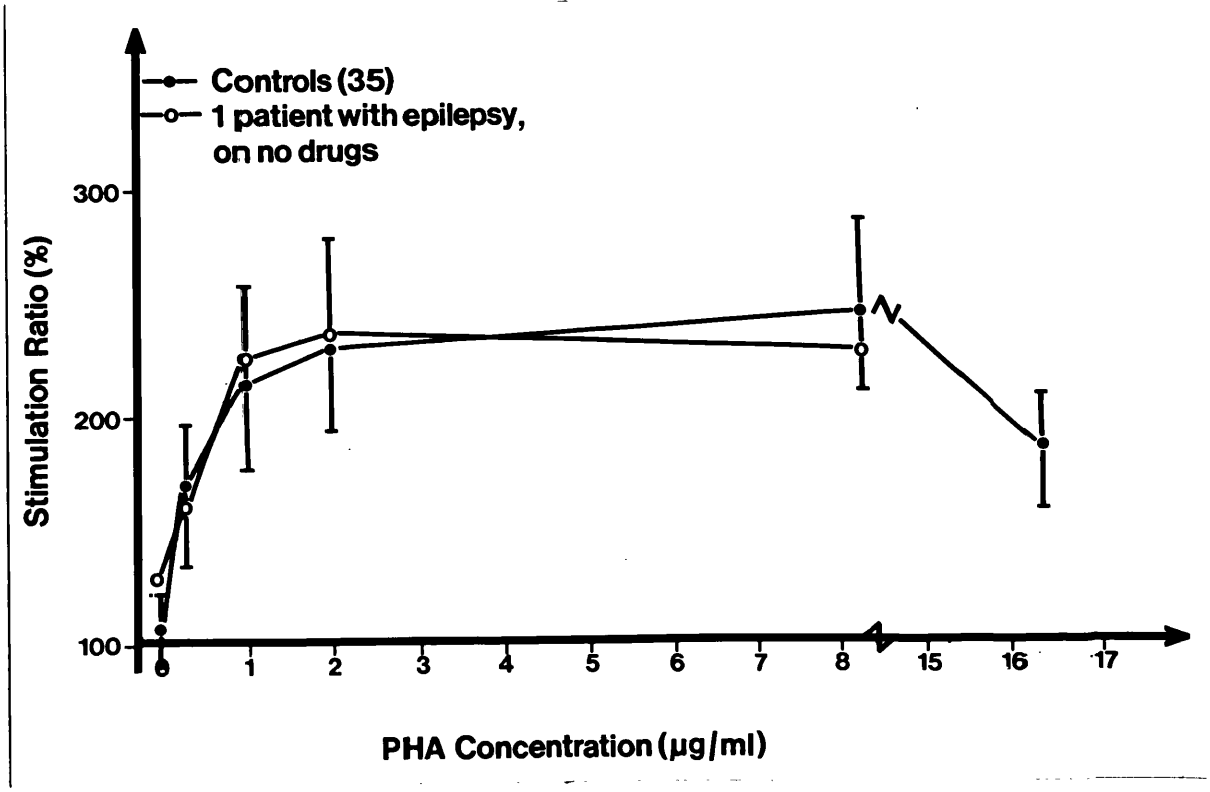

Controls (35)
Epileptic patients on phenytoin with normal IgA levels (10)
Stimulation Ratio (%)
300
200
100
0
1
2
3
4
5
6
7
8
15
16
17
P=>0·03
=>0·004
=<0·00001
=>0·26
=<0·001
PHA Concentration (µg/ml)

Fig. 20 Lymphocyte protein synthesis in epileptic patients on other anticonvulsants with normal IgA levels.

Fig. 21 Lymphocyte protein synthesis in one epileptic patient on no drugs.


Controls (35)
Epileptic patients on other anti-convulsants with normal IgA levels (10)
Stimulation Ratio (%)
300
200
100
0
1
2
3
4
5
6
7
8
15
16
17
P=>·006
P=0·18
=<0·001
=0·0005
=<0·00001
PHA Concentration (µg/ml)


Controls (35)
1 patient with epilepsy, on no drugs
Stimulation Ratio (%)
300
200
100
0
1
2
3
4
5
6
7
8
15
16
17
PHA Concentration (µg/ml)

phenytoin therapy.

Sorrell and Forbes (1975) studied the effect of phenytoin on normal lymphocytes in culture, and showed a significant depression of DNA synthesis in both PHA-stimulated, and non-stimulated cells. This immunosuppression was shown to be the result of a direct effect of phenytoin on the metabolism of lymphoid, and in particular thymus derived, cells. Carbamazepine, another anticonvulsant drug, was shown to have a similar but less potent effect. Bluming, Homer and Khiroya (1976) found that phenytoin in concentrations approximating therapeutic blood levels, also inhibited mixed lymphocyte culture proliferation. One possible explanation for these findings is that all patients on anticonvulsants have been shown to have a low serum folate level (Shakir, 1977), and this is known to affect cellular nucleic acid synthesis (Norris and Pratt, 1974), and thus the extent of lymphocyte reactivity.

Impaired T-cell function is possibly also involved in the reduced IgA levels seen in the sera of patients on anticonvulsants, since IgA function has been shown to be related to intact thymic function (Lancet, 1975). Bankhurst and Warner (1972) showed that congenitally athymic nude mice have IgA deficiency although they may have many IgA bearing B-lymphocytes. This is similar to patients with IgA deficiency secondary to phenytoin therapy (Sorrell and Forbes, 1975), who also have normal numbers of IgA bearing B-lymphocytes but low serum IgA levels. In animal experiments impaired immune responses, atrophy of thymic and lymphoid tissue and increased incidence of lymphoid tumour were produced by chronic phenytoin ingestion (Kruger and

p. 45 line 8 should read:- "an increased frequency of HLA-A2...."

Harris, 1972).

The mechanisms involved in producing these immune deficiencies are unknown, but it has been suggested by Fontana et al (1976) that a genetic factor may be operative. In a study of epileptic patients with low serum IgA, they detected a trend for the histocompatibility antigen HLA-A2. This was also demonstrated by Shakir et al (1978) who found a frequency of HLA-A2 in those patients taking phenytoin, with low IgA levels, as compared to those taking phenytoin, but with normal serum IgA. These findings strongly support the hypothesis that phenytoin causes selective IgA deficiency in genetically pre-disposed patients, whereas T-lymphocyte malfunction is a direct result of any anti-convulsant therapy.

CONCLUSIONS

The method of *in vitro* protein synthesis has been demonstrated to be a sensitive technique for measuring thymus-derived (T-cell) lymphocyte function. The technique when applied to patients (a) with brain tumours, (b) on β-blocking drugs, and (c) on anticonvulsants, demonstrated depressed lymphocyte activity.

The results obtained in patients with brain tumours and in patients with other malignancies are supported by both clinical evidence and other laboratory findings of impaired immunity. Such findings may have significance for therapy.

Lymphocytes have receptors on their surface for a large variety of substances. Impairment of the lymphocyte response in patients taking practolol, and results of *in vitro* studies involving cultures of normal lymphocytes with various β-blocking drugs shows that one of these is a β-adrenergic receptor. The blocking of this receptor caused depressed lymphocyte protein synthesis in the presence of PHA.

Finally the technique helped to elucidate the impairment of immune function in epileptic patients on anticonvulsants. The depression in T-cell activity was secondary to genetic and environmental factors and gave rise, by lack of T-cell co-operation to reduced IgA levels in some patients.

The technique of *in vitro* protein synthesis is thus a useful laboratory test which is reproducible and sensitive, and can be applied to elucidate a large number of conditions in which changes in T-lymphocyte function may be involved.

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PUBLICATION RESULTING FROM THIS WORK

Impaired thymus-derived lymphocyte function in patients with malignant brain tumour.
Menzies, C.B., Gunar, M., Thomas, D.G.T. and Behan, P.O. Clinical Neurology and Neurosurgery, Vol. 82-3 (1980), p. 157-168.