IMMUNE REACTIONS IN ACUTE VIRAL HEPATITIS

David Ian Newble BSc.(Hons), M.B.ChB, FRACP.
Department of Medicine, University of Adelaide

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STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and to the best of my knowledge and belief contains no material previously published or written by any other person, except when due reference is made in the text.

David Newble

CONTENTS

Summary

The work described in this thesis was carried out in the University Department of Medicine at The Queen Elizabeth Hospital, Woodville, South Australia during the period January 1970 - November 1973.

The thesis is introduced by a general review on humoral and cellular immune reactions in Hepatitis A and Hepatitis B. The second chapter concerns the methods used in the study. They include a lymphocyte culture microtechnique using whole blood rather than purified lymphocytes and immunofluorescent and autoradiographic techniques for the identification of B & T lymphocytes and lymphocytes undergoing blast transformation. Other methods used in the study which were in routine operation in the University or Hospital laboratories are not described in detail. These include detection of serum autoantibodies and Australia antigen, the measurement of serum immunoglobulins and complement and biochemical liver function tests.

The whole blood technique described is a modification of that reported by Junge, Hoekstra, Wolfe & Deinhardt (1970). Chapter III describes the comparison of this method with culture of purified lymphocyte preparations. This validation was considered necessary

because most studies of lymphocyte function in patients with hepatitis and in normal subjects have been performed with purified lymphocyte preparations. In normal subjects there was a good correlation between the two methods for measuring phytohaemagglutinin (PHA) induced tritiated thymidine (^{3}HT) uptake when the cultures were done in autologous serum but not when they were done in foetal calf serum (FCS). There was also a high degree of correlation when the two methods were used to measure spontaneous lymphocyte transformation in peripheral blood. The reproducibility of the results as assessed by the coefficient of variation was better for whole blood cultures than for purified lymphocyte cultures. This chapter also includes a description of the studies undertaken to determine the optimal culture conditions and preliminary work on the influence of diet and the time of collection of blood samples on PHA induced and spontaneous lymphocyte uptake of ^{3}HT. The important implications in regard to reproducibility of results are discussed.

Chapter IV concerns a study of patients with acute viral hepatitis. Serial measurements of humoral and cellular immune reactions were performed in 26 patients, of whom 17 had hepatitis A and 9 had hepatitis B. The patients were studied at weekly intervals during the

acute and convalescent periods of the illness. They were compared to normal control subjects and to 11 persons who had previously had hepatitis. With regard to humoral immunity the frequency of autoantibodies and serum levels of immunoglobulins and complement were similar to those reported by other workers. Studies of lymphocyte function showed a marked impairment of the lymphocyte response to PHA during the first two weeks after the onset of jaundice. A less marked impairment of the lymphocyte response was found to persist for at least 6 - 10 weeks. Investigation of the 11 persons with a previous history of hepatitis showed that the impairment was not permanent. The two main possible reasons for the impaired response, a direct effect on the lymphocyte by the viruses of hepatitis or the production of a serum inhibitory factor were further investigated and the results are detailed in Chapter V.

At the same time as the lymphocyte response for PHA was impaired during the first few weeks of hepatitis spontaneous lymphocyte transformation was increased. This response also occurred in other viral infections such as infectious mononucleosis and after smallpox vaccination. It seemed paradoxical that there should be a rise in spontaneous transformation at the time when PHA induced transformation was impaired. The immunofluorescent and

autoradiographic studies in Chapter VI demonstrated that there was no increase in the number of circulating B cells in the circulation during the acute phase of hepatitis. Dividing lymphocytes were present in the circulation in increased numbers and were shown by autoradiography to be large cells, probably the "atypical lymphocytes" seen in the blood films. It was concluded that these cells were T cells, that they accounted for the increase in spontaneous ^{3}HT uptake in the lymphocyte cultures and that the impaired response of the lymphocytes to PHA might at least in part, be attributed to commitment of an abnormally large proportion of the T cells as a result of hepatitis.

The effect of serum factors on the lymphocyte response to PHA was next investigated and is described in Chapter V. Serum taken from patients during the acute phase of hepatitis inhibited PHA induced ^{3}HT uptake by normal lymphocytes whilst convalescent serum did not. Moreover, lymphocytes from patients with hepatitis were also inhibited by autologous acute phase serum but not by autologous serum taken later in the course of the disease. Since washing the lymphocytes did not completely restore their response to normal, a defect in the lymphocyte itself, possible induced by virus, cannot be excluded.

It has been suggested that the competence of the

immune system in the host determines the outcome of infection with hepatitis B virus. The hypothesis suggests that in acute hepatitis the T cells react specifically against Australia antigen (Au) incorporated in the liver cell membrane with consequent liver damage but with ultimate elimination of the virus. If this is so it should be possible to detect specific cell mediated immunity to the antigen and the work of Yeung Laiwah (1971) suggested that this could be done. For this reason a study was made of the response of lymphocytes from patients with hepatitis to various Au preparations including pooled Au containing serum, a partially purified preparation and commercially prepared purified Au particles (Chapter VII). These preparations only rarely stimulated lymphocyte transformation, as measured by an increase in ^{3}HT uptake, in patients with hepatitis B and lymphocytes from patients with hepatitis A reacted in a similar manner.

Significant Contributions of the Thesis

1. The whole blood microtechnique was validated by comparison with purified lymphocyte preparations and was shown to have practical advantages over purified lymphocyte cultures. The method also proved more reproducibility than the purified lymphocyte technique.

2. Patients with hepatitis A and hepatitis B were found to have a lymphocyte response to PHA which was markedly impaired during the first two weeks after the onset of jaundice and less markedly impaired for at least 6 - 10 weeks.

3. Patients with hepatitis had an increased number of peripheral blood lymphocytes undergoing transformation as indicated by measurement of spontaneous ^{3}HT uptake and by autoradiography. The response appeared to be due to a T cell proliferation.

4. Serum from patients in the acute phase of hepatitis inhibited the PHA induced ^{3}HT uptake by normal lymphocytes and by autologous lymphocytes. The inhibitory factor(s) may play a part in the impaired response of lymphocytes to PHA in the first two weeks of the acute phase of hepatitis.

5. Australia antigen induced an increased ^{3}HT uptake in only a minority of patients with hepatitis B. A state of specific cell mediated immunity to Au was therefore not confirmed.

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CHAPTER I.

IMMUNE REACTIONS IN ACUTE VIRAL HEPATITIS;

A GENERAL INTRODUCTION AND REVIEW OF THE LITERATURE

CHAPTER I

1. General Introduction

In the last few years there has been a growing interest in the various immune reactions occuring in viral hepatitis. This interest has gone beyond the protective function of humoral and cellular immunity. Two aspects which have received increasing attention are, firstly, the modifying effects of host reactivity on the outcome of acute viral hepatitis and, secondly, the possible role of immunological events in the pathogenesis of acute hepatitis and some chronic liver diseases.

2. Hepatitis Virus

Acute hepatitis that is not due to readily identifiable causes such as infectious mononucleosis, cytomegalovirus and other known viruses, drug toxicity or hypersensitivity, is usually refered to as viral hepatitis. There is strong circumstantial evidence for a viral aetiology but the causative viruses have not been identified. It is believed that at least two viruses are involved, one causing classical "infectious hepatitis" (Hepatitis A, "short incubation") and another causing classical "serum hepatitis" (Hepatitis B, "long incubation") (W.H.O. Expert Committee on Hepatitis 1964).

In 1965 Blumberg, Alter and Visnich identified an antigen in the serum of an Australian aborigine which reacted against the serum of multiple transfused haemophiliacs and which was consequently name "Australia antigen" (Au). Two years later Au was shown to be present in the blood of some patients with acute viral hepatitis (Blumberg, Gerstley, Hungerford, London, Sutnick 1967; Blumberg, Sutnick and London 1968). Further studies demonstrated that the antigen was particularly associated with serum hepatitis (Blumberg et al 1968; Prince 1968; Okochi and Murakami 1968) and was found most readily during the incubation period and the early part of the clinical illness. It soon became evident that the Au or material closely associated with it was infective (Blumberg et al 1968) and the natural history of the disease was established by transmission experiments, particularly those conducted at Willowbrook State School (Krugman and Giles 1972). Electronmicroscopic and histochemical studies have shown that Au is associated with particles present in serum where the antigen is detected. These particles are pleomorphic and are present in three principal forms, small spherical (16 - 25 nanometres (nm) in diameter), tubular (20nm diameter and several hundred nm in length) and large spherical

(40-45nm diameter) (reviewed by Zuckerman, Taylor and Bird 1970). Most workers now believe that Au is excess viral coat protein derived from host protein and that the infectious agent in hepatitis B is a virion consisting of a very small amount of RNA-enzyme complex (Dane, Cameron and Briggs 1970; Almeida, Rubenstein and Stott 1971; Joswiak, Kloczewiak, Madalinskik, Brzosko, Nowoslwski 1971; Hirschman, Vernace and Schaffner 1971).

3. Immune Reactions in Hepatitis-Introduction

The discovery of Au and the development of techniques for its detection in serum has led to progress in the understanding of the immune reactions that occur in acute hepatitis, particularly hepatitis B. Study of these immune reactions has assumed a greater importance since Au has been found in patients with impaired immunity (Blumberg et al. 1968) and in patients with active chronic hepatitis (Wright 1972). Dudley, Fox and Sherlock (1972)[a] have suggested that the competence of the immune system in the host determines whether infection with hepatitis B virus leads to acute hepatitis, chronic hepatitis or whether the infection persists without apparent ill effects. Mackay and Popper (1973) have put forward similar views for the

pathogenesis of chronic hepatitis.

Various immunological events take place during hepatitis A and hepatitis B. More is known about the humoral immune reactions than the cellular immune reactions.

4. Humoral Immune Reactions in Hepatitis

(1) Specific Antibody

Specific antibody production in viral hepatitis has been incompletely studied as a specific antigen, Au, has only been identified in hepatitis B. The Milan antigen described by Del Prete, Constantino, Doglia, Grazina, Ajdukiewcz, Dudley, Fox and Sherlock (1970) in cases of hepatitis A is now thought to be a nonspecific reaction to liver injury (Doniach, Del Prete, Dane, Walsh 1972).

Although Au is readily detectable in the serum during the early stages of hepatitis B, the antibody to Au is usually only detectable by very sensitive techniques such as radio-immunoassay (Lauder, Alter and Purcell 1971; Coller, Millman, Halbherr, Blumberg 1971) and haemagglutination inhibition (Vyas and Shulman 1970). Most patients in whom antibody is

readily detectable have been repeatedly exposed to antigen by having multiple blood transfusions. Standard gamma-globulin preparations have little or no protective effects against hepatitis B whereas they are effective against hepatitis A (National Transfusion Hepatitis Study 1970; Krugman 1963). This suggests that, in man, Australian antigen is a weak stimulus to the humoral immune system and that specific antibody to Au plays little or no part in the recovery of the patient from the acute attack or in prevention of re-infection. This does not mean that antibody in large quantities cannot be protective and in preliminary clinical studies antisera containing high levels of antibody appear to prevent or modify hepatitis B (Krugman, Giles and Hammond 1971; Soulier, Blatix, Couronce, Benamon, Amouch and Drover 1972). The protective activity of immune gammaglobulin against hepatitis A suggests that antibody to the causative agent of this disease is produced in much larger quantities.

(2) Immune Complexes and Complement

The role of complement and complement containing antigen-antibody complexes in the pathogenesis of acute hepatitis has still to be clarified. In situations of

excess antigen immune complexes may form and may, through the complement system, produce tissue damage at their sites of deposition in vessel walls and glomeruli (Christian 1969; Cochrane 1971). The lesions closely resemble those occuring in experimental "serum sickness". The term generally used for conditions caused by antigen-antibody complexes is immune complex disease.

In hepatitis B there is usually an excess of Au in the early stages with only a minimal detectable antibody response. Circulating complexes have been demonstrated by electron microscopy (Almeida, Zuckerman, Taylor, Waterson 1969) and sera from patients with this condition have been shown to be anticomplimentary (Shulman and Barker 1969). This led Almeida and Waterson (1969) to postulate that deposition of immune complexes could account for the liver lesions of both acute viral hepatitis and active chronic hepatitis but this view has not been supported by other workers (Dudley, Fox and Sherlock 1971).

A fall in serum complement is regarded as an indication of immune complex formation. Studies of complement levels in hepatitis have been conflicting. Dudley et al (1971) reported normal levels in acute and chronic hepatitis but these and other studies can be

criticised on the basis that usually only one blood specimen was taken and serial studies were not undertaken in the same patient. Kosmidis and Leader-Williams (1972) in a serial study of complement levels in hepatitis A and hepatitis B demonstrated a characteristic pattern consisting of an early fall followed by a rise to levels above normal then returning to normal values over several weeks. This pattern was present in 29 out of 31 patients studied and there was no difference in patients with type A or type B disease. Alpert, Isselbacher and Schur (1971) demonstrated a fall in complement in patients with hepatitis who had the extra-hepatic manifestations of arthralgia and rash and in all these patients high titres of Au were detected.

Although these results are conflicting, there seems to be little firm evidence to support the hypothesis of Almeida and Waterson. Both clinically and experimentally circulating immune complexes tend to cause vasculitis and glomerulitis (Cochrane 1971; Oldstone and Dixon 1971) and hepatitis is not a predominant feature. Conversely, features of immune complex disease rarely occur in association with viral hepatitis. However, it still appears quite likely that complexes may play a role in the extra hepatic manifestations of hepatitis B.

Arthritis or arthralgia have been reported in up to 20% of cases in some series, symptoms being most prominent in the preicteric phase. In one series Onion, Crumpacker and Gilleland (1971) demonstrated a reduction in complement in the serum and joint fluid in patients with arthralgia and Au was detected in the serum of all cases and in the joint fluid where tested. Alpert et al (1971) reported similar results and also noted other features suggesting a "serum-sickness like" illness, such as an urticarial rash, in over half of their cases. Complex deposition in the lesions was not demonstrated in these cases. Gocke, Hsu, Morgan, Bombardieri, Lockshin and Christian (1971) reported the finding of Au containing complexes in the vascular lesions of 6 out of 16 cases of polyarteritis nodosa and Combes, Stastny, Shorey, Eigenbrodt, Barrera, Hull and Carter (1971) reported a case of immune complex glomerulonephritis following transfusion with Au positive blood.

(3) Immunoglobulin(Ig)Levels

Hyperglobulinaemia in viral hepatitis was first described in 1948 by Havens and Williams and was later shown to be due to an increase in the gammaglobulin fraction (Osserman and Takatsuki 1963). It was thought

to be due mainly to IgM (Hobbs 1967). More recent studies have shown a clear difference in immunoglobulin patterns between hepatitis A and hepatitis B. Wollheim (1968) showed that the hypergammaglobulinaemia was polyclonal with slightly increased IgG levels and more markedly raised IgM levels in hepatitis A but only an increased IgG in hepatitis B. These results were confirmed by the experimental transmission studies of Giles and Krugman (1969). The subsequent availability of reliable tests for Au allowed a more precise classification of patients with hepatitis. Iwarson and Holmgren (1972) studied a large series of patients with acute hepatitis where they performed serial studies of IgG, IgA and IgM. Both Au positive and Au negative patients had a marked increase in IgG lasting for many weeks with normal IgA levels. However, there was a highly significant difference in the IgM levels which were elevated in almost all cases of hepatitis A for the first 4-6 weeks of the illness whilst only one out of 40 Au positive cases had an elevated IgM. In another recent series a similar pattern was seen for IgG, IgM and IgA and in addition levels of IgD were found to be normal (Peters and Johnson 1972).

The cause of the Ig changes in hepatitis is not

understood. It is known that almost all normal circulating Ig results from antigenic stimulation primarily by bacteria but even after intense immunization with a specific bacterial antigen not all the Ig increase that occurs can be accounted for by specific antibody (Alper, Rosen and Janeway 1966). In viral infections the response is less intense and in hepatitis B it is known that the Au produces a very limited antibody response. The liver itself produces very little Ig under normal circumstances but may play a more important role in Ig catabolism (Cohen, Gordon and Mathews 1962).

Experimental studies have suggested a direct association of hypergammaglobulinaemia with liver damage. Removal of liver tissue results in a temporary increase in gammaglobulin (Aronson 1966) and liver damage in germ free rats also results in an increase in Ig. (Bauer, Paronetto, Porro and Einheber 1966). This could simply reflect a reduction of globulin breakdown in the liver but other studies suggest that other factors are concerned. Sakai, Muller-Berat, Debray-Sachs and Crevon (1970) showed that removal of liver tissue enhanced the antibody response to antigen and a similar enhancement has been reported in cirrhosis (Havens 1959). It is

possible, therefore that the liver is involved more directly in the regulation of Ig turnover. Alpers et al (1966) suggested that the liver might be involved in the regulation of the colloid osmotic pressure in the blood through its control of albumin production. A fall in albumin resulting from liver disease would lead to a compensatory increase in globulin production. This does not seem likely in view of the specifity of plasma cell function unless the liver produced a circulating regulatory factor.

Autoantibodies are produced in many liver diseases including hepatitis and it is possible that liver damage could release enough antigenic material to account for the marked Ig increase. The identifiable autoantibodies are only present in low titres and could not account for the total Ig increase but liver specific antigens have recently been described (Meyer zum Büschenfelde and Kössling 1971) and could be more antigenic. Information on this has not been published. An increase in Ig producing cells in the liver has been noted, particularly in active chronic hepatitis (Paronetto, Rubia and Popper 1962), but the number of cells does not correlate well with serum Ig levels (Hadziyannis, Feizi, Scheuer and Sherlock 1969). Lymphocyte and plasma cell infiltration of the liver occurs in acute hepatitis

(Weinbren and Stirling 1972) but no studies on Ig producing cells have been reported.

The difference in Ig patterns between hepatitis A and hepatitis B has not been explained. It may reflect immunological differences between the causative organisms the increased IgM in hepatitis A reflecting a more pronounced primary antibody response. Studies of IgM turnover have not revealed any difference between the two forms of hepatitis (Jensen 1970). It is not possible to say whether the difference in Ig patterns has relevance to the different clinical course and prognosis of the two conditions.

(4) Autoantibodies

Smooth muscle (SMA) and mitochondrial (M) auto-antibodies are frequently found in patients with active chronic hepatitis (ACH), primary biliary cirrhosis (PBC) and cryptogenic cirrhosis (CC). They are regarded as markers of an autoimmune process associated with continuing liver injury (Doniach 1972). The role of hepatitis virus as a possible trigger of such a process is of interest since Australia antigen has been found in some patients with ACH, PBC and CC (Doniach, Walker, Roitt and Berg 1970; Wright, McCollum and Klatskin 1969;

Sherlock, Fox, Niazi and Scheuer 1970; Cooksley, Powell, Mistilis, Olsen, Matthews and Mackay 1972; Prince 1971) but the casual association of hepatitis virus to chronic liver disease remains speculative.

SMA is found in over 80% of patients with viral hepatitis but is also found in similar proportions in patients with hepatitis due to infectious mononucleosis (Holborow 1972). In viral hepatitis SMA is only detectable in low titres and is present only during the acute stage (Farrow, Holborow, Johnson, Lamb, Stewart, Taylor and Zuckerman 1970). It is now thought to reflect a non-specific immune response against an actomyosin-like protein of liver cell membranes exposed by liver damage (Holborow 1972).

M antibodies are a useful diagnostic marker for PBC (Walker, Doniach, Roitt and Sherlock 1965) but are said to be present in only 2% of patients with viral hepatitis (Doniach 1972). These antibodies appear in the serum of rats after carbon tetrachloride induced liver damage (De Heer, Pinckard and Olsen 1972) once again reflecting the nonspecific nature of the response.

Antinuclear antibodies may also appear transiently in a small proportion of patients with acute viral hepatitis (Farrow et al. 1970) and in some patients with

chronic liver disease. Rheumatoid factors have also been found in acute hepatitis (Dudley, O'Shea and Sherlock 1973).

5. Cellular Immune Reactions in Hepatitis

(1) Role of Cell-Mediated Immunity

Host reaction to viral infections is a complex phenomenon but cell mediated immunity (CMI) is thought to be of critical importance. Thymus derived (T) lymphocytes responsibility for CMI reactions are the only cells known to be capable of destroying tissue containing viral antigen. Viral infections are more common and more severe in clinical situations characterised by depressed CMI. This holds true whether the immune deficiency is congenital, has arisen as the result of disease or has been induced by drugs or irradiation (reviewed by Glasgow 1970).

Since humoral immune reactions do not appear to play a major role in the pathogenesis of hepatitis, attention has been focused on the possible role of CMI in both acute and chronic hepatitis. Hotchin and Collins (1964) studying the results of neonatal lymphocytic choriomeningitis (LCM) in mice demonstrated that the animals carried large amounts of viral antigen without

apparent ill effect for several months before developing "late onset disease". This was assumed to be due to the induction of tolerance, as animals infected a month after birth died of acute hepatitis or meningoencephalitis. They drew a parallel to human serum hepatitis in which the outcome of infection may be acute hepatitis or a chronic carrier state. They suggested that the acute stage of LCM represented an immunological conflict between replicating viral antigen in the tissues and the hosts immune system producing what was essentially a homograft response. The "late onset disease" was seen as a gradual waning of the tolerance to the viral antigens. They predicted that chronic carriers of serum hepatitis virus might later succumb to chronic liver or renal disease.

The discovery of Australia antigen has enabled this hypothesis to be tested. Popper and Mackay (1972) postulated that chronic hepatitis may develop if CMI reactions fail to eliminate the antigen which persists and induces a continuous but limited immune reaction. However, not all patients with a previous history of hepatitis B who develop chronic hepatitis have detectable Australia antigen. To explain this a second mechanism was proposed whereby the virus altered host proteins and in a predisposed population an autoimmune reaction

developed.

Blumberg, Sutnick and London (1970) and Dudley, Fox and Sherlock (1972)[a] proposed similar mechanisms to explain the relation of Au to liver disease but placed a major emphasis on the immune response of the host. Dudley et al. (1972)[a] pointed out that a prospective study of hepatitis B (Nielsen, Dietrichson, Elling and Christoffersen (1971) had shown that when Au persisted after the acute episode, the majority of patients had biochemical and histological evidence of persisting or continuing hepatitis. Screening of blood donors has revealed chronic carriers of Au who have little or no evidence of liver damage although the serum from these donors is infectious. They concluded from these studies that the virus might have little or no direct cytotoxic effect and that the clinical course after exposure depended on the CMI reaction of the host. Supporting evidence comes from studies showing that patients with conditions that impair CMI are more likely to become carriers or develops subclinical disease than to develop acute hepatitis (Blumberg et al. 1967; Knight, Fox, Baillod, Niazi, Sherlock and Moorhead 1970). The hypothesis is, therefore, that three basic types of reaction may occur.

1. If the CMI system of the host is intact, the T cells react specifically against the viral antigen which is presumed to be incorporated in the liver cell membrane. This leads to necrosis of the infected cells, a consequent clinical hepatitis and finally elimination of all cells containing virus. The severity of the hepatitis depends on the number of infected cells.

2. If the CMI response is inadequate because of tolerance to the antigen or deficient T cell function, liver damage will not occur, the virus will proliferate and the patient becomes a "healthy" carrier.

3, If the T cells are partially deficient or suppressed, mild hepatitis will develop and chronic hepatitis will result if the viral antigen cannot be totally eliminated.

These hypothesis are attractive and explain many of the features of disease associated with Au but have yet to be confirmed. In one recent study no abnormality of CMI was detected in a group of Au carriers (Sutnick, London and Blumberg 1973). Information on CMI reactions in viral hepatitis is limited partly because of the lack of an animal model and partly because the tests of T cell function that are presently available for use in

man are rather crude and nonspecific.

(2) T - Lymphocyte Function in Hepatitis

Phytohaemagglutinin (PHA) induces lymphocytes to transform and divide under standard conditions of culture and it seems to act mainly on T cells or a subpopulation of T cells (Douglas, Kamin and Fudenberg 1969; Janossy and Greaves 1971; Stockman, Gallagher, Heim, South and Trentin 1971; Markley, Thornton and Smallman 1972; Wybran, Chantler and Fudenberg 1973). The response to PHA has been used widely and is considered to be a good measure of cell-mediated immunity (Oppenheim 1968).

The lymphocyte response to PHA has been shown to be impaired in the early stages of the acute phase of viral hepatitis (Table 1). Although the numbers of patients studied were small and in most cases serial studies were not done, the findings were consistently those of impairment occuring with the onset of symptoms and lasting for 1-3 weeks. There are no published studies on the time course of the changes in the incubation and preicteric phases.

Additional support for an impairment of lymphocyte response comes from Mella and Lang (1967) who showed an

Table I.1.

Studies of Lymphocyte Response to PHA in Acute Viral Hepatitis

No. of Cases	Au		Method		Serial Studies	Authors
	+	-	DNA Polymerase	3HT uptake		
9	7	2	Yes	Yes	3/9	Agarwal et al. 1971
18	5	13	No	Yes	No	Willems et al. 1969
22	-	-	No	Yes	No	Schieffarth et al. 1969
5	-	-	No	Yes	No	" Rossler et al. 1970

absence of metaphase figures in chromosome preparations from patients in the acute phase of hepatitis with a return to normal in the convalescent period, though abnormal chromosomes were noted at this time.

There are several possible explanations for this impairment of lymphocyte function.

(a) Direct effect of virus

Loss of PHA response is not restricted to patients with hepatitis but occurs in other viral infections such as infectious mononucleosis (Yam, Castoldi and Mitus 1967),

upper respiratory tract infections (Thomas, Clements and Naiman 1968) and in patients with congenital rubella (Olson, Dent, Rawls, South, Montgomery and Good 1968). Exposure of lymphocytes to certain viruses in vitro may also interfere with the PHA response. However, this is not an invariable response to viral infection (Willems, Melnick and Rawls 1969; Salaman 1969). In the instances where the PHA response was lost, virus was usually isolated from the affected lymphocytes. Although it has been suggested that the loss of PHA response in hepatitis is due to the direct effect of the virus (Willems et al. 1969; Martini, Rössler, Haveman and Dölle 1970) there is no definite evidence for this. Millman, Agarwal, Bugbee, Blumberg and Loeb (1971) were unable to demonstrate direct inhibition of lymphocyte function with a purified Australian antigen preparation and in another series, serum containing Au also failed to inhibit normal lymphocytes (Giustino, Dudley and Sherlock 1972).

(b) Serum Inhibitory Factors

The impairment of lymphocyte function in hepatitis may not be due to an inherent defect of the lymphocyte but may be a functional loss caused by an immunosuppressive factor in the serum. Mella and Lang (1967) found that small quantities of serum from patients with acute hepatitis inhibited the development of metaphase figures

in normal leucocyte cultures. This effect was not reproducible with normal serum or with serum from patients with other liver diseases. Other workers have shown that hepatitis serum may also impair the response of normal lymphocytes to PHA (Baroyan, Barinsky, and Shatkin 1970; Hsu and Leevy 1971; Newberry, Shorey, Sanford and Combes 1973) but others have been unable to confirm this finding (Willems et al. 1969; Millman et al. 1971; Kissling and Speck 1971). It is difficult to compare the results of different workers because of variations in experimental design, in the amount of serum used in the cultures and in the stage of hepatitis at which the serum was taken. Serum is stimulatory under normal circumstances in some culture systems and in one series, serum from a variety of liver disorders was said to be stimulatory to normal lymphocytes (Kissling and Speck 1971).

Substances capable of depressing DNA synthesis and lymphocyte transformation to both PHA and specific antigens are present in normal serum (Cooperband, Bondevik, Schmid and Mannick 1968; Cooperband, Badger, Davies, Schmid and Mannick 1972; Nelken 1973). One of these substances is present in the globulin fraction, is not cytoxic and is only loosely bound to lymphocytes as a simple washing procedure restores the cell reactivity (Nelken 1973).

It is possible that inhibitory or toxic substances

could be released from damaged liver cells or result from impaired liver function but little information is available in this regard. Impairment of transformation occurs in liver diseases such as PBC, ACH with cirrhosis and alcoholic cirrhosis (Giustino et al. 1972; Rössler et al. 1969; Fox, Scheuer, James, Sharma and Sherlock 1969), whilst conflicting results are reported in extrahepatic biliary obstruction (Fox et al. 1969; Newberry et al. 1973). Recent studies have shown that serum inhibitory factors are present in most of these disorders (Hsu and Leevy 1971; Newberry et al. 1973; Giustino et al. 1972). In the case of alcoholic cirrhosis lymphocytes react normally when separated from autologous plasma (Hsu and Leevy 1971). The extent of the lymphocyte inhibition is not related to serum levels of bilirubin or liver enzymes and bile salts in high concentrations do not suppress the PHA response of normal lymphocytes (Newberry et al. 1973).

(c) Inherent T cell deficiency

T cell function will be impaired if the lymphocytes fail to recognise an antigen or are inherently defective. Blumberg, Sutnick and London (1968) suggested that certain families might have an inherited autosomal recessive susceptibility to Australia antigen and Vermylen, Goethals and Van de Putte (1972) found a preponderance of certain

HL-A types in Au carriers. Certain chronic liver diseases show a preponderance for one sex and may show differences in incidence on a familial or racial basis. (Mackay and Popper 1973; Sherlock 1970). However, there is no evidence to suggest an inherited T cell deficiency which might predispose to acute hepatitis or its sequelae.

(3) Changes in the Lymphocyte in Vivo

Peripheral lymphocytes are mainly small lymphocytes but a small proportion of larger 'lymphoblastoid' or 'blast' cells (<0·5%) and cells synthesising DNA (<0·2%) are normally present. In a study of lymphoid cell responses after immunisation with a variety of antigens Crowther, Fairley and Sewell (1969) demonstrated a marked rise in blast cells and cells synthesising DNA 5-7 days after immunisation. Similar responses were seen during a variety of bacterial and viral infections, the increase in blast cells being more pronounced during the latter. Light microscopic and electronmicroscopic studies showed that the circulating blast cells were identical to the immunoblasts present in lymph nodes after antigenic stimulation.

In some viral infections such as infectious mononucleosis and infectious hepatitis, 'atypical lymphocytes' appear in the circulation in large numbers, often accounting for over 20% of the total white cell count. In hepatitis

these cells reach their peak levels on about the fifth day of illness and disappear by the fourteenth day (Havens and Marck 1946). It is mainly these cells which take up tritiated thymidine (^{3}HT) in culture and are therefore in the premitotic phase of DNA synthesis (Wood and Frenkel 1967). The atypical lymphocytes are very similar to the blast cells seen after lymphocytes are stimulated by antigen or by PHA (reviewed by Wood and Frenkel 1967). It is still not certain whether these cells are functional immunoblasts responding to specific antigen or whether they represent a non-specific response to inflammation. A recent study demonstrated an increase in DNA synthesising mononuclear cells in non infective inflammatory disorders but a more marked response in viral hepatitis (Horwitz, Stastny and Ziff 1970). The exact origin and type of these cells could not be determined but the application of methods to identify lymphocyte subpopulations has demonstrated that the atypical lymphocytes in infectious mononucleosis are T cells (Sheldon, Papamichail, Hemsted and Holborow 1973). Other workers have shown that the increased DNA synthesis in the atypical lymphocytes of infectious mononucleosis is not attributable to viral replication (Hale and Cooper 1963; Carter 1965). It would appear most likely that it indicates a specific cell mediated immune response to the

virus with proliferation of T cells. Comparable studies have not been reported for viral hepatitis. Similar findings have been reported from studies of homograft rejection crisis where an increase in the number of ^{3}HT incorporating cells is demonstrable before the rejection episode is detectable clinically (Tennenbaum, St.Pierre and Cerilli 1968; Suciu-Foca, Buda, Thiem and Suciu 1972). The cells responsible for the ^{3}HT uptake are again lymphoblasts (Hersh, Butler, Rossen, Morgan and Suki 1971).

(4) Cell Mediated Immunity to Specific Antigens

It is possible that cell-mediated autoimmune reactions to specific liver antigens or to an external antigen incorporated into liver cells, such as Au, could account for the liver damage and chronic hepatitis.

(a) Liver Antigens

Recently Meyer zum Büschenfelde and Kissling (1971) reported the isolation of liver specific proteins and were able to induce liver disease resembling ACH in rabbits by repeated injections of these proteins. Cells sensitized to the liver specific proteins were demonstrated by immunofluorescence in the livers of 85% of patients with ACH. Miller, Mitchell, Eddleston, Smith, Reid and Williams (1972), using the migration inhibition test, were

able to demonstrate significant inhibition to similar protein preparations in 69% of patients with ACH and 50% of patients with PBC but not in patients with alcoholic cirrhosis or haemochromatosis. Similar studies using crude homogenates of liver from foetal, necropsy and liver biopsy tissue have consistently shown that the homogenates may stimulate lymphocytes to transform or cause inhibition in the migration inhibition test in over 50% of patients with ACH and PBC (Tobias, Safran and Schaffner 1967; Schieffarth, Warnatz, Ottenjann and Ritter 1969; Miller et al. 1972; Bacon, Berry and Bown 1972). The changes are only detectable in the latter stages of PBC (Bacon et al. 1972). In acute infectious hepatitis no significant reactions to either liver homogenate or to liver specific proteins have been detected (Schieffarth et al 1969; Smith, Eddleston and Williams 1971).

(b) Australia antigen

The development of a CMI reaction to Au in acute hepatitis is a key feature of the hypothesis of Dudley et al. (1972)[a] discussed previously. Yeung Laiwah (1971) demonstrated a lymphocyte transformation response to serum containing Au in patients who had previously had hepatitis B but not in patients with primary biliary cirrhosis or normal controls. Dudley, Giustino and Sherlock (1972)[b]

using the migration inhibition test showed a significant inhibitory effect to both Au positive serum and Au positive liver homogenate in patients with acute hepatitis B. Serial studies performed on two patients showed that the inhibitory effect could not be detected 3 months later. Similar inhibition was found in about 50% of patients with chronic aggressive hepatitis and cirrhosis but not in patients with chronic persistent hepatitis or who were Au carriers. However, both of these studies were carried out using perparations containing many other factors as well as Au and no reports are yet available on the effect of purified Au on lymphocytes from patients with acute hepatitis.

CHAPTER II

DESCRIPTION OF METHODS

CHAPTER II

1. Introduction

In this Chapter are given descriptions of the methods used in this study. The methods developed as part of this study for lymphocyte culture and for identifying lymphocyte subpopulations will be decribed in detail. The methods used for the tests that are in routine operation in the laboratories of the University Department or the Hospital will not be discussed in detail.

2. Lymphocyte Culture Microtechnique

A modification of the whole blood microtechnique of Junge, Hoekstra, Wolfe and Deinhardt (1970) was used.

(1) Collection of Blood Samples

Peripheral venous blood was collected in disposable plastic syringes and transferred to sterile glass bottles containing 25 units of preservative-free heparin per ml. of blood (Evans Medical Ltd.). Blood was also placed in a sterile bottle to provide serum and 4ml. dispensed into E.D.T.A. for a white cell count and blood smear.

(2) Phytohaemagglutinin Induced Transformation

Phytohaemagglutinin (PHA; Wellcome; reagent grade) was

reconstituted with sterile saline. An aliquot was diluted with medium 199 (CSL) so that 3ml. of the medium contained 0·02ml. PHA. A whole blood suspension was prepared containing 20% blood, 40% serum (autologous or foetal calf serum (FCS); CSL) and 40% medium 199. Cultures were set up in triplicate in sterile plastic culture tubes. Each culture contained 1ml. of the whole blood suspension described above and 3ml. of the PHA preparation. The cultures were incubated at 37°C for 92 hours at which time 2·5μCi of ^{3}HT (Amersham; specific activity 500μCi/m Mol) was added in a volume of 0·1ml. The incubation was continued for a further 4 hours at which time DNA synthesis was terminated by holding the cultures at 4°C for 30 minutes.

(3) Spontaneous Transformation

A whole blood suspension was prepared containing 20% blood and 80% medium 199. Cultures were set up as above with 1ml. of whole blood suspension and 3ml. of medium 199. The ^{3}HT was added immediately and the cultures were incubated at 37°C for 4 hours after which time the reaction was terminated by cooling to 4°C for 30 minutes.

(4) Preparation for Counting

The cultures were transferred to 10ml. plastic centrifuge tubes with 0·9% saline (2ml.) and centrifuged

at 250g for 10 minutes at 4°C. The supernatant was removed and the cell button resuspended with 0·9 saline (4ml.) and recentrifuged. After removal of the supernatant the erythrocytes were lysed by adding 3% acetic acid (4ml.) and the cultures centrifuged. Removal of the supernatant was followed by a further saline wash after which the ^{3}HT labelled DNA protein of the lymphocytes was precipitated with 4ml. 10% trichloro-acetic acid (TCA). The cultures were allowed to stand overnight at 4°C and then centrifuged at 850g for 20 minutes. The supernatant was removed and the precipitate washed once with 5% TCA (4ml.) and twice with absolute methanol (4ml.). The supernatant was removed and the precipitate dried. The residue was dissolved in 0·5ml. Soluene (Packard) and transferred with scintillation fluid (10ml.) to glass scintillation vials. These were left in the dark at 4°C overnight and counted in a Packard Tri-Carb counter. The counts were corrected for quenching and recorded as disintegrations per minute (dpm).

3. Purified Lymphocyte Culture Technique

Purified lymphocyte suspensions were prepared using a modification of the method described by Froland and Natvig (1970). 9% Ficoll (Pharmacia) and 33·9% Hypaque (Winthrop) were mixed in a ratio of 24 parts of Ficoll to 10 parts of

Hypaque. Blood was diluted in twice its own volume of Dulbecco phosphate buffer (CSL) and 27ml. of this suspension was layered onto 11ml. of the Ficoll-Hypaque mixture in 50ml. glass centrifuge tubes. The tubes were then spun at 400g for 40 minutes and the white cell layer removed and washed three times with Dulbecco phosphate buffer (1ml.) by centrifuging for 15 minutes at 250g. The white cell suspension contained approximately 98% lymphocytes and over 98% of these were viable as assessed by trypan blue exclusion. A yield of approximately 5×10^6 lymphocytes per millilitre was obtained. The suspension was diluted with FCS so that 1×10^6 lymphocytes were added to each culture. FCS and PHA were added to the culture for measurement of PHA transformation in the same proportions as described for the microtechnique. The cultures were set up, incubated and processed in the same way as previously described except that the addition of acetic acid was not required as no red cells were present.

4. Measurement of Lymphocyte Subpopulations

It has been shown that lymphocytes can be divided into two main subpopulations, bursal or bone marrow derived (B) cells or thymus derived (T) cells. B cells have been identified by immunofluorescent and autoradiographic techniques.

(1) Immunofluorescent Technique

Lymphocyte suspensions were prepared as described under section 3. 0·03ml. fluorescein conjungated anti-human globulin and 0·1ml. FCS were added to 0·2ml. of the washed suspension. The anti-human globulin preparations used were Horse Antihuman Globulin (Roboz Surgical Instruments Co. Ltd., Washington) and Goat Antihuman IgG, IgM and IgA (Hyland Div., Travenol Labs. Inc., California). A mixture of monospecific antisera to IgG, IgM and IgA was also prepared and used in the same way. After incubation with the fluorescein conjungated antiserum at 4°C for 1 hour, the lymphocyte suspension was washed three times in the cold with phosphate buffer, centrifuging each time for 3 minutes at 250g. The suspension was then allowed to stand for 10 minutes at 37°C. Cell counts were made with a Zeiss Universal Microscope, equipped for fluorescent and phase contrast microscopy. The number of fluorescent cells in a high power field was counted and the total number of lymphocytes in the same field was counted by phase microscopy.

(2) Autoradiographic Technique

A modification of the method described by Wilson and Nossal (1971) was employed. Lymphocyte suspensions were

prepared as described previously and approximately 1 x 10^6 cells in 0·2ml. Dulbecco phosphate buffer (DPB) were incubated for 1 hour at 4°C with 0·1ml. FCS and 50µl of ^{125}I labelled antihuman immunoglobulin. The cells were centrifuged at 250g for 5 minutes and resuspended in DPB. They were then washed twice through a FCS gradient (1ml. 100% FCS, 1ml. 75% FCS in DPB, 1ml. 50% FCS in DPB) and finally resuspended in 0·1ml. FCS and smeared onto gelatin coated glass slides. The smears were fixed in a mixture of 89% methanol, 10% distilled water and 1% acetic acid for 30 minutes and then washed in distilled water for 30 minutes. After drying, the slides were dipped in Ilford Nuclear Research Emulsion Gel Form (L_4) which had been diluted 1:1 with distilled water. The slides were heated at 42°C for 1 hour and then immersed in scintilation fluid (4gm PPO and 0·3G POPOP, Koch Light Labs., England). After 24 hours immersion they were developed for 7 minutes (Ilford 1D2 Developer), rinsed for 30 seconds in distilled water and fixed for 3 minutes (Kodak 5152 fixer), all procedures being carried out at 17°C. Finally the slides were washed in running distilled water for 20 minutes and stained with Giemsa stain. Grain counts were made on 100 cells, and cells with 5 grains above background were considered labelled (Fig. II.1).

5. DNA Synthesising Cells - Autoradiographic Technique

Lymphocyte suspensions were prepared and spontaneous lymphocyte transformation cultures set up in the normal way as described under section 3. After incubation for 3 hours the cells were washed twice with DPB and smeared onto gelatin coated glass slides. The procedure was then identical to that described under section 4 (2). Cells with 5 grains above background were considered to be labelled (Fig. II.2).

6. Serum Autoantibodies

A routine screening of all sera for smooth muscle (SMA), mitochondrial (M), antinuclear (ANF), gastric parietal cell (GPC), and thyroid cytoplasmic antibodies was undertaken. The double layer technique described by Doniach, Roitt, Walker and Sherlock (1966) was used, the substrates being fixed and unfixed rat liver for ANF and M antibodies, rat stomach for SMA and GPC antibodies and human toxic thyroid for thyroid cytoplasmic antibody.

7. Australia Antigen

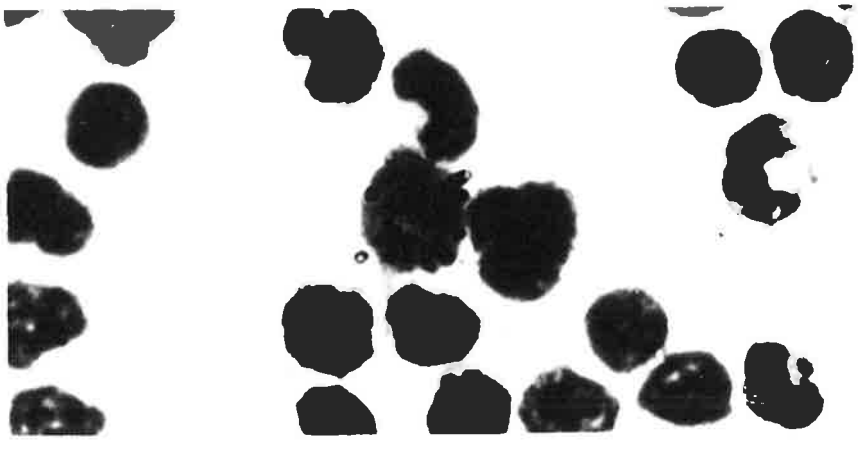
Australia antigen testing was performed using a modification of the crossover electrophoresis method described by Prince and Burke (1970) and modified by Nelson (1973) and by radioimmunoassay (Ausria kit, Abbot).

Fig. II. 1.

B Cell Autoradiograph

Fig. II. 2.

DNA Synthesising Cell Autoradiograph



8. Immunoglobulins and Complement

Immunoglobulin G, M and A were estimated on Tripartigen Immunodiffusion Plates (Behringwerke AG) and complement on M-Partigen C3c (B_1A Globulin) Immunodiffusion Plates.

9. Biochemical Liver Function Tests

All biochemical tests were performed on a Technicon SMA 12/60 Autoanalyser.

10. Statistical Methods

Some of the data obtained in the studies undertaken presented problems of statistical analysis particularly with regard to the statistical test chosen. Nonparametric methods were thought to be more appropriate in some situations. A detailed description of the methods used is given in the Appendix. Standard statistical abbreviations used in the text, in tables and on graphs are also detailed in the Appendix.

CHAPTER III

VALIDATION OF LYMPHOCYTE CULTURE METHODS

CHAPTER III

1. Introduction

Experiments were undertaken to assess the validity and reproducibility of the whole blood microtechnique by comparing it with culture of purified lymphocyte preparations. An assessment was made of the different ways of expressing the results. Studies were also made in normal subjects of some of the factors which may influence the ^{3}HT uptake by lymphocytes and which thus bear on the interpretation of the results.

2. A Comparison of The Whole Blood Microtechnique with Culture of Purified Lymphocyte Preparations

Most studies of lymphocyte function in normal subjects and in patients with hepatitis have been performed on purified lymphocyte preparations. Junge et al. (1970) described the microtechnique which was the basis of the method used in this study. He reported that the reproducibility of the technique was similar to that achieved with purified lymphocyte preparations. This work has been extended in the present study and has confirmed the findings of Junge et al. (1970) both for normal subjects and for patients with hepatitis.

(1) Patients and Methods

PHA induced ^{3}HT uptake was studied in twelve healthy

control subjects who were hospital or laboratory personnel aged between 18 and 50 years. Cultures were set up using either whole blood or purified lymphocytes as described in Chapter II. The cultures contained either 10% FCS or 10% autologous serum.

Spontaneous ^{3}HT uptake was measured in six patients with hepatitis who were part of the main study group described in Chapter IV. Serial studies were made during the course of the disease. Cultures were set up using either whole blood or purified lymphocytes. All cultures contained 10% FCS.

Results were expressed as disintegrations per minute (dpm) per million (10^{6}) lymphocytes for cultures containing purified lymphocytes and as dpm/culture and dpm/10^{6} lymphocytes in the whole blood cultures. In the latter instance the number of lymphocytes was calculated from the total white cell count and the percentage of lymphocytes in the blood smear.

(2) Results

PHA induced ^{3}HT uptake in control subjects

Table III.1. shows the mean values for PHA induced uptake in whole blood cultures and purified lymphocyte cultures. There was a significant correlation between the

TABLE III.1

Comparison of PHA uptake in Whole Blood Cultures (W.B.) with Purified Lymphocyte Cultures (Purif.L)

	PHA + FCS			PHA + Aut. Serum		
	W.B. dpm/cult.	W.B. dpm/10^6	Purif.L. dpm/10^6	W.B. dpm/cult.	W.B. dpm/10^6	Purif.L. dpm/10^6
$\bar{\chi}$	66,000	214,100	17,600	57,600	197,500	94,900
SD	19,200	48,600	9,800	24,400	67,400	47,600
η	10	10	10	10	10	10
V%	29.1	22.7	55.7	42.4	34.4	50.2

$\bar{\chi}$ = mean SD = standard deviation

η = number of subjects V% = coefficient of variation

two when culture was carried out in autologous serum and the whole blood culture results expressed as dpm/culture ($r = 0.63$; $P<0.05$) but not when results were expressed as dpm/10^6 lymphocytes ($r = 0.43$ on 9 df). There was no significant correlation between the two types of cultures when carried out in FCS ($r = 0.15$ on 9 df; $r = 0.35$ on 9 df).

The coefficient of variation was lower for whole blood cultures than for purified lymphocyte cultures (Table III.1). It was also lower for whole blood cultures containing FCS than for cultures containing autologous serum.

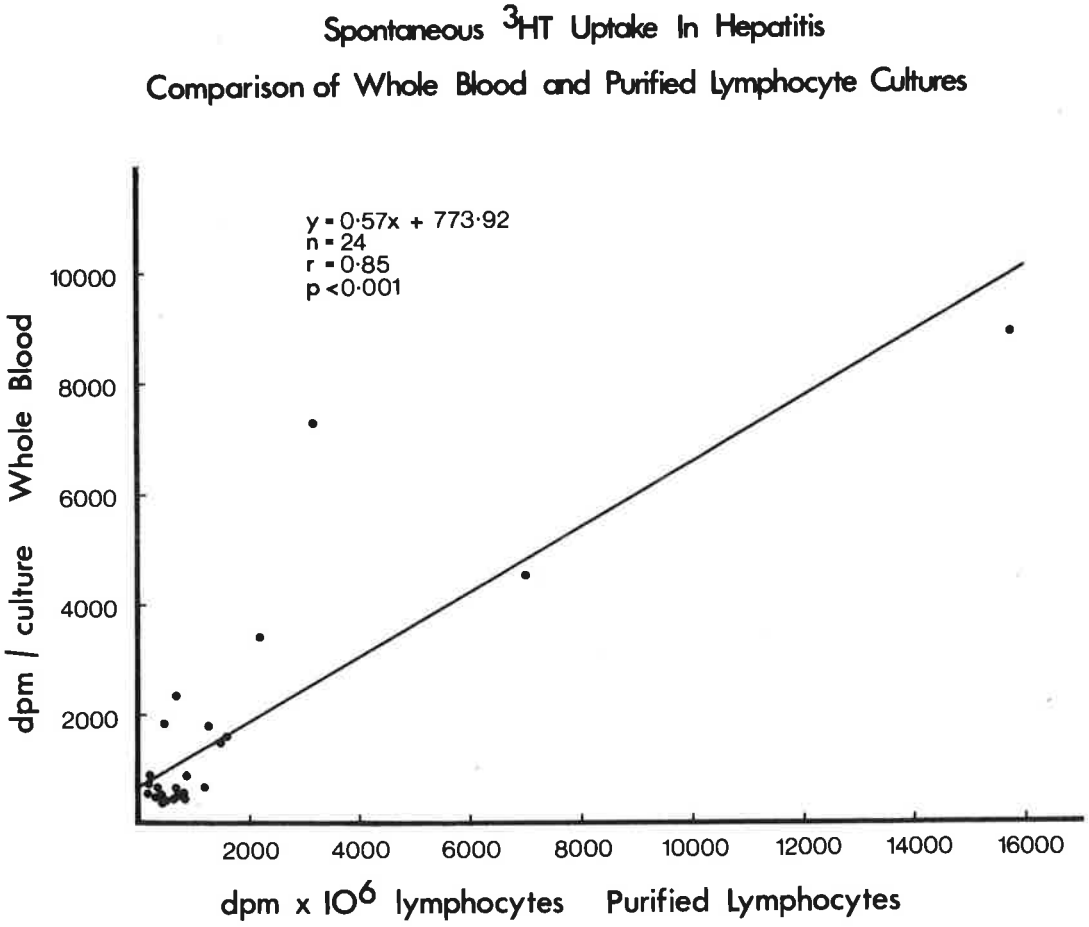
Spontaneous ^{3}HT uptake in hepatitis patients

Spontaneous ^{3}HT uptake by lymphocytes was studied in patients with hepatitis. Fig.III.1. shows that there was good agreement between whole blood cultures and purified lymphocyte cultures ($r = 0{\cdot}85$; $P<0{\cdot}001$).

(3) Discussion

The whole blood microtechnique has practical advantages in clinical studies in which the quantity of blood available for testing is often limited by the fact that blood is also required for other investigations. It is also comparatively simple in that purified lymphocyte fractions need not be prepared. On the other hand the lymphocyte count and therefore the number of lymphocytes in the culture may vary considerably in conditions such as viral hepatitis. When purified lymphocyte preparations are used the total number of lymphocytes added to each culture can be determined accurately. However, the addition of a standard number of lymphocytes to a culture need not imply that each lymphocyte or even a fixed proportion of the total number of lymphocytes has an equal chance of transforming in response to PHA or an immune stimulus. Indeed, this assumption would be unwarranted as it is now known that

Figure III 1.



Correlation of the spontaneous ^{3}HT uptake in serial whole blood and purified lymphocyte cultures in 6 patients (24 observations) with acute viral hepatitis. Each point represents the mean of triplicate cultures.

the lymphocyte population is heterogeneous and consists of two basic subpopulations of T and B cells and probably further subpopulations within these main groups. In addition the lymphocyte population which is present when the estimation of DNA synthesis is made in PHA cultures after an incubation period of 4 days probably bears little relationship to the initial lymphocyte population. It may in part account for the poor correlation between results obtained for whole blood cultures and cultures containing purified lymphocytes stimulated by PHA.

The lack of correlation between the whole blood and purified lymphocyte cultures in FCS may be due to the absence of any autologous serum in the purified lymphocyte cultures, and this lack of autologous serum also accounts for the low ^{3}HT uptake values in these latter cultures (17,600 $\pm$ 9,800 dpm/10^{6} lymphocytes) when compared to the purified lymphocyte cultures containing autologous serum (94,900 $\pm$ 47,600 dpm/10^{6} lymphocytes). As a correlation was present between whole blood cultures and purified lymphocyte cultures containing autologous serum it appears likely that both methods measure the same phenomena.

A comparison was made of whole blood cultures and cultures containing purified lymphocytes used to measure spontaneous ^{3}HT uptake. A group of patients with hepatitis was studied to provide a comparison of the

method in a situation where high and low uptake values are present. A high degree of correlation was seen in these studies giving further evidence of the validity of the whole blood technique.

In both PHA cultures and spontaneous uptake cultures there was a lower variability of ^{3}HT uptake in whole blood cultures indicating that this culture method has advantages over the purified lymphocyte technique. It has been established that immune reactions require the co-operation of T and B cells, macrophages and perhaps other cells. Lymphocyte purification techniques remove red cells, macrophages, polymorphonuclear leucocytes and with some techniques a proportion or even a whole subpopulation of lymphocytes. By contrast the normal ratio of cells in the peripheral blood is retained with the whole blood technique. This may be of some advantage in the spontaneous uptake cultures which are incubated for only 4 hours but is likely to be less so in PHA cultures which are incubated for 4 days. During this period of time many of the cells initially present in the culture have either degenerated or have undergone division.

The results reported in this section demonstrate some of the problems of lymphocyte culture techniques but indicate that the whole blood technique has definite advantages in terms of simplicity and reproducibility and

possibly also from a theorectical point of view. The results suggest that the whole blood technique is equally valid as the purified lymphocyte technique.

3. Standardisation of Culture Conditions

(1) Methods

Blood was obtained from patients with hepatitis and healthy control subjects. Triplicate whole blood cultures were set up and in each case one of the constituents of the culture was varied to determine the effect on PHA induced ^{3}HT uptake. The dose of PHA was varied between 0·1 and 100µl/ml. and the dose of ^{3}HT added prior to the termination of the culture was varied between 0·1 and 25µCi/culture. The effect of pH was assessed for cultures containing FCS and autologous serum within the range 7·0 - 7·9. The optimal time for terminating cultures was determined by removal from the incubator at twenty four hour intervals up to 5 days. The concentration of added serum was 10% for both FCS and autologous serum throughout the study.

(2) Results

P.H.A.

Peak ^{3}HT uptake values were obtained between doses of

1 - 10µl/ml. One such dose response curve is seen in Fig. III.2. Three batches of PHA were used during the study and a dose response curve was performed for each batch. There were no significant differences between the batches. On the basis of these results 5µl/ml. was chosen as the standard dose for all PHA cultures.

Tritiated Thymidine

A rapid and linear rise in culture counts was seen in the dose range 0·1 - 2·5µCi/cultures (Fig.III.3.) Further increases in the amount of ^{3}HT added did not lead to such a marked rise in culture counts. A dose of 2·5µCi/cultures was therefore used in cultures during the study.

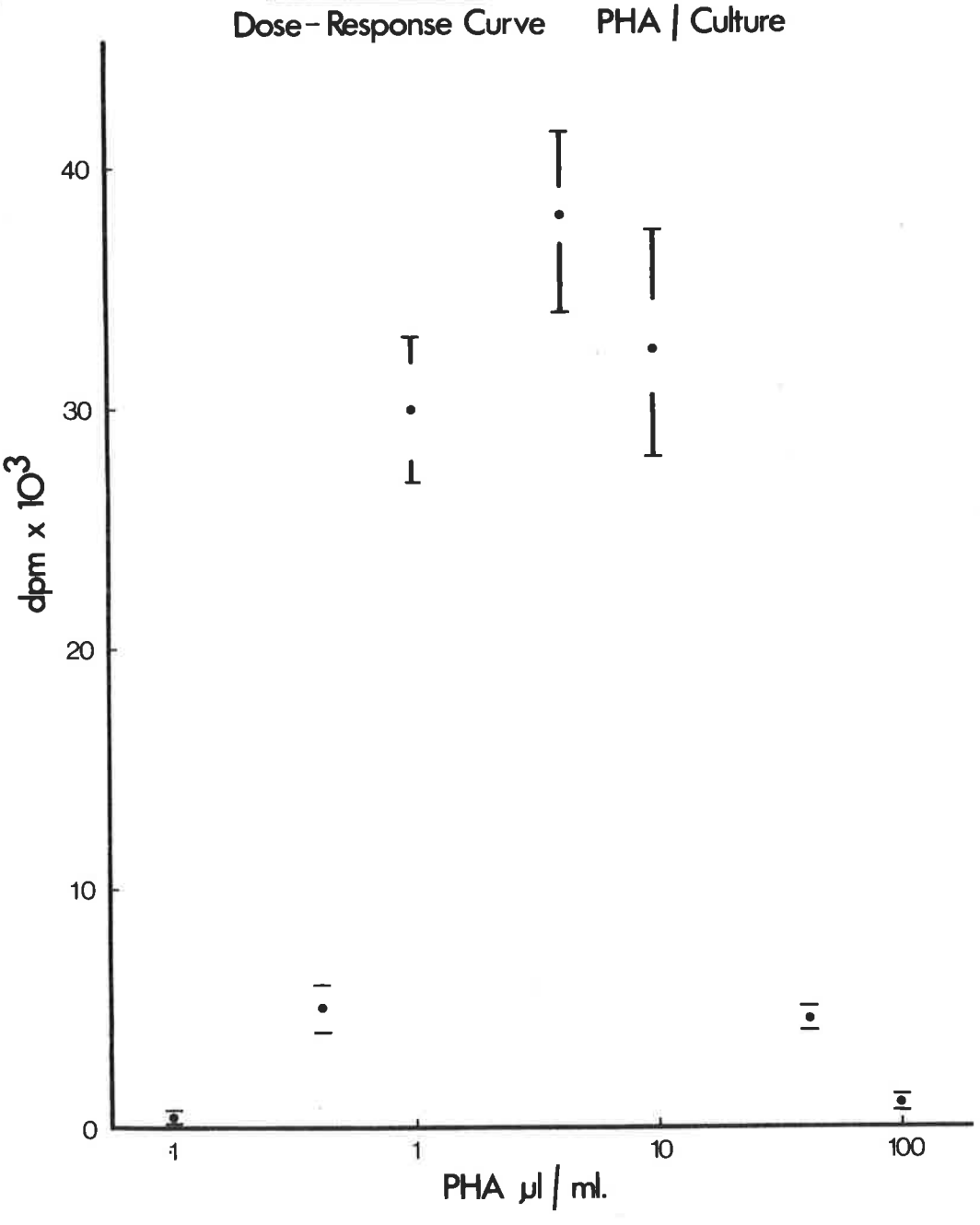
pH

In cultures containing FCS there was an increase in ^{3}HT uptake as pH values rose with peak values at pH 7·6 - 7·7 (Fig.III.4). The uptake was more variable in cultures containing autologous serum. On the basis of these results cultures were buffered at a pH of 7·7.

Length of incubation of cultures

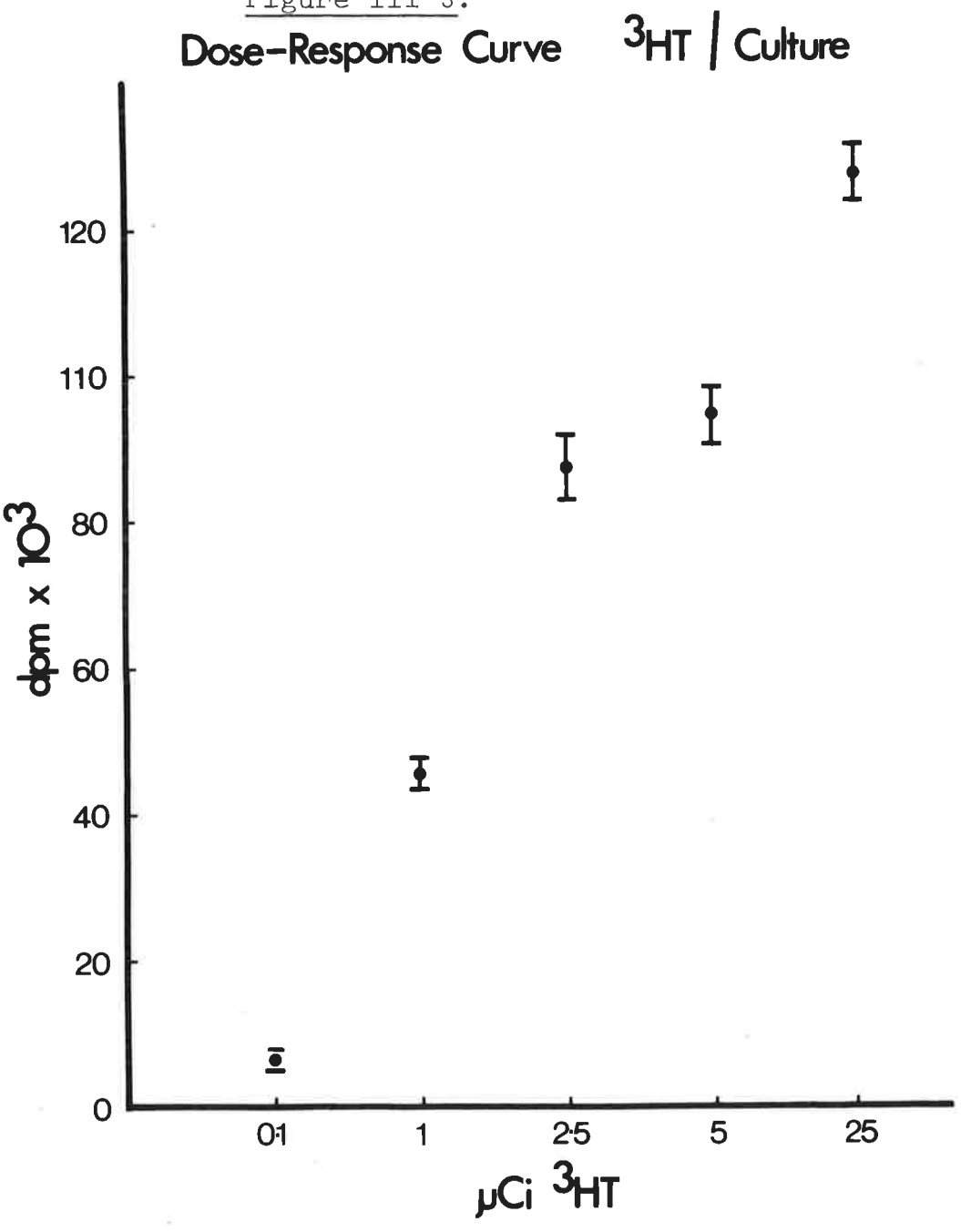
The effect of length of incubation on PHA induced ^{3}HT uptake is shown in (Fig.III.5). For cultures containing 10% FCS, peak activity was observed between 72 and 96 hours,

Figure III 2.



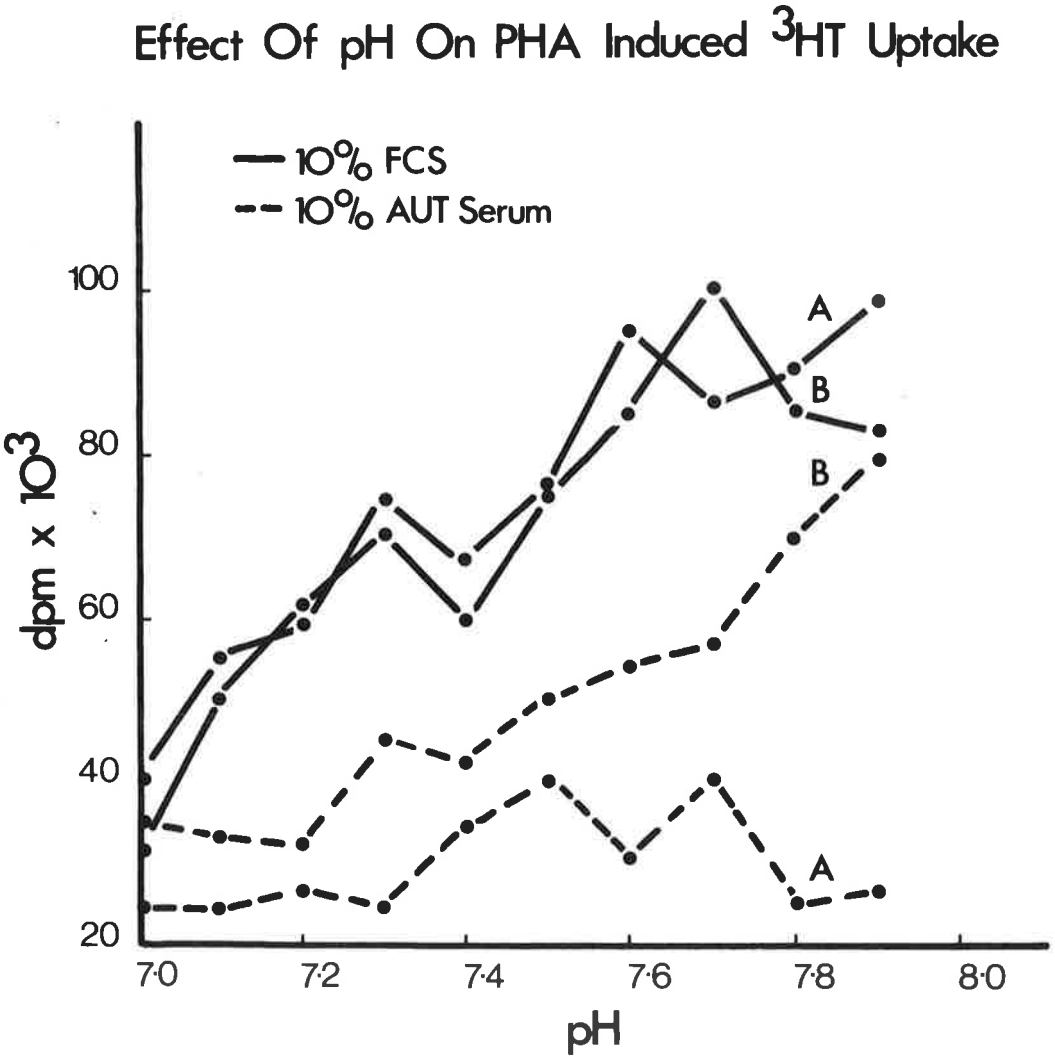
^{3}HT uptake (dpm x 10^3) in response to different concentrations of PHA in whole blood cultures from a normal subject. Cultures were terminated at 96 hours. Each point represents the mean of triplicate cultures and the vertical bars the SD.

Figure III 3.



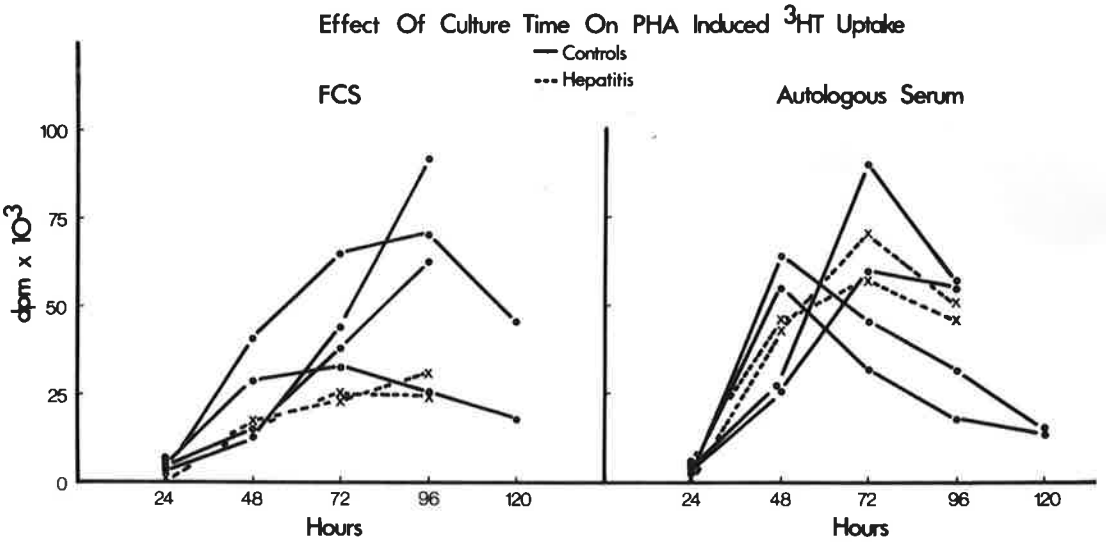
^{3}HT uptake (dpm x 10^{3}) in cultures containing PHA when measured after addition of different doses of ^{3}HT. Each point represents the mean of triplicate cultures from a normal subject. The vertical bars indicate the SD.

Figure III 4.



Effect of varying the culture pH on the PHA induced ^{3}HT uptake (dpm x 10^3) in cultures containing 10% FCS and 10% Autologous (Aut) serum. The results are shown for two normal subjects A and B. Each point represents the mean of triplicate cultures.

Figure III 5.



Effect of varying the length of culture time on PHA induced ^{3}HT uptake (dpm x 10^3) in 4 control subjects and 2 patients with acute viral hepatitis. The effect is shown in cultures containing 10% FCS and 10% Autologous serum. Each point represents the mean of triplicate cultures.

while for cultures containing 10% autologous serum the peak activity occurred between 48 and 72 hours. Because most PHA cultures were to be set up with 10% FCS, it was decided to terminate all PHA cultures at 96 hours. The results obtained in four normal control subjects and in two patients with hepatitis did not differ significantly.

(3) Discussion

These results are similar to those of Junge et al. (1970) who, however, described a later peak response to PHA. This may be explained by the lower dose and different brand (Difco) of PHA and to the higher concentration of FCS (20%) which they used. 10% serum was used in the present study because of the often limited amount of autologous serum which is available in a clinical study. There was considerable variation in the timing of the peak ^{3}HT uptake in PHA cultures in individual subjects and this applied particularly to cultures containing autologous serum. It has been suggested that the peak uptake should be determined for each subject by terminating cultures on succesive days (WHO Report 1970) but this is not practical in a clinical study in which multiple investigations are being performed. It was consequently necessary to choose a time when most subjects were likely to be close to maximum uptake and for this reason the time of 4 days was

chosen. The wide variation in PHA response in cultures containing autologous serum is discussed further in Chapter V. Spontaneous ^{3}HT uptake was determined under identical culture conditions to PHA uptake though of course no PHA was added.

4. Expression of Results

The results of whole blood cultures have been expressed throughout this study as dpm/culture rather than dpm/10^{6} lymphocytes. This has been justified on theoretical and statistical grounds.

(1) Methods

Whole blood cultures for PHA induced ^{3}HT uptake in 10% FCS were set up with blood from 53 healthy control subjects. In 46 of these subjects cultures were also set up with 10% autologous serum and in 33 of the controls, spontaneous ^{3}HT uptake was determined. Uptake values was expressed as dpm/culture and then adjusted to dpm/10^{6} lymphocytes on the basis of the white cell count and differential. The two methods of expressing the results were compared by determining the coefficient of variation (V) and the correlation coefficient (r).

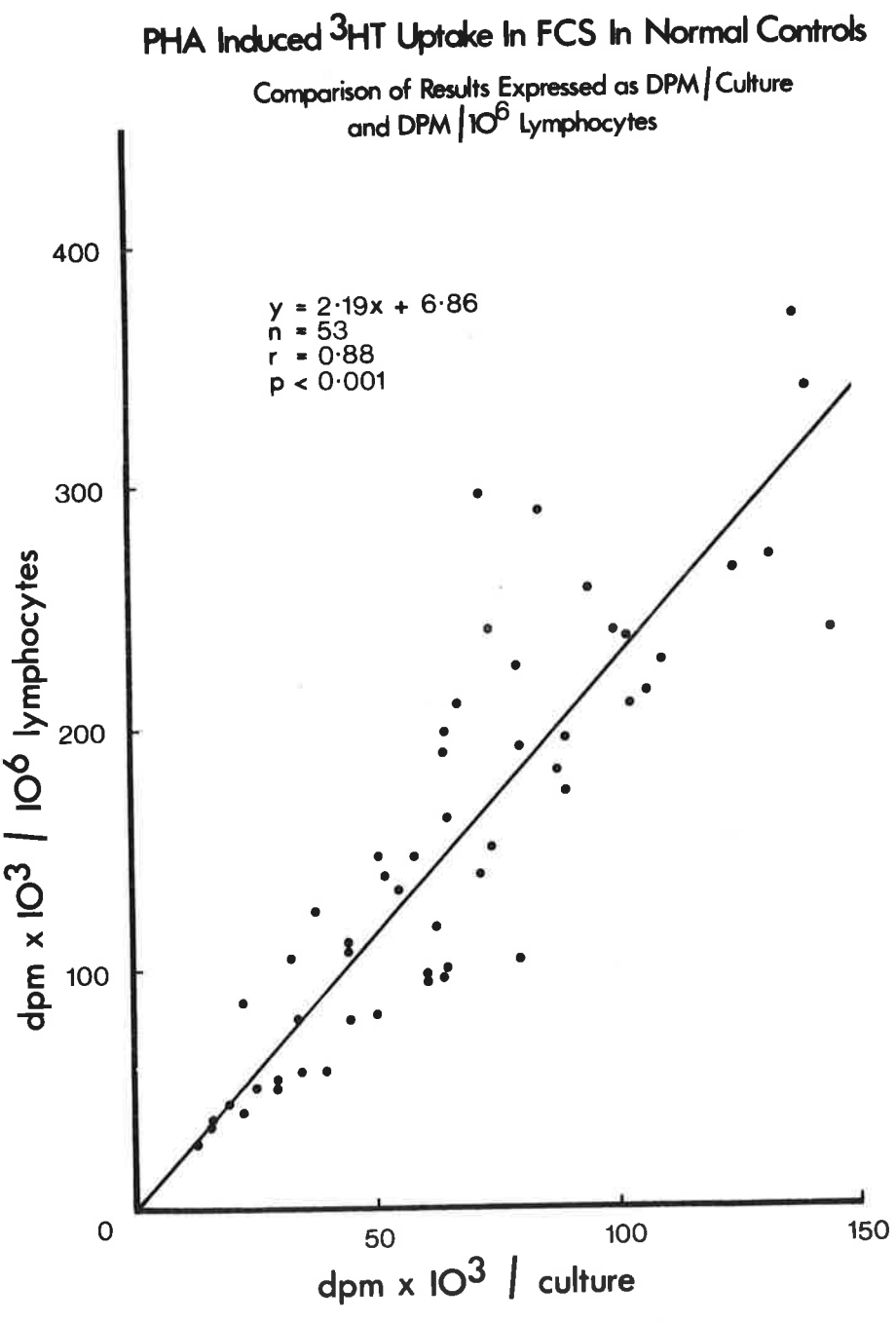
(2) Results

Table III.2 summarises the results. In the PHA cultures there is a highly significant degree of correlation ($P<0.001$) between values expressed as dpm/culture and dpm/10^6 lymphocytes (Figs. III.6 and III.7). The coefficient of variation was higher in cultures calculated as dpm/10^6 lymphocytes (Figs. 6a and 7a).

In the spontaneous ^{3}HT uptake cultures the correlation between values expressed as dpm/culture and dpm/10^6 lymphocytes was also highly significant (Fig. III.8). The coefficient of variation was lower than in PHA cultures and also demonstrated a higher coefficient of variation for values expressed as dpm/10^6 lymphocytes than for values expressed as dpm/culture (Fig. 8a).

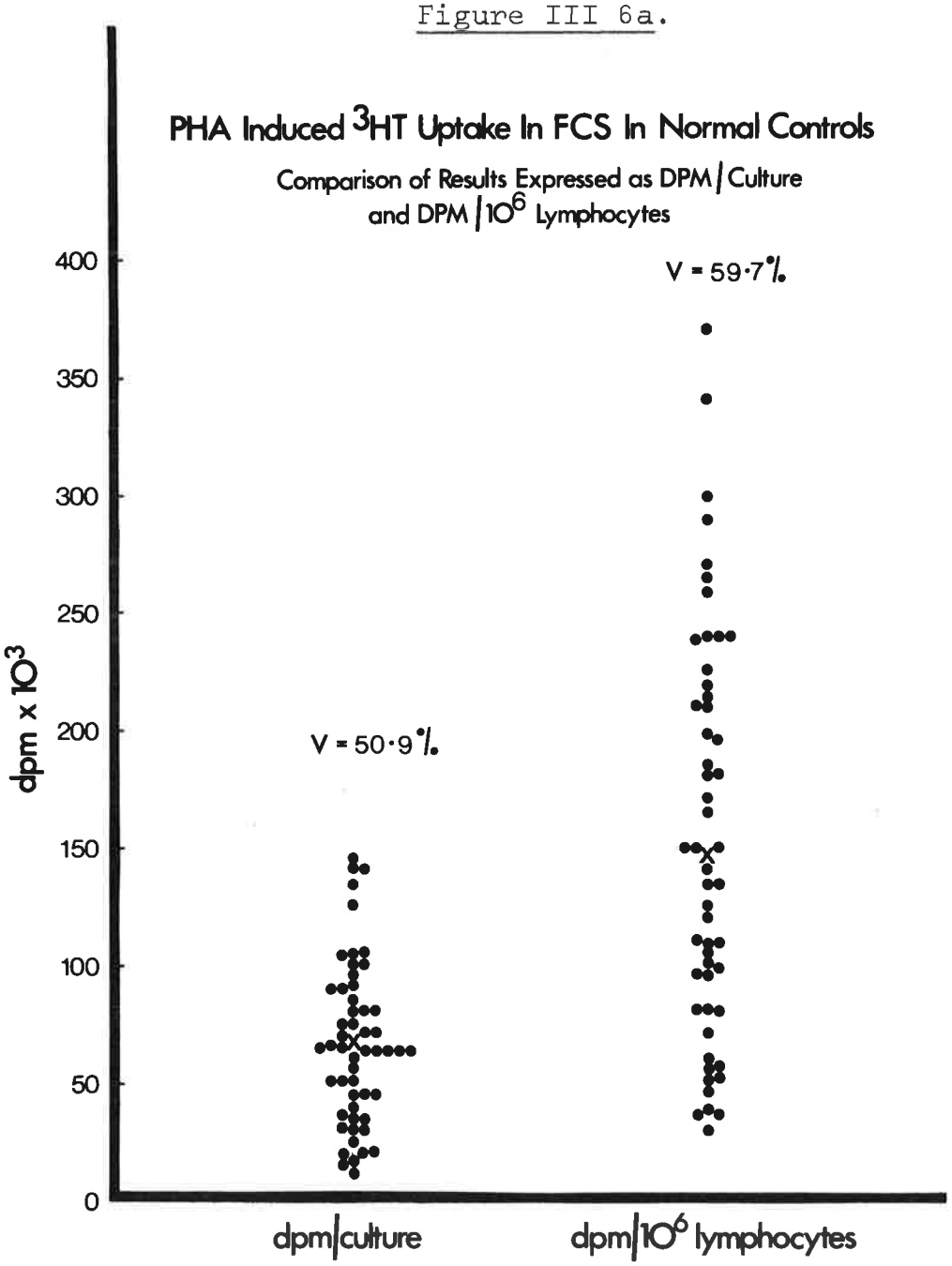
The results have also been expressed in histogram form to demonstrate the frequency distribution of results expressed as dpm/culture and dpm/10^6 lymphocytes (Figs. III.9, III.10 and III.11). Each histogram appears to show slight positive skewness. Fisher's statistics for skewness and kurtosis were calculated and the calculated and observed frequencies were compared with those expected in a normal distribution by means of Student's test. In no case did the distribution differ significantly from normal whether the results were expressed as dpm/culture or as dpm/10^6 lymphocytes.

Figure III 6.



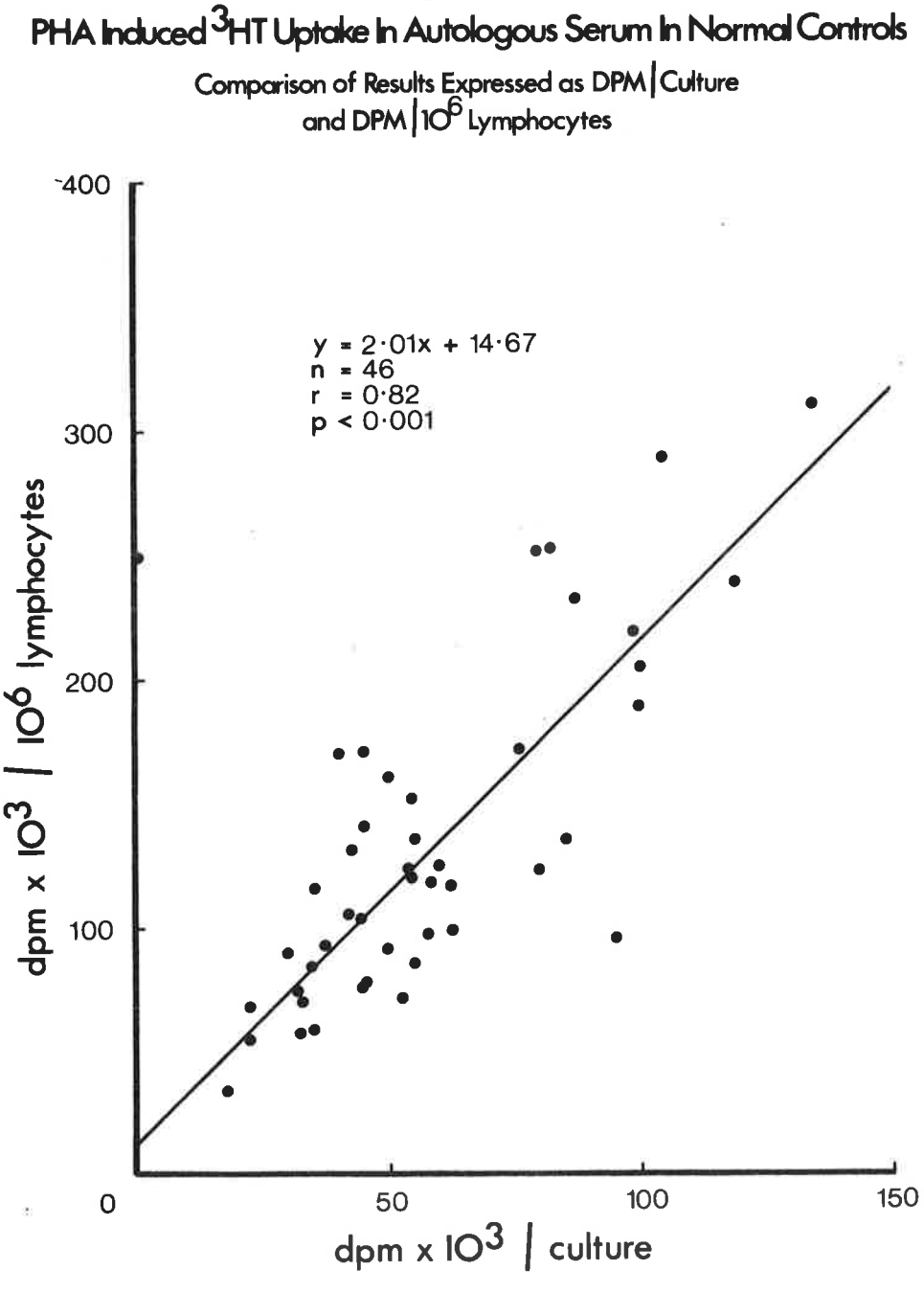
Correlation of PHA induced ^{3}HT uptake in cultures containing 10% FCS when results were expressed at dpm x 10^3 / culture and dpm x $10^3/10^6$ lymphocytes in 53 normal control subjects. Each point represents the mean of triplicate cultures.

Figure III 6a.



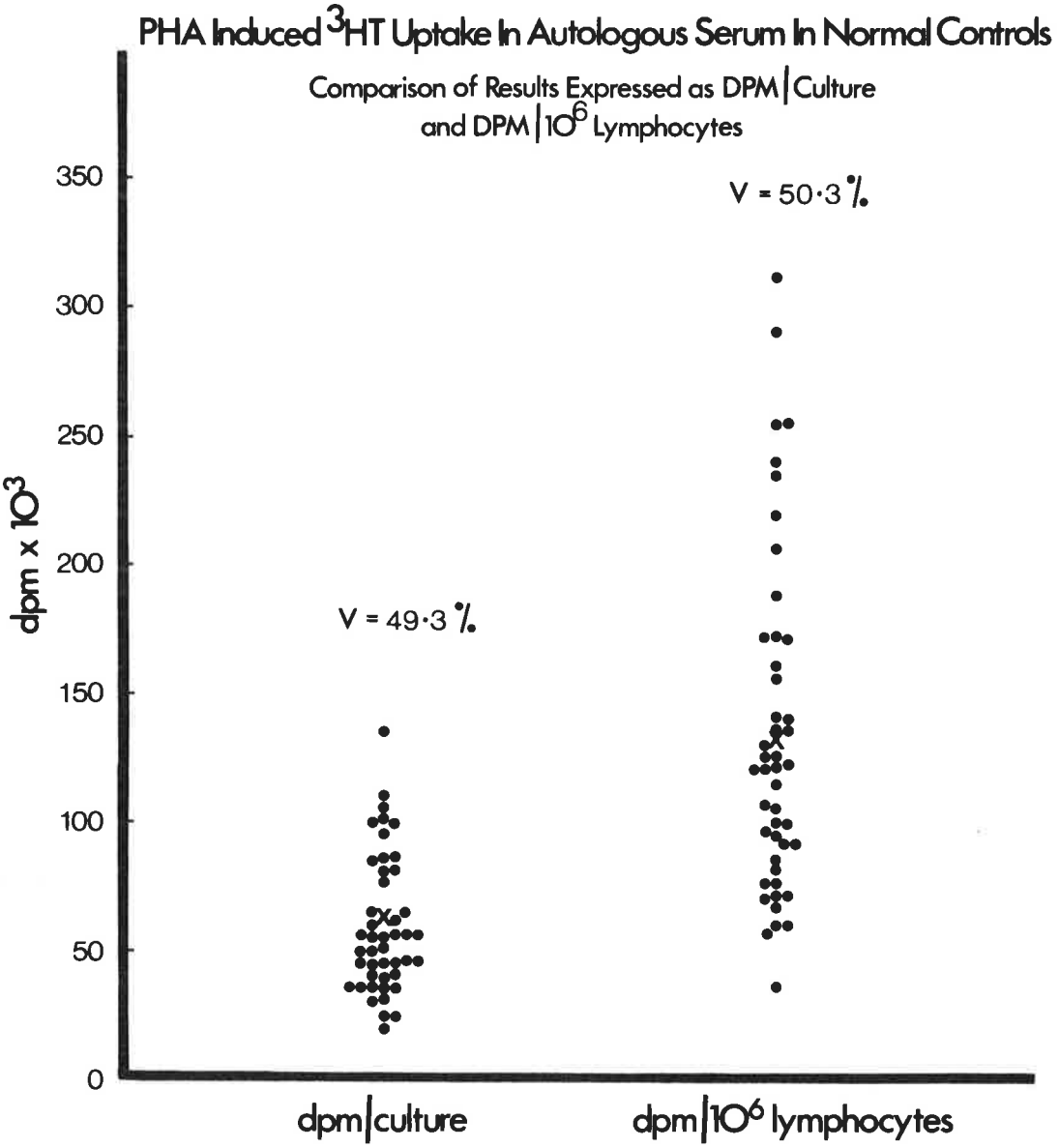
Comparison of the variability of results for PHA induced ^{3}HT uptake (dpm x 10^3) in cultures containing 10% FCS when results were expressed as dpm/culture & dpm/10^6 lymphocytes in 53 normal control subjects. Each point represents the mean of triplicate cultures.

Figure III 7.



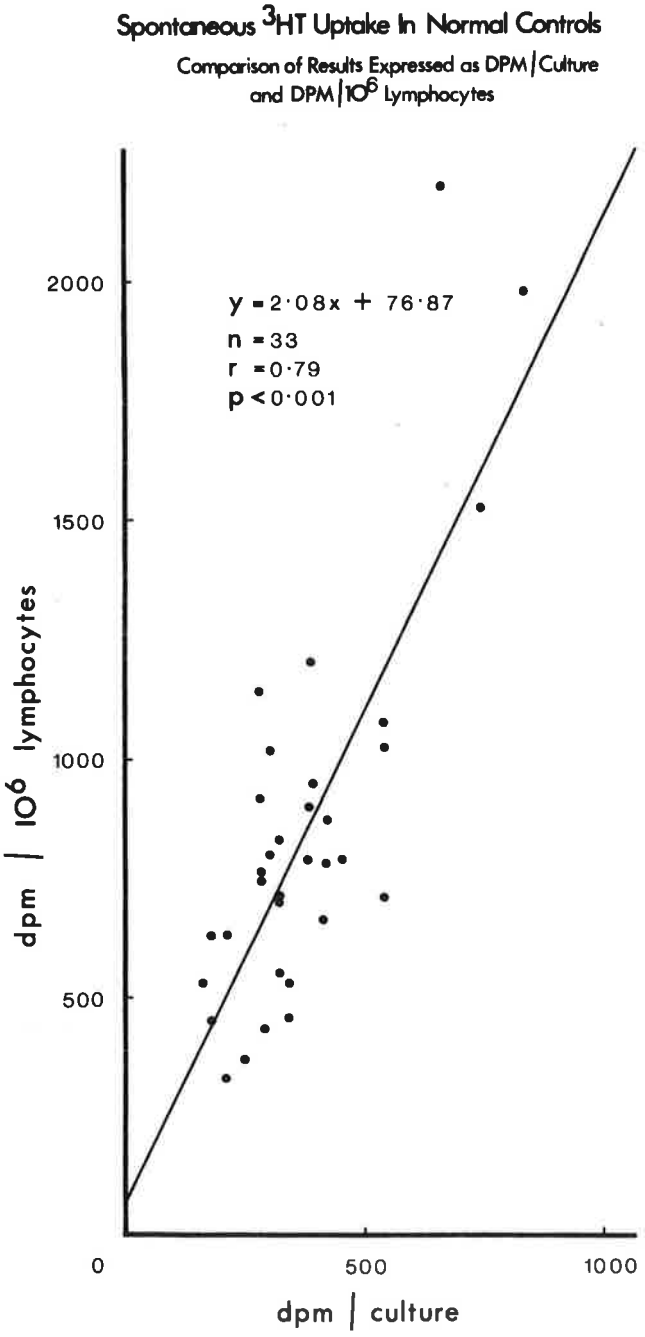
Correlation of PHA induced ^{3}HT uptake in cultures containing 10% autologous serum when results were expressed as dpm x 10^3/ culture & dpm x $10^3/10^6$ lymphocytes in 46 normal control subjects. Each point represents the mean of triplicate cultures.

Figure III 7a.



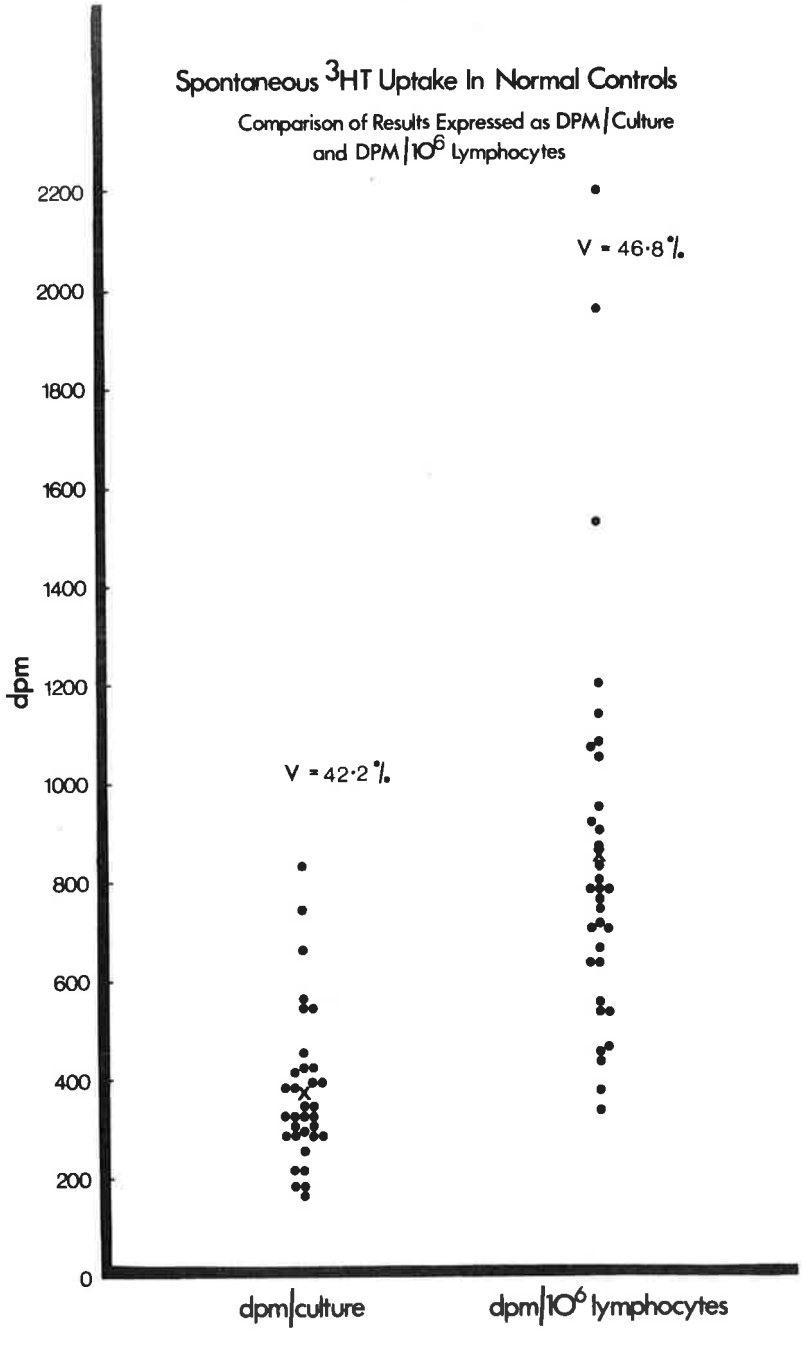
Comparison of variability of results for PHA induced ^{3}HT uptake (dpm x 10^{3}) in cultures containing 10% Autologous serum when results were expressed as dpm/culture & dpm/10^{6} lymphocytes in 53 normal control subjects. Each point represents the mean of triplicate cultures.

Figure III 8.



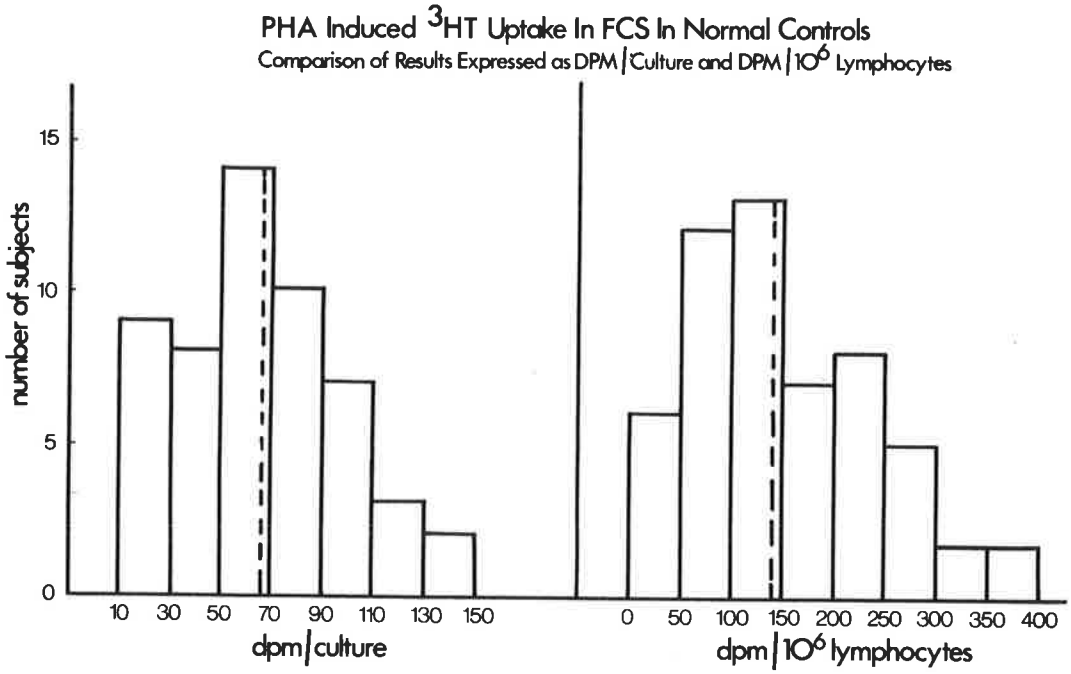
Correlation of spontaneous ^{3}HT uptake when results were expressed as dpm/culture & dpm/10^6 lymphocytes in 33 normal control subjects. Each point represents the mean of triplicate cultures.

Figure III 8a.



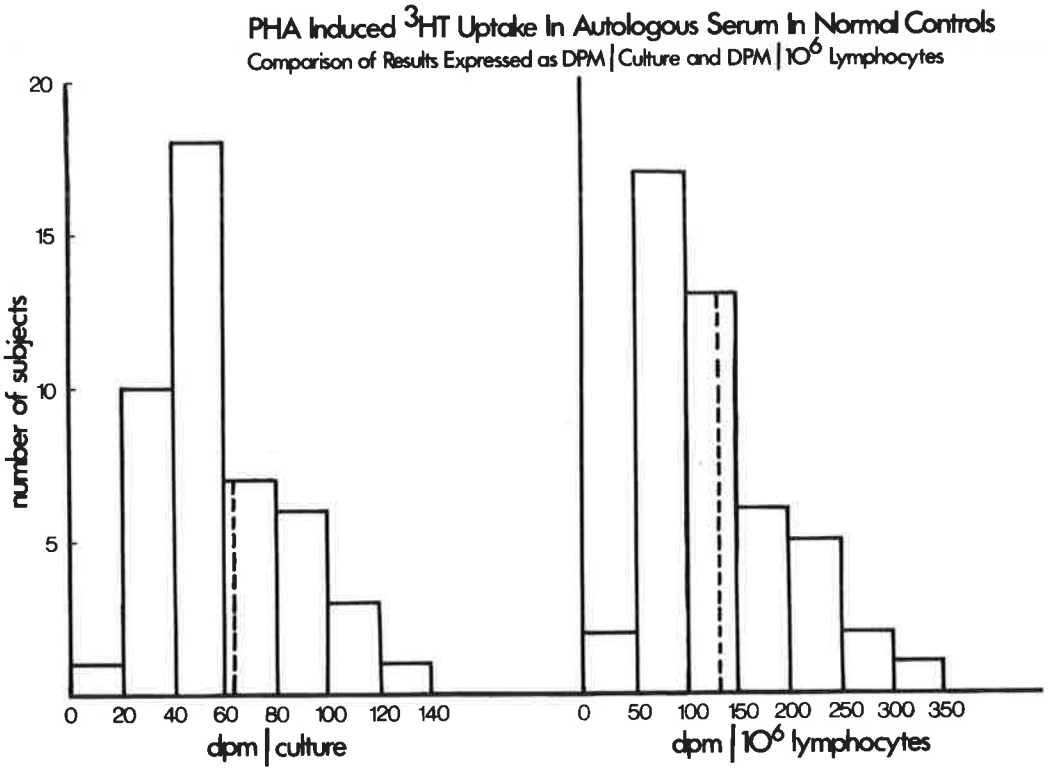
Comparison of the variability of results for spontaneous ^{3}HT uptake (dpm) when results were expressed as dpm/culture & dpm/10^6 lymphocytes in 33 normal control subjects. Each point represents the mean of triplicate cultures.

Figure III 9.



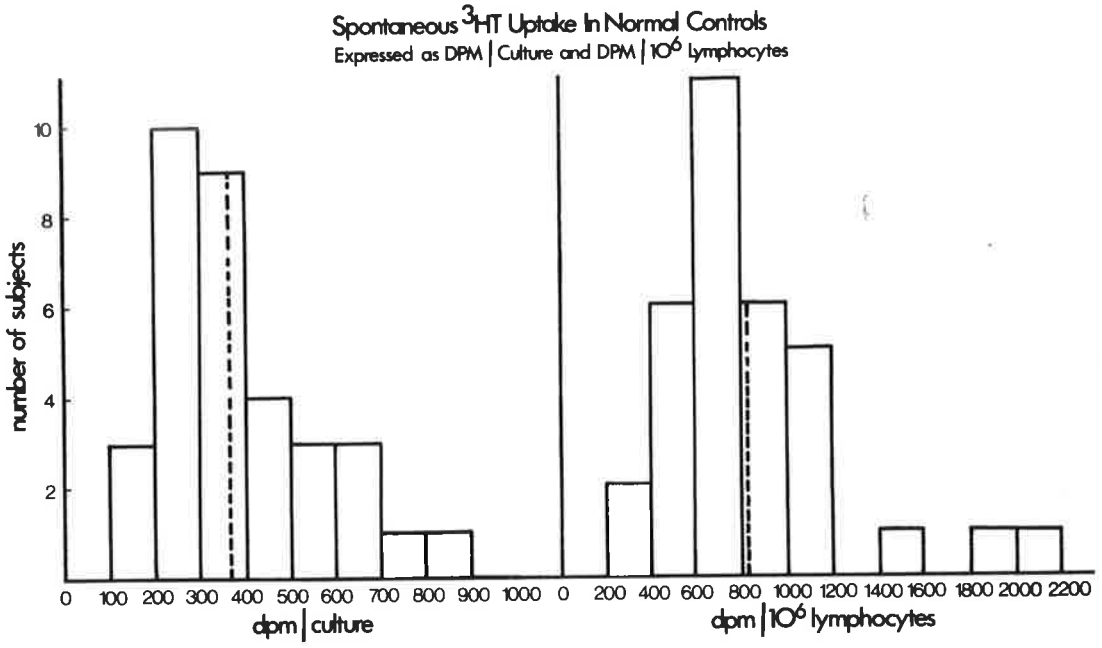
Frequency distribution for PHA induced ^{3}HT uptake in 10% FCS in 53 normal control subjects when results were expressed as dpm/culture and dpm/10^6 lymphocytes.

Figure III 10.



Frequency distribution for PHA induced ^{3}HT uptake in 10% autologous serum in 46 normal control subjects when results were expressed as dpm/culture and dpm/10^{6} lymphocytes.

Figure III 11.



Frequency distribution for spontaneous ^{3}HT uptake in 33 normal control subjects when results were expressed as dpm/culture and dpm/10^{6} lymphocytes.

TABLE III.2

Comparison of ^{3}HT uptake of Whole Blood Cultures expressed as dpm/culture and dpm/10^6 lymphocytes.

	PHA + FCS		PHA + Aut. Serum		Spontaneous	
	dpm/ culture	dpm/10^6 lympho's	dpm/ culture	dpm/10^6 lympho's	dpm/ culture	dpm/10^6 lympho's
$\bar{\chi}$	66,800	146,500	61,900	131,300	367	846
SD	34,000	87,500	30,500	61,900	155	396
n	53	53	46	46	33	33
V%	50.9	59.7	49.3	50.3	42.2	46.8

(3) Discussion

When applied to a group of healthy control subjects the whole blood technique gave results which were distributed normally. The theoretical reasons why it might be more appropriate to express results as dpm/culture rather than as dpm/10^6 lymphocytes have been discussed in an earlier section of this Chapter. There was a high degree of correlation between the two methods of expressing the results. However, for all types of cultures the scatter of results, as indicated by the coefficient of variation, was less when the results were calculated as dpm/culture. For these reasons the expression of results as dpm/culture was considered justified.

5. The Effect of Physiological and Other Factors in Vivo on Lymphocyte Cultures

The reactions of peripheral lymphocytes measured in vitro may be influenced by many in vivo factors such as intercurrent illness and drugs. However, even when such factors have been excluded, ^{3}HT uptake values, particularly in PHA cultures, show considerable variability in different individuals and even in the same individual at different times. This section describes preliminary work on the influence which food and the time of day at which blood samples are removed for culture may have on spontaneous and PHA induced lymphocyte transformation.

(1) Methods

A normal subject fasted overnight. Blood was drawn at 8.00 a.m. for cultures measuring PHA induced and spontaneous ^{3}HT uptake. On the first day a high carbohydrate (CHO) meal (CHO 155gms; Fat 1.2gms; Protein 12gms; Total calories 670) was eaten immediately after the fasting blood samples had been taken and further samples were drawn at hourly intervals until 4.00 p.m. A lunch with a high fat content (Fat 57.7gms; CHO 6.9gms; Protein 22.3gms; Total calories 640) was eaten immediately following the blood sample drawn at noon. On a subsequent day the subject fasted overnight then continued the fast throughout the day until the conclusion of the

experiment.

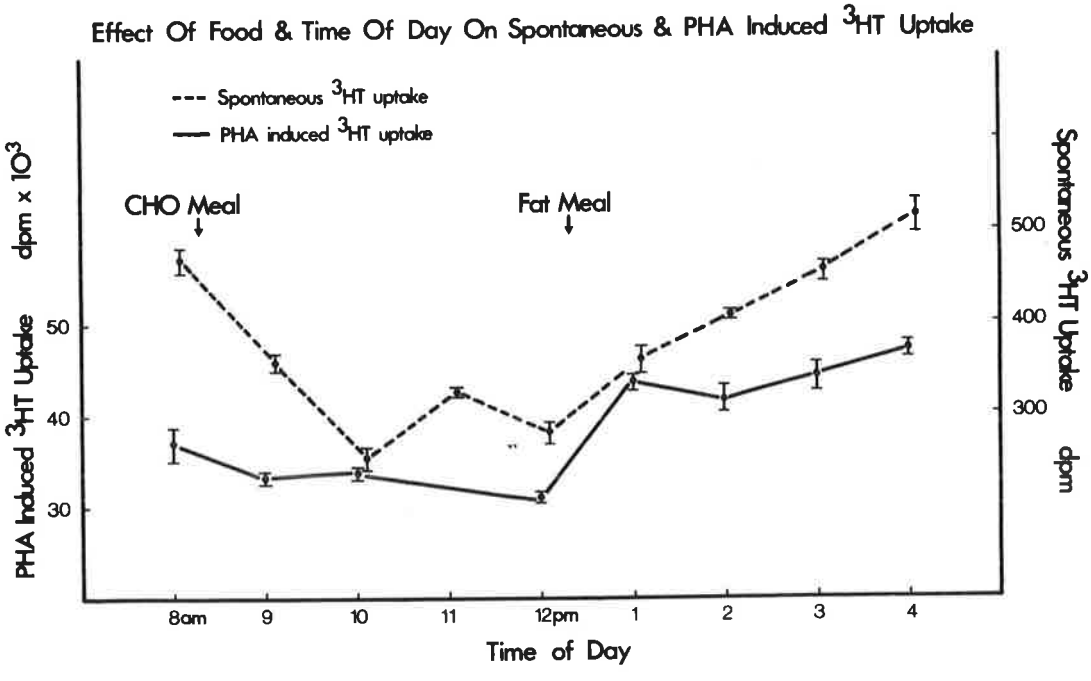
Weekly studies of PHA induced uptake were also made on lymphocytes taken at 9.00 a.m. on three separate occasions in 10 normal subjects in a fasting state. The values were compared to those obtained when blood was taken at random times on three separate occasions in a nonfasting state.

(2) Results

Figs. III.12 and III.13 show the results for the study of the single subject. There was a considerable variation in uptake values throughout the day on both fasting and nonfasting days. PHA values ranged from 30,500 dpm to 47,000 dpm and spontaneous uptake from 250 dpm to 520 dpm. The results for spontaneous uptake paralleled those of the PHA induced uptake (Fig. III.12) and showed a fall during the morning following the CHO breakfast and a progressive rise during the afternoon after the high fat lunch. Similar variations were evident in the results obtained on the fasting day but the changes were less marked (Fig. III.13).

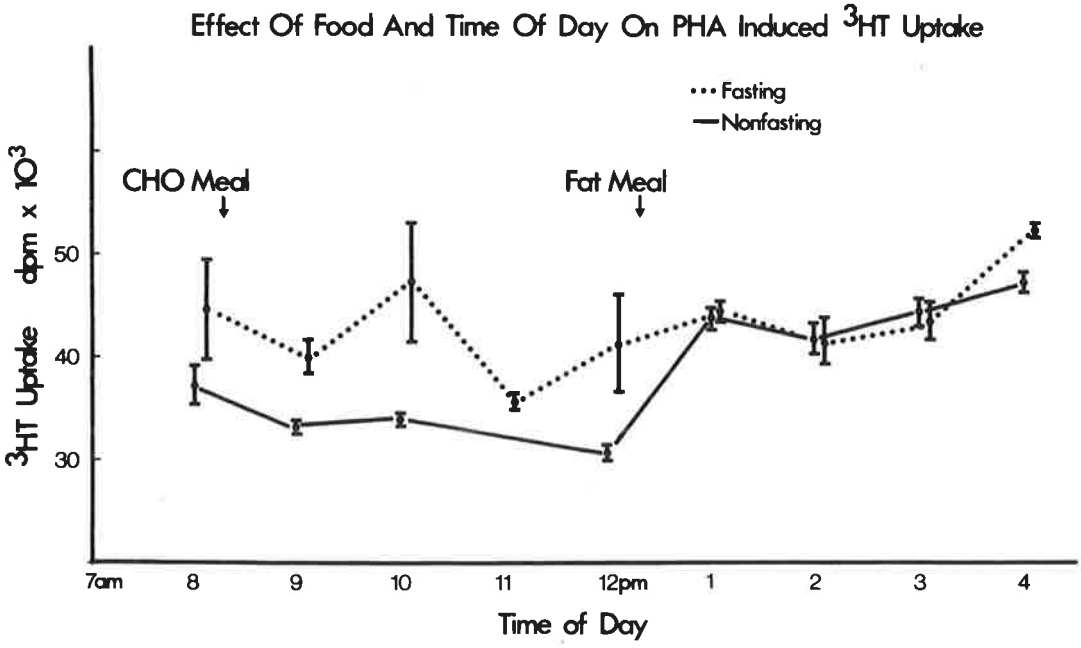
In the group of normal subjects the mean PHA uptake was 65,200 dpm with a coefficient of variation of 25.6% when blood was taken in the fasting state and 69,200 dpm with a coefficient of variation of 36.8% when blood was taken in a random fashion. For each individual the coefficient of variation was calculated for the fasting

Figure III 12.



Effect of a carbohydrate (CHO) meal, a fat meal and the time of day on spontaneous ^{3}HT uptake and PHA induced ^{3}HT uptake in a normal control subject. Each point represents the mean of duplicate cultures and the vertical bars the SEM.

Figure III 13.



Effect of the time of day on the PHA induced ^{3}HT uptake in a fasting normal control subject compared to the effect of a carbohydrate (CHO) meal and a fat meal on the uptake of the same subject. Each point represents the mean of triplicate cultures and the vertical bars the SEM.

values and the nonfasting random values. In the fasting state, the coefficients of variation ranged from 1.3 - 19.2% (mean 10.6%) and from 13.0 - 56.3% (mean 29.7%) in the nonfasting state.

(3) Discussion

The results illustrate that marked variation of ^{3}HT uptake in lymphocyte cultures may occur in an individual, not only over a period of weeks but even over a period of hours. The latter results, though preliminary, raise the possibility that short term variation may follow a pattern and may be influenced by the diet. The brisk rise in both spontaneous and PHA induced uptake after a high fat meal could reflect an increase in circulating lymphocytes resulting from an increased lymphatic flow in the thoracic duct. This increase in uptake did not occur after a high CHO meal which would not be expected to increase lymphatic flow in the thoracic duct. Moreover, the absence of a marked increase in uptake in the early afternoon during the fasting day would be consistent with this hypothesis. However, even during the fasting day there still appears to be a pattern of lower values in the morning than during the afternoon which raises the possibility of a circadian rhythm for peripheral lymphocyte numbers.

The finding that the variability of results could be significantly improved by taking the blood samples from

fasting patients at a fixed time of the day is of important practical value in improving the reproducibility of results. In the studies of patients with hepatitis described in Chapter IV all blood samples were taken between 9.00 a.m. and 10.00 a.m. However, the possible effect of food on ^{3}HT uptake was not realised at that time and thus no attempt was made to ensure that all patients were fasting.

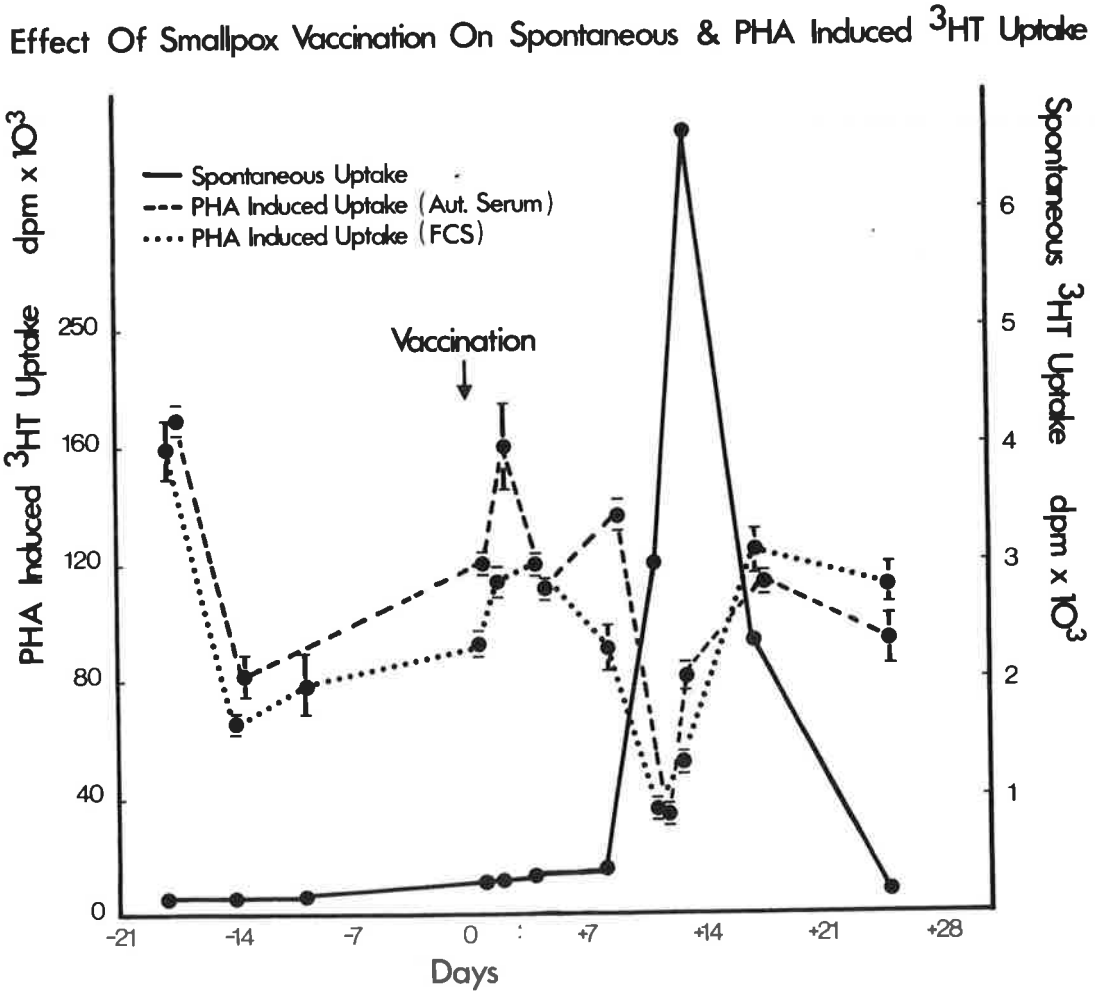
6. The Effect of Smallpox Vaccination on ^{3}HT uptake by Lymphocytes

Spontaneous and PHA induced ^{3}HT uptake was measured serially in a normal subject before and after primary vaccination with Vaccinia. The results (Fig. III.14) showed a marked rise in spontaneous uptake beginning 8 - 10 days after vaccination reaching a peak at approximately 14 days and returning to normal at approximately 21 days. The rise in spontaneous uptake was paralleled by a fall in PHA induced uptake.

Discussion

It is not uncommon to find low PHA transformation and elevated spontaneous transformation in people with intercurrent viral illness. It has been well documented that many viruses can directly effect lymphocytes in vivo and in vitro (Yam et al. 1967; Thomas et al. 1968; Olson et al. 1968; Willems et al. 1969; Salaman 1969). The apparent

Figure III 14.



Serial study of spontaneous ^{3}HT uptake, PHA induced ^{3}HT uptake in 10% FCS and PHA induced ^{3}HT uptake in 10% Autologous (Aut) serum in a normal control subject before and after Smallpox vaccination. Each point represents the mean of triplicate cultures and the vertical bars the SD.

paradox of impaired PHA response together with an increased spontaneous transformation is discussed in Chapter IV in the context of similar changes in patients with hepatitis. The experiment reported in this section was undertaken in order to determine the time course of the events following a known viral infection so that a comparison could be made with the changes in lymphocyte function observed in hepatitis.

CHAPTER IV

SERIAL STUDIES OF IMMUNE REACTIONS IN ACUTE VIRAL HEPATITIS

CHAPTER IV

1. Introduction

Serial studies of humoral and cellular immune reactions have been performed on a group of patients with hepatitis A and hepatitis B. This has involved measurement of Ig levels, autoantibodies, spontaneous ^{3}HT uptake in peripheral blood lymphocytes and ^{3}HT uptake in lymphocytes after stimulation with PHA. The results of these tests have been correlated with the patients' clinical state and liver function.

2. Patients

Three groups of patients were investigated. The main study group consisted of 26 patients admitted to hospital with acute viral hepatitis, of these 17 had hepatitis A and 9 had hepatitis B. Twelve patients with a previous history of hepatitis, 4 months to 20 years previously, constituted a control group. The third group consisted of patients with a variety of other liver disorders. Only limited studies were undertaken in this group.

3. Results

(1) Clinical Details

The clinical details of the patients with hepatitis A and hepatitis B are shown in Tables IV.1 and IV.2. All had

TABLE IV. 1.

Clinical Details of Patients with Hepatitis A.

Name	Age	Sex	Liver Biopsy	Contact	Peak Biochemical Values			Time bilirubin elevated (Weeks)
					Bil.mg/ 100ml.	SAP U/L	SGOT U/L	
D.A.	20	M	No	No	5·1	165	1120	5
S.P.	27	F	Yes	Yes	5·2	210	>1500	7
C.M.	25	M	Yes	No	3·4	295	1700	2
M.W.	34	F	Yes	No	1·8	345	830	2
J.B.	25	M	Yes	No	4·0	250	1500	3
C.L.	14	M	No	No	6·0	525	390	4
R.K.	34	M	Yes	No	6·4	130	1170	8
H.O.	43	M	Yes	No	4·7	255	480	3
J.F.	37	F	No	No	12·0	150	880	8
G.G.	51	M	No	No	9·5	230	>800	7
K.J.	34	M	No	Yes	5·7	530	>1500	4
P.W.	25	M	No	Yes	9·1	120	>1500	7
N.J.	25	M	Yes	No	6·0	365	490	10
D.G.	17	M	No	No	5·5	510	>1500	6
C.D.	21	F	No	No	12·5	185	>1500	7
A.E.	25	F	No	No	5·8	215	>1500	7
J.J.	16	F	Yes	No	7·2	220	>1500	5
				Mean	6·5	276	1168+	5·9

TABLE IV. 2.

Clinical Details of Patients with Hepatitis B.

Name	Age	Sex	Liver Biopsy	Contact	Peak Biochemical Values			Time bilirubin elevated (Weeks)
					Bil.mg/ 100ml.	SAP U/L	SGOT U/L	
G.B.	16	M	Yes	Tattoo	6·0	135	240	5
L.K.	17	M	Yes	Tattoo	5·8	260	230	5
D.T.	17	M	Yes	Tattoo	7·1	300	>200	5
R.R.	74	M	Yes	Blood Tx	19·0	170	2600	10
D.J.	26	M	Yes	S.E. Asia	6·3	160	1250	4
L.D.	21	M	Yes	Drugs	10·0	100	745	8
W.D.	26	M	No	Drugs	20·0	170	>1250	8
S.T.	19	F	No	Renal unit	3·7	150	>250	3
P.D.	22	F	No	Drugs	9·0	170	>250	6
				Mean	9·7	180	846+	6·0

a history consistent with acute hepatitis and the diagnosis was supported by the biochemical liver function tests and the course of the disease. The diagnosis was confirmed by liver biopsy in 8 of the 17 patients with hepatitis A and 6 of the 9 patients with hepatitis B. Only one patient (N.J.) had a protracted course and he was the only patient to receive corticosteroids. There was a preponderance of males in both groups.

A history of contact with a known infectious source was obtained in only 3 patients with hepatitis A, but all the cases of hepatitis B had likely contacts. Three had visited a tattoo parlour at approximately the same time, three were known to be using drugs intravenously, one had received a transfusion of Au positive blood and one girl, a nurse, had pricked a finger taking blood from an Au carrier in a renal unit. One patient had no definite contact but had visited South East Asia 3 months previously.

The control group with a previous history of hepatitis were laboratory and hospital personnel who gave a reliable history of hepatitis. Positive confirmation of the diagnosis was obtainable only in those who had the illness in more recent years. One person was known to have had hepatitis B and it is likely that this applied to three others who developed jaundice while associated with a renal unit during an outbreak of hepatitis.

The details of the group of patients with miscellaneous liver disorders are given in Table IV.3. Only single or duplicate studies were performed during the acute stages of their illness. Many of these patients were admitted with a provisional diagnosis of viral hepatitis but were later proved to have other disorders. Two patients had infectious mononucleosis with associated hepatitis. Acute pancreatitis was diagnosed at laparotomy in another patient who presented with jaundice and abdominal pain. There were two patients

with liver metastases from gastro-intestinal tract carcinoma and one of these had a positive blood test for Australia antigen. He had had surgery and transfusion before admission and may well have had associated hepatitis B. There was one patient with extrahepatic obstructive jaundice and two patients with active chronic hepatitis. In the two latter patients the diagnosis was confirmed by liver biopsy. The disease was due to oxyphenisatin in one patient whilst the other patient was a nurse from South East Asia who was a chronic Au carrier.

TABLE IV.3

Clinical Details of Patients with Miscellaneous Liver Disorders.

Name	Diagnosis	Peak Biochemical Values		
		Bil.mg/ 100ml.	SAP U/L	SGOT U/L
G.K.	Infectious mononucleosis	1.3	250	>250
A.R.	Infectious mononucleosis	7.9	310	205
L.K.	Acute pancreatitis	7.5	130	90
A.H.	Liver metastases	10.2	550	95
H.J.	Liver metastases (Au^+)	8.0	300	315
L.P.	Obstructive jaundice	9.7	290	445
L.M.	A.C.H. (Oxyphenisatin)	1.0	150	>250
J.K.	A.C.H. (Au^+)	0.7	7.5	170

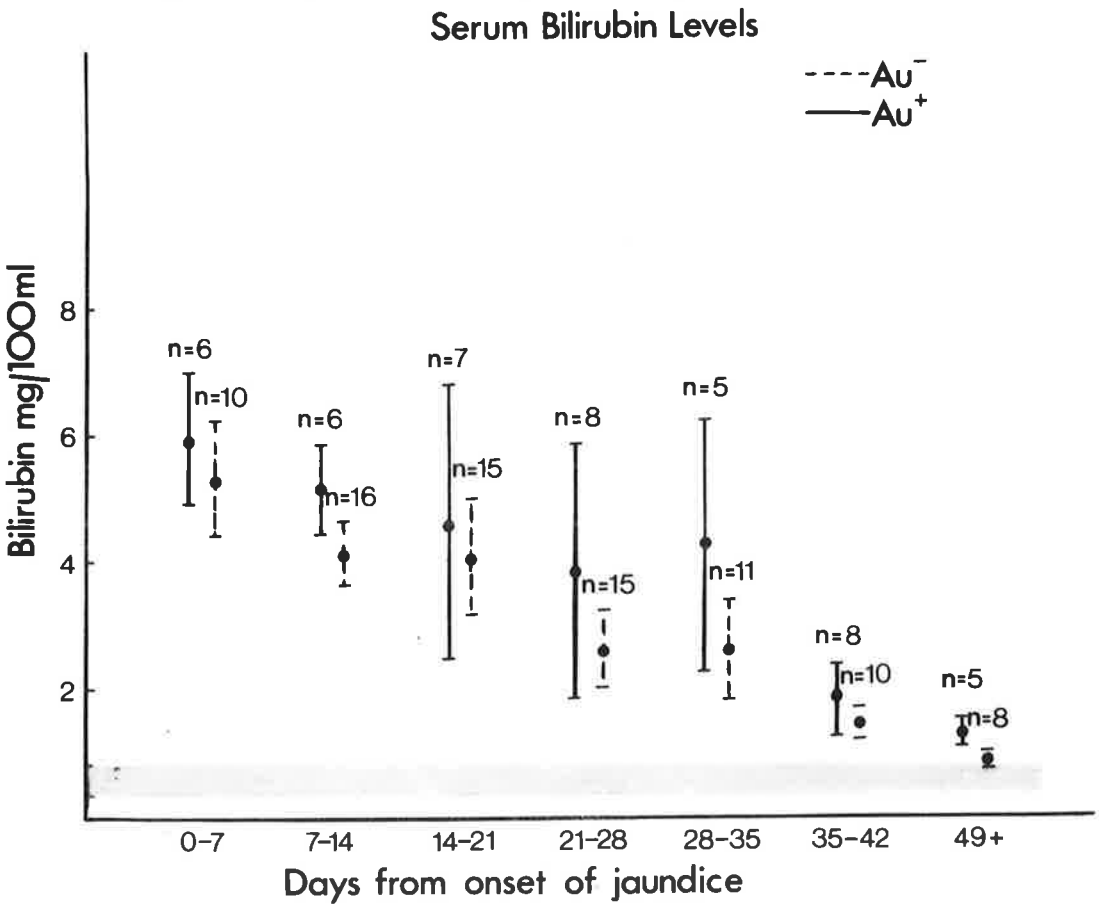
(2) Biochemical Liver Function Tests

Serial studies of serum bilirubin, alkaline phosphatase (SAP) and glutamate oxaloacetate transaminase (SGOT) were performed in all patients (Figs. IV.1, IV.2 and IV.3). The data in each case was fitted to linear and exponential time trend regression lines using the method of least squares for patients with hepatitis A (Au^-) and hepatitis B (Au^+).

Bilirubin values were best fitted to a linear time trend regression and a highly significant negative correlation was observed between bilirubin concentration and the time from onset of jaundice (Au^-: $r = -.98$ $P<.001$; Au^+: $r = -.94$ $P<.001$). There was no difference between the slopes of the regression lines ($t = 1.20$ on 12 df.) or between the intercepts tested at the midpoint of the slopes ($t = 1.91$ on 12 df) for patients with hepatitis A or hepatitis B. From the regression equations the time taken to achieve a mean level of bilirubin which was not significantly different at the 0.05 probability level from the mean value of normal control subjects was calculated to be 5 - 7 weeks after the onset of jaundice in both groups of patients.

Values for SAP were similarly best fitted to a linear time trend regression and a highly significant negative correlation was observed between SAP levels and the days from onset of jaundice (Fig. IV.2). There was no difference between the slopes of the regression lines ($t = 0.17$ on 12 df).

Figure IV 1.



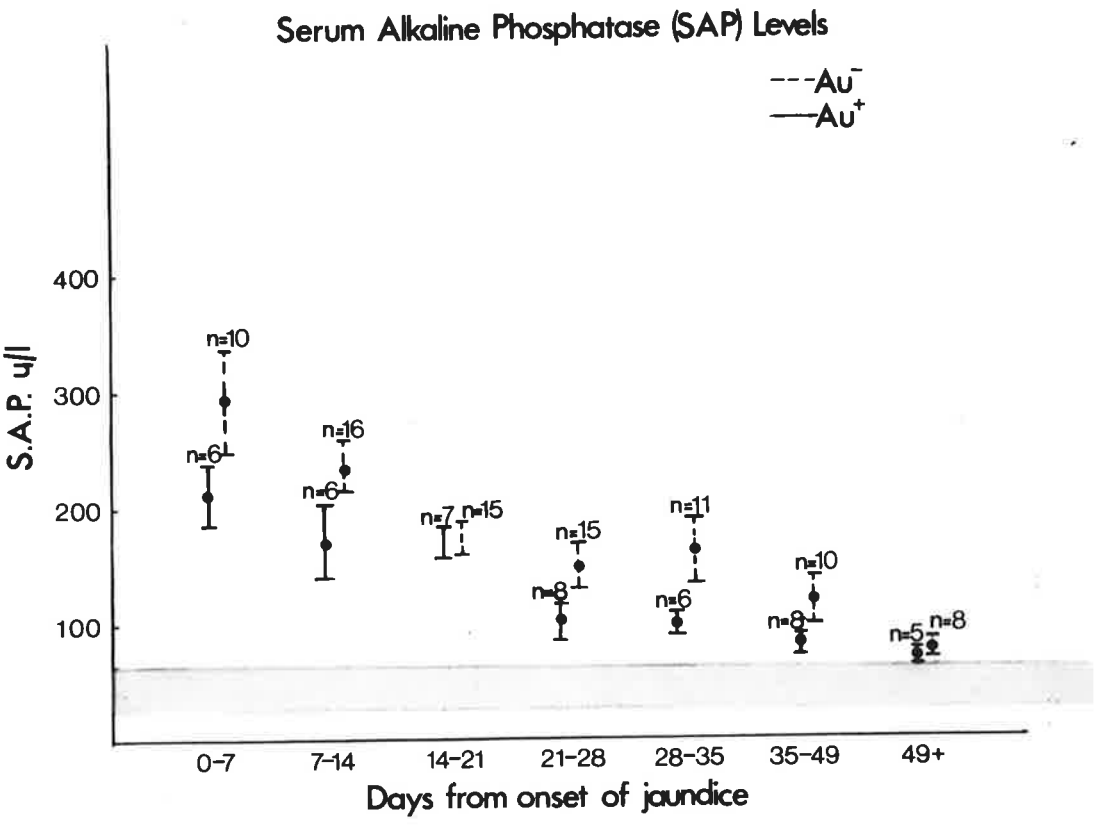
Serial study of bilirubin levels measured at intervals after the onset of jaundice in patients with hepatitis A(Au^-) & hepatitis B(Au^+). Each point represents the mean value for the number (n) of patients tested at each time interval. The vertical bars indicate the SEM. The hatched area shows the normal range (mean ± 2SD).

Regression analysis.

Au^- : $y = .71x + 5.15$ ($r = -.98$)

Au^+ : $y = .75x + 6.28$ ($r = -.94$)

Figure IV 2.



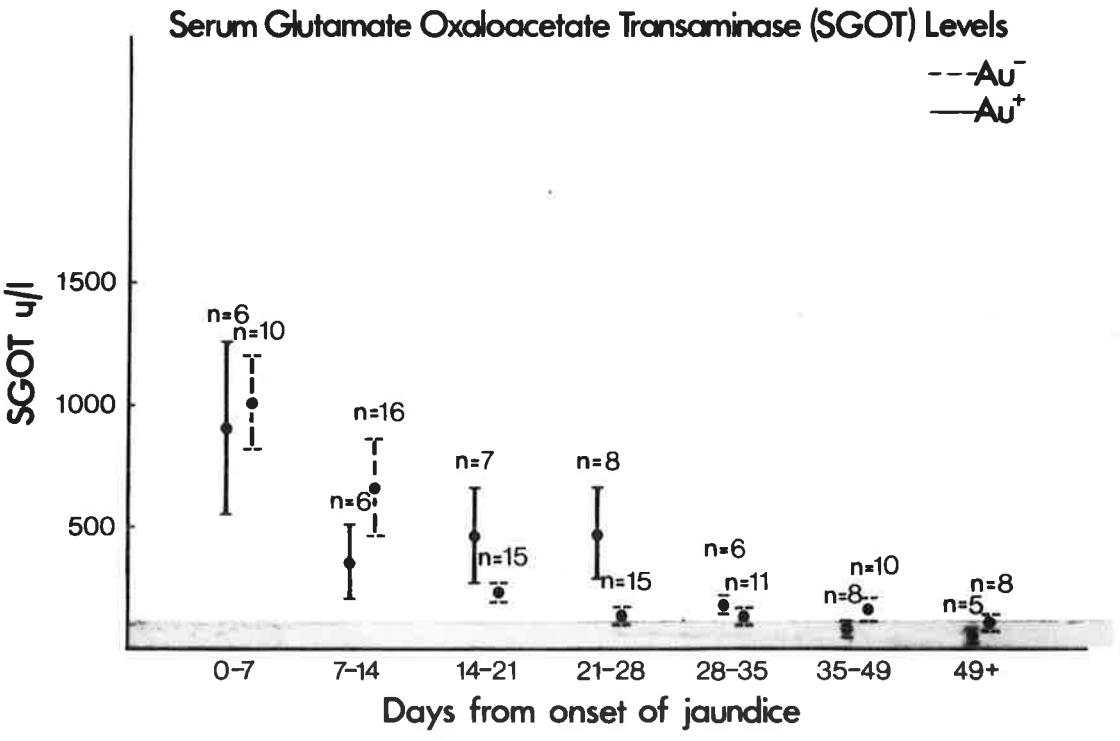
Serial study of serum alkaline phosphatase (SAP) levels measured at intervals after the onset of jaundice in patients with hepatitis A(Au^-) & hepatitis B(Au^+). Each point represents the mean value for the number (n) of patients tested at each time interval. The vertical bars indicate the $\pm$ SEM. The hatched area shows the normal range (mean $\pm$ 2SD).

Regression analysis.

Au^- : $y = -32.36x + 269.93$ ($r = -.95$)

Au^+ : $y = -22.29x + 191.20$ ($r = -.96$)

Figure IV 3.



Serial study of serum glutamate oxaloacetate transaminase (SGOT) levels measured at intervals after the onset of jaundice in patients with hepatitis A(Au^-) & hepatitis B(Au^+). Each point represents the mean value for the number (n) of patients tested at each time interval. The vertical bars indicate the SEM. The hatched area shows the normal range (mean $\pm$ 2SD).

Regression analysis.

Au^- : $\log_e y = -.41x + 6.59$ ($r = -.91$)

Au^+ : $\log_e y = -.45x + 6.83$ ($r = -.93$)

When the intercepts were tested at the midpoint of the slopes patients with hepatitis A had a higher value than patients with hepatitis B ($t = 2.28$ $P<.05$). From the regression equations the time taken to achieve a mean level of SAP which was not significantly different to the mean value of normal control subjects was calculated to be 5 - 7 weeks after the onset of jaundice in hepatitis A and hepatitis B.

SGOT values were best fitted to an exponential time trend regression. A highly significant negative correlation was observed between these values and the time from onset of jaundice (Au^-: $r = -.91$ $P<.001$; Au^+ $r = -.93$ $P<.001$). As the regression lines were exponential the midpoint of the slopes could not be validly compared. SGOT values of patients with hepatitis A were compared to those of patients with hepatitis B at the individual time intervals after the onset of jaundice using the Mann-Whitney U test. No significant differences were found. The mean SGOT levels in both groups of patients fell to a value which was not significantly different from the mean of normal control subjects 5 - 7 weeks after the onset of jaundice.

(3) Autoantibodies

Sera from all the patients with hepatitis were screened for SMA and M antibodies, antinuclear factors (ANF), thyroid cytoplasmic and GPC antibodies (Table IV.4). SMA was

TABLE IV. 4.

Autoantibodies in Hepatitis

Name	Au	Time antibody present (weeks)		
		SMA	M	ANF
D.A.	-	8	7	0
S.P.	-	4	4	0
C.M.	-	4	3	0
M.W.	-	4	4	0
J.B.	-	4	4	0
C.L.	-	5	4	0
R.K.	-	0	0	1
H.O.	-	3	0	2
J.F.	-	0	0	0
G.G.	-	5	5	0
K.J.	-	0	0	0
P.W.	-	3	3	0
N.J.	-	0	0	0
D.G.	-	5	0	0
C.D.	-	6	0	0
A.E.	-	8	8	0
J.J.	-	2	2	0
G.B.	+	1	0	0
L.K.	+	0	0	0
D.T.	+	3	3	0
R.R.	+	5	5	0
D.J.	+	4	0	0
L.D.	+	0	0	0
W.D.	+	0	0	0
S.T.	+	2	0	0
P.D.	+	0	0	0

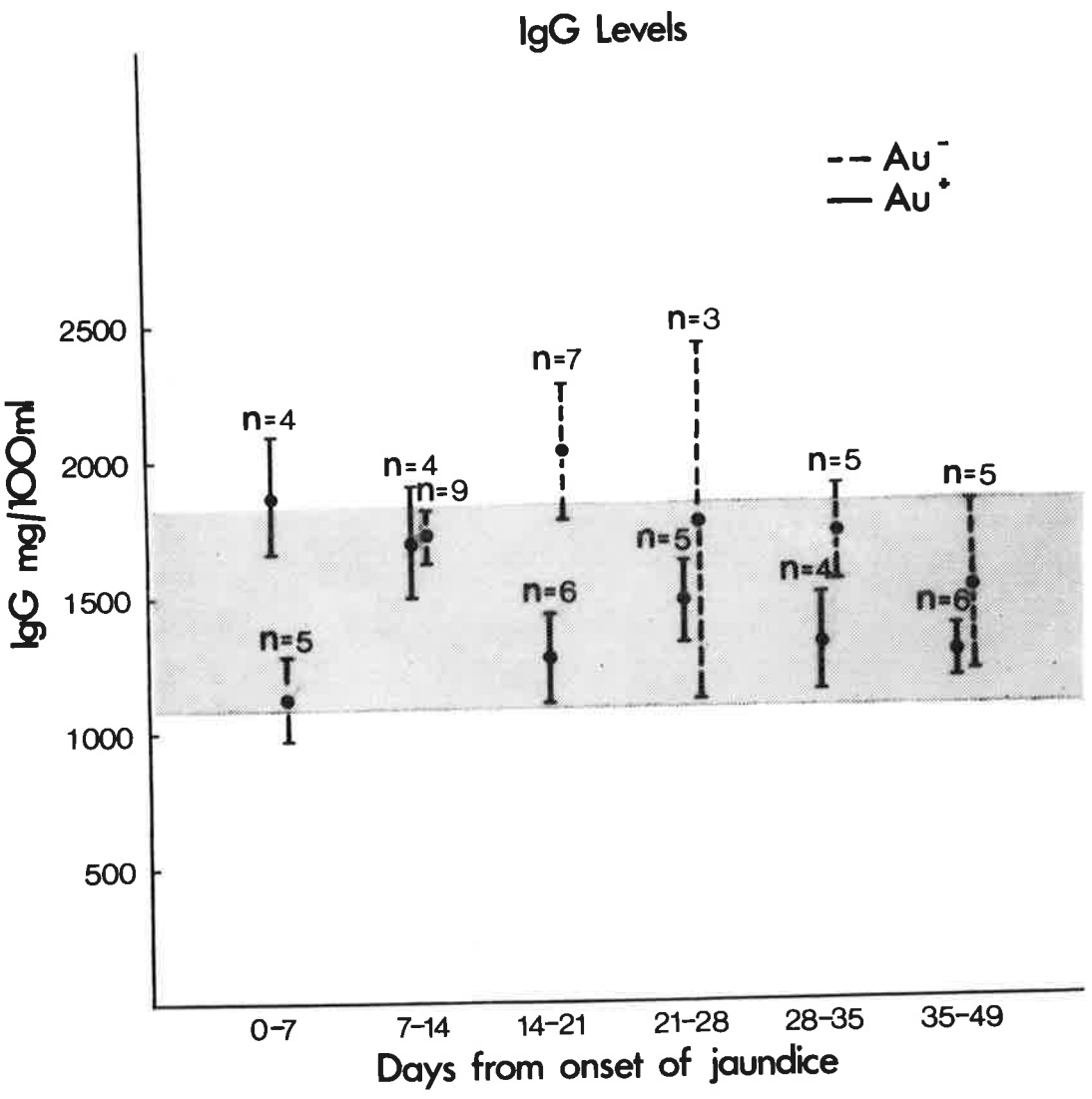
found in 76% of patients with hepatitis A and 55% of patients with hepatitis B. They were present for periods ranging from 1 - 8 weeks but in general they were present only while the liver function tests were abnormal. M antibody was also found in both hepatitis A (55%) and hepatitis B (22%). ANF was detected transiently in 2 of the 17 patients with hepatitis A. Thyroid cytoplasmic and GPC antibodies were not detected in any of the patients.

(4) Immunoglobulins and Complement

Serial determinations of IgG, IgM, IgA and Complement (β_1A) were made in a proportion of the patients (Figs. IV.4, IV.5, IV.6 and IV.7). The data in each instance, was fitted to linear and exponential time trend regression analysis using the method of least squares. The data was analysed to determine differences between patients with hepatitis A and hepatitis B.

IgG values in patients with hepatitis A showed no significant time trend regression ($r = 0.31$ on 12 df) but a negative linear time trend regression was apparent in patients with hepatitis B ($r = -.85$; $P<.02$). The highest mean value for patients with hepatitis A occurred 14-21 days after the onset of jaundice but this value was not higher than the mean of normal control subjects ($t = 2.33$ on 6 df). The highest mean value for patients with hepatitis B occurred earlier than in patients with hepatitis A (0 - 7 days) but

Figure IV 4.



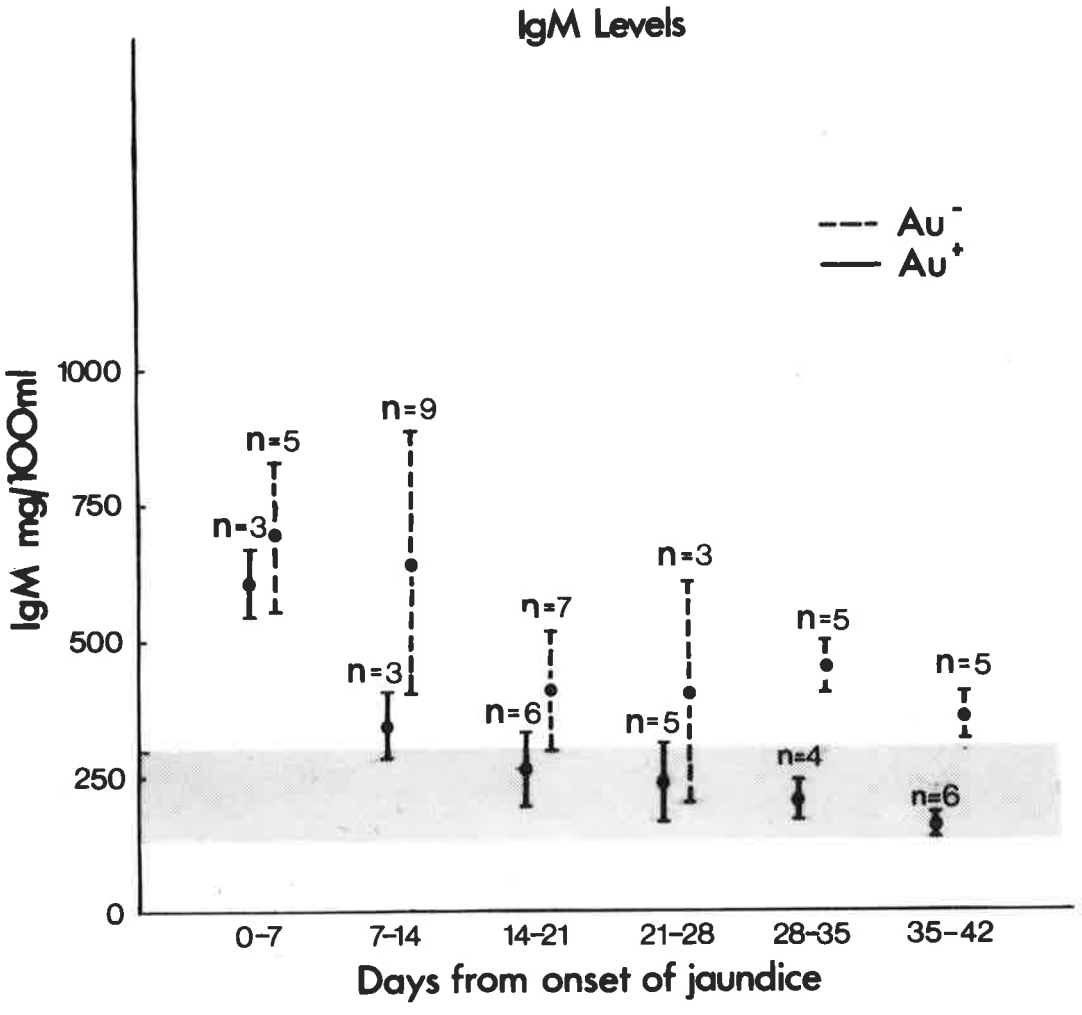
Serial study of IgG levels measured at intervals after the onset of jaundice in patients with hepatitis A(Au^-) & hepatitis B(Au^+). Each point represents the mean value for the number (n) of patients tested at each time interval. The vertical bars indicate the SEM. The hatched area shows the normal range (mean ± 2SD).

Regression analysis.

Au^- : $y = 51.29x + 1492.95$ ($r = .31$)

Au^+ : $y = -1.12x + 1741.71$ ($r = -.85$)

Figure IV 5.



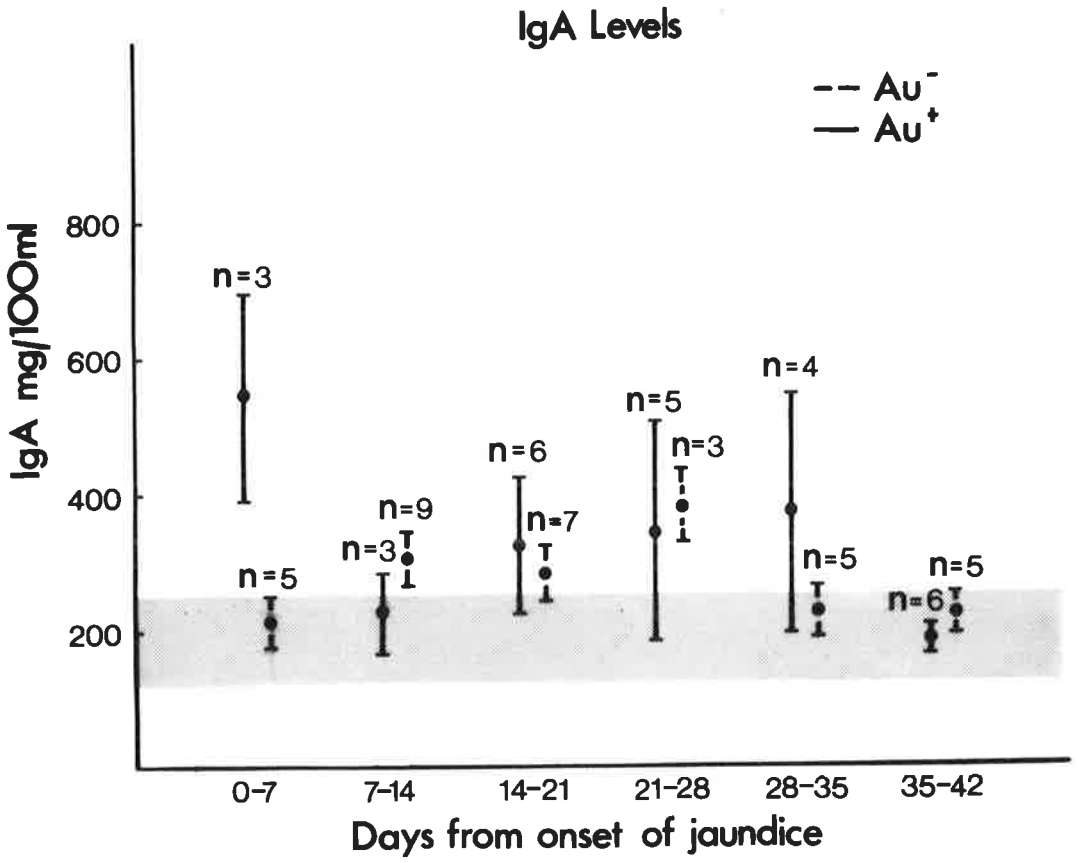
Serial study of IgM levels measured at intervals after the onset of jaundice in patients with hepatitis A(Au^-) & hepatitis B(Au^+). Each point represents the mean value for the number (n) of patients tested at each time interval. The vertical bars indicate the SEM. The hatched area shows the normal range (mean $\pm$ 2SD).

Regression analysis.

Au^- : $y = -79.8x + 668.33$ ($r = -.91$)

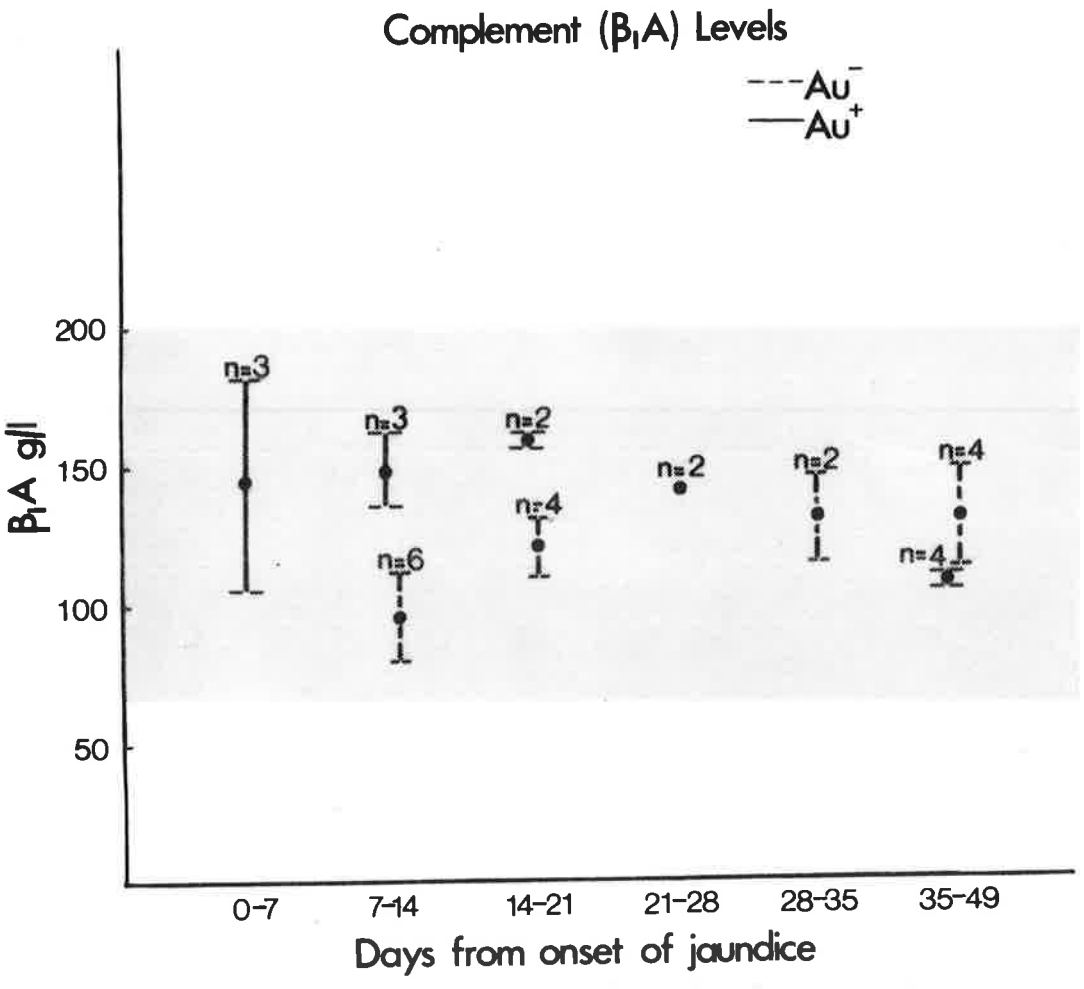
Au^+ : $y = -71.86x + 468.81$ ($r = -.89$)

Figure IV 6.



Serial study of IgA levels measured at intervals after the onset of jaundice in patients with hepatitis A(Au^-) & hepatitis B(Au^+). Each point represents the mean value for the number (n) of patients tested at each time interval. The vertical bars indicate the SEM. The hatched area shows the normal range (mean $\pm$ 2SD).

Figure IV 7.



Serial study of Complement (β_1A) levels measured at intervals after the onset of jaundice in patients with hepatitis A(Au^-) & hepatitis B(Au^+). Each point represents the mean value for the number (n) of patients tested at each time interval. The vertical bars indicate the SEM. The hatched area shows the normal range (mean $\pm$ 2SD).

there was again no difference from normal control values ($t = 1.66$ on 3 df). During the period 0 - 7 days after the onset of jaundice patients with hepatitis B had a higher mean IgG than patients with hepatitis A ($U = 2; z = 1.96; P<.025$) whereas the reverse applied during the period 14 - 21 days ($U = 37, z = 2.28, P<.01$), and for all other periods tested statistically from this time on except the period 21 - 28 days where the hepatitis A group was small and had a large standard error.

IgM levels for both groups of patients were shown to best fit a linear time trend regression (Au^-: $r = -.91$ $P<.001$ Au^+: $r = -.89$ $P<.001$). There was no significant difference between the two slopes ($t = -.008$ on 10 df). When tested at the midpoint of the slopes patients with hepatitis A had a higher intercept than patients with hepatitis B ($t = 2.37$ $P<.05$). From the regression equations the time taken to achieve an IgM level insignificantly different at the 0.05 probability level from the mean of normal control subjects was 3 - 4 weeks in patients with hepatitis B and 5 - 6 weeks in patients with hepatitis A.

There was no significant correlation between levels of IgA and the time from the onset of jaundice in patients with hepatitis A ($r = 0.06$ on 10 df) or in patients with hepatitis B ($r = 0.56$ on 10 df). Because of the small group numbers it was not valid to compare these IgA results at individual time

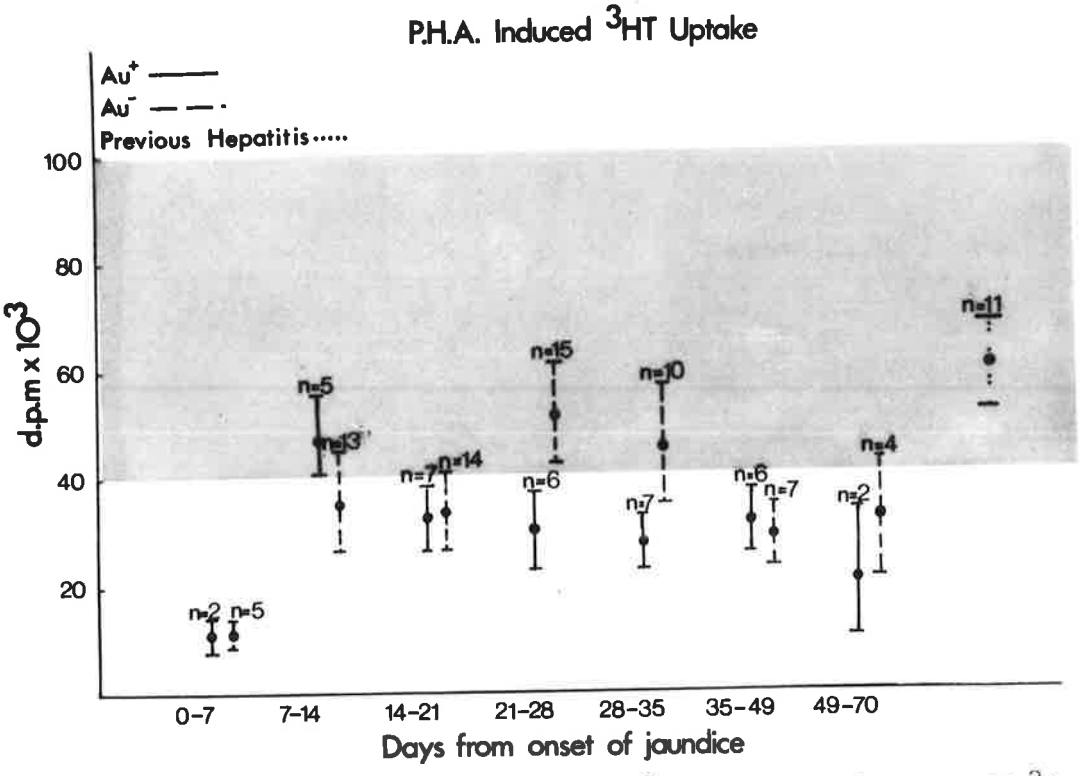
intervals from the onset of jaundice to those of the normal population. At the points where it was valid to apply the Mann Whitney U test no significant differences were found between the results obtained in patients with hepatitis A or hepatitis B.

Complement values were too few in number to be statistically analysed. All patients studied had results in the normal range. However few values were obtained within the first 7 days of the illness.

(5) PHA - Induced Lymphocyte Transformation

PHA induced lymphocyte transformation as assessed by ^{3}HT uptake was measured weekly in all patients with hepatitis during their hospital stay and 1 - 2 weekly after discharge until the liver function tests returned to normal (Fig. IV.8). Patients with hepatitis A and hepatitis B showed a similar response. The data could not be fitted to a time trend regression line. The nonparametric Mann Whitney U test was used to analyse the difference between patients with hepatitis A and hepatitis B. No significant differences were found between the two groups at any point in time after the onset of jaundice. The results were, therefore, pooled for further analysis. The mean PHA response 0 - 7 days after the onset of jaundice were lower than those obtained 7 - 14 days after the onset of jaundice ($U = 103$; $z = 1.72$; $P<.05$) and lower than the values obtained at all other periods of time

Figure IV 8.



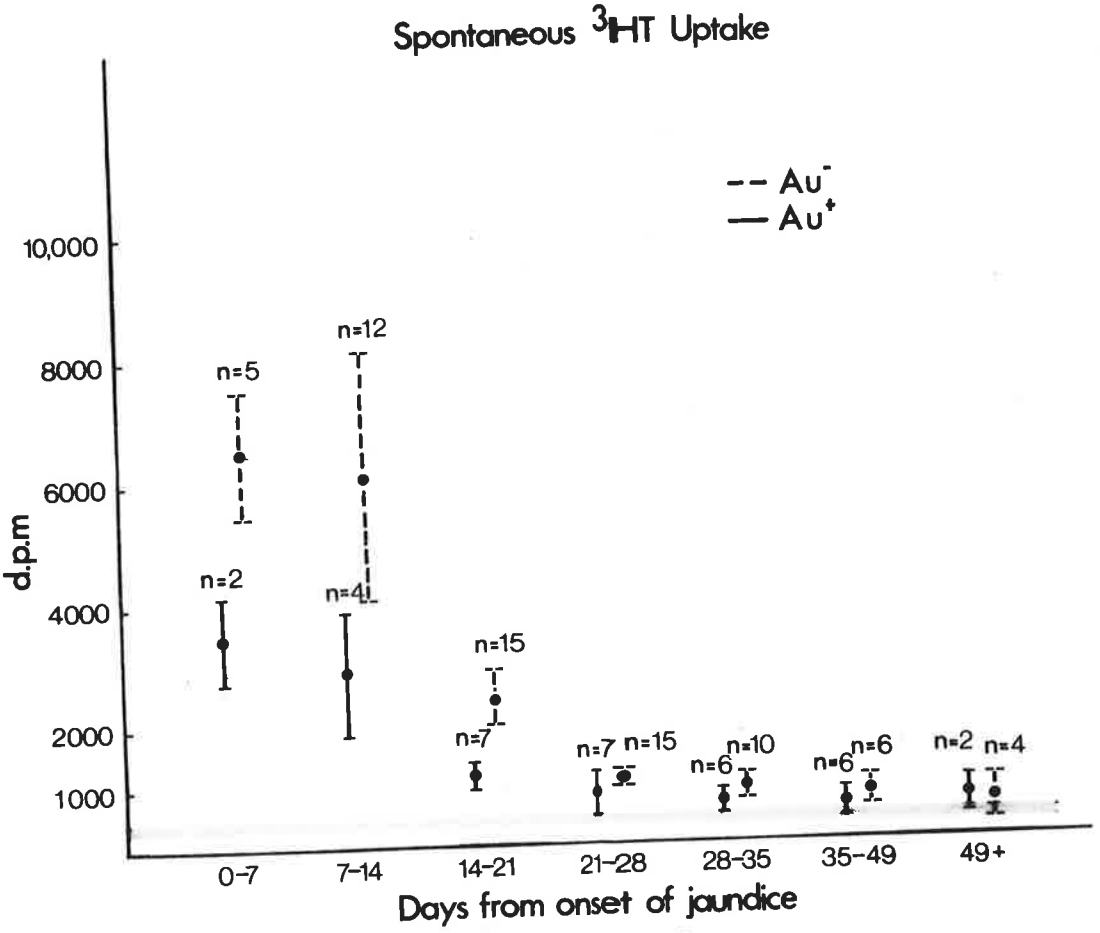
Serial study of PHA induced ^{3}HT uptake (dpm x 10^3) measured at intervals after the onset of jaundice in patients with hepatitis A(Au^-) & hepatitis B(Au^+). Each point represents the mean value for the number (n) of patients tested at each time interval. The results from a group of normal subjects with a previous history of hepatitis are indicated. The vertical bars indicate the SEM. The hatched area shows the normal range for 70 normal subjects (mean $\pm$1SD).

(P values all < or << .01). The mean value for the patients with hepatitis was highest 21 - 28 days after the onset of jaundice. The PHA induced ^{3}HT uptake results during this period of time were highly significantly lower than the results from normal control subjects (U = 1429; z = 3.93; P<.0005). It was not considered necessary to test all other points. The group of control subjects with a previous history of hepatitis did not have values significantly different from the normal control group of subjects.

(6) Spontaneous Lymphocyte Transformation

A marked elevation of spontaneous transformation was seen in both types of hepatitis (Figs. IV.9). The data was best fitted to an exponential time trend regression using the method of least squares. There was a highly significant negative correlation between spontaneous ^{3}HT uptake and time from the onset of jaundice (Au^{-}: r = -.95; P<.001; Au^{+}: r = -.91; P<.001). As the regression lines were exponential it was not possible to use the slopes to determine differences between the two groups of patients. The spontaneous ^{3}HT uptake values in patients with hepatitis A were compared with those in patients with hepatitis B at the individual time intervals after the onset of jaundice using the Mann Whitney U test and no significant differences were found. The time taken for both groups of patients to achieve a mean level of ^{3}HT uptake which was not significantly greater than the mean of the normal

Figure IV 9.



Serial study of spontaneous ^{3}HT uptake (dpm) measured at intervals after the onset of jaundice in patients with hepatitis A(Au^-) & hepatitis B(Au^+). Each point represents the mean value for the number (n) of patients tested at each time interval. The vertical bars indicate the SEM. The hatched area shows the normal range for 70 normal subjects (mean +1SD).

Regression analysis

Au^- : $\log_e y = -45x + 8.73$ ($r = -.95$)

Au^+ : $\log_e y = -32x + 7.97$ ($r = -.91$)

control subjects was 5 - 7 weeks after the onset of jaundice. One patient (NJ) did not have an elevated spontaneous transformation until later in his illness (Fig. IV.10).

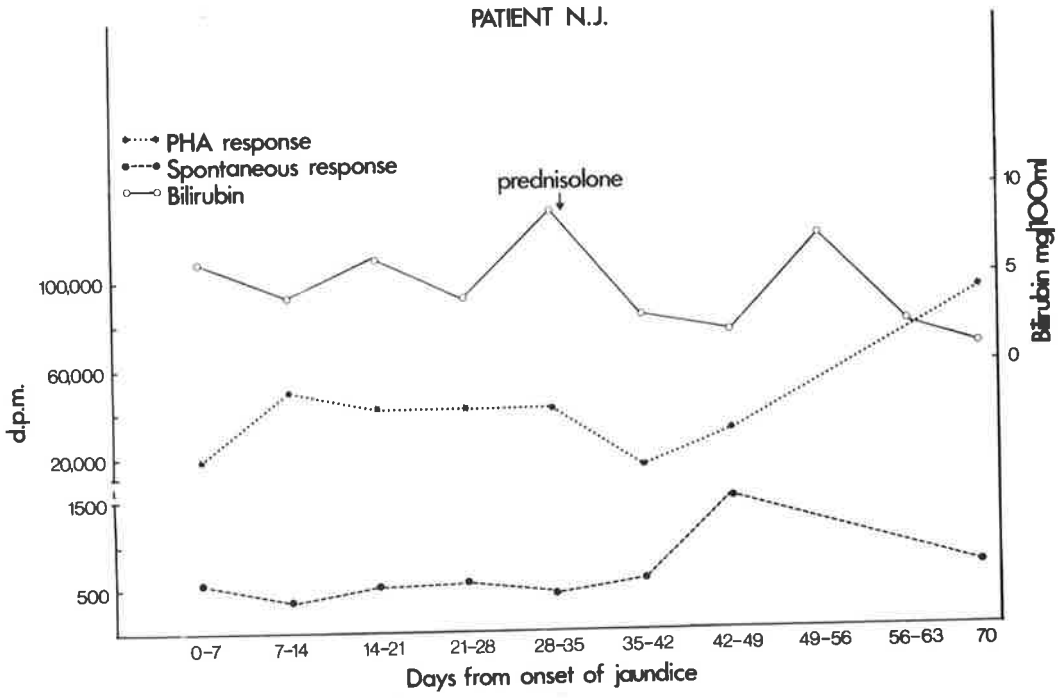
(7) PHA Induced and Spontaneous Lymphocyte Transformation in Other Liver Diseases

^{3}HT uptake with and without PHA was measured in a group of patients with a variety of liver diseases (Fig.IV.11). Two patients with infectious mononucleosis showed an identical pattern to that seen in the patients with viral hepatitis. The two female patients with ACH had normal spontaneous transformation and a depressed PHA response. The patient with acute pancreatitis showed a similar response to that seen in viral hepatitis while the patient with obstructive jaundice had a normal spontaneous transformation and a low PHA induced transformation. Two patients with liver metastases had a markedly impaired response to PHA with normal or slightly elevated spontaneous ^{3}HT uptake.

4. Discussion

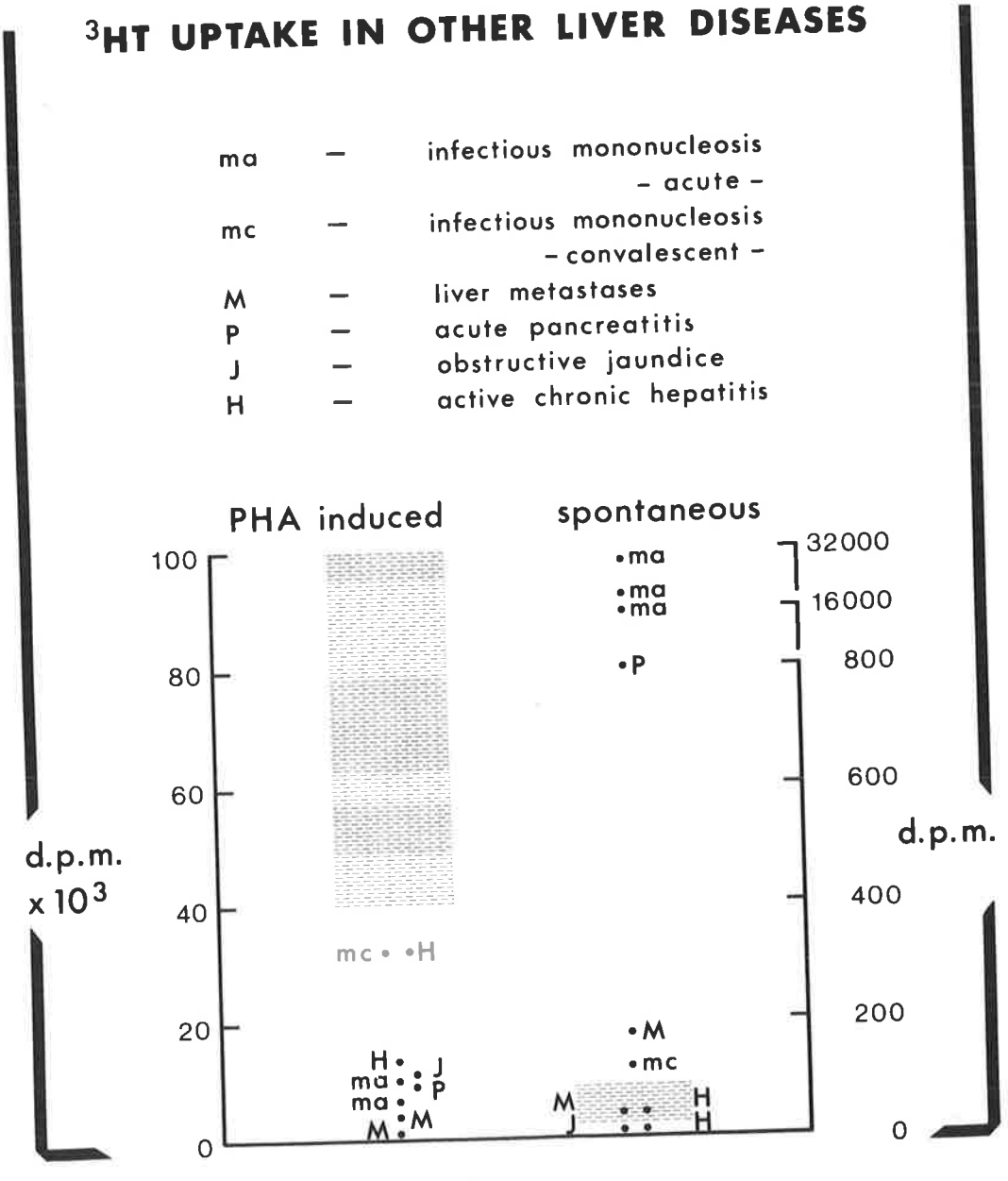
Many of the published studies of immune reactions in viral hepatitis have not been performed serially and this makes the interpretation of results difficult. The time of onset of the disease is often difficult to define and most tests of immune function are subject to marked variations

Figure IV 10.



Serial study of bilirubin level, spontaneous ^{3}HT uptake (dpm) & PHA induced ^{3}HT uptake (dpm) at intervals after the onset of jaundice in one patient (NJ) with acute viral hepatitis.

Figure IV 11.



Studies of spontaneous ^{3}HT uptake (dpm) & PHA induced ^{3}HT uptake (dpm x 10^{3}) in a variety of liver diseases.

between individuals and even in the same individual from day to day. This applies particularly to studies of lymphocyte function where the time of day, food, intercurrent illness and drugs may all lead to marked inconsistencies (see Chapter III). These difficulties can be overcome to some extent by using measurements in the same patient, by taking blood samples at the same time of the day and by having large enough study groups to allow comparison of mean values at different stages of the disease to normal control values.

(1) Clinical Details

All patients had a typical history of hepatitis with an uneventful recovery except for the patient (N.J.) mentioned previously who had a more prolonged course with an exacerbation of jaundice and elevation of enzyme levels 7 weeks after the onset of his illness. He made an uneventful recovery after receiving a course of prednisolone.

(2) Biochemical Liver Function Tests

The pattern of abnormality of biochemical liver function tests was similar for bilirubin, SAP and SGOT. Each showed a highly significant negative correlation with time from the onset of jaundice. Only in the case of SAP was there a difference in pattern between patients with hepatitis A and hepatitis B, the hepatitis A group showing higher values.

All parameters returned to normal 5 - 7 weeks after the onset of jaundice. Studies by other workers have usually shown that hepatitis B has a more prolonged course and tends to be more severe as judged by bilirubin and serum enzyme levels and by overall mortality (Sherlock 1972; Krugman and Giles 1972). This has not been confirmed for the group of patients investigated in this study.

(3) Autoantibodies, Immunoglobulins and Complement

The results of the present study are in general accord with those of other recent reports. SMA was found in 76% of the patients with hepatitis A and 55% of patients with hepatitis B and these proportions are similar to the 60 - 80% quoted in recent reviews (Doniach 1972; Holborow 1972). M antibody was also detected in a significant proportion of patients, 55% in hepatitis A and 22% in hepatitis B. These values are considerably higher than the 2% reported by Doniach (1972). The difference in these results is probably attributable to minor variations in the technique. In the method employed in this study, rat liver and stomach were the substrates used to assess the presence of M antibody whereas Doniach used human thyroid, stomach and kidney as the substrate. However, the most significant difference in the technique is that serum is routinely diluted to 1 in 4 in our laboratory compared to 1 in 10 employed by Doniach and her colleagues. M antibodies have subsequently been

measured in a similar group of patients with hepatitis when the serum was diluted 1 in 10. The value obtained in patients with hepatitis A was 12% and 0% in patients with hepatitis B. These are similar results to those obtained by Doniach et al. In all cases, SMA and M antibody were only detected transiently during the acute stages of hepatitis and though they may be markers of liver injury do not appear to play any specific part in the pathogenesis of the disease.

The immunoglobulin levels in the patients with hepatitis were similar to those reported by Peters and Johnson (1972), Thompson, Carter, Stokes, Geddes and Goodall (1973) and Iwarson and Holmgren (1972). Mean IgG levels were highest from 14 - 21 days after the onset of jaundice in hepatitis A but this elevation was not significant. In hepatitis B the mean value was highest from 0 - 7 days and though the elevation did not reach statistical significance, the overall pattern was one of a progressive fall in IgG. The values in patients with hepatitis B are higher than those with hepatitis A ($P<.025$) during the first 7 days but the reverse applies at a later stage. Similar results can be seen in the much larger series of Iwarson and Holmgren but the authors did not comment on this feature. It may be a reflection of the longer pre-icteric phase and earlier immune response in hepatitis B.

In the present study IgM levels were significantly

elevated in both types of hepatitis. In hepatitis A the values were raised for a period of 5 - 6 weeks but during only the first 3 - 4 weeks of hepatitis B. The values were higher in hepatitis A. Peters and Johnson (1972) reported a mildly elevated IgM in about half of their cases of heptaitis B but this was not confirmed by Iwarson and Holmgren (1972). Thompson et al. (1973) reported IgM levels significantly higher than control values in hepatitis A and hepatitis B. In all series, however, there was a striking elevation of IgM levels in hepatitis A compared to hepatitis B. The reason for this is not known but it may indicate that the virus of hepatitis A is a more potent stimulus of the primary immune response or that the primary immune response in hepatitis B occurs earlier and was waning at the time the study was performed. Although autoantibodies found in hepatitis belong to both IgM and IgG classes, and the incidence of autoantibodies in this study was higher in hepatitis A, the titres were generally low and would not by themselves account for the raised IgM levels. Moreover previous studies have shown no correlation between Ig levels and autoantibodies (Walker, Doniach, Willette, Cameron and Dane 1970), but the possible immunogenic effect of liver specific antigens released as a result of liver damage has not yet been elucidated.

Mean IgA levels were not significantly elevated in hepatitis A or hepatitis B. However, the numbers in the

present study were small and the mean levels in hepatitis B were markedly effected by one patient who had very high levels throughout his illness. IgA levels were transiently elevated in the series reported by Thompson et al. (1973) but not in the other series (Iwarson and Holmgren 1972; Peters and Johnson 1972).

Serum C_3 values were within normal limits supporting the findings of Dudley et al. (1971). However, few patients were studied during the first 7 days after the onset of jaundice, the period during which Kosmidis et al. (1972) demonstrated a transient fall in C_3 levels. This shortcoming also applies to the series of Dudley et al. (1971). No patients in this study had any of the extra-hepatic manifestations said to be associated with low serum complement (Alpert et al. 1971).

(4) Lymphocyte Studies

The studies of PHA induced and spontaneous transformations yielded results, some of which had not been reported previously. The response to PHA of lymphocytes from patients with hepatitis has been studied by several authors Table I.1. All agree that the response is impaired for the first 1 - 3 weeks after the onset of symptoms. The results from this study, in which serial values were obtained, show that not only is there a marked impairment of the PHA response during the first week but that there is also a less marked impairment

which persists for at least 6 - 10 weeks. Blumberg et al. (1968) suggested, on the basis of epidemiological studies, that there might be a genetic predisposition to infection with hepatitis B virus based on a deficiency in the immune system and it is known that immune deficiency predisposes to prolonged carriage of Australia antigen. As detailed studies of immune function have so far not been undertaken in patients with a past history of viral hepatitis or in the families of such patients the suggestion by Blumberg et al. (1968) cannot be excluded. Accepting the fact that PHA induced transformation may be a rather crude test of T cell function, it was shown in the present study that a group of patients with a past history of hepatitis did not have cell mediated immune function significantly different from a control group without such a history.

The prolonged impairment of the PHA response demonstrated in this study is most likely to be due to a direct effect of the virus or to production of a serum inhibitory factor. Both of these possibilities were investigated and the results are discussed in Chapter V.

The increase in spontaneous lymphocyte transformation seen in patients with hepatitis during the first 3 - 4 weeks after the onset of jaundice is similar to that which occurs in other viral infections such as infectious mononucleosis. In this latter disease the increased activity

has been attributed to the atypical lymphocytes present in the circulation. It seems paradoxical that there should be a marked rise in spontaneous transformation which can be presumed to represent increased numbers of lymphocytes dividing in response to an immune stimulus, and at the same time a reduction in response to PHA. There are two possible explanations. Firstly assuming that the spontaneous transformation reflects mainly T cell activity, the proportion of commited T cells may be sufficiently large to reduce significantly the number of unstimulated T cells available to transform in response to PHA. This possibility is supported by the work of Sheldon et al. (1973) who identified the atypical lymphocytes in infectious mononucleosis as T cells. The second possibility is that the increased spontaneous ^{3}HT uptake is due to proliferation not of T lymphocytes but of B cells or even some other cell type in the peripheral blood. Similar increased spontaneous transformation occurs before human allograft rejection. (Pagé, Posen, Stewart and Harris 1971; Hersh, Butler, Rossen, Morgan and Suki 1971; Tennenbaun, St. Pierre and Cerille 1968). It has been attributed by these authors to an increase in number of circulating lymphoblasts but others have claimed it is not related to the immune response but due to the presence in the circulation of large numbers of myeloid cells consequent on therapy with large doses of corticosteroids

(Dimitriu, Debray-Sachs, Descamps, Sultan and Hamburger 1971). Experimental studies in rats which received no immunosuppressive therapy showed a serial rise in circulating lymphoblasts rather than myeloid cells. (Suciu-Foca, Buda, Theim and Suciu 1972). Suciu-Foca et al. (1972) also demonstrated that humoral antibody was not detectable until late in the rejection process. An increase in spontaneous transformation also occurs during infections in patients with agammaglobulinaemia (Horwitz et al. 1970). In hepatitis, the peripheral blood does not usually contain primitive myeloid cells but in view of the rise in serum immunoglobulins a proliferation of B cells is possible. Further studies have been undertaken to elucidate this problem. (Chapter VI).

Few conclusions can be drawn from the studies undertaken on the patients with the liver disorders other than viral hepatitis. The results in the two patients with infectious mononucleosis are similar to those seen in viral hepatitis. This would suggest that the immune responses in hepatitis A or B are not specifically related to the causative viruses of hepatitis. The nonspecificity of the response is further emphasized by the results in the one patient with acute pancreatitis and in the subject who had a smallpox vaccination where both had increased spontaneous transformation and an impaired PHA response. The findings in the two patients with active chronic hepatitis are of

some interest. In neither patient was the low PHA response associated with an increase in spontaneous transformation. This would be consistent with the hypothesis of Dudley et al. (1971) which suggests that chronic hepatitis might occur if viral antigen persisted because of an impaired cell mediated immune response. However, a much more extensive study of such patients is required particularly in the early stages of the disease process. Only one patient (N.J.) with hepatitis had a more prolonged illness than expected and he had a normal spontaneous transformation. The PHA response was impaired but not as markedly as in the other patients. It could be argued that he initially failed to mount an adequate immune response with a consequent prolongation of the disease. Later in the course of his illness, bilirubin and enzyme levels rose suddenly and this time there was a rise in spontaneous transformation. He then made a rapid and uneventful recovery. Interpretation is made difficult by the fact that he was given corticosteroids a week before the rise in spontaneous transformation was detected. Further serial studies on patients in whom hepatitis follows a prolonged course would seem worthwhile.

No firm conclusions can be drawn from the results of patients with liver metastases. The PHA response was markedly impaired, more so than in any of the other patient groups studied. Impaired immune responses have been well documented in patients with advanced malignancy (Harris and

Bagai 1972). One patient had a slightly elevated spontaneous transformation but also had Australia antigen in his serum and may have had associated hepatitis.

CHAPTER V

EFFECT OF SERUM FACTORS ON THE LYMPHOCYTE RESPONSE TO PHYTOHAEMAGGLUTININ IN ACUTE VIRAL HEPATITIS

CHAPTER V

1. Introduction

It was shown in Chapter IV that the response of lymphocytes to PHA is markedly impaired during the acute phase of viral hepatitis and less markedly impaired for a period of at least 6 - 10 weeks after the onset of jaundice. This Chapter describes studies carried out on patients with hepatitis A and hepatitis B to determine the effect of serum factors on the PHA response of both normal and patients lymphocytes.

2. Patients and Methods

20 patients were studied, 12 with hepatitis A and 8 with hepatitis B. These patients were part of the main study group described in detail in Chapter IV.

(1) Effect of hepatitis serum on normal lymphocyte function

Serum was collected from the 20 patients during the first two weeks after the onset of jaundice (acute phase serum). The mean collection time of acute phase sera was 10 $\pm$ 4 days for patients with hepatitis A and 12 $\pm$ 5 days for patients with hepatitis B. Further blood samples were collected after liver function tests had returned to normal (convalescent serum).

Normal lymphocytes were obtained from six healthy donors. Cultures containing PHA were set up using the micromethod previously described. For each patient with hepatitis there was one triplicate set of control cultures containing 10% FCS, one set containing 10% FCS with an additional 5% acute phase serum and one set containing 10% FCS with an additional 5% convalescent serum.

(2) Effect of autologous serum on lymphocyte function

The effect of autologous acute phase serum on the patients own lymphocytes was studied in 12 cases, 7 of hepatitis A and 5 of hepatitis B. Patients were studied at weekly intervals for a period of 6 weeks. Two sets of triplicate cultures containing PHA were made up with blood from each patient. One set contained 10% FCS and the other set contained 10% autologous serum. This patient group was compared to a normal control group of 70 subjects and a control group of 11 subjects, described in Chapter IV, with a previous history of hepatitis.

(3) Effect of washing on lymphocyte function

Washing experiments were performed to investigate the role of serum factors in the lymphocyte response to PHA. Blood from 6 patients with hepatitis and from 7

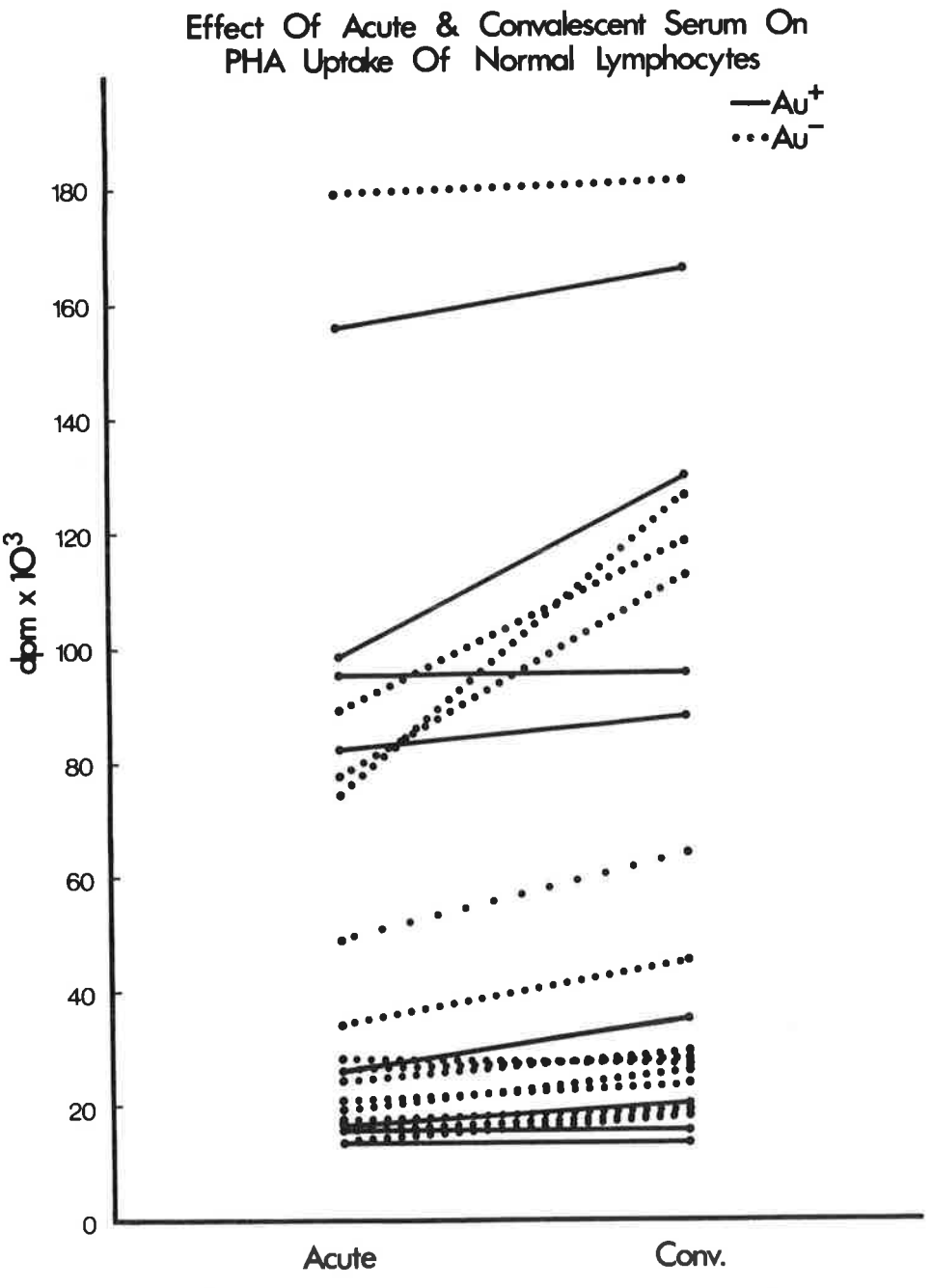
subjects from each control group was washed three times with Dulbecco phosphate buffer (DPB, CSL) and then reconstituted to its original volume with either FCS or autologous plasma. The washed and reconstituted blood was used for cultures with PHA and 10% FCS as already described. The cultures which had been reconstituted with autologous plasma therefore contained the same concentration of autologous plasma as the original blood sample. Cultures reconstituted with FCS were assumed to contain only trace amounts of autologous plasma.

3. Results

(1) Effect of hepatitis serum on normal lymphocyte function

The PHA induced transformation was lower when the normal lymphocytes were incubated in acute phase serum than when they were incubated in convalescent serum (Fig. V.1). Because of the inherent variability of the data nonparametric methods were used for statistical analysis. Two related samples were being compared so the tests selected were the Sign Test applying the binomial expansion and the Wilcoxon Matched Pairs Ranks Test. The PHA transformation was significantly lower in the presence of acute phase serum for patients with hepatitis A (Sign test $P<.001$, Wilcoxon test $P<<.01$) and for patients with hepatitis B (Sign

Figure V 1.



Effect of acute phase serum & convalescent serum from patients with hepatitis A(Au^-) & hepatitis B(Au^+) on the PHA induced ^3HT uptake (dpm x 10^3) of lymphocytes from normal control subjects.

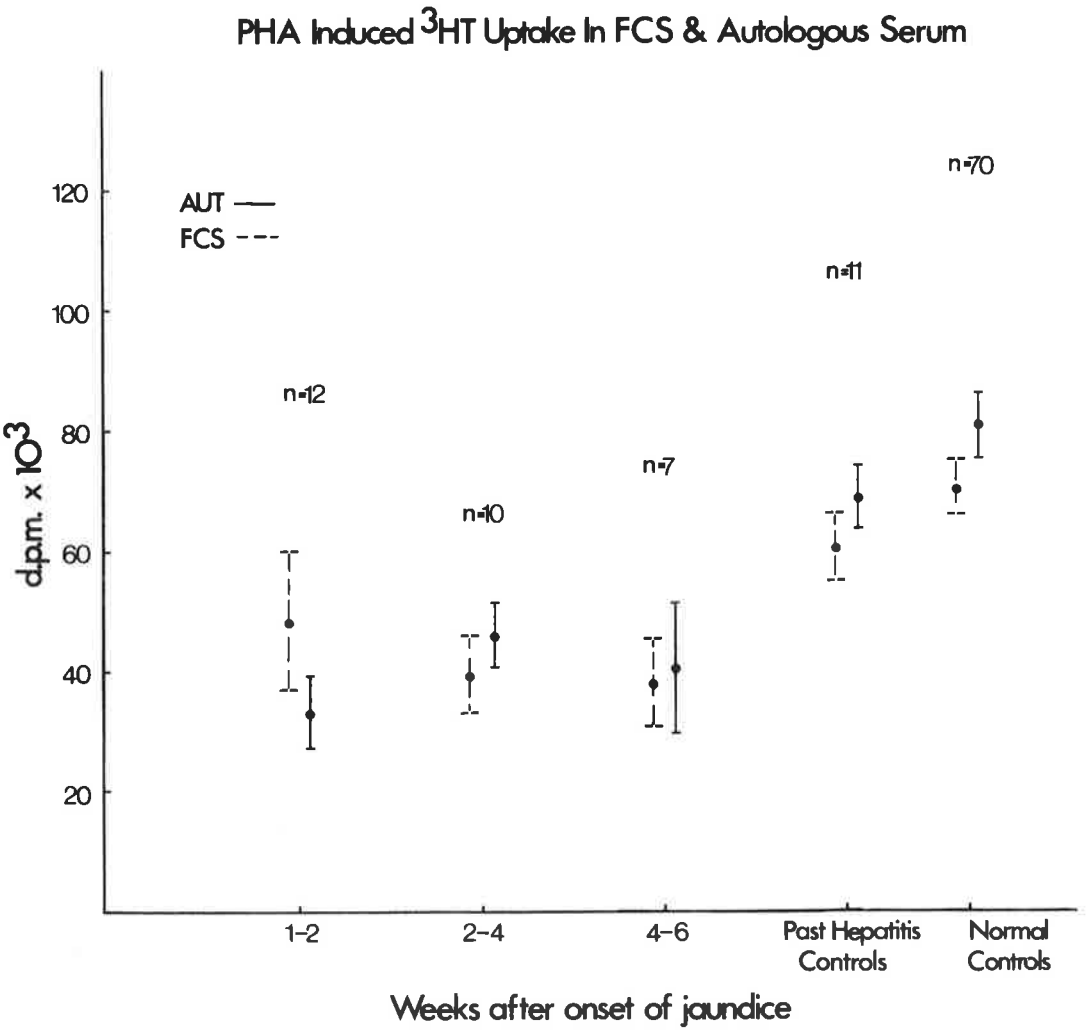
test P<.03; Wilcoxon test P<.02).

(2) Effect of autologous serum on lymphocyte function

Autologous serum had a very variable effect on PHA induced ^{3}HT uptake of lymphocytes from 70 normal control subjects (Table V.1) with 36 subjects showing a higher uptake in autologous serum and 34 showing a higher uptake in FCS. There was no significant difference between the mean uptake in autologous serum or in FCS (Paired t = 0.30 on 69 df). Similar results were seen in cultures from subjects with a past history of hepatitis (t = 0.64 on 10 df) and in patients with acute hepatitis 1 - 2 weeks (t = 1.41 on 12 df), 2 - 4 weeks (t = 1.23 on 10 df) and 4 - 6 weeks (t = 0.37 on 6 df) after the onset of jaundice (Fig. V.2). There was no significant difference in the results obtained in patients with hepatitis A and hepatitis B and because of the limited numbers studied they were included in one group for analytical purposes.

Lymphocytes from the 12 patients with acute hepatitis showed a significantly lower PHA induced ^{3}HT uptake than those from normal subjects when cultured in FCS or in autologous serum (Table V.1 Fig. V.2). This effect was apparent 1 - 2 weeks after the onset of jaundice (FCS t = 2.56 P<.02; Autologous serum t = 4.07 P<.001), 2 - 4 weeks after the onset of jaundice (FCS t = 3.15 P<.01; Autologous serum

Figure V 2.



Effect of 10% FCS & 10% Autologous (Au^+) serum on the PHA induced uptake (dpm x 10^3) of patients with viral hepatitis at intervals after the onset of jaundice. The results are compared to those obtained in control subjects with a past history of hepatitis & normal control subjects with such a history. Each point represents the mean value for the number (n) tested at each time interval. The vertical bars indicate the SEM.

TABLE V. 1.

Effect of 10% FCS and 10% Autologous serum

on PHA induced ^{3}HT uptake

Subjects	No	Uptake in 10% FCS (dpm)	Uptake in 10% aut. serum (dpm)
Normal controls	70	69,800 ±31,000	79,800 ±39,000
Past hepatitis controls	11	60,000 ±26,000	69,000 ±36,000
Acute hepatitis 1 - 2 weeks	12	43,900 ±37,500	32,500 ±19,200
Acute hepatitis 2 - 4 weeks	10	39,500 ±19,600	45,500 ±17,900
Acute hepatitis 4 - 6 weeks	7	37,600 ±18,800	39,700 ±29,100

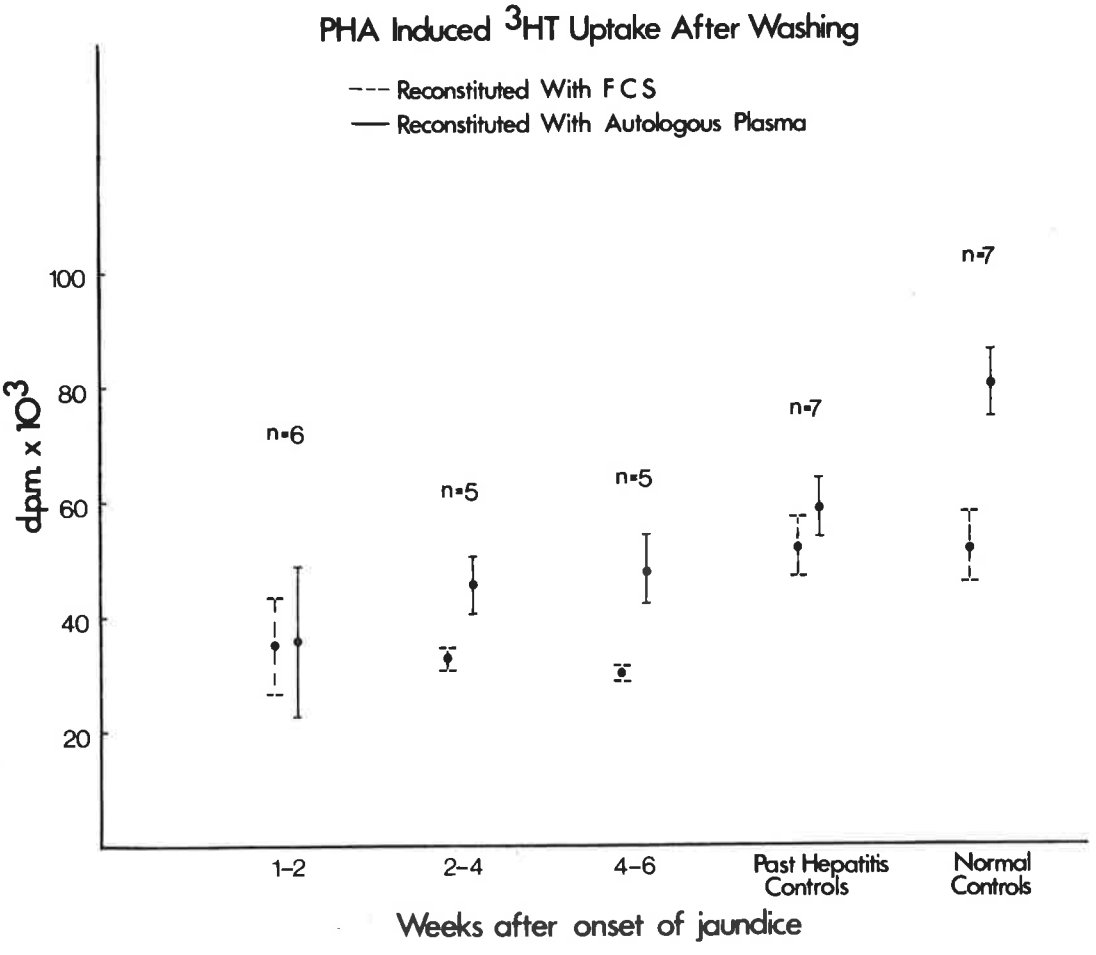
t = 2.71 P<.01) and 4 - 6 weeks after the onset of jaundice (FCS t = 2.07 P<.01; Autologous serum t = 2.61 P<.02). These statistical results were confirmed by the Mann Whitney U test. The Kruskal Wallis One Way Analysis of Variance was applied to compare results in the cultures obtained at different time intervals after the onset of jaundice. No significant differences were present in cultures containing FCS (H = 3.24 P<.2) or containing autologous serum (H = 0.18 P<.5).

The group of control subjects with a previous history of hepatitis did not differ significantly from normal control subjects when PHA induced ^{3}HT uptake was measured in FCS (t = 0.98 on 79 df) and in autologous serum (t = 0.85 on 79 df).

(3) Effect of washing on lymphocyte function

In 6 of the 7 normal subjects in whom the blood was washed before culture, reconstitution with autologous plasma led to a higher PHA induced ^{3}HT uptake than on reconstitution with FCS (Table V.2). The mean ^{3}HT uptake values for cultures reconstituted with autologous plasma was higher than for cultures reconstituted with FCS (Paired t = 3.20 P<.02). This difference was not apparent in the 7 control subjects with a previous history of hepatitis (Paired t = 2.42 on 6 df) (Table V.3 Fig. V.3).

Figure V 3.



Effect of washing blood and reconstituting the blood with FCS or Autologous plasma on the PHA induced ^{3}HT uptake (dpm x 10^3) of cultures set up with 10% FCS. The effect was studied in patients with viral hepatitis at intervals after the onset of jaundice, in control subjects with a past history of hepatitis & normal control subjects without such a history. Each point represents the mean value for the number (n) tested at each time interval. The vertical bars indicate the SEM.

TABLE V.2

Number showing higher or lower ^{3}HT uptake in cultures containing blood washed and reconstituted with autologous plasma Vs cultures containing blood washed and reconstituted with FCS

Subjects	Aut plasma>FCS (number)	Aut plasma<FCS (number)
Normal controls	6	1
Past hepatitis controls	6	1
Acute hepatitis 1 - 2 weeks	2	4
Acute hepatitis 2 - 4 weeks	4	1
Acute hepatitis 4 - 6 weeks	4	1

TABLE V. 3.

Effect of 10% FCS and 10% Autologous serum on the PHA induced ^{3}HT uptake in Hepatitis A and Hepatitis B

Time from onset of jaundice	Hepatitis A		Hepatitis B	
	10% FCS	10% aut serum	10% FCS	10% aut serum
1 - 2 weeks	49,100 ±47,300 (n = 7)	30,700 ±17,500 (n = 7)	36,600 ±12,500 (n = 5)	35,100 ±21,000 (n = 5)
2 - 4 weeks	41,200 ±24,000 (n = 6)	45,200 ±21,200 (n = 6)	36,800 ± 9.200 (n = 4)	45,900 ±11,200 (n = 4)
4 - 6 weeks	42,100 ±19,200 (n = 5)	48,000 ±30,600 (n = 5)	26,300 ±11,300 (n = 2)	18,800 ±11,200 (n = 2)

The washing procedure itself did not appear to interfere with ^{3}HT uptake since this was not significantly different for cultures containing unwashed blood and cultures washed and reconstituted with autologous plasma. This applied both to the control groups and to the patients with hepatitis (Table V.3). The mean ^{3}HT uptake for the cultures which had been washed and reconstituted with FCS, and which therefore contained no autologous plasma, was lower than the uptake for unwashed cultures. With respect to patients with a past history of hepatitis the mean uptake for the cultures reconstituted with autologous plasma was lower than that for the normal control group ($t = 2.58$ $P<.05$). However, these subjects did not differ significantly from normal subjects in regard to the ^{3}HT uptake of cultures containing unwashed blood or cultures washed and reconstituted with FCS.

Compared to the two control groups the patients with acute viral hepatitis showed a decrease in ^{3}HT uptake in washed and unwashed cultures. A higher uptake in the presence of the small amount of autologous plasma used to reconstitute the blood was seen in 4 of the 6 patients during the first two weeks of illness (Table V.2). The mean uptake was significantly impaired for cultures contained blood washed and reconstituted with autologous serum ($t = 2.95$ $P<.02$) but not for cultures washed and reconstituted with FCS ($t = 1.56$ on 11 df). At the subsequent time intervals of

2 - 4 weeks and 4 - 6 weeks after the onset of jaundice the ^{3}HT uptake was equally impaired regardless of the reconstituting medium ($P< or <<.05$). The paired t test was used to compare the effect of FCS and autologous serum as the reconstituting media in the patients with hepatitis at the different time intervals and no statistically significant differences were found. The values obtained in these washing experiments were also analysed by the non-parametric Mann Whitney U test and identical results were obtained.

4. Discussion

The impaired PHA response of lymphocytes from patients with viral hepatitis was described in Chapter IV. The impairment was most marked during the first two weeks after the onset of jaundice but was present for at least 6 - 10 weeks. The results obtained in persons with a previous history of hepatitis indicated that it did not persist for longer than 3 months. It could be due either to a defect in the lymphocyte itself and this defect could be induced by the virus or alternatively it could be due to a factor in the lymphocyte's environment. In this part of the study an attempt was made to determine the effect of serum factors on the lymphocyte response in hepatitis.

Serum from patients with acute hepatitis, taken during the acute phase, inhibited the ^{3}HT uptake of normal lympho-

cytes by comparison with convalescent serum. This inhibitory effect was most marked and highly significant in patients with hepatitis A. Serum from the patients with hepatitis B produced a similar effect but the magnitude of the inhibition was less. A comparison of the effect of acute phase serum and convalescent serum with the FCS control cultures suggested that acute phase serum was inhibitory.

The results of the present study confirm the work of previous authors (Baroyan et al. 1970; Hsu and Leevy 1971; Newberry et al. 1973) who also described a factor in the serum of patients with acute hepatitis which inhibited the PHA response of normal lymphocytes. However, on present evidence, it cannot be assumed that the factor is specific to hepatitis or even that it plays a significant role in vivo. Inhibitory substances have been found in the serum of normal individuals (Cooperband et al. 1968; Nelken 1973) and in the serum of patients with a wide variety of other diseases (Newberry et al. 1973; Hsu and Leevy 1971; Gatti 1971). It would therefore be of interest to establish whether acute phase hepatitis serum is also inhibitory to autologous lymphocytes.

Studies of lymphocyte function using autologous serum present certain difficulties. Results of such studies in normal subjects demonstrated that the number of subjects who had lower and higher ^{3}HT uptakes when compared to the

uptake in FCS were almost equal. It was shown in Chapter III that PHA stimulated ^{3}HT uptake occurs earlier in cultures which contain autologous serum than in cultures which contain FCS. Individual variations in the response to PHA in the presence of both autologous serum and FCS were also demonstrated. Despite these limitations some conclusions can be drawn from the studies of patients with hepatitis.

The serial studies in patients with hepatitis of the effects of 10% autologous serum and 10% FCS showed that the lymphocyte response to PHA was impaired in both environments. However, the effects of autologous serum and FCS were not identical. The former was relatively inhibitory compared to the latter during the first and second weeks. In contrast to this the ^{3}HT uptake in FCS did not alter throughout the 6 week period. However the numbers studied were small and the results are not statistically significant. However they raise the possibility that a factor or factors may be present in autologous serum of patients with hepatitis which can alter the PHA response and which are inhibitory during the first two weeks. The FCS cultures contained the small proportion of autologous serum present in the original blood. The prolonged impairment of the PHA response in these cultures could therefore also reflect the presence of a serum inhibitor.

Experiments performed by other workers on inhibitors

present in normal blood (Nelken 1973) and in cirrhosis (Hsu and Leevy 1971) have shown that such inhibitors can easily be removed from lymphocytes by simple washing and that washing restored the PHA response of the lymphocytes to normal. In the present study blood from normal subjects was washed and reconstituted with autologous plasma or FCS. The blood was used to set up cultures in 10% FCS. One set of cultures could therefore be regarded as having no autologous serum present and the other set as having the small amount of autologous plasma present in the original blood amounting to approximately 2.5% of the final culture volume. As there was no reduction in the ^{3}HT uptake in the latter cultures when compared to an identical set of cultures which had not been washed, the washing procedure did not impair the lymphocyte response. In normal control subjects the washed cultures which contained no autologous serum had a lower uptake than those reconstituted with autologous serum indicating that in normal subjects even small amounts of autologous serum facilitate the lymphocyte response to PHA. The results obtained in the subjects with a past history of hepatitis were similar but the facilitatory effect of autologous serum was less marked than that seen in the normal control subjects. The difference in response was significant and raises the possibility of a defect inherent in the lymphocyte or of a prolonged presence of an

inhibitor of lymphocyte function as a result of past hepatitis. It would seem justified to confirm this difference in a larger study as both possibilities have important implications.

Lymphocytes which had been obtained from patients with hepatitis, washed and reconstituted in FCS, showed significantly lower ^{3}HT uptakes than lymphocytes treated in the same manner but obtained from normal persons. Cultures washed and reconstituted with autologous plasma showed similar uptakes to the unwashed cultures. They were both significantly lower than those from normal subjects but the uptake values were lower, but not significantly so, in cultures reconstituted with autologous serum than in cultures reconstituted with FCS during the first two weeks than during the succeeding weeks.

The main fact which emerges from the washing experiments is that lymphocytes from patients with hepatitis cultured in the absence of autologous serum showed low uptakes. Washing did not restore the lymphocyte responsiveness to PHA suggesting the possibility of a defect in the lymphocyte itself and possibly induced by hepatitis virus.

5. Summary

The experiments reported in this chapter confirmed that the PHA response of lymphocytes from patients with

hepatitis was impaired for at least 6 - 10 weeks after the onset of jaundice. It would appear that there are two reasons for this. Firstly, the response was impaired whether the cells were cultured in the presence of autologous serum or in the absence of autologous serum and could not be restored to normal by washing the cells. This strongly suggests a defect in the lymphocyte present during hepatitis. Secondly, serum taken from patients during the first two weeks of the illness was inhibitory to normal and autologous lymphocytes.

CHAPTER VI

STUDIES OF LYMPHOCYTE SUBPOPULATIONS

CHAPTER VI

1. Introduction

The serial studies of lymphocyte function in patients with hepatitis, described in Chapter IV, showed an increase in spontaneous transformation during the first 3 - 4 weeks after the onset of jaundice. Lymphocyte response to PHA was impaired during the same period and for a further 3 - 6 weeks. Two explanations were suggested for this combination. Firstly, that the increase in spontaneous transformation might reflect a T cell response to infection and that the proportion of T cells committed might be large enough to reduce the number of unstimulated T cells available to transform to the extent of impairing the ^{3}HT uptake in response to PHA. Secondly, the increase in spontaneous transformation might be due to a proliferation of B cells or some other type of cell in the peripheral blood. This Chapter describes the studies undertaken in an attempt to identify the cells responsible for the increase in spontaneous transformation.

2. Patients and Methods

Twenty seven healthy control subjects taken from among hospital and laboratory staff and 6 patients from the main hepatitis study group described in Chapter IV, were investigated. The latter patients, two of whom had

hepatitis B, were studied serially during the acute phase and early convalescent period. The proportion and type of B cells present in the peripheral blood were estimated by the immunofluorescent and autoradiographic techniques described in Chapter II. These estimations were performed on purified lymphocyte preparations. The total number of B cells was calculated from the proportion of B cells and the white cell and differential counts. Whole blood cultures were set up at the same time to measure spontaneous ^{3}HT uptake.

A granulocyte preparation was produced in one patient studied during the acute phase of the illness. During the preparation of the suspension of purified lymphocytes for B cell studies, red cell and granulocytes pass through the FCS gradient. In one patient these were recovered and layered onto a Ficoll/Hypaque mixture to remove the red cells as described in Chapter II. A white cell suspension containing 98% granulocytes was thus obtained. The cells were then used to set up spontaneous transformation cultures.

The number and type of cells in the peripheral circulation active in spontaneous DNA synthesis were estimated using the autoradiographic technique described in Chapter II.

3. Results

(1) B lymphocytes

More studies were done with the immunofluorescent technique than with the autoradiographic technique. Table VI.1. shows the proportion and calculated total number of B cells using the former technique. Patients with hepatitis had a significantly lower proportion of B cells than did normal subjects only during the first two weeks of their illness ($t = 2.30$; $P<0.05$). In the later stages of the disease this proportion rose towards normal. There was no statistically significant difference in the calculated total B cell numbers in patients with hepatitis and normal subjects. The results were very similar when B cells were measured by the autoradiographic technique although the number studied was smaller (Table VI.2). There was a correlation between the results obtained with the two different methods ($r = 0.71$; $P<0.05$).

When monospecific antiglobulin sera were used the proportions of IgG, IgA and IgM labelling lymphocytes were found not to be significantly different from normal (Table VI.3). The sum of cells labelled by each monospecific Ig conjugate was higher than the number labelled with the polyvalent conjugate.

TABLE VI. 1.

B Cells in Hepatitis: Immunofluorescent Technique

	Normals		1-2 Weeks		2-4 Weeks		4-6 Weeks	
	%	Total/ µl	%	Total/ µl	%	Total/ µl	%	Total/ µl
$\bar{X}$	15·2	303	10·8	284	11·7	281	12·6	351
SD	4·5	150	2·5	81	1·8	65	1·8	106
n	27	27	6	6	5	5	5	5
Spont. ^{3}HT	370 ± 150		4,500 ± 2,800		900 ± 490		530 ± 49	

TABLE VI. 2.

B Cells in Hepatitis:

Immunofluorescent and Autoradiographic Techniques

	1 - 2 Weeks				2 - 4 Weeks			
	%		Total/µl		%		Total/µl	
	Fluor.	Autor.	Fluor.	Autor.	Fluor.	Autor.	Fluor.	Autor.
$\bar{\chi}$	12·8	12·6	330	316	11·3	10·9	334	311
SD	0·7	2·5	71	62	1·6	1·5	131	86
η	4	4	4	4	6	6	6	6

TABLE VI 3.

Percentage of IgG, IgA and IgM Labelling Lymphocytes in Hepatitis.

		Normals	1-2 Weeks	2-4 Weeks	4-6 Weeks
IgG	$\bar{\chi}$	9·8	9·8	7·6	6·0
	SD	2·0	4·4	4·0	2·9
IgA	$\bar{\chi}$	5·7	5·7	4·1	4·8
	SD	2·4	3·3	1·0	2·0
IgM	$\bar{\chi}$	9·1	10·6	7·0	8·5
	SD	2·3	4·2	0·8	1·0
η		9	6	5	6

(2)Granulocytes

The patient in whom spontaneous ^{3}HT uptake of a granulocyte preparation was studied was in the acute phase of hepatitis. The spontaneous ^{3}HT uptake of her lymphocytes was elevated when measured in whole blood cultures (3,420 $\pm$ 76 dpm). The uptake in the granulocyte preparation was very low (110 $\pm$ 13 dpm/10^{6} granulocytes).

(3)Spontaneously Transforming Cells

^{3}HT autoradiographs were available in 5 patients with hepatitis studied during the first 3 weeks after the onset

of jaundice. The proportion of cells labelled was 11·0 ± 7·6% during the first week and 3·7 ± 2·8% during the 2nd. and 3rd. weeks. The labelled cells were all large cells.

4. Discussion

The proportion of B cells in normal control subjects as estimated by the fluorescent technique is smaller than that reported by other workers (Wilson and Nossal 1971; Stjernswärd, Jondal, Vanky, Wigzell and Sealy 1972). This probably reflects differences in the technique but the significant correlation between the fluorescent and autoradiographic techniques confirms the reliability of the method. There is a significant reduction in the proportion of B cells in patients with hepatitis in the first two weeks after the onset of jaundice but the total number of cells was not reduced. This reflects the lymphocytosis present in the acute phase of the illness. Spontaneous ^{3}HT uptake was high in the patients studied during this period and it would thus appear that the elevation of spontaneous uptake is not due to proliferation of B cells.

The study of IgG, IgA and IgM labelling cells was inconclusive. The percentage of cells within each group was lower in the 2 - 4 week and 4 - 6 week periods than the values in normal subjects but not significantly so. The

results in the 2 patients with hepatitis B were similar to those in the 4 patients with hepatitis A, although the former had a lower proportion of IgM labelled cells than in the latter. IgM levels are known to be lower in hepatitis B than in hepatitis A.(Chapter IV). A larger study of Ig labelling cells in the two types of hepatitis would therefore be of interest.

The sum of the cells labelled by the nonspecific Ig conjugates was higher than the number labelled with the polyvalent conjugate. This phenomenon might be due to cross reactivity of the monospecific conjugates but this was excluded when the specifity of the conjugates was confirmed by immunoelectrophoresis. The polyvalent conjugate contained IgG, IgM and IgA so should have labelled all the B cells present. It is possible that the higher labelling reflects either nonspecific adsorption of Ig on the surface of lymphocytes not removed in the washing procedure or labelling of macrophages.

In their studies on spontaneous ^{3}HT uptake during homograft rejection, discussed in Chapter 4, Dimitriu et al. (1971) attributed the increased spontaneous ^{3}HT uptake to the presence of myeloid cells consequent on corticosteroid therapy. In hepatitis the peripheral blood picture shows a lymphocytosis with many atypical lymphocytes and no evidence of a "shift to the left" of the granulocytes which

might suggest the presence of primitive granulocytes. No patient included in the present study received corticosteroids in the acute phase of the disease. Moreover, spontaneous ^{3}HT uptake in a granulocyte preparation was very low in the one patient studied at a time when the whole blood culture spontaneous ^{3}HT uptake was high. It is therefore possible to exclude the granulocyte series as a significant contributor to the increased spontaneous DNA synthesis which is seen in the acute stage of hepatitis.

Autoradiography using ^{3}HT confirmed that there was indeed an increase in the proportion of cells active in spontaneous DNA synthesis circulating in the peripheral blood of patients with hepatitis. These cells were large blast like cells and were probably identical to the atypical lymphocytes present in the peripheral blood. These results confirm those of other workers (Horwitz et al. 1970; Wood and Frenkel 1967). In infectious mononucleosis these cells have been identified as T lymphocytes (Sheldon et al. 1973).

The results of the studies reported in this Chapter support the suggestion that the elevation of spontaneous transformation in acute hepatitis is due to a proliferation of T cells rather than B cells and that these prolificating T cells are probably the atypical lymphocytes seen in the peripheral blood smear.

CHAPTER VII

LYMPHOCYTE REACTIONS TO AUSTRALIA ANTIGEN

CHAPTER VII

1. Introduction

Dudley et al. (1972)[a] suggested that a cell mediated immune reaction to Au could account for the clinical manifestations of hepatitis B. Evidence to support this came from Yeung Laiwah (1971) who demonstrated lymphocyte transformation in response to Au containing serum in patients who had previously had hepatitis B. Using the leucoyte migration inhibition technique, Dudley et al. (1972)[b] showed that both Au positive serum and liver tissue homogenates from Au positive patients produced an inhibition of migration in patients with hepatitis B, though this was not detectable 3 months after the onset of the disease. However, neither of these studies included patients with hepatitis A and in neither were purified preparations of Au used. For these reasons it was decided to investigate the effect of various preparations of Au on the lymphocytes of normal subjects and of patients with hepatitis A and hepatitis B.

2. Patients and Methods

(1) Patients

Lymphocytes were obtained from patients with hepatitis A and hepatitis B and from normal persons. The

latter were healthy members of laboratory staff.

(2) Australia antigen preparations

Three preparations were used during this study. The first consisted of serum taken from patients with hepatitis B during the acute phase of their illness when Au was detectable by the crossover electrophoresis method described in Chapter II. The pooled serum was strongly Au positive and 0·15ml. of this serum was added to each culture.

The second preparation was partly purified. Au containing blood obtained from the Red Cross Blood Transfusion Service was spun at 3,000 rpm for 30 minutes and the supernatant removed. This supernatant was then spun at 22,000G for 2 hours, the supernatant removed and the pellet of Au particles resuspended in Dulbecco phosphate buffer (DPB). The presence of Au particles was confirmed by electron microscopy and the protein concentration was determined by the Biuret method. During this part of the study two similar preparations were used, Preparation I and Preparation II. These were produced from differing pools of Au positive blood. Dose response curves were determined for each patient.

The third preparation consisted of purified Au particles obtained commercially (Electro Nucleonics Labs.

Inc., Bethesda, U.S.A.) and contained $2 \cdot 0 \times 10^{14}$ particles/ml. An aliquot was removed and diluted with DPB. Further dilutions were made in medium 199 so that particle concentrations of $0 \cdot 01 \times 10^{6}$, $0 \cdot 1 \times 10^{6}$, 1×10^{6}, 100×10^{6} and 100×10^{9} could be added to the cultures in a standard volume.

(3) Lymphocyte cultures

Two sets of triplicate whole blood cultures were set up as described in Chapter II. To one set was added the Au preparation and the other set acted as a control. Cultures were terminated after 5 days incubation as other workers in the laboratory had previously established this to be the time of maximum ^{3}HT uptake in lymphocytes stimulated by specific antigen.

Cultures containing the first Au preparation made from hepatitis serum were set up using lymphocytes from 5 patients with hepatitis B three to four weeks after the onset of jaundice. Studies using the partly purified preparations I and II were performed on a total of 5 patients with hepatitis B, one patient with hepatitis A and 2 normal subjects. Two of the patients with hepatitis B were still mildly jaundiced, two were in the early convalescent period and one was in the late convalescent

period. The single patient with hepatitis A was studied during the early convalescent period.

The final group of experiments using the commercial purified Au particles were performed on 5 patients with hepatitis B and one patient with hepatitis A. One patient was still jaundiced and the remainder were in the early convalescent period. The results from these 6 patients were compared with those obtained in 3 normal control subject.

3. Results

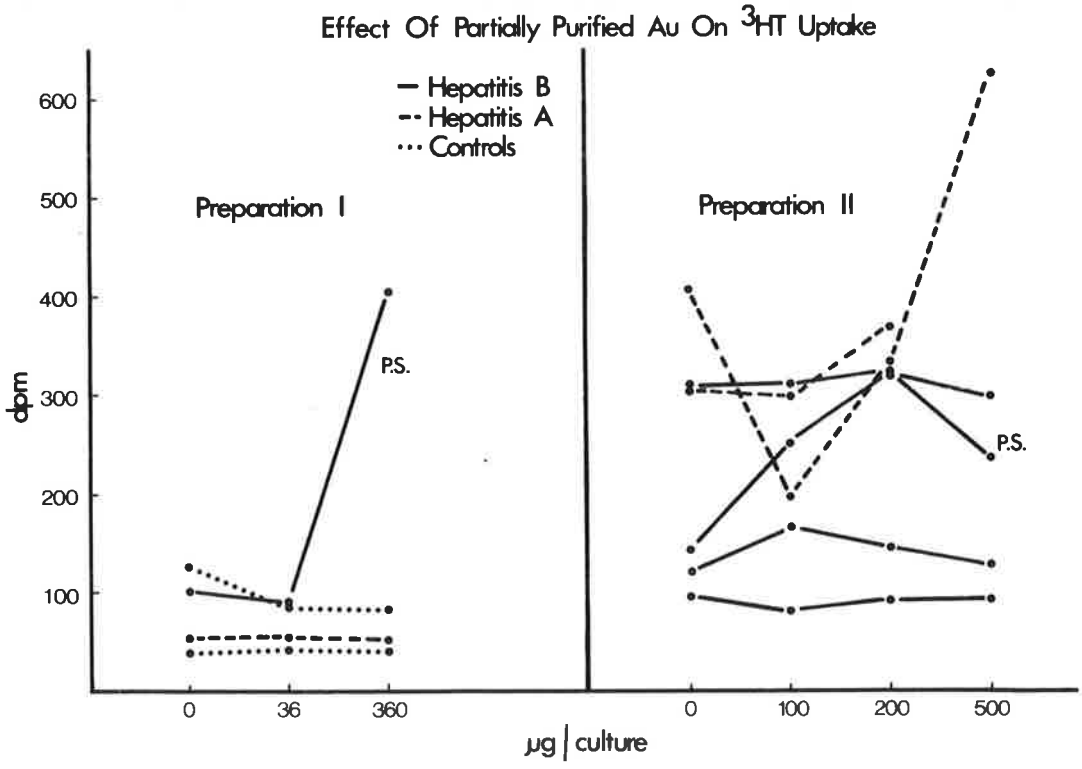
(1) Pooled hepatitis B serum

The results seen in Table VII.1 show that this Au containing preparation had little effect on the ^{3}HT uptake. A small rise in ^{3}HT uptake was detected in 2 patients but was not significant. In one patient (L.K.) a marked inhibitory effect was produced.

(2) Partially purified Au preparations

In the initial experiments with Preparation 1, a marked increase in ^{3}HT uptake was noted in the one patient (P.S.) who had had hepatitis B 3 months previously (Fig.VII.1). This effect was not seen in two normal control subjects and in the one patient who had had

Figure VII 1.



Effect of different doses of two partially purified Australia antigen (Au) preparations on the ^{3}HT uptake (dpm) of lymphocytes from patients with hepatitis A, hepatitis B & normal control subjects. Each point represents the mean of triplicate cultures. P.S. refers to one patient (see text).

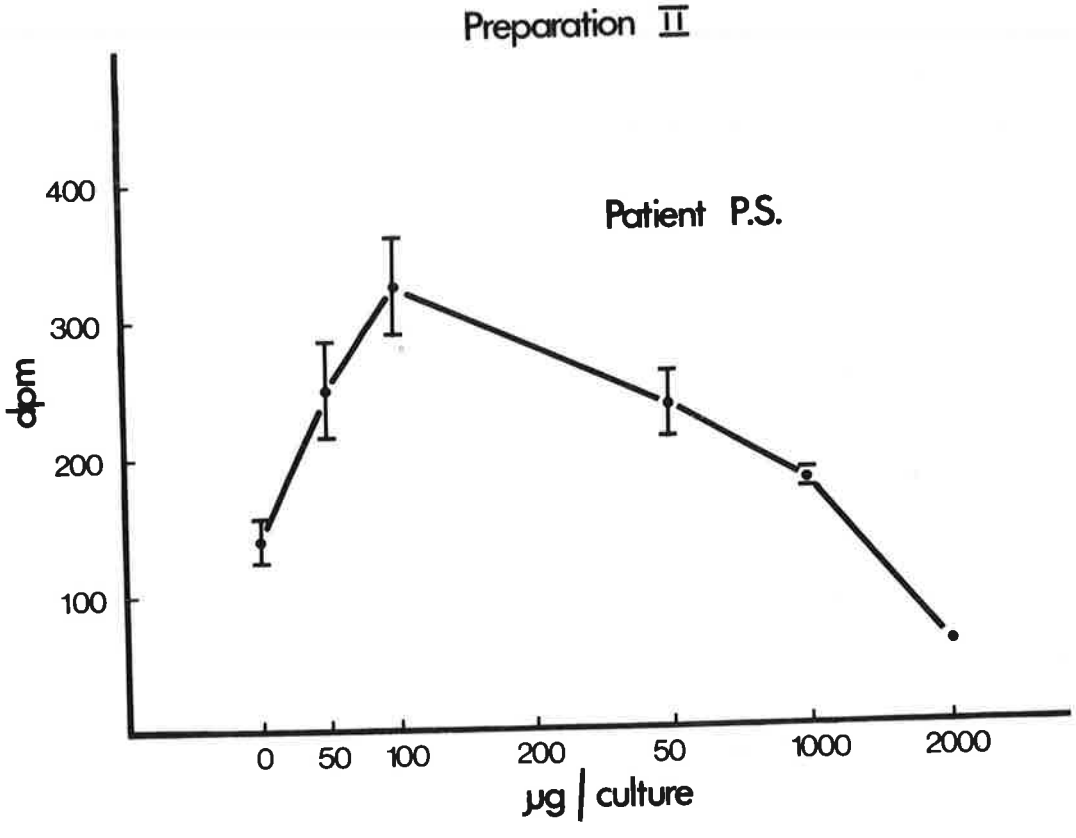
TABLE VII.1.

Effect of hepatitis serum positive for Au on ^{3}HT uptake of patients with hepatitis B, 3 - 4 weeks after the onset of jaundice.

Patient	Control (dpm)	Au (dpm)	Bilirubin
G.B.	123 ± 20	144 ± 16	2·1
L.K.	134 ± 30	46 ± 3	1·0
S.A.	164 ± 1	149 ± 30	0·9
S.A.	244 ± 50	220 ± 6	0·6
G.H.	167 ± 10	141 ± 10	1·0
G.H.	66 ± 19	53 ± 11	0·6
M.M.	147 ± 18	180 ± 37	1·5

hepatitis A. The same patient (P.S.) was used to establish a dose response curve for Preparation II (Fig.VII.2) and on the basis of this curve amounts of 100µg, 200µg, and 500µg protein/culture were used in further studies (Fig.VII.1). Of the 4 patients with hepatitis B, only the one patient (P.S.) showed a significant increase in ^{3}HT uptake. One of the patients with hepatitis A showed a marked decrease in uptake with a dose of 100µg/culture but both patients with hepatitis A showed an increased uptake at the higher doses.

Figure VII 2.



Effect of different doses of a partially purified Australia antigen (Au) preparation (II) on the ^{3}HT uptake (dpm) of lymphocytes from one patient (P.S.). Each point represents the mean of triplicate cultures & the vertical bars the S.D.

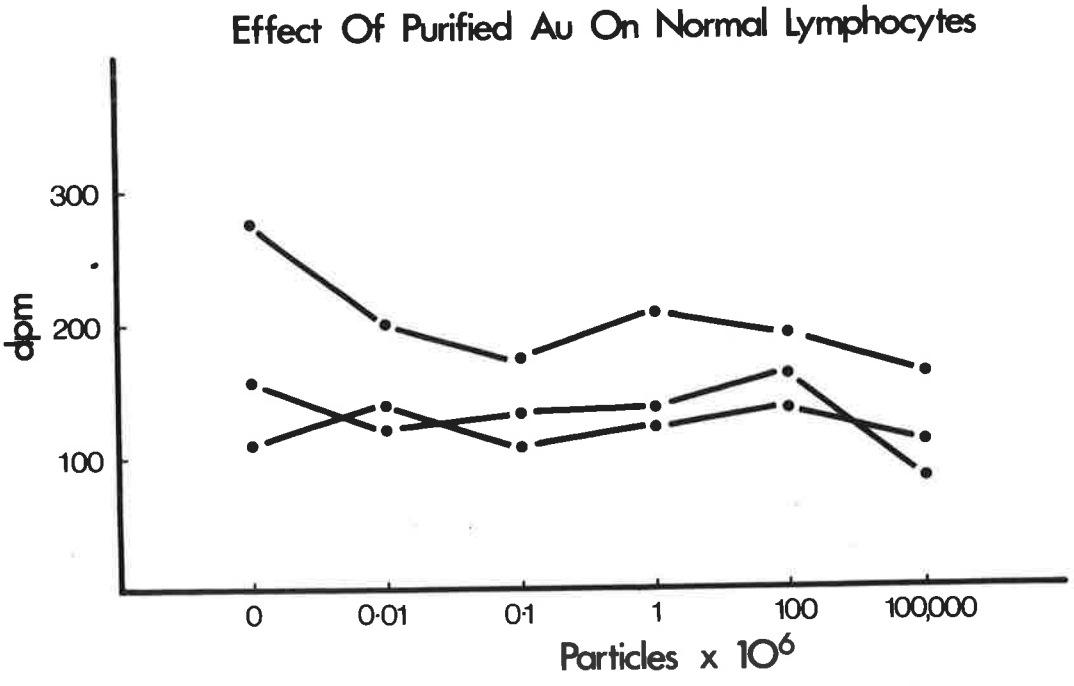
(3) Purified Au particles

Purified Au particles appeared to produce a mild inhibitory effect on cultures from normal subjects (Fig.VII.3). Similar responses were seen in 4 of the 5 patients with hepatitis B (Fig.VII.4). A marked inhibition was seen in one patient with hepatitis A.

4. Discussion

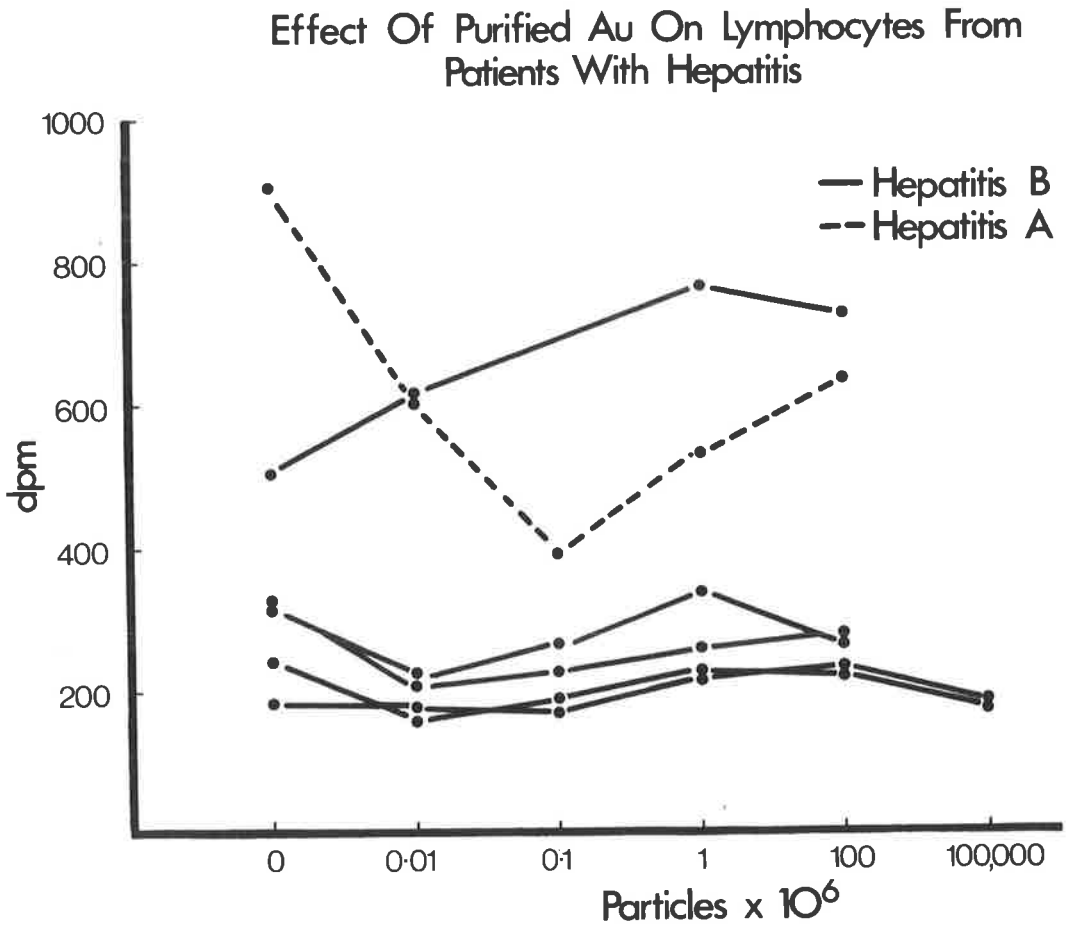
The results show that Au containing preparations will only rarely stimulate lymphocyte transformation, as measured by an increase in ^{3}HT uptake, in patients with hepatitis B and that some patients with hepatitis A may react in the same manner. These results conflict with those of Yeung Laiwah (1971) and Dudley et al. (1972)[b]. However, the Au preparations which these workers used consisted of Au containing whole serum and the variable effect that human serum has on lymphocyte cultures has already been demonstrated in Chapter 5. Both Yeung Laiwah (1971) and Dudley et al. (1972)[b] studied only patients with hepatitis B whom they compared to normal subjects and to patients with chronic liver disease but not to patients with hepatitis A. The specificity of the response obtained by these workers is therefore not certain. To support the hypothesis of Dudley et al. (1972)[a] it would

Figure VII 3.



Effect of different doses (particles x 10^6) of a commercially prepared purified Australia antigen (Au) preparation on the ^{3}HT uptake (dpm) of lymphocytes from three normal control subjects.

Figure VII 4.



Effect of different doses (particles x 10^6) of a commercially prepared purified Australia antigen (Au) preparation on the ^{3}HT uptake (dpm) of lymphocytes from 5 patients with hepatitis B and 1 patient with hepatitis A.

be necessary to establish a response to Au in only those patients who had had hepatitis B. The demonstration by Dudley et al. (1972)[b] that liver homogenates from patients with hepatitis B can produce inhibition in the leucocyte migration test is interesting but may also not be entirely specific. Because of its important implications it needs confirmation, particularly in the light of recent studies by Meyer zum Büschenfelde et al. (1971) and Miller et al. (1972) who have isolated an organ specific protein in normal liver which can induce a CMI response in CAH and PBC.

The failure to induce lymphocyte transformation specifically in patients with hepatitis B with purified Au particles would appear to exclude this antigen as a major determinant of CMI reactions in hepatitis. It is possible that the purification procedures altered antigenic determinants responsible for lymphocyte activation but not the Au determinant. Subtypes of Au have been described (Nielson and Le Bouvier 1973) and it is possible that hepatitis B in the patients whose lymphocytes did not react had been due to Au of a different antigenic subtype to that used for the studies in vitro. This is unlikely because the preparations used in this study came from differing and pooled sources.

The studies reported here and also those of Dudley et al. (1972)[b] and Yeung Laiwah (1971) were performed on limited numbers of patients. Now that purified Au preparations are available it would seem worthwhile to carry out serial studies in a larger series of patients with hepatitis and other liver diseases using lymphocyte transformation techniques and more specific tests of CMI, such as the leucocyte migration inhibition test, in an attempt to provide direct evidence for or against the hypothesis proposed by Dudley et al. (1972)[a].

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APPENDIX

STATISTICAL METHODS

PARAMETRIC TESTS

1. Characterisation of frequency distributions

Characterisation of frequency distributions was carried out using Fisher's g_1 (skewness) and g_2 (kurtosis) statistics (Bliss, 1967).

2. Student's t test

Significance of difference between the means of two populations was performed for paired and unpaired data using Student's t test as described by Dixon and Massey (1969).

3. Regression analysis

Regression analysis, including correlation coefficient and tests of significance between slopes and intercepts, were carried out as described by Mather (1964). Since the biological results being evaluated could be expected to show an exponential fall with time, it was decided to perform regression analysis on untransformed and logarithmically transformed data. The simple linear form used in the analysis was $y = mx+c$ and for the exponential form $\log y = x \log m+\log c$. The decision to accept the linear and exponential

relationship was made on the value of the correlation coefficient.

NON PARAMETRIC TESTS

In small samples with a wide range of values, comparisons were made using non-parametric tests. In these cases it was difficult to define the exact nature of the population distribution. The following tests were used as described by Siegel (1956).

1. Mann Whitney U test

   The Mann Whitney U test is performed on ranked data. It is one of the most powerful non-parametric tests and a most useful alternative to the parametric t test when it is wished to avoid the assumptions of the t test.

2. The Sign Test and the Wilcoxon Matched Pairs Signed Ranks Test

   These tests were used in the situation where two related samples were being analysed and where the data itself covered a wide range giving no indication as to the nature of the population distribution.

3. Kruskal Wallis One Way Analysis of Variance

   This test was applied in the situation where inferences had to be made from more than two independent populations.

All calculations were performed on a Wang 601 programmable electronic calculator with output typewriter.

STATISTICAL ABBREVIATIONS

SD	Standard deviation
±	Infers SD
$\bar{X}$	Mean
SEM	Standard error of the mean
P	Probability
df	Degrees of freedom
m	Slope
c	Intercept
r	Correlation coefficient
V or V%	Coefficient of variation
t	Statistic for evaluating probability in Student's t test
U	Statistic in Mann Whitney U test
z	Statistic for evaluating probability in Mann Whitney U test
H	Statistic used in Kruskal Wallis One Way Analysis of Variance by ranks
g_1 & g_2	Fisher's statistics for skewness and kurtosis

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