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Aspects of Monocyte Function in Cirrhosis

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CHAPTER 1

HISTORICAL REVIEW AND GENERAL INTRODUCTION

(a) CIRRHOSIS

"The liver, reduced to a third of its ordinary size, was, so to say, hidden in the region it occupied; its external surface, lightly marmellated and wrinkled, showed a greyish yellow tint, indented, it seemed entirely composed of a multitude of small grains, round or oval in form, the size of which varied from that of a millet seed to that of a hemp seed. These grains, easy to separate one from another, showed between them no place in which one could distinguish any remnant of liver tissue itself; their color was fawn or a yellowish russet, bordering on greenish; their tissue, rather moist, opaque, was flabby to the touch rather than soft and on pressing the grains between the fingers one could not mash but a small portion; the rest gave to the touch the sensation of a piece of soft leather"

So wrote Laennec in his classical description of cirrhosis in 1819; but even earlier; various workers such as Vesalius and Morgagni had described shrunken nodular livers. References to hardened livers are also to be found in early Greek and Egyptian literature.

Laennec recognized that the cirrhosis was in some way related to alcohol, a fact also previously recognized by Vesalius (1543) and Baillie (1793). Despite this early link it is sobering to realize that it is only relatively recently that direct proof that excessive alcohol intake resulted in cirrhosis was obtained from studies in baboons. Similarly while the relationship of cirrhosis to alcohol has become much clearer from epidemiological studies comparing alcoholic intake and the prevalence of cirrhosis in various communities (Lelback, 1976) the reasons why some patients develop cirrhosis but others do not while apparently drinking the same amount of alcohol is not known. Attempts to explain this phenomenon have been unconvincing and have ranged from coexisting malnutrition and vitamin deficiency (Hall and Morgan, 1939); genetic factors (Sherlock, 1970) and sex linkage (Spain, 1945). Even infections such as syphilis and tuberculosis (Fleming, 1942) have been implicated, this because they are so commonly encountered in cirrhotic patients. It is now realized that these factors are mainly coincidental and dependent on the life style of the alcoholic patient and have no direct role in the pathogenesis of the disease.

The first detailed histological description of the cirrhotic liver was probably that of Carswell (1838). Laennec had considered that the fibrous septa in the cirrhotic liver were new structures resulting from new connective tissue formation, and thus the concept of fibrogenesis as being an integral part of the cirrhotic process has been with us for more than a century. Although this was clarified by Rossle (1930) in his definition of cirrhosis which still holds good today [(1) hepatic parenchymal necrosis, (2) active formation of connective tissue, (3) regeneration of the liver cell in disorientated nodular fashion)], we have come little further along the path towards greater understanding as to why this fibrogenesis occurs in the way that it does. The more recent advances in hepatology have come as a result of the ability to obtain specimens of liver from living patients for histological review. Liver biopsy was probably first performed by Ehrlich (Von Frericks, 1884) but was developed into a clinically useful procedure by Schupfer (1907) and Josefson (1921). These and later studies have lead to various classifications of liver disease and to a greater understanding of them. This has been helped by advances in the fields of biochemistry, and more recently, immunology.

(b) IMMUNOLOGY

In brief the history of immunology can be divided into four main periods. Firstly immunity, secondly serology, thirdly immunochemistry, and fourthly seroimmunology. Important dates in the history of the four sections are summarized in Table I.

(1) Immunity Against Infectious Diseases

It has long been known that infectious disease rarely attack the same individual twice. Human to human vaccination was in practice in the eigthteenth century against smallpox. Jenner used a cow-produced vaccine which protected against smallpox and Pasteur in the mid eighteenth century produced the first antibacterial vaccines.

(2) Serology

Early on in the development of vaccination much attention was given to both the humoral and cellular basis for immunity. Earliest interest centered on the humoral system and it wasn't until the late nineteenth

CHAPTER I. Table I: EARLY LANDMARKS IN IMMUNOLOGY

1721	Lady Montaga	Intrahuman vaccination
1798	Edward Jenner	Bovine vaccination
1880	Louis Pasteur	Attenuation of chicken cholera bacillus
1884	Elie Metchnikoff	Discovery of phagocytosis
1885	Louis Pasteur	Rabies vaccinaton
1890	Robert Koch	Koch phenomena and delayed hypersensitivity
1890	Emil Behring	Antitoxins
1896	Marc Gruber + Herbert Durham	Agglutination tests
1901	Karl Landsteiner	Blood groups
1902	Charles Richet + Paul Portier	Anaphylaxis
1903	Maurice Arthus	Local hypersensitivity
1905	Clemens Von Pirguet	Serum sickness disease
1929	Michael Heidelberger	Quantitative chemical serology
1932	Gaston Ramnon	Toxiod immunisation
1938	Elvin Kabat	Antibodes as gamma-globulins
1942	Albert Coons	Immunofluorescence
1946	Jacques Oudin + Orjan Outcherlony	Immunodiffusion
1953	Peirre Grabar	Immunoelectrophoresis
1957	Alick Isaacs	Interferon
1958	Jean Dausset	Human leukocyte antigens
1958	Peter Medawar	Tolerance phenomenon
1958	Rodney Porter	Immunogloblulin structure
1959	James Gowans	Lymphocyte function
1962	Jacques Miller	Effect of neonatal Thymectomy

century and early twentieth century that tests such as agglutination and precipitation techniques were developed and allowed further study of humoral immunity.

(3) Immunochemistry

Following the recognition of antigens and antibodies; allowing for the introduction of blood transfusion techniques, interest in this field grew quickly. In the 1950's and early 1960's the structure and function of immunoglobulins was described.

(4) Cellular immunology

Although Koch first described the delayed hypersensitivity reaction in 1890, the importance of the lymphocyte and the cellular basis of the delayed hypersensitivity reaction, did not emerge until the 1960's. Since which time lymphocytes have been divided into two basic subgroups (ie) T-cell responsible for cell mediated immunity and the B-cell responsible for humoral ability. Further differentiation into many other subclasses has now becone possible each with differing function.

(5) The Monocyte

The phagocytic action of monocytes has long been recognized, but the importance of monocyte as regulators of the immune response has been recognized only relatively recently. Following the discovery of antibodies and complement, interest waned, until recently, when the regulatory aspects of the monocyte on the lymphocyte became apparent. It is interesting to note that the number of publications on lymphocytes outnumber those on monocytes 5:1 and it is no coincidence that this ratio is similar to that of lymphocytes to monocytes in peripheral blood.

IMMUNOLOGY OF LIVER DISEASE

Most interest in this field has concerned chronic active hepatitis. Soon after its description (Waldenstrom, 1950) it became apparent that there was a high frequency of multisystem disease in these patients e.g. Sjögrens syndrome, thyroid disease, and arthritis (Golding, 1973), all

of which have a putative autoimmune pathogenesis. This link was further clarified by the realization that chronic active hepatitis patients were more likely to be HLA B_8 positive than controls (Mackay and Morris, 1972) and that this antigen was also encountered more often than could be expected in a number of other putative autoimmune conditions.

Patients with chronic active hepatitis, and indeed other forms of chronic liver disease, were found to have marked hyperglobulinaemia and increased levels of immunoglobulins (Feizi, 1968). They were also found to exhibit autoantibodies to a range of different antigens (Doniach et al, 1966). In only the most recent reports have a specific antigen or antibodies been described namely liver specific lipoprotein (Targe-Jensen et al, 1977) and even the specificity of this antibody has been questioned by some and certainly remains to be clarified (Chisari, 1980). As well as evidence of an over-active humoral response, evidence has emerged of impaired cell mediated immunity in patients with various forms of liver disease with reduced in vivo skin testing and in vitro lymphocyte transformation response to plant mitogens. Similarly, some evidence suggests that lymphocyte subpopulations are altered in the cirrhotic patients with reduced numbers of circulating lymphocytes (Dehoratious, et al. 1974). Studies on cell mediated immunity to liver specific lipoproteins have resulted in conflicting results but the most recent studies have suggested that abnormal responses are not confined to patients with chronic active hepatitis but are also found in other groups of cirrhotic patients and occasionally in controls (Thestrup-Peterson, 1976). Attempts to define the pathogenesis of chronic active hepatitis have led to various studies of lymphocyte cytotoxicity in these patients. These reports have also been conflicting and have not always proved reproducible. Peripheral blood lymphocytes from patients with chronic active hepatitis have been shown to be cytotoxic for autologous liver cells (Wands and Isselbacher, 1978; Geubel, 1976), Chang cells (Wands et al., 1975) and autologous hepatocytes (Thompson et al., 1974) and there is some evidence to suggest that the cytotoxicity mirrors the clinical activity of the disease (Cochrane et al, 1978). It appears that the cytotoxicity is non T-lymphocyte dependent (Vergani et al., 1979) and there is evidence that it is HLA dependent (Vogten, 1979).

It has proved difficult to convincingly tie all these observations together into a rational theory for the autoimmune basis for hepatocyte injury but two theories have emerged. One suggests that there is a failure of the host to eliminate the antigen e.g. hepatitis B virus; the other that the production of an autoantibody in response to an antigen results in damage to the liver cell. Thus, Dudley, Fox and Sherlock (1972) suggest that the competence of the cellular mediated response determines whether an infection is self-limiting or persists, whilst Eddleston and Williams (1974) suggest that as well as the T-cell response to the viral antigen on the surface of the affected hepatocyte, elimination of the virus is also dependent on a viable B cell function and further suggest that the excessive antibody production seen especially in patients who develop chronic active hepatitis may be due to defective suppressor T-cell function. Recently weak suppressor activity has been demonstrated in chronic active hepatitis (Hodgson et al., 1978) and since comfirmed by others. In systemic lupus, a disease closely related to chronic active hepatitis, the excess immunoglobulin production, which is a feature of the disease, has been shown to be due to this weak suppressor activity.

In other forms of liver disease (e.g.) alcoholic liver disease or drug-induced liver disease controversy remains as to the part played by an immunological reaction. Alcoholics have impaired cellular immunity and it has been suggested that there may be an immunological response possibly against alcoholic hyalin, especially in alcoholic hepatitis (Levy, Chen, and Zeterman, 1975). Autoantibodies are also found in alcoholic cirrhotics but are less common than in chronic active hepatitis. Further support for a possible autoimmune factor in alcoholic liver disease has emerged from the recently reported differences in presentation in men and women, the disease being more severe in women who are known to have an increased incidence of autoimmune disease. On the other hand there is no established HLA linkage to HLA B8 or any other tissue antigen. Recently impaired generation of suppressor cells has been shown in patients with alcoholic hepatitis (Kawanashi et al., 1981). Similarly in the alcoholic there may be an association between the presence of liver membrane lipoprotein and the histological finding of piece meal cirrhosis (Perperas et al, 1981).

MONOCYTE AND MACROPHAGE KINETICS

Metchnikoff (1905) and Aschoff (1924), using histological methods, were the first to demonstrate that monocytes developed into tissue macrophages. More direct evidence that peripheral monocytes were indeed the precursor was obtained by Lewis (1925). Ebert and Florey (1939), using rabbit ear chambers, confirmed this by showing that monocytes containing phagocytosed carbon particles migrated from the blood vessels into connective tissue and formed tissue macrophages. Hence it is now well recognized that the cell line of the mononuclear phagocyte, consisting of monoblasts, promonocytes, and macrophages (Van Furth, 1968) all originate from the same cells. This assumption has not been universally accepted and various workers have suggested that macrophages are derived from other mesenchymal cells, lymphocytes, or that they are self-replicating cells (Rebuck and Crowley, 1955). Staining differences between macrophages and monocytes, showing a different distribution of peroxidase staining (Daems et al, 1976), and the absence of labeled macrophages in unlabelled partners of parabiotic rats (Volkman, 1976) were used to argue against a monocyte origin of these tissue macrophages. Indeed there is little doubt that hepatic macrophages do have the potential for renewal (North, 1969; Souhami and Bradfield, 1974). Chimaeric studies (Shand and Bell, 1972; Balner, 1963, Bell and Shand, 1972), labelling studies with monocyte depletion (Van Furth and Cohn, 1968), and investigations on bone marrow cultures (Goodman, 1954) have now satisfied most experts in the field that macrophages do derive from the bone marrow and that monocyte-macrophage systems can be viewed as the continuum of progressively more mature cells, beginning with monoblasts and promonoblasts within the bone marrow. After two or three cell divisions promonocytes mature into monocytes which probably only circulate in the blood for as little as two hours before migrating into tissue and differentiating into mature macrophages. As the monocyte enters the tissue there are histological changes and the cells enlarge reaching 5 to 10 times their original size. Dense granules (lysosomes) and other cytoplasmic structures became more prominent (Cohn et al., 1964 and 1965). Accompanying these changes are increases in the content of lysosome enzymes including acid phosphatase, B glucosamidinase, lysozyme and aryl-sulphatase (Nichols and Bainton, 1973; Bennett and Cohn, 1966).

In humans it is difficult to obtain adequate numbers of tissue macrophages to study. Attempts have been made to study alveolar, peritoneal and gut macrophages. This involves long and tedious enzymatic and mechanical methods of separation and it is difficult to be sure that these are not altering function. Monocytes are easily obtained from peripheral blood and it would be satisfactory if their function mirrored that of tissue macrophages. Unfortunately , to date, very little work has been done in this field but studies comparing the function of tissue macrophages obtained from different sites have shown differences in bactericidal activity (Pavillard, 1963), chemotaxis (Ward, 1968), immunogenicity of ingested antigens (Cohn, 1965) and in other functions, and a clear cut relationship between macrophages from different sites seems unlikely. Even macrophages produced from circulating monocytes by incubation on plastic for several days show differing effects on the modulation of lymphocyte proliferation as monocytes cause enhancement while the macrophage cause suppression (Rinhart et al., 1979).

This functional diversity between tissue macrophages can be attributed in part to their further differentiation into more specialized cells and does not necessarily argue against their common site of origin (Walker, 1976). We do not know if a depression of a function of the circulating monocytes mirrors that of the tissue macrophages either qualitatively or quantitatively. It is probable that there is at least some similarity but with circulating monocytes having such a short lifespan in the blood stream it is always possible that rather than seeing altered function of peripheral blood monocytes in various disease situations one is simply studying a different cell population due to an altered turnover rate and that abnormalities in different diseases indicate altered kinetics.

One problem is accurate identification of monocytes. Study of mononuclear cells by light, or phase contrast, microscopy help to define the cell as an immature or mature mononuclear phagocyte but the application of morphological criteria alone does not allow for accurate identification of all monocytes. Thus stains utilizing enzyme activities e.g. peroxidase and esterase (Braunstein et al., 1968) and functional capacities e.g. endocytosis (Carr,1973) are required for certain identification.

FUNCTION OF THE MONOCYTE/MACROPHAGE SYSTEM

General Introduction

Perhaps the best understood function of these cells is their action as scavengers, removing dead cells and cellular debris. Thus monocytes are encountered at sites of tissue damage and repair. These cells also form, in partnership with the more quickly acting neutrophils, a defence mechanism to microbial infection, ingesting and killing bacteria and fungi. The monocyte is the prime defence mechanisms in various intracellular infections such as tuberculosis or leprosy. The cell must first arrive at the site of injury or infection and thus one function is to migrate to the involved area and this is probably the result of release of a soluble chemotactic factor by the injured tissue.

The monocyte/macrophage also interacts with lymphocytes in both the afferent and efferent limb of the immune response and thus plays a role both in cellular and humoral responses.

More recently defined are their function as controller of granulopoesis and fibrogenesis and a crucial role in iron metabolism. Similarly the monocyte-macrophage is important in host response to tumour and is the site of synthesis of certain proteins. These main functions will be dealt with separately below.

Chemotaxis

One mechanism that could account for the local accumulation of macrophages is the unidirectional migration of cells along the concentration gradient of a chemotactic substance. Most studies in this field have resulted from the quantitative measurement of *in vitro* chemotaxis using the methods initially described by Boyden (1962). This technique essentially involves the movement of cells through porous filters from an upper chamber containing cells to the lower containing the chemotactant. An 8 mm pore filter is used for monocytes. Either the number of cells which have gone through the filter and appear on the lower surface, or the distance moved by the leading front of the cells after a short incubation period can be used (Zigmund and Hirsh, 1973).

Investigations into the nature of this chemotactant have shown that inflammatory agents such as endotoxin or immune complexes activate complement and cleave a number of C components (Nelson, 1974). It

appears that a fragmentation product (c5a)of the fifth component of complement may be important as demonstrated both in vivo and in vitro (Shin, 1968; Snyderman et al., 1971). The importance of C5a as chemotactant has also been shown by gel filtration and fractionating experiments and the use of monospecific antibodies (Hausman et al., 1972). These observations on chemotaxis are better established for neutrophils than for the monocytes (Wissler et al., 1972) but probably also apply to monocytes. It is known that the proteolytic enzyme released by damaged cells is responsible for the cleavage of complement (Ward and Hill, 1970) and may initiate chemotaxis. Normal serum has been shown to be a chemotactant (Borel and Sorkin, 1969) and the substance proved to be heat labile and to have a molecular weight in the range of 90,000 daltons. Lymphokines produced by lymphocytes exposed to antigens also have chemotactant qualities (Ward et al., 1959). The migration of cells is an active progress requiring energy and depends on myofilament and myofibril formation as well as actin polymerization (Boxer, 1974).

Unfortunately in vivo studies using skin windows have not proved practical or useful for studying monocytes.

Phagocytosis and Bacterial Killing

Once the monocyte-macrophage has reached the site of infection it can begin processing the microbe. Serum factors are vital and coating of the bacteria with immunoglobulin or complement (opsonization) enables the bacteria to adhere to the macrophage utilizing the IgG and complement receptor sites on the macrophage. Bacteria are ingested and phagocytic vacuoles are formed. Metabolic changes occur within the macrophage and there is lysosomal degranulation with release of the active enzyme into the phagocytic vacuole resulting in killing of the organism (Lehrer, 1975; Territo and Cline, 1975).

There are two methods of killing the micro-organism. One is related to the cell's metabolism, the other depends on entry of micro-bacteriacidal constituents into the phagocytic vacuole during the process of degranulation. Phagocytosis results in an increase in the cell's peroxidase metabolism and in its oxygen consumption and leads to the production of hydrogen peroxide, superoxide anion (Barbior et al, 1973) and identifiable chemiluminescent molecules (Allen et al., 1972).

There is also increased oxidization via the hexose monophosphate shunt. These changes result in some direct toxicity and some secondary toxicity due to reaction with the peroxidase enzyme (Klebanoff, 1968).

Methods measuring oxygen consumption and superoxide production (Lehrer and Cline, 1969; Klebanoff and Pincus, 1971) have been introduced to investigate this function and the nitroblue tetrazolium test (Park et al, 1968) enables indirect measurement of glucose oxygenation via the hexose monophosphate shunt. It should be stressed that most of the above observations have emerged from studies on the neutrophil but are also applicable to the macrophage (UCLA Conference, 1978).

The exact role of the antimicrobial substances released from the granules has also been studied. These substances include phagocytin, lysosyme, chymotrypsin like enzymes, and a variety of other substances (Davis, 1976). Stimulation by various substances can result in increases in the production of these substances and this can be measured. From these studies the concept of the activated macrophage has emerged (see below). Unfortunately, although there is a vast mass of literature on this subject, very little more is known today than when phagocytosis was first described over a hundred years ago.

Tests of monocyte function in vivo have mostly depended on the clearance for the blood stream of inactive substances by macrophages and have not proved very useful.

Monocyte Lymphocyte Interaction

The interaction between monocytes, macrophages and lymphocytes is now well established (Machaness, 1970; Unuanue, 1980). Unfortunately because of technical obstacles, and the presence of many variables, it has been difficult to fully elucidate the problem. Monocytes appear to affect the afferent limb by means of antigen processing (Rosenthal, 1980; Unuanue, 1980) and the efferent limb by acting as effector cells (Nathan et al, 1980). Rigorous depletion of adherent cells [mainly monocytes but some lymphocytes (Boyum, 1968)] reduces the proliferative response of non-adherent cells to mitogens (Schmidtke and Hatfield, 1976; Taniguchi et al., 1977), to soluble antigens and to allogeneic lymphocytes (Rode et al., 1974). On the contrary in situations where there is reduced lymphocyte proliferation, removal of glass adherent

cells (i.e.) reducing the number of monocytes present, has been shown to return function towards normal (Berlinger, et al, 1976). Thus, it appears that the macrophages can either enhance or suppress lymphocyte proliferation in response to these stimuli. There is also evidence suggesting that monocytes behave differently to macrophages in these situations (Rinhart et al., 1979). The macrophage is a principal site of control by the immune response gene and antigen handling is inadequate if the lymphocytes and macrophages are not histocompatible (Rosenthal, 1980).

In the efferent loop, macrophages collect, catabolize and eliminate certain immunogenic materials whilst concentrating and retaining other molecules. There is evidence that a small fraction of the immunogenic substances remain in its original native state to aid further responses (Unanue and Ceroltina, 1970) and may remain so for a considerable time. The response of the macrophage to the antigen can be affected by many different factors (Salmon et al., 1971). The mechanism of transfer of information is unclear, but there is evidence that close contact is vital as the monocytes are surrounded by lymphocytes forming an "immunogenic island" (Lipsky and Rosenthal, 1975). The RNA associated antigen may be important in this situation (Adler, 1966). Indeed early studies (Fishman, 1969) suggested that, in the case of peritoneal macrophages, three populations may be present. 1) scavengers; 2) binding antigen; 3) interactive with lymphocytes with formation of RNA antigen complexes. It should be stressed that this interaction between the monocytes and lymphocytes is not a one way step and certain lymphocytes release lymphokines, some which are probably identical to the "macrophage activating factor" (MAF) described by David (1975). Lymphocytes also produce the high affinity antibodies (cytophilic antibodies) which can modulate the affect or function of the monocytes (Mitchel et al, 1973; Pells and Den Otter, 1974). Other substances such as 'specific macrophage activating factors' have also been identified (Evans, 1972) and it should be emphasized that much of the work in this field is not always reproducible. As well as affecting the response, monocytes seem to also be important for lymphocyte viability in culture (Pierce et al., 1974). Similar results can also be achieved by the addition of 2 mecaptoethanol to the mixture. Activated macrophages also produce lymphocyte activation factor (interleukin 1) which enhances the

response to various antigens and mitogens (Gery and Walesman, 1972). Thus, in vitro at least, there is evidence that macrophage direct lymphocytes and lymphocytes direct macrophages. The exact role in vivo remains to be further clarified. Monocytes may also be important in the human mixed lymphocyte response since monocyte depletion diminishes this response (Twomey et al., 1980). This may, however, be an artificial observation as the results differ if the assays are performed in round bottom tube wells or in flat bottom wells (Grier et al., 1980).

Host Response to Malignancy

It is becoming apparent that the monocyte plays an important role in the host response to invasion by tumor (Keller et al, 1975; Remington et al, 1974). Nonspecifically activated macrophages can identify and kill syngeneic tumour cells (Hibbs et al, 1972; Churchill et al, 1975) both in vivo and in vitro (Fidler, 1974). Circulating monocytes also have the ability to kill tumor cells (Holterman et al, 1974). Interaction between the monocyte and the lymphocyte may also be important e.g. the production of cytophilic antibodies. Furthermore the degree of macrophage infiltration of various tumors may have prognostic significance (Eccles and Alexander, 1974).

Role in Granulopoesis and Fibrogenesis

Macrophages are the main source of colony stimulating activity (CSA), a substance vital for culture of granulocyte colonies in vitro (Chervenick et al, 1972). Possible feedback mechanism could therefore exist but in vitro evidence of this is lacking except for the observation of erythroblastic islands observed in bone marrow consisting of a central monocyte surrounded by nucleated red blood cells (Ben-Ishay and Yoffey, 1972). The monocyte may play a role in fibroblast proliferation (Korn et al., 1980) and as such may play a role in the pathogenesis in various fibrotic conditions, e.g. in the lung (Hepplestein and Styles, 1967) or liver (Posselwaite, 1976).

Synthesis of Bioactive Compounds

The ability of the monocyte to synthesize bioactive substances has only recently been discovered and monocytes are thought to synthesize complement components including procoagulant factors, plasminogen

activaters, transferin, interferon, and a colony stimulating activator (Epstein, 1975; Gordon et al., 1974; Rivers et al., 1975). The importance of these substances in normal and abnormal physiological states remains to be determined, but the monocytes may play an especially important role in iron metabolism (Brink et al., 1976).

MONOCYTES IN DISEASE

Quantitative Aspects

Normal monocyte counts ranges from 0-9% of the total white blood count. In terms of absolute numbers, this represents 300-450/mm^3 in adults. More are found in children and there is a gradual decline in numbers with age (Munan and Kelly, 1979). There is slight diurnal variation. The most common causes of monocytosis are shown in Table II. As potent antibiotics have emerged, fewer cases of monocytosis due to infections are encountered than previously reported (Meldonado and Hanlon, 1965; Cassileth, 1979). It is assumed that the monocytosis is, particularly in infection, a secondary physiological response. Monocytosis has been reported in cirrhotic patients (Maldonado and Hanlon, 1965) but is likely to be due in these cases to superimposed infection, to which these patients are prone.

Qualitative Aspects

Experiences with tests of monocyte functions are in their infancy and abnormalitites in disease are not yet fully documented. Thus, many reports of abnormal monocyte function in disease must await supporting evidence before they are accepted without question. Similarly, with the possible exception of the Chediak-higashi syndrome (Gallin et al, 1975), abnormal monocyte function is probably often secondary to the disease. Monocyte chemotaxis has been shown to be defective in patients with mucocutaneous candidiasis (Snyderman et al., 1973, Van der mere et al., 1978; van Furth, 1978), in lung (Kay and Mere, 1978) and breast cancer (Snyderman et al., 1978) and malignant melanoma (Hedley and Currie, 1978). Reduced bacteriacidal activity has been reported in chronic granulomatous disease (Davis, et al., 1968) and other abnormalities in sarcoidosis (Douglas, et al., 1977) and Crohn's disease (Mee, et al, 1980).

TABLE II: CAUSES OF MONOCYTOSIS

1. Haematological disorders (51%)
   (eg) Preleukaemia, myeloproliferative diseases, multiple myeloma, haemolytic anaemia

2. Collagen disorders (19%)
   (eg) Rheumatoid arthritis, SLE, Polyarteritis nodosum, temporal arteritis

3. Malignant disease (8%)

4. Infections (6%)
   (eg) TB, SBE, brucellosis, syphilis, Rickettsial diseases, Malaria, trypanosomiasis, typhoid fever, any acute infection.

5. Inflammatory bowel disease (8%)

6. Miscellaneous and unexplained
   (eg) sarcoidosis, drug reactions

(after Maldonado and Hanlon, 1965)

Defects of monocyte function have been described in chronic neutropenia (Greenwood et al, 1978), myeloperoxidase deficiency (Lehrer, 1975) and in patients on high dose corticosteroid therapy (Rinhart et al, 1975).

More recently there have been reports of abnormal function in pyoderma gangrenosum (Norris et al, 1978), following trauma (Miller et al, 1979) and burns (Lloyd and Levick, 1978), in mycosis fungoides (Norris, 1979) and other lymphoma patients (Lab et al, 1978). Abnormalities have also been reported in patients with pulmonary tuberculosis (Campbell, 1979).

The monocyte has been incriminated in the etiology of serum sickness nephritis (Hunsicher et al., 1979), glomerulonephritis (Cotron,

1978) and rheumatoid arthritis (Kay, 1979). A specific monocyte arthritis has been described, although this is probably a reactive change to an unknown stimulus (Brawer and Cathcart, 1979). Similarly, abnormalities have been described in psoriasis (Bar-Eli et al., 1979).

Defects in monocyte suppressor activity have been reported in SLE (Markonson et al, 1978, 1980) and in sarcoidosis (Goodwin et al; Hunyard, 1979). There is some evidence that monocyte depletion in these patients results in a return to normal of the previously reduced proliferative response to PHA.

In cirrhosis there are limited reports of reduced chemotaxis (Deneo et al, 1973), reduced phagocytosis (Hassner et al, 1978) and increased levels of lysosomal enzyme production (Ganguli et al, 1978).

As yet there is no place for monocyte testing in clinical practice as interpretation is difficult. In breast cancer, where the defect has been shown to recover following excision of the primary tumor (Norman, 1979), there is some unconvincing evidence that these tests may have a prognostic value (Taylor, 1979). Defects in the monocytes might account in part for the decreased lymphocyte function in cancer patients (Wood et al, 1979). It is interesting to note that most of the conditions listed above, in which defective monocyte function has been demonstrated, are conditions in which there is increased susceptibility to infection. It is possible, therefore, that these tests may pick up those patients with an increased risk of developing infections. Interestingly, but unexplained, in mice there is correlation between the health and hair condition with the number of circulating monocytes present (Van Waarde, 1979).

Attempts to improve monocyte function therapeutically have not been very successful. Ascorbic acid may improve chemotactic function in the Chédiak-Higashi syndrome (Gallin et al., 1979) and levamisole may have similar effects (Wright et al., 1977) and also affect monocyte-lymphocyte interaction (Kazura et al., 1979).

THE CONCEPT OF THE ACTIVATED MACROPHAGE

Following observations that macrophages obtained from patients with infections (e.g.) tuberculosis (Lurie, 1942) showed enhanced function, Machanass (1964) coined the phrase "activated macrophage" for these cells showing enhanced activity (1964). This state of activity had,

however, been recognized by Metchnikoff (1905). These cells show increased adherence and spreading, are larger with increased ruffling of the membrane and have increased powers of phagocytosis, greater respiratory activity (Karnovsky and Lazdine, 1978), increased glucose metabolism (Stubbs, 1973) and contain increased amounts of acid hydrolases (Saito, 1965). Along with the increased function, some measurements showed decreased values (e.g.) superoxide production (Karnovosky and Lazdine, 1978; Cohn, 1978). Activated cells also show increased tumor cytotoxicty (Fink, 1977). The secretion of plasminogen activator may be the best indicator of assessing macrophage activity (North, 1978).

In vivo activation can be produced by injection with BCG, listeria (Machaness, 1962) or killed corynbacteria. Despite early claims to the opposite (Machaness, 1964), it soon because apparent that this increased ability of macrophages to kill bacteria was not specific to the priming organism but was nonspecific and general (Pullinger, 1936). More recent evidence, however, would suggest that the nonspecific resistance and delayed sensitivity is short lived compared to the specific activity (Machaness, 1964) and is T-cell dependent (Lane and Unanue, 1972).

In vitro it is also possible to activate macrophages and monocytes and indeed it was the discovery that normal macrophages could be activated by incubation with sensitized lymphocytes that led to the discovery of lymphokines (Simon et al., 1972). Monocytes can also be activated by endotoxin, polynucleotides, oils and immune complexes as well as many other substances. Even simple adherence to glass or plastic has some effect (Rabinovitch et al., 1973). It is an attractive but unproved theory that the activation is important in the physiological response to infections. Nonspecific inflammation results in some of the adaptations seen in 'activation' and these changes may result in improved function. Once inflammation is controlled the stimulus is reduced and a feedback situation exists. It is also suggested that this response to initial inflammation may be required for the further response of the macrophage to lymphokines (Nogueria et al, 1977).

As far as the monocyte is concerned, similar changes occur on glass adherence and many of these changes are similar to the normal pattern of further differentiation of the monocyte into a macrophage.

AIMS OF THESIS

It is hoped to investigate monocyte function in patients with cirrhosis and, by studying various other disease groups, to gain some insight into the significance and relative importance of any abnormalities discovered. The first section (Chapter 2 and 3) studies monocyte function and assesses the importance of the overspill phenomenon on monocyte function in cirrhosis. The second part (Chapters 4 and 5) involve investigation into the role of monocytes in immunoregulation in patients with liver disease and includes details of the interrelationship of defects of this system with the HLA phenotype and T cell suppressor activity. Chapter 6 investigates the possibility that the abnormal monocyte suppressor activity found in cirrhosis may be related to abnormal fibrogenesis and Chapter 7 investigates the possibility that the hormonal changes occuring in cirrhosis might also influence immunoregulation of their function. In Chapter 8 a disease group, namely inflammatory bowel disease patients, is included and the opportunity is taken to study the effects of sulphasalazine on immunoregulation in these patients. Also included in this chapter is information on immunoglobulin production by lymphocytes from patients with Crohn's disease as these act as a disease control group for the study in Chapter 9. In Chapter 9 the pathogenesis of the hypergammaglobulinaemia associated with liver disease is studied with particular reference to the possibility that this results either from nonspecific activation of the B cells or from abnormal monocyte mediated suppressor cell activity.

CHAPTER 2

MONOCYTE FUNCTION IN CIRRHOSIS

A variety of defects in the cellular immune system have been described in liver disease, but the literature concerning monocyte function in cirrhosis is scarce. The pre-disposition of patients with alcoholism and cirrhosis to infection (Stilman, 1924; Green and Kass, 1964; Whipple and Harris, 1950) is well recognised, and alcoholics may show varying degrees of leucopenia (Eichner, 1972) and leukocyte functional derangement (Brayton et al, 1970). Von Epps and co-workers (1975) showed that inhibitors to both leukocyte and monocyte chemotaxis was present in 50% of patients with alcoholic liver disease and that the severity of the defect correlated with serum IgG and particularly IgA concentrations. DeMeo (1972) also showed chemotactic inhibitory substance activity in serum from 19 of 22 patients with alcoholic, at least three different chemotactic inhibitors being present. Hassner and colleagues (1981) demonstrated reduced phagocytosis and killing of Candida albicans in patients with cirrhosis and suggested the presence of a serum inhibitory factor. On the contrary Ganguly et al. (1978) suggested that the production of acid hydrolase enzymes from monocytes was increased in cirrhotics, suggesting that the cells were "activated". These studies of monocyte function in cirrhosis are of interest not only because they may account for the increase susceptibility of patients to infection but also because they may be an indicator of Kupffer cell function (Reiner et al, 1981).

It has been postulated that, in cirrhosis, antigens absorbed from the gastrointestinal tract may not be removed by the failing liver either through shunting of portal blood, or because of impairment of Kupffer cell function. These antigens may then result in continuous immunostimulation leading to immunosaturation and a reduced response to further superimposed infection (Bjorneboe et al, 1972; Triger et al, 1972). These antigens could be similar to the inhibitory substance discussed above.

The purpose of this study is to try to define more accurately monocyte defects in patients with cirrhosis and to investigate the possibility of an overspill phenomenon contributing to the abnormalities found in cirrhosis.

PATIENTS AND METHODS

Patients

Only patients with biopsy-proven liver disease were studied. Where indicated below, and where numbers permitted, the patients were classified as "active" or "quiescent" on the basis of the biopsy, biochemical, and clinical assessment. The former were mainly in-patients, the latter out-patients. Age and sex-matched controls were selected from hospital staff and from patients attending a day-ward for minor surgery. A standard full blood count and differential white count was performed. Eleven alcoholic patients with no evidence of hepatitis or cirrhosis on biopsy and 6 patients with obstructive jaundice were also studied. All studies were approved by the district ethical committee.

Culture Conditions

A mixed mononuclear cell preparation (MMC) containing approximately 30% of monocytes was obtained from fresh venous heparinized (10 U/ml) blood by density centrifugation following layering onto lymphocyte preparation medium (Flow Laboratories, UK). This was then washed 3 times in Hanks Buffered Salt Solution (HBSS). A monocyte-enriched preparation (MEC) was obtained by adherence to plastic Petri dishes for two hours at which time nonadherent cells were removed. The adherent cells were then washed and harvested using a rubber policeman and then washed again. Viability was confirmed by exclusion of Trypan blue. Cytospin preparations were stained with either Giemsa-Wright or non-specific esterase and the MEC preparation was confirmed to contain approximately 90% monocytes. Any preparation with a viability of less than 90% or which contained less than 80% of monocytes was discarded.

Tests of Monocyte Function

1. Macrophage precursors

The macrophage precursor assay described by Currie and Hedley (1977) was used. In brief, MMC (2×10^6 per ml) in RPMI 1640 (+ 25 mmol HEPES and antibiotics) containing 50% autologous serum was added in 100 ul volumes to the wells of a plastic microplate. Plates were incubated at 37° in 5% CO_2 in humid air for seven days, when the wells were washed

free of unattached cells with HBSS and 50 ul of a .01M citric acid solution containing 1:2000 crystal violet added to each well (Sanford et al, 1950). The plates were allowed to stand 30 minutes at room temperature at which time the detached nuclei were counted in a haemocytometer. This assay is thought to indicate the number of circulating monocytes present which have the capacity to mature into tissue macrophages.

2. Monocyte spreading

Spreading was measured using the method described by Territo and Cline (1977). In brief, 0.5 ml of a 4 x 10^6 cell/ml MMC preparation was placed over glass coverslips in sterile plastic multiplates (Lux Scientific Corporation). After 1, 24, and 48 hours coverslips were removed, gently washed in HBSS and fixed in 2% buffered glutaraldehyde for 5 minutes. The coverslips were then inverted on to oil drops in glass slides, taking care to avoid drying. The longest axis of the cells was measured using phase-contrast microscopy with an oil-immersion objective lens (each reading mean of 20). This ability to spread is increased when the monocytes are 'activated' and is probably a measure of cell maturity.

Nitro-blue tetrazolium reduction

The redox dye nitro-blue tetrazolium (NBT) is reduced by the action of NADPH oxidase. The product is the insoluble coloured crystalline formazan. The assay is therefore an indirect measurement of the hexose monophosphate shunt. The method described by Hedley and Curry (1978), itself an adaptation (Baehner and Nathan, 1968) was employed. In brief, 150 ul of a MMC suspension containing 10^6 mononuclear cells was placed in 2-ml polypropylene tubes. As a phagocytic stimulus, 50 ul of .79 u latex polystyrene beads (Sigma) diluted in 1/100 in RPMI 1640 was added and 50 ul of RPMI 1640 added to control tubes. Following incubation at 37° for 15 minutes, 25 ml of a 4 nmol solution of NBT (Sigma) in 314 nm sucrose were added. After a further one hour incubation, the reaction was stopped by adding one drop of 1/10 N HCl to the tubes. The formazin was then extracted by adding 500 ul of Dioxan to the cell button and incubation at 70° for 20 minutes. Following centrifugation at 2000 g for 15 minutes, the clear supernatant was read at 520 cm on a spectro-fluorometer, using Dioxan as a blank. A standard curve was prepared by

reducing doubling dilutions of NBT with 150 mmols of ascorbic acid (Segal and Peters, 1975).

Phagocytosis and bacterial killing of staph aureus

The method used was based on that described by Territo and Cline (1977) with minor modifications (Verbrugh et al, 1978; Steigbigel et al, 1974). The method below produced the most reproducible results in preliminary studies. Staph. aureus was cultured overnight in a brain/heart medium and diluted to a final concentration of approximately 2 x 10^6 bacterial colony-forming units per ml (CFU-ml). This was divided into aliquots, snap-frozen and stored at -70° for later use. For phagocytosis, 0.125 ul of a MMC preparation in a concentration of 4 x 10^6 cells was added to 0.125 ul of autologous serum and 0.125 ul of bacterial suspension in a 12 x 75 mm. plastic tube. Following incubation at 37°C for one hour, a cytospin preparation was prepared and the number of bacteria ingested by 100 monocytes counted (mean of five readings). To measure bacterial killing 200 ul of bacterial solution was put in 12 x 75 mm plastic tube. To this was added 200 ul autologous serum and 500 ul of a mixed mononuclear cell preparation containing 4 x 10^6 cells/ml. This was incubated for one hour at 37°C., sonicated to disrupt cells and release bacteria (confirmed microscopically) and diluted 1:1000. The number of CFU/ml was then measured by culturing on blood-agar plates for 24 hours. The percentage killing was calculated from the equation: % killing = A - B/A, where A is CFU/ml for the control cultures, i.e. tube containing only bacteria and no cells, and B is CFU/ml count for tube containing bacteria and cells.

Chemotaxis

Chemotaxis assays were performed by a modification of the Boyden chamber technique (Boyden, 1962) as described by Wilkinson (1973). Serum chemotactic activity was produced by incubating fresh frozen human AB serum with zymosan (Sigma Chemical Co. 0.5 mg/ml serum). A mixed mononuclear cell preparation of 1 x 10^6 cells in 0.5 mls) was added to the top chamber with no added serum. Distance moved by monocytes through an 8 u millipore filter was measured after incubation at 37°C for four hours in a CO_2 incubator using the leading front counting technique as described by Zigmond and Hirsch (1973). The mean of ten readings was recorded. An assessment of random movement was obtained by

the measurement of migration of mononuclear cells exposed to RPMI 1640 alone (containing no zymosan treated serum). This measurement was subtracted from the movement induced by the zymosan-treated serum to obtain the measurement of chemotaxis.

Acid hydrolase production

Production of the acid hydrolase N-acetyl-B-glucosaminidase (NAG) and B-glucuronidase (BG) was measured using the methods described by Ganguly (1978). HEPES buffered RPMI 1640 supplemented with 10% fetal calf serum (SRPMI 1640) was used for cell maintenance to each of six wells of a sterile plastic multiplate (Lux Scientific Corporation) monocytes enriched cells (1.5×10^6) in SRPMI 1640 were added and allowed to adhere to the surface by incubation at 37°C in a 5% carbon dioxide fully humidified atmosphere for two hours with occasional agitation. After incubation, supernatant was discarded and three of the wells were filled with 1.5 ml of an endotoxin solution and three with S-RMPI 1640 alone. The plates were incubated for a further two hours and the supernatants discarded, the cells washed three times in HBSS to remove the endotoxin and refilled with 1.5 ml of S-RPMI 1640. Following a further 24 hour incubation, supernatants were collected from each well, centrifuged to remove any contaminating cells, and stored in plastic tubes at -20°C. Adherent cells were removed from the plastic surface of each well with a rubber policeman into 1.5 ml of sterile ice-cold distilled water and the cell suspension was rapidly frozen and thawed eight times to lyse the monocytes. It was not possible to carry out a cell count before lysis, and we have assumed that the cell number after incubation bore a constant relationship to the starting inoculum in each case. The total protein concentration of cell lysate was estimated by the method of Lowry et al. (1951) and was used as an index of cell mass (Beutler et al, 1976). A sensitive assay for NAG and BG was developed from the spectrofluorometric method of Beutler et al. (1976). The endotoxin solution Salmonella typhosa 0901 (Difco Laboratories) was prepared in .85% sodium chloride at a concentation of 1 mg/ml and diluted with RPMI 1640 medium to give a working concentation of 50 ug/ml.

Further experiments

Monocytes from a normal human volunteer were incubated at 37°C for 4 hours in paired specimens of serum (20%) from the controls and from cirrhotics. The cells were then washed 3 times prior to measurement of chemotaxis and bacterial killing. In these experiments the bacterial killing and phagocytosis was performed in fetal calf serum. In further experiments chemotaxis and bacterial killing were measured on cells from cirrhotic patients and incubated with control serum (conditions as above) and compared to the results obtained by those incubated in autologous serum. To investigate the overspill phenomenon, paired specimens of portal serum and venocaval serum were collected. These were obtained from patients undergoing vascular reconstructive surgery. None of these patients had clinical or biochemical evidence of liver disease. The effect of the paired serum specimens was measured on chemotaxis and bacterial killing properties of monocytes from healthy volunteer donors (methods as for studies comparing cirrhotic and control serum).

Statistics

The student t test was used throughout. The Mann-Whitney U test was also used for compairing nonparametric data. Probability values of less than 5% were considered to be significant. All assays were performed in triplicate and were coded and read without knowledge of the patients' clinical details. The results are expressed as mean ± SEM. Interassay variability was less than 15% for all the assays.

RESULTS

Not all the tests of monocyte function were measured concurrently. Table 1 shows the total number of patients studied, the nature of the liver disease, and the total number of monocytes in the blood film and number of adherent cells recovered for each group. There was no significant difference between controls and cirrhotics. Although the number of macrophage precursors was slightly reduced in the cirrhotics ($9.2 \pm 3.6 \times 10^4$/ml for the controls, and $8.8 \pm 4.2 \times 10^4$/ml for the cirrhotics, N=16), the difference did not reach statistical significance. The results for monocyte spreading in controls (N=15) were 12.4 u ± 2.4, 17.4 u ± 2.2, and 19.4 u ± 3.4 at time 0, 24, and 48

hours respectively. Corresponding values for the cirrhotics (N=15) were 12.8 u ± 2.7, 14.4 u ± 1.8, and 16.8 u ± 3.4. The differences were small and only reached statistical significance at 24 hours ($p.< 05$). The reduction of NBT was 12.2 ± 4.0 x 10^{-15} per unstimulated monocyte, and 14.2 ± 3.6 x 10^{-15} per monocyte stimulated with latex for the controls, and 9.6 ± 3.8 x 10^{-15} and 12.4 ± 4.2 x 10^{-15} per monocyte for the cirrhotics. Thus, NBT reduction tended to be lower in the cirrhotics, but again the difference did not reach statistical significance. Both controls and cirrhotics responded to the latex beads in a similar fashion and the increase expressed as a percentage was not significantly different in the two groups.

Results of phagocytosis, bacterial killing and chemotaxis are shown in Table 2. Both phagocytosis and chemotaxis were significantly reduced in the cirrhotics. Although reduced, the overall difference in killing between the cirrhotics and the controls did not reach statistical significance, but, comparing those with active disease (N=10) there was a signficant reduction (29% ± 3.8 v. 40% ± 3.6, $p<.02$), suggesting that the more severe the disease, the greater the loss of function. In the case of bacterial killing, in a separate group of 15 patients, incubation of cells from cirrhotic patients in control serum resulted in marked improvement of function compared to the results in autologous serum (41% ± 4.2 and 12% ± 6 respectively ($p<.02$)) suggesting that the difference is due to serum inhibitory factors. Similarly for chemotaxis there was a significant reduction in the cells from cirrhotics when pre-incubated in autologous serum, but if these were pre-incubated with control serum chemotaxis was not significantly different from the controls (34u ± 5.6 vs. 37.4u ± 4.2).

The result for the production of NAG and BG are shown in Tables 3 and 4 which show values in supernatants and the cell button respectively and demonstrate the results of stimulation with endotoxin. In general, levels in supernatant and cells were decreased in cirrhotics compared to the controls. Differences were more marked for the NAG than for BG, where very low levels were recorded throughout. Results from 11 alcoholic patients without significant liver disease are also shown, and these values were not significantly different from control values. Table 5 shows the results for enzyme production divided into patient grouped

CHAPTER 2. Table 1. Numbers of monocyte and glass adherent cells in patients and controls (Mean ± SEM)

	No. Patients	Absolute No. of Monocytes in blood film (no/ml)	No. of glass adherent cells Recovered from 50 ml blood X 10^6
Controls	35	538 ± 42	7.0 ± 3.4
Chronic active hepatitis	10	594 ± 54	6.0 ± 4.5
Alcoholic cirrhosis	25	512 ± 48	6.6 ± 4.5
Alcoholic non cirrhotic	11	594 ± 84	8.2 ± 5.01
All other cirrhotics	16	524 ± 34	5.37 ± 6.7
Obstructive jaundice	6	582 ± 39	7.9 ± 5.6
All cirrhotics	51	528 ± 56	6.1 ± 5.6

CHAPTER 2. Table 2. Results of Chemotaxis, bacterial phagocytosis and killing in cirrhotics and control patients

Mean ± SEM

	Chemotaxis (u)	Phagocytosis No./100 cells	Killing (%)
	n = 24	n = 16	n = 30
Cirrhotic Patients	12 ± 6 u p < .02	123 ± 18.4 p < .05	34 ± 4 NS
Controls	39.2 ± 3.8 u	149 ± 28.4	40 ± 3.6

CHAPTER 2. Table 3. Enzyme Release by Human Monocytes Measured in Supernatant

Mean + SEM
nmols/ug.prot./hr

		NAG		BG	
		Unstim.	Stim.	Unstim.	Stim.
Control	35	2.3 (.26)	2.5(.291)	.13 (.06)	.14 (.012)
All cirrhotis	35	1.55 (.3)*	1.8 (.4)*	.099(.03)**	.124(.09)
Chronic active	10	2.4 (.4	2.4 (.4)	.11 (.04)	.12 (.02)
Alcoholic + cirrhosis	15	1.2(.119)***	1.6 (.28)**	.079(.005)*	.091(.009)
- cirrhosis	11	2.0 (.35)	2.1 (.39)	0.076 (.01)	0.09 (.016)
Other cirrhotics	10	1.2 (.15)*	1.6 (.25)	.12 (.03)	.18 (.05)
Obstructive jaundce	5	1.9 (.24)	2.2 (.28)	.16 (.04)	.17 (.02)

* $P < .05$ ** $P < .02$ *** $P < .01$

CHAPTER 2. Table 4. Enzyme Cell Content of Monocytes

Mean + SEM
nmols/ug.prot./hr.

		NAG		BG	
		Unstim.	Stim.	Unstim.	Stim.
Controls	35	.48 (.06)	.52 (.07)	.09 (.1)	.11 (.02)
All cirrhotics	35	.36 (.06)**	.46 (.06)	.06 (.01)*	.085 (.02)
Chronic active	10	.49 (.28)	.64 (.09)	.06 (.01)	.06 (.05)
Alcoholic + cirrhosis	15	.23 (.05)**	.26 (.05)***	.06 (.01)**	.07 (.01)
- cirrhosis	11	.43 (.08)	.45 (.11)	.07 (.01)	.08 (.01)
Other cirrhotics	10	.39 (.08)	.69 (.09)	.06 (.01)*	.06 (.01)
Obstructive jaundice	5	.25 (.08)	.66 (.28)	.09 (.015)	.12 (.05)

* P < .05 ** P < .02 *** P < .01

CHAPTER 2. Table 5. Production of Enzymes by Human Monocytes (unstimulated) in Patients Grouped by Severity of the Disease (mean + SEM nmols/ug prot/hr.)

		Supernatant NAG	Cells	Supernatant BG	Cells
Controls	35	2.3 (.35)	.48 (.07)	.13 (.02)	.09 (.01)
Acute alcoholic hepatitis	4	1.3 (.36)*	.55 (.1)	.08 (.017)	.094 (.03)
Active cirrhosis	10	1.34(.132)**	.29 (.06)**	.079 (.06)*	.057(.024)
Quiescent cirrhosis	17	1.95 (.27)	.446(.115)	.132(.029)	.069(.012)
Cirrhosis on steroids	8	2.67 (.5)	.65 (.25)	.113(.016)	.086 (.02)

* $P < .05$ ** $P < .02$

CHAPTER 2. Table 6. Effect of control and cirrhotic serum; portal and systemic specimens; and mixing experiments on monocytes obtained from a normal volunteer (mean + SEM)

The systemic/portal serum results were performed at different times and on different cells than the control/cirrhotic serum. Mixture refers to pooling of the relevant samples using equal volumes of each.

	Chemotaxis (u)		Bacterial Killing (%)	
Control serum (n=20)	28.4 ± 3.2		17.4 ± 3.8	
Cirrhotic serum (n=20)	14 ± 4.5	(P<.05)	7.5 ± 3.5	(P<.02)
Mixture (n=15)	16 ± 54	(P<.05)	8.4 ± 4.2	(P<.05)
Systemic serum (n=18)	49.2 ± 2.7		65 ± 8.4	
Portal serum (n=18)	45.8 ± 2.2	(P<.05)	29.2 ± 4.2	(P<.02)
Mixture (n=15)	46.2 ± 2.4	(NS)	34.6 ± 6.8	(P<.05)

on clinical grounds. Those with the severest disease, especially those with alcoholic hepatitis, were found to have the lowest levels, whilst those with quiescent disease were found to have near normal values. The small group of patients with cirrhosis on steroids were found to have slightly increased levels, although not statistically significant, and this group included most, but not all, the patients with chronic active hepatitis. Studying the chronic active group as a whole, levels were not significantly below normal values.

A comparison of the effects of paired specimens of cirrhotic and control serum in shown in Table 6. Both chemotaxis and killing are significantly reduced by pre-incubation in cirrhotic serum compared to the controls. Also in this table are shown the values for pre-incubation of normal monocytes with paired specimens of portal and systemic serum. Portal serum also results in significanlty reduced chemotaxis and killing compared to the systemic serum. Mixing of cirrhotic with control and portal with systemic serum in equal volumes resulted in reduced chemotaxis and killing in the same range as those obtained for the cirrhotic and portal specimens respectively.

DISCUSSION

We have attempted to test various aspects of monocyte function in patients with cirrhosis and have demonstrated that this function is generally reduced. Although the changes were modest and did not always reach significance, results in cirrhotics were consistently reduced. Changes reached significance for chemotaxis, bacterial killing, monocyte spreading and bacterial phagocytosis. The number of monocytes present in cirrhotics was not different from the control population, but the results could be interpreted to suggest that the ability of these cells to mature into macrophages is less in cirrhotics than in the controls. Failure to show more clearcut differences between the cirrhotics and controls may be due to the inadequacies of currently available techniques. This reduced function appears to be mainly due to a serum inhibitor as incubating cirrhotic cells with normal serum resulted in some improvement in their function. Experiments in mixing cirrhotic and control serum showed that monocyte function was still reduced, and this would suggest the presence of an active inhibitor rather than a deficiency situation. Unfortunately the nutritional status of the patients was not assessed. It has previously been shown that there is defective bacterial opsonization in patients with cirrhosis (Wyke et al, 1980; Munoz et al, 1977), but previous work has differed in respect to the nature of this defect. Some have found, as we have, that there appears to be an active inhibitor of chemotaxis and phagocytosis present (Von Epps et al, 1975; Demeo et al, 1972; Hassner et al, 1981). Others, based on the results of mixing and dilution experiments, have suggested that a relative deficiency state exists, possible of complement, and that this accounts for the reduced osponization in cirrhotics (Wyke et al, 1980). In our view the reduced phagocytic function is unlikely to be simply due to a defect of opsonization, as after preincubation with the appropriate serum, the actual phaygocytosis was performed in fetal calf serum in these experiments. Similarly if this was a defect of opsonization the defect would not be present on mixing the two sera.

The results would suggest that the monocyte defect is secondary to the cirrhosis and not dependent on the underlying aetiology, as it was present in all groups of cirrhotics to some extent. The enzyme production by the group of alcoholic patients, in whom there was no

cirrhosis or hepatitis, was also not different from the controls. In chronic active hepatitis patients with cirrhosis, monocyte function was also reduced but all but 1 patient was taking corticosteroids, which, in the short-term at least, are known to depress monocyte function (Rinehart et al, 1975). Long-term corticosteroid therapy has less effect on monocyte function, but we cannot exclude the possibility that the results obtained in our patients with chronic active hepatitis may be artificially lowered by the corticosteroid therapy, and that this may explain some of the differences obtained between our results and those previously obtained in a small group of patients in our laboratory (Ganguly et al, 1978).

While most of the evidence suggests that the reduced function is due to the presence of an inhibitory factor. The NAG and BG production results might suggest that the cells themselves are abnormal, as these were performed in fetal calf serum. The production of these enzymes probably mirrors the ability of the monocytes to differentiate into macrophages (Musson et al, 1980) and further evidence to show there is a relative failure of maturation of these cells is obtained from the spreading experiments. Hassner recently reported (1981) that treatment of cirrhotic monocytes with trypsin (to remove all traces of serum factors) resulted in a return of monocyte functon to normal. Thus, although the cells were washed carefully, it is probable that these defects in enzyme production could also be accounted for by an effect of a serum factor carried over by the cells. However, we cannot exclude the possibility that there is a shift in the population of circulating monocytes in cirrhosis, although the findings of normal numbers of circulating monocytes which function normally in control serum makes this unlikely.

For enzyme production and NBT reduction, the monocytes were studied before and after stimulation either by endotoxin or latex and, in both cases, the response of the cells to this stimulation was not significantly different from that of the controls. Thus, the theory of hyperstimulated cells becoming immunosaturated and unable to react to fresh stimuli has not been substantiated and it would appear that these cells are capable of a normal response, at least <u>in vitro</u>. Endotoxaemia may be common in cirrhotics (Triger et al, 1978; Nolan, 1975) and

assuming the in vivo effects are similar to the in vitro effects, a state of 'monocyte activation' might have been expected. The relevance of the concept of the activated macrophage (North, 1978) is unknown but it is a term used to indicate a state of increased activity of the cells which occurs on exposure to various stimuli including endotoxin. Contrary to our expectation it appears that in cirrhosis the cells are less 'activated' and that the serum contains 'deactivating' substances. Activated macrophages also excrete collagenase (McCarthy et al, 1980) and thus this mechanism may be important in the instigation or perseverence of increased collagen deposition in the liver and hence the development of cirrhosis.

We have shown that normal cells exposed to portal vein serum show reduced chemotaxis and bacterial killing compared to those exposed to simultaneously taken systemic serum specimens. The mixing experiments would also suggest that there is an active inhibitor present. Thus, rather than the monocyte defect being explained on the basis of a constant stimulation by antigens resulting in a failure to respond to fresh stimulation, our results would suggest that inhibitory substances (probably gut related antigens) bypass, or are not sufficiently removed by, the failing Kupffer cells in the cirrhotic liver. We have further investigated this by measuring immune complexes and endotoxin levels in the portal systemic serum and comparing them to the effect of the individual specimen on the monocyte responses. These studies suggest that it is immune complexes rather than endotoxin which have the most marked effect (Chapter 3).

Ferluger (1978) has suggested that monocytes, which are presumed to be Kupffer cell precursors, may be important in certain types of liver damage. He has described an animal model where mice, primed with C. parvum and exposed to endotoxin, developed acute liver failure. He suggests that monocytes recruited into the liver by the C. parvum may be activated by the endotoxin to release potentially toxic substances. He further suggests that the same mechanism may be operative in hepatitis. We have recently provided further evidence for this from in vitro studies in rats (Tanner et al, 1981) but contrary to our original expectation (Ganguly et al, 1978) that enzyme release from monocytes might be increased in cirrhosis, and thus a possible indicator of

ongoing liver damage, the finding that enzyme release is reduced does not support this theory of hepatocyte damage in the patient groups studied. Crucial to this is the unanswered question regarding the relationship of peripheral monocyte function to that of Kupffer cells. If such a relationship exists our findings suggest that Kupffer cell function is reduced in cirrhotics. Thus the role may be reversed and the failing Kupffer cells may be unable to protect the hepatocyte from further damage. This finding is in keeping with recent in vivo studies of Kupffer cells activity (Waddle et al, 1980) suggesting reduced function in cirrhosis.

In conclusion, we have demonstrated wide-ranging defects in monocyte function in patients with cirrhosis. This appears to be secondary to the presence of cirrhosis and is probably not of primary immunopathogenic importance. The abnormality may be due to the presence of an active inhibitor which possibly originates from the portal venous system due to failure of the filtering mechanism of the normal liver. The defects in monocyte function together with previously shown defects in neutrophil function and other abnormalities including defective serum bactericidal activity (Fierer et al, 1979), may all play a role in the susceptibility of these patients to infection. It is possible that nutritional deficiencies (O'Keefe et al, 1980) in part explain the abnormalities, but this would not seem to explain the presence of an active inhibitor substance.

CHAPTER 3

IMMUNE COMPLEXES IN PORTAL AND SYSTEMIC SERUM AND THEIR EFFECT ON MONOCYTE ACTIVATION - HUMAN AND RABBIT STUDIES

There is some evidence that the Kupffer cells of the liver act as a filter, removing antigens, bacteria and immune complexes originating from the gut. Failure of this mechanism may account for the increased incidence of bacterial and dietary antibodies (Triger et al, 1972) found in liver disease, and may contribute to the associated hyperglobulinaemia (Triger and Wright, 1973).

Following our finding of increased acid hydrolase production by peripheral monocytes from patients with various conditions including liver disease (Ganguly et al, 1978), we have investigated the possibility that factors normally present in the portal vein and which have spilled over into the systemic circulation in patients with liver disease could be responsible for this abnormality. We have compared the effect on human peripheral blood monocytes of serum from the portal vein with that from the inferior vena cava, and correlated the activity with the levels of immune complexes, immunoglobulins and the presence or absence of endotoxin.

Preliminary results from a rabbit model are also presented.

Patients and Methods

Patients. The study was approved by the Ethical Committee of the University of Southampton. Blood was simultaneously taken from the portal vein and the inferior vena cava in 16 patients at laparotomy. Two patients were undergoing colectomy for inflammatory bowel disease, four cholecystectomy for cholelithiasis and the remaining ten vascular reconstructive surgery. None of the patients had biochemical or clinical evidence of liver disease. Because of the difficulty of obtaining adequate specimens from humans, 16 rabbits were sacrificed and blood taken simultaneously from the portal vein and the hepatic vein. From the rabbits, blood was divided into two aliquots, one for collection of monocytes from both portal and systemic circulations, and the other for collection of serum. A single healthy human volunteer provided normal peripheral monocytes for all comparative studies. The method for enzyme producing assays are described in Chapter 2.

Isolation of Monocytes. A mixed mononuclear cell preparation was obtained from fresh heparinized blood by density centrifugation using lymphocyte preparation medium (Flow Laboratories, U.K.) as described elsewhere. Monocyte enriched cells were obtained by adherence to plastic Petri dishes for two hours. The nonadherent cells were discarded and the adherent cells washed and then harvested using a rubber policeman. Viability was assessed by exclusion of Trypan blue and preparations with a viability of less than 90% were discarded. Both preparations were stained with Giemsa-Wright and non-specific esterase (Horwitz et al, 1977), and this showed an approximate 30% and 90% monocyte preparation respectively.

Endotoxin Assay

Portal and systemic sera were assayed for endotoxin using a standard limulus assay (Levin and Bang, 1969) (Mallinkrodt) after removal of potential inhibitors by chloroform (Levin et al, 1969). This assay detects endotoxin at a concentration of .005 ug/ml.

Statistics. The paired t test was used for statistical analysis. Probability values of less than 5% were considered significant.

Immunoglobulin, complement and immune complex assay. The freshly collected whole blood was clotted at 37°C and, following centrifugation, serum aliquots were examined for cryoglobulins at 4°C after 24 and 48 hours as these are known to interfere with the assay. The serum was then snap-frozen in liquid nitrogen and stored at -70°C prior to assay. Immune complexes were measured by a modification (Hammond et al) of the nephelometric method (Deaton et al, 1976) used for the immunoglobulin assay (Creighton et al, 1973), with polyethylene glycol precipitation and specific antisera to the IgA, IgM, IgG, C3 and Clq components of the complex. All antisera was obtained from Hyland Travenol Lab., U.S.A. Sera to be assayed were rapidly thawed at 37°C and 0.75, or 1.0 ml, duplicate samples were precipitated with polyethylene glycol (PEG, molecular weight 6000, BDH Laboratories, U.K.) in 0.02 M Tris EDTA buffer at pH 8.0 to a final concentration of 2%. The sera and PEG were mixed and left overnight at 4°C. The resultant precipitates were

centrifuged at 1000 g and the supernatants discarded. Care was taken to drain off all liquid by tube inversion onto absorbent tissue. The precipitates were washed twice by thorough resuspension in 1 ml volume of PEG, allowing 15 minutes before centrifugation and removal of the supernatant. The washed precipitates were redissolved in the original serum volume of Tris buffer and assayed for immunoglobulin and complement.

The original sera and redissolved PEG precipitates were assayed by measuring the light scatter at 623 nm with a Hyland PDQ laser nephelometer. After addition of appropriate antibody, the redissolved PEG precipitates were assayed at dilutions to give scatter readings that could be compared to that produced by reference sera (Hyland). After corrections for dilution, the amount of immuno globulin or complement in the original serum sample was compared to that in the PEG precipitate.

RESULTS

The values of N-acetyl-B-glucosaminidase (NAG) produced by human monocytes exposed to paired portal and systemic serum from humans and rabbits are shown in Table 1. In both cases the incubation with portal serum resulted in more enzyme production, particularly in the supernatant, than incubation with the paired systemic serum. Figure 1 shows the differences between individual cases of portal and systemic serum, with the paired specimen results joined together. Table 2 shows the value of NAG production by monocytes obtained from the rabbit portal and systemic blood produce more of the enzyme when obtained from portal blood than when obtained from systemic blood. This suggests that the short period that the cells are in the portal sera prior to separation results in some degree of activation. Table 3 shows the values for immune complexes, complement and immunoglobulins detected in human portal and systemic serum. A comparison of portal and systemic levels in individual cases is shown in figures 2 and 3. Higher levels of immune complexes, particularly of the IgG class, were found in the portal serum than in systemic serum. Differences in the immunoglobulins were small, the only one reaching statistical significance being in IgA ($p < 0.05$). There was no difference between complement content. Immune complexes were not increased in the two patients undergoing

CHAPTER 3. TABLE I

Effect of treating human monocytes with systemic and portal serum from humans and rabbits. Production of NAG (nmol/50/ul/hr mean + SEM).

		Systemic	Portal	P
Human (14)	cells	48 (± 3.2)	50 (± 3.2)	NS
	supernatant	72 (± 5.2)	92 (± 6)	< .05
Rabbit (13)	cells	13.63 (± 1.19)	14.9 (± .96)	< .01
	supernatant	2.1 (± .19)	2.7 (± .235)	< .05

CHAPTER 3. TABLE II

NAG production of monocytes obtained from rabbit portal and systemic blood (nmol/50/ul/hr) mean + SEM.

	Systemic	Portal	P
Unstimulated	17.57 ± 2.4	19.8 ± 3.5	< .02
Stimulated cells Endotoxin	23.6 ± 3.4	27.2 ± 3.9	< .02

CHAPTER 3. TABLE III

Immune complexes and immunoglobulins in portal and systemic blood (mean + SEM).

	Systemic	Portal	Significance
Immune complexes mg/dl			
IgG	7.14 (1.14)	10.46 (.53)	$p < .01$
IgA	1.35 (1.19)	2.74 (.89)	$p < .05$
IgM	5.14 (.09)	7.7 (.12)	$p < .05$
C_3	3.07 (.37)	4.13 (.36)	$p < .01$
Clq	13.36 (1.29)	14.89 (1.9)	NS
Immunoglobulin g/litre			
IgG	9.1 (.05	8.8 (.05)	NS
IgA	2.5 (.27)	2.8 (.3)	$p < .05$
IgM	.713 (.1)	.737 (.11)	NS
Complement component			
C_3	1.51 (.08)	1.68 (.16)	NS

surgery for inflammatory bowel disease compared to the other patients studied.

Endotoxin was detected in one human systemic serum specimen and no portal specimens, but was found in five rabbit portal serum and two systemic serum specimens. There was no correlation between the amount of enzyme produced and the presence or absence of endotoxin in the specimens. Figure 4 shows correlation between enzyme production by monocytes exposed to portal and systemic serum and the immune complex content of the serum. There was good correlation between enzyme production and immune complexes detected by antisera to IgA ($r = .687$, $p < .001$) IgG ($r = .384$, $p < .05$) and C3,)$r = .504$, $p < .01$), but not with the anti-IgM or anti-Clq antiserum.

DISCUSSION

Our findings of higher levels of immune complexes in portal serum than in systemic serum suggest that even in the fasting state complexes derived from the gut are being removed or inactivated by the liver. The differences between portal and systemic serum are likely to be greater in the non-fasting state and might also have been abrogated by the effect of general anaesthesia, which is known to suppress reticulo-endothelial function (Saba and DiLuzio, 1969). In our system, technical problems have been kept to a minimum by using simultaneously taken and identically treated paired specimens throughout, thus enabling direct comparison between the two specimens.

The role of the liver in removing immune complexes has previously been demonstrated only experimentally by the infusion of complexes into the portal vein of the rat (Thomas and Vaez-Zadeh, 1974). Recently studies in mice have suggested that the uptake of immune complex is a function of both hepatocytes and non-parenchymal cells (Hopf et al, 1981). The modestly increased immunoglobulin A level in portal serum is consistent with the known predominance of IgA-producing lymphocytes in the gut and Peyer's patches and the relative paucity of IgG-producing cells (Shearman et al, 1972). It is thus perhaps surprising that the most marked difference in immune complexes is found in the IgG and not the IgA class, but this is consistent with the observation that most antibodies to dietary components are of the IgG class (Jewell and Truelove, 1972).

Our results from humans are supported by the results of those from our rabbit model, although the two are not strictly comparable. The short exposure of cells obtained from the portal or systemic system to the relevant autologous serum also showed that portal serum "activated" the cells compared to systemic serum.

It is not possible to be sure which factors in portal serum are responsible for the activation of monocytes. Lymphokines (Klimetzek and Sorg, 1977), antigen-antibody complexes (Cardella et al, 1974), complement components, endotoxin and other bacterial products are all capable of activating monocytes (North, 1978: Mundsdotter and Weir, 1980). None of the portal specimens from the human, but five out of the 16 rabbit portal specimens contained endotoxin. These results are

compatible with other reports (Jacob et al, 1977; Pyrtz et al, 1976; Bailey, 1970), which have failed to show conclusively that portal endotoxaemia occurs in cirrhotics under normal circumstances. Those specimens containing endotoxin did not produce more monocyte activation than those without endotoxaemia. Unfortunately, the limulus test for endotoxin is not quantitative and may be too insensitive to assess minor changes, but our results would suggest that endotoxin is not the most important factor in the activation of these monocytes. Similarly, there was no difference between the complement levels as measured between the two paired specimens. The correlation between immune complexes and enzyme production is consistent with previous *in vitro* work, showing that enzyme release is triggered by immune complexes (Cardella et al, 1974; Mee et al, 1979). We cannot exclude the possibility that other variables between portal and systemic serum might be having an effect on monocytes.

Our finding of increased levels of immune complexes in portal serum provides *in vitro* evidence that Kupffer cells do act as a filter, removing substances including soluble immune complexes. Unfortunately, only by a study of cirrhotic patients would it be possible to see the effect of cirrhosis on this action and hence prove beyond doubt these substances spill over into the systemic circulation under certain circumstances. The importance of the soluble immune complexes is not known, but we have shown that they may be affecting the body's immune response by their effect on monocytes.

In contrast with the physiological effect of Kupffer cells in sequestering antigens reaching them by the portal vein, it is possible that recruited and activated tissue macrophages may play a direct role in the pathogenesis of hepatic injury. This is supported by the work of Ferluga and Allison (1978), who have produced an animal model in which hepatic necrosis appears to be dependent upon the activation of monocytes recruited into the liver by the prior injection of *Corynebacterium parvum*. They speculate that the liver damage results in the release of hepatoxic substances by macrophages that have been activated by endotoxins, and postulate that other substances, such as immune complexes, may be similarly operative. We have recently provided some evidence from *in vitro* studies to support this concept (Tanner et

al, 1981). In the present study, we have demonstrated that potential monocytes activators are present in higher concentration in the portal vein in man, and it is possible that they could be important in the perpetuation of some forms of liver disease, more particularly if they act selectively on monocytes recruited into the liver from extrahepatic sites.

Recent work has shown increased levels of immune complexes in various forms of liver disease, including primary biliary cirrhosis, chronic active hepatitis and alcoholic liver disease (Callard et al, 1975; Thomas et al, 1977; Thomas, 1977). It is possible that these increased levels could in part be explained by complexes from the portal vein spilling over to the systemic circulation.

CHAPTER 4

IMMUNE REGULATION AND HLA TYPES IN CHRONIC HEPATITIS

It has been postulated that the pathogenesis of chronic active hepatitis (CAH), a disease of a etiological heterogeneity, is mediated by both humoral and cell-mediated immune mechanisms directed, at least in some patients, against hepatocyte membrane proteins (Vergani, 1979). Investigations of immunoregulatory function in patients with CAH measuring in vitro proliferative responses and immunoglobulin synthesis have demonstrated deficient suppressor T-cell functon (Hodgson, 1978; Tremolada, 1980; Kakumu, 1980; Chisari, 1981; Coovadia, 1981), but the importance of these findings in the pathogenesis of CAH is not clear. It is not known whether defects in immune regulation are primary or secondary, are limited to a sub-group of CAH patients with autoimmune features or are seen predominantly in patients with specific HLA phenotypes, i.e. HLA B8 and/or DW3, which are stongly associated at the population level with hepatitis B-negative CAH.

The present studies were undertaken to define the relationship between various suppressor cells activities and the HLA B8 and B12 phenotypes in patients with chronic hepatitis.

MATERIALS AND METHODS

Patients: Patients with hepatitis B-negative or B-positive CAH, or with chronic persistent hepatitis (CPH), were studied after giving informed consent according to guidelines of the Committee on Human Experimentation of the University of Vermont. Clinical details are shown in Table 1. The diagnoses of CAH and CPH based on morphological criteria (International Group, 1977) were established by liver biopsy.

Preparation of Peripheral Blood Mononuclear Cells: Fresh heparinized (10 U/ml) venous blood was layered on top of an equal volume of Ficoll/Hypaque (specific gravity 1.077) and centrifuged for 30 min at 600 x g. The mononuclear cells were recovered and washed twice with phosphate buffered saline before resuspension. Cell suspensions were made in RPMI-1640 with 25 mM HEPES supplemented with penicillin (100 U/ml), streptomycin (100 mcg/ml), and 2 mM glutamine (RPMI-PSG).

HLA A and B Serotyping: HLA-A and B antigens were identified by the Amos modification of the microlymphocytotoxicity method (Amos, 1965). Using this technique 31 A and B antigens were identified using at least 2 antisera to define each specificity.

Concanavalin A (Con A) Suppression of T-cell Activity (Con ASST):

Con A suppression of T-cell activity was based on the method of Shou et al. (Shou, 1976) which measures the degree of inhibition of Con A-induced ^{3}H-thymidine incorporation into responder T-lymphocytes by Con A generated suppressor T-cells. In order to generate suppressor T cells, the peripheral blood mononuclear cells were adjusted to a concentration of 5 X 10^{6} in RPMI-PSG and incubated with 20% human AB serum in 1 ml aliquots in round bottomed tubes with 60 ug/ml Con A for 48 hr. Identical cultures were incubated without Con A for subsequent addition to responder lymphocytes so that the "non-suppressed" Con A-induced ^{3}H-thymidine incorporation ~~of the~~ of the latter could be assessed. Aliquots were then recombined, pelleted and the cells suspended in fresh RPMI-PSG plus 50 ug/ml mitomycin C and incubated for 30 min. The cells were washed three times in alpha methyl-d-mannoside to remove Con A and then resuspended in RPMI-PSG prior to reincubation with responder lymphocytes.

Because degree of suppression of responder lymphocytes may be dependent in part on the responder cells themselves, 2 allogeneic donors were used as sources of Con A responder cells. In later experiments, isogeneic cells were used also as Con A responders to avoid the problem of possible HLA restriction.

As noted, suppressor activity was measured by the inhibition of Con A (7 ug/ml) stimulated ^{3}H-thymidine incorporation in peripheral blood mononuclear responding cells by Con A generated suppressor cells. Suppressor cells (10^{5}) and responder cells (10^{5}) were incubated in 0.2 ml/well with 20% human AB serum for three days at 37°C in a humidified 5% CO_2 atmosphere. Cells were labelled with 1 uCi/well ^{3}H-thymidine 18 hr prior to harvesting on glass-fibre filters with a semi-automatic suction harvester apparatus (Skatron). Dried filters were counted in minivials by liquid scintillation spectrometry in a Packard Tri-Carb.

The data are expressed as mean cpm from triplicate cultures and the degree of suppression calculated according to the following formula:

$$\% \text{ suppression} = 1 - \frac{cpm_s.\text{Con A} - cpm_s.0}{cmp_c.\text{Con A} - cmp_c.0} \times 100$$

in which cpm_s.Con A is mean cpm of ^{3}H-thymidine incorporation in responder cell cultures containing Con A (in addition to responding cells) generated suppressor cells and Con A, cpm_s.0 is ^{3}H-thymidine incorporation in cultures containing Con A generated suppressor cells but no Con A, cpm_c.Con A is ^{3}H-thymidine responder cell incorporation in responder cell cultures containing preincubated control cells and Con A, and cpm_c.0 is ^{3}H-thymidine incorporation in responder cell cultures containing preincubated control cells but no Con A.

Non-Induced Suppressor T-Cell System (NSS): Non-induced suppression of T-cell activity was based on the method of Hong et al. (unpublished observations) which measures the ability of irradiated T lymphocytes obtained from the patient to inhibit a mixed leukocyte culture (MLC). Cells from the same responder and stimulator in the MLC were used in all assays. Each well contained 10^5 responder cells, 10^5 irradiated stimulator cells, 10^5 irradiated patient cells ("suppressor T cells") and 25% human AB serum; control cultures contained 10^5 irradiated responder cells in lieu of irradiated suppressor cells. The cultures were incubated for 6 days at 37°C in a humidified 5% CO_2 atmosphere before labelling with 1 uCi/well ^{3}H-thymidine 18 hr prior to harvesting on glass-fiber filters in a Skatron apparatus. Dried filters were counted in minivials by liquid scintillation spectrometry in a Packard Tri-Carb.

Suppression was calculated from the difference between mean cpm obtained in MLC's containing patient suppressor cells and control MLC's which contained only irradiated responder cells by the following formula:

$$\% \text{ suppression} = 1 - \frac{cmp_s}{cpm_c} \times 100$$

in which cpm_s is the mean cpm of the MLC in the presence of patient irradiated suppressor cells and cmp_c is the mean cpm of the MLC in the presence of irradiated responder cells.

Prostaglandin Suppressor System (PgSS): Suppression of T-cell activity by prostaglandin-producing mononuclear cells is based on the method of Goodwin et al. (Goodwin, 1977) which utilizes indomethacin in

its role as a prostaglandin synthetase inhibitor to enhance phytohemagglutinin (PHA)-stimulated ^{3}H-thymidine incorporation in vitro. Peripheral blood mononuclear cells were adjusted to 10^5 cells/well and incubated with 1 ug/well PHA - P (Burroughs Welcome) with and without 250 ng/well indomethacin (Merck, Sharp & Dohme). Wells contained a final volume of 0.25 ml/well, 20% AB serum and RPMI-PSG medium. Cultures were incubated for 3 days at 37°C in a humidified 5% CO_2 atmosphere before labelling with 1 uCi/well ^{3}H-thymidine 18 hr prior to harvesting on glass-fiber filters in a Skatron apparatus. Dried filters were counted in minivials by liquid scintillation spectrometry in a Packard Tri-Carb.

The data are expressed as mean CPM from triplicate cultures with suppression calculated according to the following formula:

$$\% \text{ suppression} = 1 - \frac{\text{CPM-PHA}}{\text{CPM-PHA-IND}} \times 100$$

in which CPM-PHA is the mean CPM in the absence of indomethacin and CPM-PHA-IND is the mean CPM in the presence of indomethacin.

Statistical evaluation was performed by analysis of variance. If the F values indicated statistically significant differences, group comparisons were made by a Neuman-Keuls test to determine which specific differences were present.

RESULTS

Suppressor T-cell activity measured by the Con A-induced suppressor system using a single individual (ELK) as a source of allogeneic responder cells is illustrated in Fig. 1A. Comparison of the whole group revealed considerable overlap but the values for patients with hepatitis B-negative CAH (51.4±8.3) ($\bar{X}$±SE), hepatitis B-positive CAH (47.2±16.3) or CPH (80.5±5.1) were not statistically significantly different from values obtained in controls (57.3±5.3). As illustrated in Fig. 1B, however, mean suppressor cell activity for patients with hepatitis B negative CAH (6.4±9.0) was diminished ($p < .001$) compared

CHAPTER 4. Table 1: Clinical Data on 32 Patients with Cronic Hepatitis

Pt.	Sex	Age	HLA	Rx	ANA/ASMA	Blood Tx	HBsAg/ HbcAb/ HbsAb	Alb	Glob	G Glob	SGPT
CHRONIC ACTIVE HEPATITIS											
LF	F	26	A1 Aw23 B8 B14	0	4+/ +	0	0/0/0	4.0	5.1	2.9	454
RN	F	22	A1 Aw31 B8	0	0/4+	0	0/0/0	2.7	4.1	2.9	104
IB	F	46	Aw28 B14	0	4+/0	0	0/0/0	4.4	3.2	2.3	106
MJ	F	56	A2 Aw30 B12 B18	0	3+/4+	0	0/0/0	4.3	3.8	2.4	89
PM	M	77	A2 Aw30 B5 B12	0	4+/0	0	0/0/0	3.7	3.0	1.4	44
EB	F	63	A1 Aw23 B8 B12	0	0/0	0	0/0/0	3.3	4.3	1.9	14
RR	F	67	A3 A29 B7 b12	P	+*/-	0	0/0/0	4.6	3.8	-	38
DC	F	57	A1 A28 B5 B14	P	+*/-	0	0/0/0	3.9	2.5	0.8	64
HL	F	60	A1 Aw24 Bw22Bs35	P	+/0	0	0/0/0	2.9	9.9	1.5	22
EF	F	63	A11 B7 B12	P	+*/-	0	0/0/0	3.4	4.7	3.3	44
MS	F	43	A3 Aw24 B27 B40	P	+*/-	0	0/0/0	3.4	3.8	1.3	123
DH	F	24	A1 Aw30 B8 B13	P	0/0	0	0/0/0	3.4	2.2	-	83
DC	M	64	A3 Aw24 B13Bw35	P	2+/0	0	0/0/0	3.1	5.3	3.0	54
RA	M	32	A1 B8	PA	+*/-	0	0/0/0	4.2	3.2	1.9	45
BM	M	71	A2 A11 B18 B40	P	+*/-	+	0/0/0	2.5	2.8	-	76

Pt.	Sex	Age	HLA	Rx	ANA/ASMA	Blood Tx	HBsAg/ HbcAb/ HbsAb	Alb	Glob	G Glob	SGPT
CHRONIC ACTIVE HEPATITIS (continued)											
MM	M	57	A2 A9 B5 Bw4	P	-/-	+	0/0/0	4.0	3.2	-	89
WF	F	64	A29Aw32 B14 B17	P	0/3+	+	0/0/0	3.9	2.6	1.4	134
AB	F	66	Aw30Aw32 B13 B40	P	0/0	+	0/0/0	4.1	3.3	1.3	91
RM	M	41	Aw24A29 B12 B40	0	0/0	DA	0/+/0	4.2	3.8	2.3	156
LB	M	41	A2 Aw24 B12 B14	0	0/0	DA	0/+/0	4.5	3.7	1.8	155
MB	M	64	A11 B12 B35	R	0/0	Acu	0/+/+	4.3	3.5	1.0	40
CN	M	24	A28 A29 B5 B12	P	0/0	0	+/+/0	3.4	2.9	0.9	55
AV	M	52	A11Aw24 B18w35	P	-/-	+	0/+/+	3.6	-	-	-
EK	M	65	A11Aw24 B5 Bw21	P	0/0	0	0//0	4,7	2,6	0.6	23
CHRONIC PERSISTENT HEPATITIS											
LM	F	22	A1 Aw24 B8 B40	0	0/0	0	0/0/0	3.9	4.1	-	107
MC	M	27	A11 A29 B12 B14	0	0/0	0	0/0/0	4.8	2.6	1.2	87
EW	F	30	A1 Aw32 B8	0	0/0	DA	+/+/+	4.2	3.9	2.2	165
DB	F	24	A2 B12Bw35	0	0/0	DA	0/+/0	4.5	3.7	2.2	144
LP	F	27	A1Aw24 B8 B17	0	0/0	DA	0/0/+	4.8	3.0	1.8	101
TV	M	29	A1 A2 B7 B15	0	0/0	DA	0/+/+	4.3	2.8	1.5	74

Pt.	Sex	Age	HLA	Rx	ANA/ASMA	Blood Tx	HBsAg/ HbcAb/ HbsAb	Alb	Glob	G Glob	SGPT
CHRONIC HEPATITIS (continued)											
PR	M	35	A11A26 B5 B7	0	0/0	DA	0/+/+	4.6	3.4	1.0	263
AM	M	30	A2 B17Bw35	0	0/0	DA	0/+/+	4.5	2.9	-	125

Rx: P - prednisone, PA - prednisone, azathioprine. 0 - None.

ANA/ASMA: Antinuclear Antibody/Anti-Smooth Muscle Antibody.

ANA/ASMA: 4+ 1:640, 3+ 1:320, 2+ 1:160, + 1:80, 0 1:80.
+* previously positive

Blood Tx: + - blood transfusions, DA - intravenous drug abuse, Acu - acupuncture.

Hepatitis B Markers: ABsAg - Hepatitis B surface antigen. HBcAb - Hepatitis B core antibody, HBsAb - Hepatitis B surface antibody, + - positive, 0 - negative.

Alb: Serum albumin, g/dl

Glob: Serum globulin, g/dl

G Glob: Serum gamma globulin, g/dl

SGPT: Serum glutamic-pyruvic transaminase (alanine aminotransferase), IU/L -: Not determined.

with control values (52.8±6.0) when another individual (DDW) was used as a source of allogeneic responder cells. In order to avoid this problem of possible HLA restriction of the cell-to-cell interaction required in this regulatory response, we also performed experiments in which isogeneic cells were used as the responder cell population. Experiments performed in patients with CAH and CPH (Fig. 1C) revealed no statistically significant differences comparing values from patients with chronic hepatitis and those from controls. No differences were apparent comparing treated and untreated patients in any of the assays.

Fig. 2 depicts the results of the Con ASST in patients with chronic hepatitis using 1-3 allogeneic responder cell populations with patients grouped according to whether or not these phenotypes included the HLA antigen B8, B12 or neither. The values obtained in patients possessing B8 or B12 was within the range of those obtained from patients not possessing either of these antigens. Statistical evaluation by analysis of variance revealed no significant differences among groups. In general, the magnitude of suppression was more dependent upon the responder cells selected than on the HLA phenotypes. The values obtained were consistent with those seen in control groups. The percent suppression observed in patients possessing HLA B8 was similar to those we have determined in normals with HLA B8 and patients with celiac disease possessing HLA B8 (unpublished observations).

Suppressor T-cell activity measured by the noninduced suppressor cell system in patients with CAH is shown in Figure 3. Although mean suppressor activity in patients with hepatitis B negative CAH was lower than controls, the difference was not significant ($.05 > p < .1$). However, compared with CPH, the value was depressed ($p < .01$). Again, when values were compared, depending on the presence of HLA B8, B12 or neither in the phenotype of the donor, no statistically significant differences were found.

Increased PgSS activity was found in patients with either hepatitis B-negative CAH ($p < .005$) or hepatitis B-positive CAH ($p < .05$) compared with controls while no differences were noted between patients with CPH and controls (Fig. 4). When results were compared among patients with chronic hepatitis dependent on the presence of HLA B8, B12 or neither of these phenotypes, no significant differences were present.

There was no significant correlation between PgSS and either NSS activity ($r = .20$), Con ASST activity using ELK ($r = .12$), or DDW ($r = .04$) as sources of responder cells. Similarly, when the 10 patients with the highest PgSS activity were contrasted with the 10 lowest values, no differences were present in the magnitude of T-cell suppressor function as measured by Con ASST or NSS activity.

DISCUSSION

Subsequent to Allison's hypothesis (Allison, 1971) that alteration of T-cell control plays a role in the pathogenesis of autoimmune disorders, Eddleston and Williams (Eddleston, 1974) proposed that the autoimmune reaction of hepatitis B-negative CAH was secondary to decreased suppressor T-cell activity. Furthermore, because of the increased frequency of HLA B8, which had been reported in populations of patients with hepatitis B-negative CAH (Mackay, 1972), they postulated that this histocompatibility antigen might serve as a marker for a specific defect in suppressor T-cell function, although there was no good evidence in man that genes on chromosome 6 controlling immune regulation were linked to genes coded in the HLA locus. Galbraith et al. (Galbraith, 1976) reported increased levels of antibodies to rubella, measles, nuclei and smooth muscle in patients with CAH with HLA B8 or B12, but these results were not confirmed by Lindberg et al. (Lindberg, 1977; Lindberg, 1979). Since that time, alterations in immunoregulatory T-cell activity have been demonstrated in CAH (Hodgson, 1978; Tremolada, 1980; Kakumu, 1980; Chisari, 1981; Coovadia, 1981) and there has been confirmation of the increased frequency of HLA B8. HLA Dw3 which is found in linkage disequilibrium with B8 is also increased in CAH (Opelz, 1977). This is the first study in which investigation of immune regulation has been performed in conjunction with HLA typing.

The recent description of a prostaglandin-producing monocyte suppressor cell system by Goodwin and associates (Goodwin, 1977) has underscored the complexity of the suppressor cell network, which is thought to physiologically inhibit the immune response. They suggest that regulation of the Con ASS might be controlled by the PgSS (Goodwin, 1980), that lymphocytes from normal subjects with HLA B12 are less inhibited by prostaglandins *in vitro* (Staszak, 1980) and that increased

activity of the PgSS may contribute to cellular hyporesponsiveness in patients with Hodgkin's disease and sarcoidosis (Goodwin, 1977; Goodwin, 1979).

Data from published studies of suppressor T-cell deficiencies in patients with CAH do not permit interpretation with regard to whether or not the defect is primary or represents an epiphenomenon. Reversal of suppressor T-cell defects in patients whose CAH is in remission on anti-inflammatory therapy or during recovery (Hodgson, 1978; Tremolada, 1980; Chisari, 1981) suggests that at least in some patients the defect may not represent an immunological antecedent of disease. In addition, it does not appear that decreased suppressor cell activity is seen only in patients with autoimmune features but is seen also in patients who appeared to have virus-induced CAH.

The results of the present studies point to some difficulties involved in interpreting in vitro suppressor cell systems which involve measurements of Con ASS on T-cell activity. First, the required cell-to-cell interactions may be dependent on the HLA phenotypes of the interacting cells. A dissociation between results of studies which measure Con ASST using a responder system where DNA synthesis is mitogen-induced (Con A) as compared to MLC studies was observed. This has previously been noted by Hodgson (Hodgson, 1978), prompting him and others to use a single individual as the source of allogeneic responder cells. As demonstrated by the present studies however, depending on the individual chosen, interpretation of results may differ. In our studies using DDW as a source of responder cells, one would conclude that a significant proportion of patients with heptatitis B-negative CAH have weak suppressor T-cell activity while such a conclusion cannot be reached using ELK as a source of allogeneic responder cells (Fig. 1). This difference in allogeneic responders might explain the differences between our study and that of Hodgson et al (Hodgson, 1978).

No differences were found using an isogeneic responder system although fewer patients were tested. In contrast to those findings Chisari et al (Chisari, 1981) did find decreased suppressor T cell activity using isogeneic responder cells in 6 of 12 assays in 4 patients with hepatitis B CAH. The reasons for the differences are not apparent. Chisari used a 96 hr induction time compared with 48 hr in our study and it is conceivable that during the additional 48 hr of activation the

suppressor cell population measured, and/or other cell populations, are affected differently.

Using a non-induced suppressor T-cell assay, we noted no significant depression of suppressor activity in any of the groups. This may reflect the heterogeneity of different suppressor systems measuring different populations of suppressor cells which in part are dependent on different cell-to-cell interactions according to the reacting cells in the MLC.

In searching for a subgroup of patients with CAH in which weak suppressor T-cell activity might play a role in pathogenesis, it is clear that there is no difference in Con ASST activity or NSS activity comparing patients with HLA B8, HLA B12, or those with neither of these antigens. The magnitude of suppression in the Con ASST activity is more dependent on the allogeneic responder cell population used than on the HLA type of the patient donor of the suppressor cell (Fig. 2). The results in figure 2 are from patients with CAH and CPH, and it should be noted that none of the patients with hepatitis B CAH possess HLA B8. Thus, these data do not support the concept that a gene coding for weak suppressor T-cell activity is linked to HLA B8 or B12. Since initiation of our studies, investigations of suppressor T-cell function have shown alterations in patients with acute and chronic hepatitis of both hepatitis B positive or negative forms. Thus, it is not surprising that HLA B8 or B12 did not discriminate between patients with decreased suppressor T-cell function of various etiologies. Because we have not completed D locus typing, we cannot say with certainty whether or not linkage between a "weak suppressor" gene and a gene for DW3 may occur, but the linkage disequilibrium between B8 and DW3 makes this unlikely.

Within families, however, it is possible that a gene coding for weak suppressor cell activity may be linked to a specific HLA gene and family studies will be needed to search for such linkage. Along these lines, it should be noted that <u>in vitro</u> cellular cytotoxicity of lymphocytes from hepatitis B-negative and positive CAH to ^{51}Cr labelled pigeon erythrocytes coated with a human liver specific lipoprotein was greater in patients possessing HLA DW 3 or DRW3 (Vogten,1979; Kastelan, 1979). In comparing suppressor cell activity of CAH patients with HLA B8 to normals with HLA B8 and to patients with coeliac sprue with HLA B8, we have observed no difference in the range of values obtained

(unpublished observations), which also suggests that weak suppressor cell activity is not merely a manifestation of HLA B8.

The results of the present studies which indicate increased activity of a PgSS in patients with CAH provide further evidence that immunoregulatory function is altered in patients with CAH. These findings may reflect increased suppression by a population of prostaglandin-producing suppressor cells in vivo in hepatitis B-negative or B-positive CAH patients. It is possible that this increased PgSS activity is in part responsible for the decreased proliferative responses to nonspecific antigens observed in vitro (Wands, 1975) in patients with CAH. The magnitude of the PgSS response does not appear to be dependent on HLA B8 or B12 in these patients and it is not clear whether or not this increase in presumed monocyte-mediated depression is a quantitative or qualitative effect or may represent a sensitivity of lymphocytes to prostaglandin-producing cells. Our data do not support the concept that the PgSS controls the Con ASST response since no correlation between the results of the suppression measured by these systems was observed.

The findings of increased PgSS activity which like the ADCC is thought to reflect activity of a monocyte suppressor cell are in apparent contrast to those of Chisari et al (Chisari, 1981) who reported decreased activity of an adherent cell suppressor population in patients with Hepatitis B positive CAH. It is possible that the differences noted using these methods also reflect heterogeneity of suppressor cell population, in this case monocyte suppressors. Alternatively these results may be dependent on the stage of disease, in particular on the presence of cirrhosis. Further studies (Chapter 5) suggest that increased monocyte suppressor cell activity appears to be seen only once cirrhosis has developed.

CHAPTER 5

STUDIES ON LYMPHOCYTE HYPORESPONSIVENESS IN CIRRHOSIS: THE ROLE OF INCREASED MONOCYTE SUPPRESSOR CELL ACTIVITY.

INTRODUCTION

Patients with cirrhosis are especially susceptible to infection and have been shown to have reduced cellular immunity manifested by a reduced response to skin test reagents (O'Keefe et al, 1980) and hyporesponsive lymphocytes *in vitro*. Reduced lymphocyte transformation has been shown to be present in many types of liver disease including viral hepatitis, chronic active hepatitis, primary biliary cirrhosis and alcoholic liver disease. The cause of this reduced response is unknown, but much of the lymphocyte hyporesponsiveness may be attributable to circulating inhibitory factors since unidentified substances found in the serum of cirrhotic patients have been shown to affect lymphocyte responses *in vitro* (Mella et al, 1970; Hsu et al, 1971; Fox et al, 1973; Newberry et al, 1973; MacSween et al, 1973; Wands et al, 1975; Nakao et al, 1975; Brattig et al, 1976; Young et al, 1979; Feighery et al, 1980) and phagocyte function (DeMeo et al, 1972, Wyke et al, 1980). This inhibitory effect appears to be dependent on the degree of hepatocyte damage and/or the presence of cirrhosis, rather than on the underlying etiology of the liver diseases (Young et al, 1979).

It has been suggested that defects in the immuno-regulatory system play a role in the development of chronic hepatitis. Reduced suppressor cell activity may allow a cellular and/or humoral immunological response, possibly mediated against hepatic membrane proteins, to occur and to persist resulting in hepatocyte damage (Vergani et al, 1979). Reduced T-lymphocyte suppressor activity has been demonstrated in chronic hepatitis using assays of T-lymphocyte proliferation (Hodgson et al, 1978; Chisari et al, 1981) and B-cell immunoglobulin production (Kakumu et al, 1980). More recently, development of monoclonal antibodies has allowed preliminary morphological studies of the suppressor:helper cell ratio (Routhier et al, 1980). Although these abnormalities in T-cell suppressor cell function may explain the hypergammaglobulinemia (Stobo, 1979) seen in cirrhosis they do not explain the lymphocyte hyporesponsiveness.

Increased activity of a prostaglandin-producing suppressor cell system (PgSS), thought to be mediated by the monocyte, has been demonstrated in conditions such as sarcoidosis (Goodwin et al, 1979) and Hodgkin's disease (Goodwin et al, 1977). In these patients and also those with systemic lupus erythematosus (Marlenson et al, 1980), this may in part account for the decreased lymphocyte responsiveness. More recently we have documented such an abnormality in patients with chronic active hepatitis (CAH) (Krawitt, et al, 1981). In this study we have investigated the possibility that similar monocyte suppressor cells may explain the cellular hyporesponsiveness seen in cirrhosis. We utilized the PgSS and the adherent cell suppressor system (ACSS), also thought to be monocyte-mediated. We further studied the effects of monocyte depletion on lymphocyte response and the possibility that the inhibitory effect of serum from patients with cirrhosis might be explained by an effect on the monocyte.

METHODS

Patients

Patients were selected from outpatient clinics and from the wards. Disease was proven by liver biopsy except in 1 patient with primary biliary cirrhosis. Controls were selected mainly from hospital or laboratory staff and were similar in age range and sex ratio to the patients. The study conformed to the guidelines of, and was approved by, the Committee on Human Experimentation at the University of Vermont.

Preparation and Culture of PBMC.

Peripheral venous blood was drawn into syringes containing preservative free heparin (10 Units/ml). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Ficoll/Hypaque and were then washed 3 times in phosphate buffered saline. All suspensions were made in RPMI-1640 with 25 mM HEPES. (Gibco Laboratory), supplemented with penicillin (100 units/ml), streptomycin (100 ug/ml) and 2 mM glutamine.

For the prostaglandin-producing suppressor system, the method used was similar to that described by Goodwin, et al. (1977). To individual wells of a flat bottom microtitre plate (Costar 6.4 mm well diameter) was added 50 ul containing 1×10^5 of PBMC. To this was added 50 ul of

decomplemented human AB serum, 1 ug/well of purified phytohemaglutinin (PHA-P) (Burroughs Welcome) and either indomethacin (IND) 250 ng, or medium alone, to make a final volume of 250 ul/well. Following incubation at 37°C in 5% carbon dioxide in a fully humidified atmosphere for 72 hours, one uCi/well of ^{3}H thymidine (New England Nuclear Sp Act 6.7) was added. Following a further 18 hours incubation, cells were harvested on glass fiber filters using a Skatron semiautomatic suction harvester apparatus. Dried filters were then counted in minivials by liquid scintillation spectrometry in a Packard Tri-Carb.

For the adherent cell suppressor system the method was similar to that described by Laughter and Twomey (1977). PBMC from the patients and controls were exposed to 7000 rads, effectively killing lymphocytes but leaving monocytes relatively undamaged. The 7000 rads used was considerably higher than the 2500 rads used by Laughter and Twomey, but preliminary studies showed that in our system this dosage was required to prevent a proliferative response of the stimulator cells. The surviving cells were then recounted and increasing numbers of the irradiated cells (1×10^5, 2×10^5, 4×10^5, 8×10^5) were then added as stimulator cells to form a mixed leukocyte culture with 1×10^5 PBMC obtained from a single normal subject acting as the responder cells. Each well contained 25% human AB decomplemented serum and was made up to a final volume of 200 ul per well. These were then incubated, labelled with ^{3}H thymidine after 6 days, and counted after a further 18 hour culture as indicated above.

Monocyte Depletion

PBMC were incubated in plastic petri dishes for two hours, at which time the non-adherent cells were removed, washed and counted. Esterase-stained cyto-spin preparations before and after depletion showed that monocyte cells fell from 24.8% ± 8 and 23.5% ± 6 in the cirrhotics and controls respectively to 6.4% ± 4 and 5.9% ± 6. The differences between cirrhotics and controls were not significant.

Presentation and Analysis of Data

Results of the response to the mitogens are expressed as counts per minute ± 1 standard deviation. Each reading was a mean of triplicate

readings, each triplicate being generally within 10% of the mean. The % suppression was calculated from the formula (1-A/B) x 100. For the PgSS, A is the mean CPM in the absence of indomethacin and B is the mean CPM in the presence of indomethacin. For the ACSS, A is response to 8 x 10^5 cells and B is a response to that number of cells giving the maximum response. Although in the original description of the ACSS the highest CPM were obtained at 2 x 10^5 stimulator cells, this was not always the case in our experiments. One-way analysis of variance and the Student t test were used in statistical analysis. Probability values of less than 5% were considered significant.

RESULTS

Patients

A total of 26 patients with CAH, 8 with alcoholic liver disease and cirrhosis (ALD), 4 with other forms of cirrhosis (3 primary biliary cirrhosis (PBC), 1 drug-induced cirrhosis) and 8 with chronic persistent hepatitis (CPH) were studied. Some patients were tested on more than one occasion. Forty-six controls were also studied. Of the CAH group 16 had evidence of cirrhosis on liver biopsy and 10 had no such evidence. Five were positive for HB_s ag while 21 were negative. Three of the CPH patients were positive. The age range of the patients was 22 to 75 years (mean 48), and for the controls, 23 to 70 years (mean 42).

The Prostaglandin-Producing Suppressor Cell

The % change in the proliferative response on adding indomethacin was -4.1% ± 24.3 for the controls and was significantly higher for the CAH group (+13.4% ± 22.9, $p<.001$), ALD (+27.9% ± 24.5, $p<.05$) and the other cirrhotic group (+18.1% ± 9.5, $p<.025$). These results for individual patients are shown in figure 1, which also shows that the values from CPH patients (1.9% ± 18.7) were not significantly different from the controls. There was no significant correlation between the response to PHA and the change induced by adding indomethacin ($r = .184$). Thus, the response to indomethacin was independent of the initial proliferative response to PHA.

In the CAH group there was no significant difference in the % suppression between those that were HB_s positive and those that were HB_s

negative (10.8% ± 10.2 versus 14.4% ± 19.9), but those who had progressed to cirrhosis had significantly higher values than those who had not (17.7 ± 20.6% vs. 6.0 ± 7.3%, $p < .05$) (see Fig. 2).

Two patients underwent testing on 3 separate occasions and the values were 9.4, 11.4, 13.5% and 6.5, 9.3, and 59.0%. A similar scatter was found in those patients who underwent testing on 2 occasions. Neither in these patients nor in the group as a whole was there a clear-cut relationship between the results and the severity of disease or drug therapy (see Fig. 2). We repeated the PgSS 8 times on 2 healthy controls at weekly intervals and the results expressed as mean ± SD were 1.3% ± 6.6 and 1.9% ± 3.4 showing that in health there is little variation.

Monocyte Depletion and the PgSS

Twenty patients with biopsy proven cirrhosis (7 ALD, 1 PBC, 12 CAH) and 15 controls were studied. As shown in Figure 3, although the response of the controls to PHA-P tended to be slightly increased by monocyte depletion, there was no significant difference between the counts obtained before and after monocyte depletion (133,130 cpm ± 56,390 vs 147,360 cpm ± 48,650). This is similar to results obtained by others (22). On the contrary, patients with cirrhosis demonstrated a highly significant increase in the response to PHA-P after monocyte depletion (48,940 cpm ± 27,510 vs 88,000 cpm ± 47,370, $p < .005$ in autologous serum and 66,390 cpm ± 36,700 vs 112,440 cpm ± 56,210, $p < .01$, in AB serum). Thus monocyte depletion significantly increased the response of the lymphocytes to the mitogen PHA-P. There was a significant correlation between the PgSS and the change on monocyte depletion ($r=.6583$ $p < .01$, Figure 4). Furthermore when the PgSS was performed in these same cirrhotics using monocyte depleted cells, the effects of indomethacin were abolished and under these circumstances the results were not significantly different from monocyte-depleted controls (0.8% ± 4.3 and 1.1% ± 3.8 respectively).

Effect of Serum Factors on PgSS

As shown above mixed mononuclear cells from the cirrhotic patients showed a reduced response to PHA-P in autologous serum compared to that

seen with AB serum ($p < .02$) but no such difference was found for the control patients. Twenty patients with cirrhosis and 16 controls were tested for their response to indomethacin in autologous and in AB serum. In the cirrhotic patients, the % change in autologous serum was significantly greater than that in AB serum (31.9% ± 30.2 vs 20.5% ± 25.2, $p < .02$), while no such difference was found in the controls (2.4% ± 5.4 vs. 2.2% ± 4.8) (Fig. 3). This suggested the possibility that part of the known inhibitory effect of cirrhotic serum might be blocked by the addition of indomethacin. We therefore measured the response to PHA-P of cells from a normal volunteer in the presence of 16 specimens of cirrhotic serum and 16 control specimens. The mean response of these cells in cirrhotic serum was 127,200 CPM ± 34,650 and with the addition of indomethacin, 153200 CPM ± 38460. The mean change on adding indomethacin was therefore 29% ± 3.7. In the control serum the respective values were 161,600 CPM ± 28,360 and 155,960 CPM ± 23,510 with a mean change of -3.4% ± 4.5 (Fig. 5). Thus the significant reduction in the response to PHA-P achieved by incubation of normal cells in cirrhotic serum compared to the control serum (127,200 CPM ± 34,650 vs 161,600 ± CPM ± 28,360 $p < .02$) was abolished by the addition of indomethacin (153,200 CPM ± 38,460 vs. 155,960 CPM ± 23,510) (Fig. 6). There was no correlation between the PgSS of the cirrhotic patients in autologous serum and the effect of the serum on the normal cells ($r = .264$).

To try to confirm that the inhibitory effect of cirrhotic serum may be mediated via the monocyte we also studied the response to PHA-P after monocyte depletion. Unlike the response of normal PBMC, there was no significant difference between those results obtained by incubating normal monocyte- depleted cells in cirrhotic serum and to those obtained by adding control serum (94,720 CPM ± 25,530 and 92,360 CPM ± 18,200) (Fig. 6). It should be stressed that these latter two experiments were performed on separate occasions and are not directly comparable.

Adherent Cell Suppressor System

The results for 11 patients with cirrhosis and 16 controls are shown in Figure 7. The age range of the cirrhotics was 24-73 years (mean 42) and the controls 24-64 years (mean 46). In general the counts

were higher for the control patients than for the cirrhotic patients at all concentrations of stimulator cells and were statistically significantly different at concentrations of 1×10^5 and 8×10^5 cells ($p < .05$). The % suppression obtained was 57.7% ± 27.3 for the cirrhotic patients and 36.2% ± 18.4 for the control patients ($p < .025$). When the test was repeated on monocyte-depleted cells the suppressor effect was abolished confirming that the suppression was caused by cells, presumably monocytes, which have been removed by the depletion techniques. The relevant values at increasing concentration of stimulator cells ($n = 11$) for the monocyte depleted controls were 6.4 ± 1.2, 6.0 ± .8, 5.6 ± .6 and 6.3 ± .5 (all CPM $\times 10^{-4}$).

DISCUSSION

We have shown that in the presence of indomethacin there is a significantly increased lymphocyte response to PHA-P in patients with cirrhosis, indicating increased activity of a prostaglandin-producing suppressor cell, probably a monocyte (Goodwin et al, 1977, although possibly a glass-adherent T-lymphocyte (Rogers et al, 1980). Not all individuals with cirrhosis had increased prostaglandin producing suppressor activity and some fell into the normal range. The increased activity appears to be only seen when cirrhosis has developed since it was not found in patients with CPH or CAH without cirrhosis. This effect of indomethacin was abolished by monocyte depletion supporting the theory that it is monocyte-mediated. In those patients in whom more than one result was obtained, there was no close relationship to drug therapy, disease activity or HB_s ag status. Although there was variation in the results when repeated at intervals, results in cirrhotics were consistently higher than those of controls. The observation that the PgSS activity appears to be secondary to cirrhosis suggests that it is not of primary immunopathogenic importance in the development of chronic hepatitis but it may partially explain the known increased susceptibility of these patients to infection. Similarly it may be a marker of altered macrophage-fibroblast interaction and be important in the pathogenesis of the liver fibrosis as there is evidence that both monocytes (Korn et al, 1980) and prostaglandins (McCarthy et al, 1980) may be implicated in the control of fibrogenesis (discussed further in Chapter 7).

Further support for the importance of the monocyte in the reduced lymphocyte response is obtained from the observation that monocyte depletion significantly increased the response to PHA-P in cirrhotics while having little effect on the normal controls. Moreover, there was a good correlation between the effects of monocyte depletion and the PgSS. There was also significantly greater suppression as measured by the ACSS in cirrhosis compared to the controls and this suppression was abolished by monocyte depletion. The lower counts obtained at each concentration of stimulator cells in the ACSS indicates a reduced mixed leukocyte culture reaction (MLC), another indication of in vitro cellular hyporesponsiveness. The cause and significance of this reduced MLC is unclear but similar abnormalities have been shown in malignancy (Berlinger et al, 1976) and inflammatory bowel disease (Fiske et al, 1980) and in these conditions, removal of macrophages has been shown to reverse the depressed MLC response.

The increased sensitivity to indomethacin of lymphocytes from cirrhotic patients in autologous serum compared to AB serum led us to investigate the possibility that the known inhibitory effect of serum from cirrhotics may be partly reversed by the addition of indomethacin. We found that PBMC obtained from a healthy volunteer were significantly less responsive to PHA-P in cirrhotic serum compared to control serum and that the effect of IND on these cells in the cirrhotic serum was much greater than in control serum. Furthermore this inhibitory effect of cirrhotic serum was not present when monocyte-depleted cells were studied. Thus, it would seem that the inhibitory effects of cirrhotic serum may in part be monocyte-mediated and prostaglandindependent. High levels of circulating prostaglandins have been shown to be present in cirrhosis (Zusman et al, 1977) and these may have originated from cells of the monocyte cell series.

Using an immunoglobulin-producing assay, Kakumu and colleagues (1980) failed to show any monocyte suppressor activity in patients with acute viral hepatitis. Our results do suggest the presence of such activity in cirrhosis, but are not inconsistent with their findings as we found no abnormality in patients with CPH or CAH who had not developed cirrhosis. Chisari and colleagues (1981) suggest that there is reduced adherent-cell suppressor activity in both acute and chronic

hepatitis but studied only 4 patients with biopsy-proven chronic hepatitis and did not comment on the presence of cirrhosis. Results of the ACSS are dependent in part on HLA antigen disparity between the responder and the stimulator which might explain these apparently conflicting findings. Our results better explain the lymphocyte hyporesponsiveness seen in cirrhosis and are consistent with the results obtained with the PgSS. The observation that the PgSS is independent of the initial proliferative response may indicate that other factors are involved including reversible suppressor influences (11), although there is also considerable variation in normal responses and it is impossible to say what is "normal" for a single individual. Unlike sarcoidosis and systemic lupus erythematosus, where monocyte suppressor cells have also been found, there is no absolute monocytosis in cirrhosis, but there is other evidence of reduced monocyte function.

Previous investigations into the cause of the inhibitory effects of serum from cirrhotics have produced conflicting results in different systems. Some suggest that the effect is due to the presence of active inhibitory substances (Feighery et al, 1980) but others suggest a deficiency state (Wyke et al, 1980; Van Epps et al, 1975), perhaps in part nutritional (O'Keefe et al, 1980). Our results favour the former, namely the presence of active inhibitors as the effect is reversed by the addition of indomethacin. The observation that the monocyte suppressor cell activity is found only in the group of patients with established cirrhosis may indicate that this may be due to an overspill, or shunting-phenomenon of substances from the portal vein which, under normal circumstances would have been removed by the liver.

It is tempting to suggest a possible role for indomethacin in the treatment of patients with chronic liver disease, particularly those who prove susceptible to recurrent infection. There is some evidence that in common variable immunodeficiency (Goodwin et al, 1978) there is partial reversal of the cellular immune defect with indomethacin, but the tendency of indomethacin to cause fluid retention in cirrhotics would be a significant factor and may contraindicate the trial of indomethacin in these patients (Boyer et al, 1979).

In conclusion, these results suggest that glass-adherent suppressor cells play an important role in the depressed cellular immunity seen in some patients with cirrhosis. The inhibitory effect of cirrhotic serum appears to be monocyte-mediated and prostaglandin-dependent.

CHAPTER 6

EFFECTS OF TESTOSTERONE, OESTRADIOL AND PROGESTERONE ON IMMUNE REGULATION

Clinicians have long realized that several connective tissue diseases are much more frequently encountered in females than in males and that pregnancy can dramatically alter the disease course. Pregnancy is a time of considerable endocrine change and it is possible that alterations in the circulating levels of various sex hormones may account, in part, for some of these observations. An important role for sex hormones is further suggested by a number of other observations including the increased resistance of females to some bacterial infection (Wheater and Hurst, 1961), higher immunoglobulin levels in young women than in either older women or men (Rhodes et al, 1969), and the observation that oral contraceptives result in increased levels of cirulating IgM (Bole et al, 1969). Augmentation of the antibody response in women compared to men to E. coli (Paty et al, 1976), measles (Michaels and Rogers, 1971), rubella (Spencer et al, 1977), brucella (Rhodes et al, 1969) and hepatitis B virus (London and Drew, 1977) has also been described. Auto-antibodies are much more frequent in women than in men (Hooper et al, 1972) and sex differences in in vitro lymphocyte cytotoxicity (Santoli et al, 1976) and proliferation (Barnes et al, 1974) have been described.

Perhaps the best studied disease known to be associated with abnormal immunoregulation is systemic lupus erythematosus (SLE), in which the sex ratio in adults is usually about 9 females to 1 male (Kornreich, 1976) but is only about 3 to 1 in children. SLE is also more common in Klinefelter's syndrome (Stern et al, 1977) than in normal males. In both children and patients with Klinefelter's syndrome, hormonal differences are much less marked than in normal adults. Studies of the NZB mouse, an animal model for SLE, have also shown that female mice have more severe disease than males and that the prognosis in females is improved by the administration of testosterone (Roubinian et al, 1977). Similarly castration or oestrogen therapy results in male mice dying earlier of the disease (Melez et al, 1978). Both in the animal model and in humans with the disease, defects in suppressor cell function have been described (Talal, 1976) and may be a factor in the immunopathogenesis of the disease.

The purpose of this study was to investigate possible actions of sex hormones on aspects of *in vitro* immunoregulatory function. We have therefore studied the effects of testosterone, progesterone, and oestradiol on the Con A-induced suppressor cell system which is mediated by T lymphocytes and on the prostaglandin-producing suppressor cell system which is thought to be mediated by monocytes.

Subjects

A total of 17 healthy hospital and laboratory staff were studied. Nine were male (age range 28 - 55) and 8 were female (age 23 - 55). All gave informed consent and the study conformed to the guidelines of the Committee on Human Experimentations at the University of Vermont. The methods for Con A-induced suppressor cell assay have been described previously.

The Effects of Hormones

The concentrations used for testosterone was 12 ng/ml, for oestradiol was 40 ng/ml and for progesterone was 20 ng/ml. These concentrations were chosen because they approximate upper normal values seen in adults. The original stock solutions were made in ethanol but the final concentration of alcohol was only .0001 mg/ml which, in preliminary experiments, had no effect on lymphocyte responses. We studied the effects of these three hormones on (1) the response of lymphocytes to the mitogen PHA (2) the generation of suppressor cells by Con A (1st incubation period of the ConASST) and (3) the proliferative response of the responder cells (the second incubation period of the ConASST). The effects of incubation of cells during the first incubation period with the three hormones was also studied in the absence of Con A. These cells were incubated for 48 hours in the presence of the hormones and treated exactly as those cells in the CON-ASST assay and added to isogeneic responder cells. The effects of the hormones on the PgSS were studied by addition of the hormones (at the same time as indomethacin) and comparing the results with the normal response.

CHAPTER 6. Table 1

	Mitogen Response		Manipulation of the 1st Incubation Period of the ConASST				Manipulation of the 2nd Incubation Period of ConASST	
	PHA Alone	PHA + Prog	Medium Alone	Prog Alone	Con A Alone	Con A + Prog	Con A Alone	Con A + Prog
Male N = 8	139386 ±38478	149760 ±54297	47441 ±21805	43905 ±18399	19963 ±13109 N = 4 58%±13.5	14813 ±8374 N = 4 67%±14	19963 ±13109 N = 4 58%±13.5	21136 16942 N = 4 59%±10
Female N = 7	182292 ±45498	176259 ±43052	61798 ±38489	48479 ±19969	20179 ±10773 N = 4 68%±18	14613^{XX} ±8673 N = 4 78.4%±10.2	20179 ±10773 N = 4 68.8%±18	18843 ±9352 N = 4 75%±5
Total N = 15	155889 ±44229	159697 ±49013	53182 ±29219	45734 ±18473	20044 ±11462 N = 8 62%±15	14738^{XX} ±7847 N = 8 71%±13	20044 ±11462 N = 8 62%±15	19939 ±13225 N = 8 65%±13

^{XX} $p < .005$

The effects of addition of progesterone (Prog) on mitogen response and the two incubation periods of the CONASST.

Values expressed as CPM and % suppression (mean ± SD)

CHAPTER 6. Table 2

	Mitogen Response		Manipulation of the 1st Incubation Period of the ConASST				Manipulation of the 2nd Incubation Period of ConASST	
	PHA Alone	PHA + Test	Medium Alone	Test Alone	Con A Alone	Con A + Test	Con A Alone	Con A + Test
Male N = 9	103211 ±55264	99969 ±54184	49252 ±14693	46349 ±15587	27340 ±115230 N = 5	32470 ±10805 N = 5	27340 ±115230 N = 5	26371 ±13899 N = 5
				6.0%±18	44%±22	37%±14	44%±22	40%±23
Female N = 9	87203 ±84502	94612 ±87316	60617 ±21640	49023^{xx} ±19328	25551 ±10751 N = 5	25079 ±20242 N = 5	25551 ±10751 N = 5	30145 ±10124 N = 5
				15%±13	59%±21	60%±36	59%±21	52%±33
Total N = 17	95482 ±66679	97763 ±67215	54600 ±19398	47606^{xx} ±16936	26764 ±9521 N = 10	28774 ±15785 N = 10	26764 ±9521 N = 10	28052 ±11789 N = 10
				12%±22	51%±21	48%±29	51%±21	46%±24

^{xx} $p < .005$

The effects of addition of testosterone (Test) on mitogen response and the two incubation periods of the ConASST

Values expressed as CPM and % suppression (mean ± SD)

CHAPTER 6. Table 3

	Mitogen Response		Manipulation of the 1st Incubation Period of the ConASST				Manipulation of the 2nd Incubation Period of ConASST	
	PHA Alone	PHA + Oest	Medium Alone	Oest Alone	Con A Alone	Con A + Oest	Con A Alone	Con A + Oest
Male N = 8	103211 ±55264	102908 ±56065	49252 ±14693	46972 ±15799	27340 ±115230 N = 5 44%±22	26614 ±6909 N = 5 47%±16	27340 ±115230 N = 5 44%±22	26371 ±13897 N = 5 47%±17
Female N = 9	87203 ±84562	92425 ±83432	49023 ±19328	51561 ±20205	25551 ±10751 N = 5 59%±21	26190 ±20277 N = 5 50%±29	25551 ±10751 N = 5 59%±21	26190 ±26279 N = 5 60%±29
Total N = 17	95482 ±66679	98592 ±66383	47606 ±16936	49131 ±12578	26764 ±9521 N = 10 51%±22	26320 ±14303 N = 10 54%±24	26764 ±9521 N = 10 51%±22	26281 ±16389 N = 10 53%±24

The effects of addition of oestradiol (OEST)on mitogen response and the two incubation periods of the ConASST

Values expressed as CPM and % suppression (mean ± SD).

Statistical Analysis

Statistical analysis was performed by the paired student-t test. Probability values of less than 5% were considered significant.

RESULTS

The results of the effects of testosterone, progesterone and oestradiol on the responses of lymphocytes to the various manipulations are summarised in Tables 1, 2 and 3. At the concentrations used, these hormones did not significantly alter the response of the lymphocytes to the mitogen PHA. Similarly addition of the hormones to the second incubation period of the ConASST did not have any effect on the proliferative response of the responder cells to Con-A and the suppressor cell activity was unaltered. In contrast, the addition of progesterone to the Con A generation of suppressor cells (1st incubation) resulted in significantly greater suppression than that achieved by Con A alone ($P< .005$), while neither of the other two hormones produced statistically significant differences (fig. 1). The effects of cells pre-incubated with hormones in the absence of Con A on the isogeneic responder cells are shown in figure 2. Those cells pretreated with testosterone resulted in significant suppression ($P<.005$) of the responder cells but neither of the other two hormones had any such effect. The Con A-induced suppression in our small group tended to be slightly greater in the female patients than in the male patients but this difference did not reach statistical significance.

No significant difference was observed when the hormones were added to the PgSS. The results for the PgSS were 3.5% ± 11, 0.4% ± 18, 2.5% ± 9 and 1.5% ± 15 in medium alone, testosterone, oestradiol and progesterone respectively.

DISCUSSION

We have found that Con A generation of suppressor cells is enhanced by the addition of progesterone but not by testosterone or oestradiol. Pre-incubation with the hormones alone resulted in testosterone generating modest, but highly significant, suppressor cell activity, whilst progesterone and oestradial had no such effect. There was no evidence that any of the hormones affected the prostaglandin-producing suppressor cell system.

Although at first sight the effects of the hormones alone, and on Con A-induced suppressor cell activity, appear to be inconsistent, the results may reflect more than one process. Although the concentrations of the hormones used in our assays were not high enough to inhibit mitogen responses, all three hormones at higher concentrations are known to be inhibitory (Wyle and Kent, 1977). Hormones with these inhibitory properties might therefore be expected to reduce the generation of suppressor cell activity non-specifically as has been shown for hydrocortisone (Knapp and Posch, 1980). If this were so, the findings of normal, and not reduced Con A induced suppressor cell acitivty, in the presence of the hormones may still indicate a specific effect of the hormones on suppressor cells. The differences in these two systems remain unexplained but the effects of the hormones in the absence of Con A may well be more physiological, and of greater clinical relevance, as antigens encountered in vivo are unlikely to resemble Con A.

Although Con A-induced suppression in our small group tended to be greater in females than in males, this was not statistically significant and the individuals were not matched for age and other variables. Although other studies have not stressed differences between sexes, it may be of relevance that in a family study of patients with SLE, 12 of the 13 first degree asymptomatic relatives of patients with SLE found to have weak suppressor cell activity were women (Miller and Schwartz, 1979). It is also noteworthy that other diseases known to be associated with weak suppressor cell activity are also predominantly diseases of women.

To our knowledge there are no directly comparable studies on the effects of these hormones on immune regulation. The observation that testosterone increases suppressor cell function is consistent with the reduced immune responses that have been described in males compared to females and also with the observation that the heightened immune response in female mice can be reproduced by castrating male mice as long as the thymus gland is intact (Eidinger and Garret, 1972). The humoral response of castrated male mice is even greater than females and one interpretation of this is that testosterone resulted in either increased suppressor cell or reduced helper cell activity (Eidinger, 1972). A recent report showing that the incidence of autoantibodies in alcoholic cirrhotics inversely correlates with testosterone levels is

further supporting evidence (Gluud et al, 1981). Another study investigating the immunoregulatory effects of oestrogens has shown that serum from rats treated with oestradiol contains a factor which inhibits most lymphocyte responses in vitro but that this effect can be abolished by thymectomy (Stimson and Hunter, 1980).

Our findings would in part explain some of the observed clinical manifestations in diseases in which defects of suppressor cell function have been noted. Thus the effects of testosterone could explain why adult males appear to be more resistent to developing autoimmune diseases than females and why children and patients with Klinefelter's syndrome behave differently. Less convincingly the increase of mitogen-induced suppressor cell activity found in the presence of progesterone could explain, in part, why patients with SLE may improve dramatically during pregnancy but relapse post partum (Garnenstein and Pollak, 1962), co-inciding with rising and falling progesterone levels respectively. It is also possible that "weak" suppressor cell function in women with autoimmune disease might reflect on inability to generate a progesterone-dependent suppression of immune response. It should be noted, however, that we have studied only one aspect of the immunoregulatory changes which occur in pregnancy and the influence of foetal suppressor T lymphocytes which spill over into the maternal circulation may also be important (Olding and Oldstone, 1974).

These results do offer some support for the so-far unconfirmed concern over the effects of the contraceptive pill on the late development of auto-immune disease (Bole, 1969). Unfortunately the possibility of extension of these findings to clinical studies is limited because of the side effects associated with the use of these hormones in vivo.

In conclusion our findngs indicate that sex hormones, at physiological dosage, have an effect on in vitro immunoregulation. These findings are compatible with some clinical observations and other previously reported in vivo and in vitro effects. Endocrine changes do occur as a result of cirrhosis and these might therefore account for some of the abnormalities of immunoregulation found in cirrhosis.

CHAPTER 7

SUPPRESSOR CELL ACTIVITY IN PROGRESSIVE SYSTEMIC SCLEROSIS

Progressive systemic sclerosis (PSS) is a generalized disorder of unknown etiology characterized by inflammatory, fibrotic and degenerative changes, associated with vascular lesions, in skin and other organs. The observation that the disease usually affects young females and is associated with a high incidence of autoantibodies (Davis et al, 1976) and with other autoimmune diseases has led to suggestions that the disease involves "autoimmune phenomenon". Decreased numbers of circulating T cells (DeJesus et al, 1975; Gupta et al, 1979), a depressed lymphocyte proliferative response to mitogens (Salem et al, 1976) and evidence of sensitization of lymphocytes to skin extracts (Kondo et al, 1976) have also been described. Recently, the possibility that alterations in immune regulation play a role in the pathogenesis of the disease has been suggested by conflicting reports suggesting an imbalance in circulating T lymphocyte cell populations (Gupta et al, 1979; Inoshita et al, 1981) using methods dependent on surface membrane markers.

In this study we have studied functional suppressor cell activity using three different assays. Because defects in these suppressor systems have been described in a variety of other putative autoimmune conditions the findings of similar defects in patients with PSS would support arguments that defects in the immunoregulatory response are important in the pathogenesis of the disease.

Patients and Methods

Subjects: Fifteen patients fulfilling the diagnostic criteria for systemic sclerosis (Masi, 1980) and 19 healthy controls were studied. The clinical details, relevant drug therapy and HLA typing where available are shown in Table 1. The age range of the controls was 22-60 (mean 45) years. As a disease control, included to detect possible effects of anti-inflammatory drugs, a group of 12 rheumatoid arthritis (RA) patients who were taking anti-inflammatory drugs was also studied. All 12 were taking non-steroidal anti-inflammatory drugs (Aspirin 8, Naprosyn 4, Indomethacin 4) and 4 were also taking corticosteriods. The age range of these patients was 26-70 (mean 54) years.

Statistical analysis was performed by one-way analysis of variance and the unpaired student t test. Probability values of less than five percent were considered significant.

RESULTS

The results for the two assays of suppressor T cell activity are summarized in Table 2. Although Con A-induced suppression tended to be less in PSS than controls the differences did not reach statistical significance. In each group only one assay fell 2 SD below the normal mean. The other values were within the normal range. There was also no significant difference in activity between the controls and the scleroderma patients in the NSS system. Significantly increased prostaglandin-producing suppressor cell activity was demonstrated in PSS compared to the controls (12.7% $\pm$ 11.5 vs. 2.4% $\pm$ 8.6 $p<.01$). The values for the individual patients for the PgSS are shown in Figure 1 which also shows the results obtained in the patients with RA. Results from patients with RA were not significantly different from controls. Numbers did not allow the study of the effect of drugs on the response.

DISCUSSION

Our findings of normal T lymphocyte suppressor cell activity in PSS do not support arguments which favor a defect in immunoregulatory T cells as being important in the pathogenesis of the disease. Previous studies employing immunoglobulin membrane markers have produced conflicting results. Gupta and colleagues (1979) report that the number of Tg (suppressor) cells is increased in PSS but Inoshita and co-workers (1981) suggest that these cells are reduced in number. Furthermore, the latter workers using a functional test based on the proliferation of B cells suggest that there is an increased helper: suppressor activity ratio. These conflicting results are difficult to interpret but could be explained by effects of drug therapy, technical differences or the specificity and sensitivity of the methods. Furthermore, unlike many diseases in which defects of immunoregulatory function have been described there is no known association of HLA B8 or DW3 with PSS (Majsky et al, 1979; Giordano et al, 1979). Methods of preparation of

CHAPTER 7. Table I: Details of patient studies.

PT	AGE	SEX	INVOLVEMENT	ANTINUCLEAR ANTIBODY	SPECIFIC DRUG THERAPY	HLA TYPE
1	25	F	Raynaud's, skin	-ve	Indomethacin	--
2	25	F	Raynaud's, skin, renal	1/160	Naprosyn	A3, B12, B15
3	42	F	Raynaud's, skin, joints, lungs.	1/80	Indomethacin	A2, B5, B40
4	26	F	Raynaud's, skin, esophagus.	1/320	prednisolone colchicine	A26, Aw32, B27, Bw21
5	61	F	CREST syndrome	-ve	Naprosyn	---
6	71	F	Esophagus, skin, Raynaud's	1/80	Aspirin	A2, B7, B8
7	42	F	Skin, esophagus, Raynaud's, renal.	1/320	Aspirin	---
8	35	F	Skin, Raynaud's, esophagus.	-ve	penicillamine colchicine	---
9	48	F	Raynaud's, skin, esophagus	1/2560	Indomethacin	A1, A12, B8 B35
10	46	F	Raynaud's, skin, esophagus.	1/80	Naprosyn	Aw23, Aw31, B12

CHAPTER 7. Table I continued

PT	AGE	SEX	INVOLVEMENT	ANTINUCLEAR ANTIBODY	SPECIFIC DRUG THERAPY	HLA TYPE
11	61	F	Raynaud's, skin esophagus	1/320	penicillamine colchicine	A1, A3, B8 B14
12	60	F	Raynaud's, skin, esophagus	-ve	colchicine	A1, Aw23 B14, Bw21
13	59	F	CREST syndrome	1/320	Aspirin	---
14	51	F	Raynaud's, skin, esophagus.	1/2800	Indomethacin	A1, A2, B8 B40
15	24	F	Raynard's skin esophagus.	-ve	penicillamine colchicine	A1, A28, B8 BW35

CHAPTER 7. Table II: Results of suppressor T cell activity in PSS

	% SUPPRESSION	
	CONTROLS	SCLERODERMA
CON A-INDUCED SUPPRESSOR CELL SYSTEM		
Responder 1	70.8± 23.7 (7)	63.3± 32.0 (5)
Responder 2	67.3± 19.4 (13)	54.3± 54.5 (9)
Responder 3	62.8± 17.9 (9)	58.4± 29.3 (6)
Isogeneic	66.1± 14.9 (12)	56.7± 31.0 (7)
NON-INDUCED SUPPRESSOR CELL SYSTEM	-102.0± 91.7 (17)	-105.4± 140.2 (14)

The figure in parenthesis indicates number of studies in each group. Values represent mean + SD. Responders 1-3 were from 3 different allogenic individuals.

mononuclear cells, ConA induced suppressor cell assay, the non-induced suppressor cell assay and the prostaglandin producing suppressor cell assay are described in Chapter 4.

We have shown significantly increased activity of a prostaglandin-producing monocyte suppressor cell system in a group of patients with PSS. Although we have included a "disease control" group to try to exclude the possibility that the results might be influenced by therapy, and the results obtained in this group were not significantly different from normal, we cannot completely exclude this possibility. Increased prostaglandin-producing suppressor cell activity has previously been shown in sarcoidosis (Goodwin et al, 1979) and Hodgkin's disease (Goodwin et al, 1977) and we have recently described similar changes in inflammatory bowel disease (Chapter 7) and cirrhosis (Chapter 5). This indirect evidence that monocytes are producing increased amounts of prostaglandins may have wide ranging implications as prostaglandins are known to have a variety of functions in immune regulation. Mononuclear cells produce prostaglandins in response to in vitro stimulation (Ferraris et al, 1974) and prostaglandin E has been demonstrated to inhibit antibody (Zimecki et al, 1976) and lymphokine (Bray et al, 1974) production as well as T cell proliferation (Goodwin et al, 1977). Prostaglandins also reduce the mixed leukocyte culture response (Darrow et al, 1980) and macrophage tumor cell killing (Taffet et al, 1981) and may induce T lymphocyte suppressor cells (Goodwin et al, 1977).

Although our findings do provide some evidence of altered immunoregulatory function in patients with PSS, an alternate explanation might relate to a primary defect in collagen formation. There have been reports of increased collagen production (Leroy, 1974) and decreased collagenase levels (Brady, 1975) in the skin of patients with PSS. On the contrary, the skin may actually be thinner than normal and the total collagen content is probably reduced at least in end stage disease (Black et al, 1970). Monocytes and prostaglandins (Korn et al, 1980) have been implicated in fibrogenesis. Prostaglandin E may reduce new collagen formation by reducing fibroblast proliferation (Korn et al, 1980) or by increasing collagenase production (McCarthy et al, 1980) and this can be abolished by the addition of prostaglandin inhibitors such as indomethacin to the system (McCarthy et al, 1980). Thus it is

possible that the increased PgSS in PSS does not reflect an altered immunoregulatory system, but represents an appropriate response to a defect in the regulation of fibrogenesis. As prostaglandins may inhibit collagen formation this response, or negative feedback, may be a compensatory mechanism attempting to reduce excess collagen production. This theory is also compatible with the findings of increased PgSS activity in cirrhosis (Chapters 4 and 5) and sarcoidosis (Goodwin et al, 1979) conditions in which abnormal fibrosis also occurs and could explain why the actual organ content of collagen is not increased in these conditions. Similarly, fibrotic reaction may occur as a feature of inflammatory bowel disease and Hodgkin's disease.

Drug therapy including indomethacin has failed to significantly halt the progression of PSS. If the increased PgSS activity is indeed a compensatory reaction, and prostaglandins are important mediators of collagen formation, the use of prostaglandin inhibitors may have the unwanted effect of speeding progression of the disease. To our knowledge, the effect of prostaglandin inhibitors has not been fully investigated in PSS.

In conclusion, we have demonstrated no changes in lymphocyte suppressor cell activity but an increase in prostaglandin-producing suppressor cell activity in PSS. These findings may indicate abnormal immunoregulation or a compensatory response to altered fibrogenesis.

CHAPTER 8

SUPPRESSOR CELL ACTIVITY IN INFLAMMATORY BOWEL DISEASE

PART I. FUNCTIONAL T-CELL ACTIVITY

The findings of various abnormalities of the immune response in inflammatory bowel disease have led a number of investigators to suggest that immune mechanisms are involved in the pathogenesis of the disease and several analogies have been drawn with diseases in which immune, or autoimmune, mechanisms are known to be involved (Sachar et al, 1980; Kraft, 1979; Whorwell et al, 1976). Following the discovery that lymphocytes could be subdivided into several subgroups, including suppressor and helper T-cells, Hodgson and co-workers (Hogdson et al, 1978) described reduced suppressor T-cell activity in inflammatory bowel disease and suggested that this defect might allow for potentially damaging immune responses, directed against a variety of candidate antigens, to continue unchecked. Similarly there have been preliminary reports that intestinal lymphocytes from patients with Crohn's disease exibit abnormal suppressor T-cell activity compared to those from controls (Goodacre et al, 1980; Fiocchi et al, 1979; Fiocchi et al, 1981). As the mode of action of sulphasalazine, a drug of known benefit in inflammatory bowel disease, is unknown, we postulated that it may have an action on immunoregulatory function. The aim of this study was to try to confirm the presence of reduced T-cell suppressor cell activity in Crohn's disease and to investigate the possibility that sulphasalazine or its metabolites, may exert beneficial effect by an action on this limb of the immunoregulatory system.

Patients and Methods

Subjects: 18 patients with Crohn's disease were studied. All had had their diagnoses confirmed radiologically or histologically and the disease had been present for at least three months. Fourteen were in remission, four were in relapse. Six were taking corticosteroids and eight, sulphasalazine. A total of 20 healthy volunteers from hospital and laboratory staff served as a control group. The two groups were of similar age range and sex distribution. All patients gave informed consent and the study conformed to the guidelines of the Committee on Human Experimentations at the University of Vermont. Methods as in Chapter 4.

Effects of Sulphasalazine and its Metabolites.

Sulphasalazine (SS), sulphapyridine (SP) and 5-amino-salicylic acid (5ASA), all supplied by Pharmacia, were used at a concentration of 100 mcg/ml. Their effect on the generation of suppressor cells in the preincubation period of 48 hours was assessed, both by themselves in lieu of the Con A and also with coincubation with Con A. The cells were otherwise treated exactly as for the standard Con A assay. The concentrations of the drugs used approximate serum levels achieved in clinical practice. The effects of the drugs were measured only in the isogeneic system.

Statistical Analysis.

The Student t test was used for statistical analysis. The unpaired t test was employed for comparing groups and the paired t test used for comparing the *in vitro* effect of the drugs. Probability values of less than 5% were considered significant.

Results.

The results of both suppressor T cell assays in patients and controls are shown in Table 1. There was no difference between Con A-induced suppressor cell activity using either of the 2 allogeneic responders or in the isogeneic system. Similarly, there was no difference in the non-induced suppressor cell activity. There was no clear cut relationship to therapy or disease activity. None of the CD patients were outside 2 S.D. of the mean of the normals. The 4 patients in relapse (all hospitalized and on treatment with corticosteroids) also showed normal Con A-induced suppressor activity (68% ± 14, 54% ± 28 and 72% ± 32 for responder I and II and for isogeneic cells respectively). The respective values for the 8 patients on sulphasalazine were 64% ± 26, 58% ± 24 and 68% ± 14.

The effects of the addition of sulphasalazine and its metabolites SP and 5-ASA on the generation of suppressor cells are shown in Table 2, which indicates the effects of the drugs alone, and in Table 3, which indicates the effects of adding the drug with Con A during generation of suppressor cells. The addition of the drugs to the system during the 48 hour generation period made no significant difference either in the patients with Crohn's disease or in the controls whether added alone or with the Con A.

CHAPTER 8. Table 1

	Control (n = 20)	Crohn's
Con A induced suppressor activity		
Allogeneic Responder 1	67.2 ± 19.4	68.9 ± 25.3 (16)
Allogeneic Responder 2	62.8 ± 17.9	58.5 ± 23.7 (14)
Isogeneic Responder	54.7 ± 21.3	60.2 ± 25.2 (14)
Non-induced Suppressor Activity	- 102 ± 109	- 88 ± 117 (18)

Results of suppressor cell assays in controls and patients with Crohn's disease. Results expressed as % (mean ± SD). Figure in parenthesis indicates number studied.

CHAPTER 8. Table 2

	Medium alone	SS	SP	5-ASA
Patient (n = 9)	31125 ±1515	27976 ±2156	28561 ±1642	23487 ±2005
Controls (n = 8)	56243 ±2584	52234 ±3307	50864 ±2686	53957 ±3415

Effects of adding sulphasalazine (SS) and its metabolites, Sulphapyridine (SP) and 5-aminosalicylic acid (5-ASA) to the preincubation period in lieu of Con A. The methods were otherwise similar to the isogeneic Con A suppressor cell assay. Results expressed as CPM (mean ± SD).

CHAPTER 8. Table 3

	Con A alone	Con A + SS	Con A + SP	Con A + 5 ASA
Crohn's (n = 9)	50.8 ±14.2	44.4 ±28.2	48.5 ±28.7	59.8 ±20.7
Control (n = 8)	38.9 ±21.1	41.5 ±11.5	37.4 ±28.7	41.2 ±19.5

Effects of adding sulfasalazine (SS) and its metabolites sulfapyridine (SP) and 5-aminosalicylic acid (5-ASA) to Con-A preincubation in the Con-A isogeneic suppressor cell system. Results expressed as % (mean ± SD).

Discussion.

Our results showing normal Con A-induced suppressor cell activity in Crohn's disease are in direct contrast to those of Hodgson (Hogdson et al, 1978) who found reduced Con A suppressor cell activity in 4 out of 5 patients with active Crohn's disease. The only other report of abnormal Con A-induced suppression in Crohn's disease is that of Knapp who found reduced levels in 8 patients in relapse but normal activity in 6 patients in remission (Knapp et al). Although we used different concentrations of Con A, particularly during the first incubation, this should not account for the differences and the methods are otherwise similar. We did not encounter the problems of leuco-agglutination reported by Hodgson at this higher dose which is the concentration used in the original report of the method (Shou et al, 1976). It is noteworthy that the range of suppression generated in our normal controls was similar to that reported by Hodgson and that we have shown reduced suppressor cell activity in chronic active hepatitis using this system (Chapter 4). The assay is in part HLA dependent and results do differ between different responders (Chapter 4). To avoid this problem, unlike the previous workers who used only one allogeneic responder, we have used two allogeneic responders, as well as isogeneic responder cells. We have also found no significant difference using a non-induced assay

of suppressor cell activity. It therefore seems most likely that the previously reported abnormalities of Con A-induced suppression indicate a transient abnormality which is related to some variable such as disease severity or drug therapy.

The failure of this study to show any reduced suppressor T-cell activity in any of our responder systems is strong evidence against there being a primary defect in Crohn's disease. Further evidence to support this emerges from a study of the conflicting reports of both increased T-cell suppression of immunoglobulin synthesis (11) and reduced T-cell suppression as measured by a non-induced assay of suppressor T-cell activity dependent upon the observation that suppressor T-cells are inactive after a short culture period (Victorino et al). Similarly, there are reports of increased (Fiocchi et al, 1981) and reduced (Goodacre et al, 1980) Con A-induced suppressor cell activity by intestinal mononuclear cells. More recently, techniques employing surface markers and monoclonal antibody techniques have been introduced to differentiate between helper and suppressor T lymphocytes. To our knowledge only one group has employed these techniques in Crohn's disease and report increased T_M (helper) but normal T_G (suppressor) cells (Victorino et al, 1980). This would also seem to support our finding of normal suppressor T cell activities although the relationship of these assays to the functional assays is not straightforward (Victorino et al, 1980). In other diseases, notably scleroderma, directly conflicting results were reported using similar techniques of membrane markers by two different groups (Gupta et al, 1979; Inoshita et al, 1981) and we must await further confirmation of these findings in Crohn's disease.

Despite claims to the contrary, drug therapy in Crohn's disease remains relatively empirical and corticosteroids and sulphasalazine are the two major drugs employed. Corticosteroids have anti-inflammatory properties and are known to be immunodepressant. Hydrocortisone inhibits the generation of suppressor cells when added to the Con A system, although this may be a non-specific effect (Knapp et al, 1980). The mode of action of sulphasalazine is unknown but it has been suggested that its beneficial effect is due to anti-inflammatory (Das et al, 1973), antibiotic (West et al, 1974), or anti-prostaglandin (Sharon

et al, 1978) activity. In this study no effect of sulphasalazine on the aspects of immune regulation studies were detected either in vitro or in vivo. The concentrations of the drugs used in this study were similar to those which are known to inhibit lymphocyte proliferation in vitro, and it is therefore perhaps surprising that the addition of the drug to the Con A system did not inhibit the induction of suppressor cells. However other studies on the effect of inhibitory drugs have shown that those drugs which inhibit mitosis or protein synthesis do not inhibit the response, while only those that inhibit microtubule or microfilament function have such an inhibitory effect (Shand et al, 1980). The observation that 5-ASA, which had been shown in several studies to be the active moiety of the drug (Azad et al, 1977; Van Hees etal, 1980; Klotz et al, 1980), does not behave differently to SP, which is thought to be an inert carrier, is further support for the lack of any effect of this drug on the T cell suppressor cell systems studied in this report. It has previously been shown that SS does not affect the total number of circulating T or B cells present although helper/suppressor cell ratios were not studied in this report (Thayer et al, 1979).

In conclusion, we have not been able to confirm that there is abnormal Con A-induced suppressor T-cell activity in Crohn's disease. The growing number of conflicting reports of both increased and decreased suppressor T cell activity, together with our findings, suggests that any defect is likely to be a secondary phenomenon. In this study there is no evidence either in vivo or in vitro that sulphasalazine, or its metabolites, exert their beneficial effects by an action on this limb of the immunoregulatory system.

PART 2. PROSTAGLANDIN PRODUCING SUPPRESSOR CELL ACTIVTY

The discovery of increased levels of prostaglandin-like material in rectal biopsy material (Harris et al, 1978; Sharon et al, 1978; Smith et al, 1979; Rampton et al, 1980) and the fecal content (Gould, 1976) of patients with inflammatory bowel disease (IBD) have led to suggestions for a possible role for prostaglandins in the pathogenesis of the disease (Moore et al, 1978). Reports of the beneficial effect of indomethacin in conditions such as post-radiation colitis (Mennie et al, 1975), food allergies (Buisseret et al, 1978), and irritable bowel disease (Rask-Madsen et al, 1978), have stimulated further interest in the effects of prostaglandin synthetase inhibitors in IBD. Similarly,

although the mode of action of sulphasalazine is unknown, it has been postulated that sulphasalazine itself may act as a prostaglandin synthetase inhibitor (Moore et al, 1978).

Following the description of prostaglandin producing suppressor cells, sensitive to indomethacin (Goodwin et al, 1977), in a variety of conditions including sarcoidosis (Goodwin et al, 1977) and Hodgkin's disease (Goodwin et al, 1977), we have used this system to study the in vitro effect of indomethacin (IND), hydrocortisone (HC), and sulphasalazine (SS) and its metabolites sulphapyridine (SP) and 5-amino salicylic acid (5-ASA) on peripheral blood mononuclear cells (PBMC) from patients with IBD. We hoped to be able to compare and contrast the actions of the drugs to gain insight into the mode of action of sulphasalazine, particularly to investigate the possibility that it may act as a prostaglandin synthetase inhibitor.

Patients

Forty-nine patients with IBD and 34 controls were studied. All patients were classified on evidence gained from radiographic studies, sigmoidoscopy and biopsy material. Patients were classified on clinical grounds as being in an active stage of the disease or in remission. A record of the patient's medication was taken with particular reference to sulphasalazine and corticosteroid use. The study conformed to the guidelines of, and was approved by the Committee on Human Experimentation at the University of Vermont. Methods as previously described in Chapter 4.

MITOGENS AND DRUGS

Preliminary data showed that the optimum concentration of purified phyto-hemagglutinin-P (PHA-P) was 1 ug/well, Concanavalin-A (Con-A) 5 ug/well and Pokeweed (PWM) 10 ug/well and these concentrations were used throughout. Similarly, optimum concentrations for IND appeared to be 250 ug/well, as reported by Goodwin in his original description of the assay (Goodwin et al, 1979). The concentration of hydrocortisone used was 250 ug/well. Incubation of PBMC with increasing doses of SS and its metabolites showed that the response to PHA-P fell off rapidly with increasing drug levels, indicating toxicity. For these experiments, concentrations similar to pharmacologically achieved blood levels were

chosen which resulted in minimal reduction of the response to PHA-P in control and were 25 ug/well for SS and its two metabolites SP and 5-ASA.

PRESENTATION AND ANALYSIS OF DATA

Results of the response to the mitogens are expressed as counts per minute, plus or minus one standard deviation. Each reading was a mean of triplicate experiments. The reading in each triplicate was generally within 10% of the mean. The effects of the drugs on the response to PHA-P was recorded as a percentage of the original response to PHA-P in the absence of the drug. One-way analysis of variance and Student's t test were used for statistical analysis.

RESULTS

Thirty-four patients with Crohn's disease (CD), 15 patients with ulcerative colitis (UC), and 34 controls were studied. Twenty-five of the patients had active disease and 24 were in remission. Eleven were taking SS alone, 19 prednisone alone, and 18 were on no treatment. The remaining patient was taking both drugs. The results of the response to PHA-P, Con-A, PWM, and the effects of IND, SS, SP and 5-ASA are shown in Table 1 and Figure 1. There was a significant reduction in the response of PBMC to PHA-P in Crohn's disease compared to controls ($p < .05$) but otherwise there was no difference between controls, CD and UC in the response to the other mitogens.

As illustrated in Table 1 and Figure 2, there was a highly significant increase in the response to indomethacin, indicating increased prostaglandin-producing cell activity in the CD group (13.47% ± 23.1) and UC group (7.8% ± 12.4) compared to the controls (2.1% ± 16.8, $p < .01$ and $<.02$ respectively). There was no significant difference between the patients and controls in their response to HC. SS caused a significantly greater decrease in ^{3}H thymidine uptake in Crohn's disease (-20.4% ± 24.4) and UC (-24.2% ± 25.1) compared to controls (-13.3% ± 11.52, $p < .05$). This relationship was also present for 5-ASA ($p < .05$) but not for SP. In a further group of disease controls (chronic active hepatitis 4, coeliac disease 7, scleroderma 3), the effects of SS, SP and 5-ASA were -11.7% ± 11.4, -11.6% ± 8.46 and -11.9% ± 16.1 respectively and these results were similar to those obtained in the normal controls and significantly less than those in IBD for both SS and 5-ASA ($p < .05$).

Table 2 shows the results obtained in patients grouped according to disease activity and drug therapy. There was no significant difference between these groups. The response to HC, SS, SP and 5-ASA from patients with a positive in vitro response to indomethacin was similar to that from patients with a negative response (Table 3). Thirty of the patients had been HLA A and B typed, and we were able to evaluate the effects of indomethacin with reference to HLA types in these patients. We looked specifically at those that were B12 positive (n = 7) and compared them with those who were B8 positive (n = 6) and those that were positive to neither of these antigens (n = 17). Results were 12.4% ± 6, 12.8% ± 14.6 and 14.2% ± 29.9 for the 3 groups. These values were not significantly different from one another.

Finally, we studied the effect of adding SS and IND at the same time on the PHA-P response in 16 patients (Figure 3). In these patients, IND alone resulted in an increase of 11.4% ± 13.42 and SS alone a decrease of 21.5% ± 18.36, values that were comparable to those obtained in the entire patient population. Addition of IND to PBMC incubated with SS resulted in a reduction of the PHA-P response to only 8.35% ± 14.1. This was significantly less than for SS alone ($p < .001$), showing that SS does not abolish the in vitro effect of IND. Simultaneous incubation of IND with HC did not result in the same effect, the results being similar to incubation with HC alone (-17.2% ± 16.1 vs-18.4% ± 13.9).

DISCUSSION

We have shown that patients with inflammatory bowel disease have increased indomethacin-sensitive, prostaglandin-producing suppressor cells compared to the control group. This is consistent with, and could explain, the known mild reduction in the lymphocyte response to mitogens in patients with inflammatory bowel disease (Sachar et al, 1973; Whorwell et al, 1976). Although not investigated in our patients, previous studies have shown that this suppressor cell is probably a prostaglandin-producing monocyte (Rask-Madsen et al, 1978; Goodwin et al, 1977; Goodwin et al, 1979), data compatible with the growing number of reports of "activated macrophages" in inflammatory bowel disease (Meuret et al, 1978). Whether the increased activity of this suppressor cell system in IBD is primary and of importance immuno-

CHAPTER 8(2). Table 1: Results of mitogen and drug responses in IBD and control subjects.

Pt. Group	Total Number Studied	PHA Response CPM Mean ± SD	ConA Response CPM Mean ± SD	PWM Response CPM Mean ± SD	IND % Control	HC % Control	SS % Control	SP % Control	5 ASA % Control
Total Controls	34	96377 ±87864	34862 ±19578	16769 ±16454	2.12 ±16.8	-19.8 ±23.2 (n=16)	-13.3 ±11.52 (n=15)	-6.9 ±10.8 (n=16)	-5.3 ±18.6 (n=15)
Crohn's Disease	34	71488* ±38797	34819 ±23063	19680 ±14945	13.47*** ±23.1	-19.6 ±28.3 (n=31)	-20.4* ±24.4 (n=30)	-10.3 ±16.2 (n=30)	-19.4* ±22.3 (n=32)
Ulcerative Colitis	15	76224 ±40732	30873 ±21392	20134 ±10268	7.8** ±12.4	-28.3 ±14.1 (n=11)	-24.2* ±25.1	-8.6 ±15.9	-30.3* ±20.2

*p < .05
**p < .02
***p < .01

Compared to the control group

CHAPTER 8(2). Table 2: Results of mitogen response and effects of IND, SS and HC in different patient groups

Pt. Group	No.	PHA Response CPM Mean ± SD	ConA Response CPM Mean ± SD	PWM Response CPM Mean ± SD	IND % Control	SS % Control	HC % Control
Active Disease	25	71554 ±31520	33240 ±15442	18434 ±12040	15.2 ±21.8	-21.1 ±25.4	-19.4 ±15.8
In Remission	24	80417 ±32480	36424 ±18432	18240 ±14840	10.1 ±26.3	-21.7 ±23.4	-24.4 ±12.4
Sulphasal-azine Only	11	78243 ±36554	31277 ±12987	12517 ±8816	9.4 ±10.7	-16.2 ±21.1	-27.6 ±11.2
Prednisone Only	19	77653 ±32711	30072 ±25443	27593 ±24990	19.0 ±24.2	-21.6 ±24.0	-18.3 ±15.3
No Treat-ment	18	77910 ± 3995	42078 ±24917	18083 ±9830	12.8 ±13.4	-26.4 ±21.6	-22.0 ±16.4

CHAPTER 8(2). Table 3: Comparisons of the effects of SS, SP, and HC on response to indomethacin

Group	No.	SS % Control	SP % Control	5 ASA % Control	HC % Control
Positive response to Indomethacin	39	-18.3 ± 16.9	-11.3 ± 13.5	-22.6 ± 14.4	-19 ± 17.4
Negative response to Indomethacin	10	-21.8 ± 16.4	-13 ± 11.8	-27.4 ± 17.3	-25.3 ± 10.5

pathogenetically, or is an epiphenomenon cannot be determined from this study. The observation that levels were similar in patients with active or quiescent disease suggests that it is primary, although its occurrence in other conditions (Goodwin et al, 1977; Goodwin et al, 1979) suggests that it may be a secondary phenomenon. We failed to confirm the suggestion, based on studies in normal controls, that this system is HLA-dependent (Staszak et al, 1980).

Our results suggest that neither sulphasalazine nor corticosteroids exert their therapeutic effect by affecting this limb of the immunoregulatory system. The results might explain the increased levels of prostaglandins that have been found in rectal biopsies in patients with inflammatory bowel disease, as it may be macrophages and not the colonic epithelial cells which produce the prostaglandins. The presence of increased prostaglandin-producing suppressor cell activity suggests a possible role for indomethacin in IBD patients and provides further justification for trials with drugs such as indomethacin, although the observation that concurrent incubation of PBMC with IND and SS reduces the effects of SS, suggests that these two drugs may have antagonistic, although possibly unrelated, modes of action (Moore et al, 1978). Indeed, SS may act on the monocyte and influence lymphocyte response, possibly by prostaglandin release. Thus, in clinical trials the effect of indomethacin on patients not concurrently taking sulphasalazine should receive special attention. It should also be remembered that if the increased PgSS is a compensatory response then blocking it with indomethacin may have a detrimental effect.

Reports have suggested SS may inhibit prostaglandin synthetases (Gould, 1976; Moore et al, 1978). Our results do not support this, but suggest that it has an immunosuppressant effect which is not dependent on the response to IND. Furthermore, this <u>in vitro</u> effect of IND is not blocked by coincubation with SS. Corticosteroids are also thought to have antiprostaglandin properties, possibly by reducing the availability of substrate for prostaglandin synthesis (Floman et al, 1976). The <u>in vitro</u> response to HC that we observed does not support the suggestion that the main therapeutic role of corticosteroids in IBD is to reduce prostaglandin synthesis. The coincubation experiments are difficult to interpret but suggest that HC, SS and IND all have different modes of activity.

In our view, it is of interest that the reduction of lymphocyte response caused by SS is greater in IBD patients than in controls, particularly as its effect seemed to be most marked for its metabolite 5-ASA, which appears to be the active metabolite (Azad Khan et al, 1977; Van Hees et al, 1980; Klotz et al, 1980) and is not seen with SP. However, the difference is small and previous work on the effect of SS on lymphocyte responses has failed to show this specificity in IBD (Das et al, 1973; Rubinstein et al, 1978; Thayer et al, 1979). Although our finding needs to be confirmed by others, the results are compatible with the hypothesis that SS acts on the monocyte which is known to circulate in increased numbers in IBD.

The response to mitogens of patients receiving sulphasalazine was not reduced compared to those on no treatment. This might argue against an immunosuppressant effect, but neither was there a significant reduction in patients on corticosteroids, which is known to be a potent immunosuppressant. Drug levels of sulfasalazine are particularly high in colonic mucosa and it may be that a local immunosuppressant effect accounts for the beneficial effect of the drug.

In conclusion, the results suggest that there is an increased population of prostaglandin-producing suppressor cells in IBD, which may play a role in the immunopathogenesis of the disease. There is no evidence in this system that sulphasalazine acts as a prostaglandin synthetase inhibitor. Trials of indomethacin in IBD will help to determine whether this suppressor cell system is of immunopathogenic importance but should take into account the possibility that IND and SS have different and possibly antagonistic modes of action in vivo as well as in vitro. These findings are supported by recent studies on gastrointestinal smooth muscle (Hoult et al, 1980) which also show that the two drugs have opposite effects.

PART 3. IMMUNOGLOBULIN PRODUCTION BY LYMPHOCYTES

Although several immunological abnormalities have been described (Kraft et al, 1980) in inflammatory bowel disease (IBD), none offers a unifying explanation for the immunopathogenesis of the disease. Because of its prime role in intestinal defence mechanism, a defect in IgA secretion has been proposed (Meuwisse et al, 1976; Brooks, 1981; Green et al, 1975). Such an underlying abnormality might accunt for the familial

occurrence of the disease and its almost invariable recurrence after apparently curative segmental surgery. In support of this hypothesis a weak clinical association between Crohn's disease and IgA deficiency has been reported (Söltojt et al, 1972; Eggert et al, 1969). Furthermore, the relative increase of cytoplasmic IgG and IgM compared to IgA in lymphocytes in diseased bowel (Baklien et al, 1975) might indicate a compensatory reaction to an underlying deficiency of IgA function. In apparent contradiction, increased levels of circulating IgA and IgM immunoglobulin and normal intestinal juice IgA levels have been reported in Crohn's disease (Jones et al, 1976; Bolton et al, 1974). However, it is not clear whether these observations reflect an underlying abnormal immune response to the antigen load or a normal response to chronic infection and the elevated IgA levels could be masking a relative defect.

We have used a sensitive Enzyme-Linked Immunosorbant Assay (ELISA) technique to measure *in vitro* immunoglobulin synthesis by circulating lymphocytes to further investigate this hypothesis. We have also studied the effects of indomethacin as we have previously shown increased prostaglandin-producing suppressor cell activity in IBD using a T-cell proliferative assay (Chapter 8, part 2). Sulphasalazine, a drug of known benefit but unknown mode of action in IBD and its metabolites sulphapyridine and 5 aminosalicylic acid, were also studied to assess their potential for altering immunoglobulin production.

METHODS

Subjects

A total of 31 patients with IBD were studied. Twenty-six had Crohn's disease and 5 ulcerative colitis. Ten were in clinical remission and 21 in relapse. Sixteen were taking prednisolone at the time of study, 5 sulphasalazine and 3 were taking both of these drugs. Five were on no treatment. There were 10 males and 21 females. The age range was from 12 to 62 years. All cases had characteristic radiological findings and the diagnosis was confirmed histologically in most patients. Thirty-one healthy laboratory and hospital staff served as a control populaton. Twelve of these were male and 19 female. The age range of the controls was 22 to 58 years. Both patients and controls gave informed consent and the study conformed to the guidelines

of the Committee on Human Experimentation of the University of Vermont. Peripheral blood mononuclear cells were obtained as previously described.

Culture and Assay for Immunoglobulin Determination

Immunoglobulin synthesis in vitro was assayed as previously described (Ershler et al, in press). Briefly, 10^6 PBMC were cultured in sterile 12 x 75 mm test tubes (Falcon) in 2 mls of RPMI-PSG supplemented with 10% fetal calf serum. For each subject cultures were established with 50 mcg of pokeweed mitogen (GIBCO) (PWM), a T-dependent B-cell mitogen (Keighley et al, 1976) which stimulates immunoglobulin synthesis. The effects of indomethacin, sulphasalazine, sulphapyridine, and 5-amino salicylic acid on the in vitro stimulated immunoglobulin production were studied as indicated below. Cultures were incubated at 37°C in a fully humidified atmosphere in 5% CO_2 for 7 days. Upon completion of the culture period, the specimens were centrifuged and the supernatants removed and stored at -20°C for later assay of immunoglobulins. IgG, IgA, and IgM concentrations in the supernatent of the lymphocyte cultures were measured by an ELISA technique modified (Ershler et al, in press) from that described by Engvall (1971). For this, polystyrene micro-titer plates (Dynatech, Alexandria, Va.) were coated with anti-human IgG, IgA or IgM (Cappel Laboratories, Cochranville, Pa.). Samples of the supernatant, diluted in PBS supplemented with .05% v/v Tween-20 (PBS-T) (Sigma, St. Louis) were incubated in triplicate overnight at 4°C. After a brisk wash with PBS-T alkaline-phosphatase conjugated goat anti-human IgG (Miles Yeda, Rehouet, Israel) was added and the plates incubated overnight at 4°C. Following an additional wash, p-nitrophenylphosphate (Sigma St. Louis), was added and the product of the enzymatic cleavage of this compound measured spectrophotometrically at 405 nm (Dynatech, manual ELISA plate reader). Serial dilutions of standard immunoglobulin preparations served as a standard on each plate and from thich the sample IgG values were calculated. Similar methods employing the appropriate antisera are applied for IgA and IgM.

Mitogens and Drugs

The optimum concentration for PWM proved to be 50 mcg per culture. Concentration of indomethacin was 1 ug/ml and the concentration for sulphasalazine and its metabolites 100 ug/ml. These levels were chosen because they approximate serum levels and are similar to those we have previously used. Preliminary studies showed that increasing doses of sulphasalazine and its metabolites resulted in markedly reduced immunoglobulin production suggesting toxicity. For indomethacin, the effect on immunoglobulin synthesis (% suppression) was calculated from the formula:

$$1 - \frac{A}{B} \times 100$$

in which A indicates immunoglobulin production with indomethacin added and B that produced in the absence of Indomethacin.

Presentation and Statistical Analysis of Data

The final values were the mean of triplicate assays. These values were usually within 10% of the mean. Values were expressed as mean ± standard error. The unpaired Student t test was used for comparison of group data and the paired Student t test for analysis of the effects of the drugs on lymphocyte response.

RESULTS

In Fig. 1 the results of the immunoglobulin production assay are demonstrated. There was significantly increased IgA and IgM production by unstimulated lymphocytes in the IBD patients compared to controls ($p < .02$). The differences for IgG did not reach statistical significance. There was considerable individual variation in the response to PWM and there was no significant difference for PWM stimulated IgG and IGM production between the patients and the controls. Lymphocytes from patients with IBD, however, produced significantly less IgA than controls ($p < .05$) in response to PWM. Table 1 shows that in patients with active disease the unstimulated production of all 3 classes of immunoglobulins was significantly elevated compared to controls ($p < .02$). Values in patients in remission were not significanlty different from controls. In Table 2 results of PWM

stimulated immunoglobulin production are reported for patients grouped according to disease activity and drug therapy. Drug therapy made no apparent difference although numbers were small. As seen with unstimulated levels, the highest immunoglobulin levels were found in patients in relapse.

In vitro drug effects are shown in Figs. 2 and 3, for 28 patients and 27 controls respectively. Indomethacin resulted in increased IgG, IgA and IgM production in the IBD patients but not in the controls, suggesting an active prostaglandin-producing suppressor cell system in these patients. The % suppression for IBD patients and for controls respectively were 24% ± 18 vs -38% ± 24 for IgG ($p < .05$), 81% ± 36 vs -57% ± 28 for IgA ($p < .02$) and 17% ± 16 vs -8% ± 14 ($p < .05$) for IgM. Sulphasalazine and its metabolites produced no statistically significant difference.

DISCUSSION

We have found that in vitro IgA and IgM production by unstimulated PBMC from patients with IBD is significantly greater than controls. IgG production is also increased but only reached significance in those patients in relapse. These findings correlate with the reported observation of increased circulating IgA levels in IBD, but contrast with the observation that IgM levels tend to be reduced (Jones et al, 1976; Bolton et al, 1974). This apparent contradiction may be accounted for by a increased loss or degradation of IgM. Unstimulated levels were highest in those patients in relapse, an observation that suggests persistent stimulation by unknown, but probably gut related, antigens.

The failure of IBD patients to increase IgA production upon stimulation by PWM may indicate a failure of the lymphocytes to differentiate into plasma cells and to produce IgA in response to certain stimuli. This may be a primary defect and of immunopathogenic importance, but may equally be a secondary phenomenon perhaps due to alterations of lymphocytes occuring during their intestinal circulation. The number of circulating lymphocytes containing cytoplasmic IgA is increased in IBD (Strickland et al, 1974) suggesting that the defect is in synthesis or release of IgA. It is not known whether the effects of PWM are comparable to antigens such as cholera toxin which increases gut IgA secretion in vivo (Hamilton et al, 1980). As discussed above, a

CHAPTER 8(3) Table 1. Immunoglobulin production by unstimulated lymphocytes in controls and patients with IBD divided on the basis of disease activity. Results expressed as ng immunoglobulin per culture (mean ± SEM).
*$p < .02$ compared with the normal control values.

Group	No.	IGG	IGA	IGM
Normals	31	150±27	84±22	119±25
IBD Remission	10	110±60	163±32	402±153
IBD Relapse	21	309±101*	264±80*	639±35*

CHAPTER 8(3) Table 2. Immunoglobulin production by PWM-stimulated lymphocytes in controls and in patients with IBD as a whole and divided into groups on clinical grounds. Results expressed as ng immunoglobulin per culture (mean ± SEM).
^{X}*$p < .05$ compared with normal control values.

	No.	IGG	IGA	IGM
Normals	31	865±131	1090±315	1108±189
IBD total	31	787±135	337±61^{X*}	1536±340
IBD no therapy	5	1039±267	198±59	147±959
IBD on sulphasalazine	7	727±171	246±74	1104±596
IBD on prednisolone	16	735±227	380±100	1901±503
IBD remission	10	698±199	226±48^{X}*	1295±506
IBD relapse	21	901±181	395±85^{X}*	1656±438

relative defect in IgA function would explain many of the features of IBD. IgA inhibits the binding of microorganisms to mucus membranes (Williams et al, 1972) and a deficiency may explain the finding of an increased incidence of adhesive feacal coliforms in ulcerative colitis (Dickinson et al, 1980). Reduced salivary IgA production has also been reported in Crohn's disease (Basu et al, 1975).

Further elucidation of our findings might be obtained by extension of these studies to family members of patients with IBD, to determine if a defect in IgA production is a genetically determined immunological antecedent to disease. In this regard there have been conflicting reports of HLA linkage in family studies of IBD patients (Schwartz et al, 1980; Eade et al, 1980) and secretory IgA deficiency has been reported in asymptomatic first degree relatives (Engstrom et al, 1978).

Assays of immunoglobulin synthesis have only recently been utilized in patients with IBD. Elson, et al. (1981) have demonstrated normal PWM-stimulated IgM production in patients with mild or inactive Crohn's disease using an assay that was designed to investigate suppressor cell activity, but IgA and IgG were not measured. MacDermott and colleagues (1981) studied 6 patients with IBD and found increased unstimulated production of immunoglobulins, as well as a relative failure of stimulated IgG , IgA and IgM production. The wide individual variation in their small sample did not allow for statistical analysis.

The <u>in vitro</u> effects of indomethacin on immunoglobulin production appear to be similar to those observed when measuring T-cell proliferative responses (Chapter 8, part 2). The increased response to indomethacin in patients with inflammatory bowel disease suggests the presence of increased prostaglandin-producing suppressor cell activity. It is possible that this increased prostaglandin-producing suppressor cell activity accounts, in part, for the defective IgA production although if this were the case production of IgG and IgM would also be expected to be reduced. Sulphasalazine and 5-ASA, its putative active metabolite, tended to reduce immunoglobulin production, which also correlates with our earlier observations in studies of proliferative responses. It is probable, therefore, that sulphasalazine, unlike indomethacin, does not act as an anti-prostaglandin, at least in these systems.

In conclusion we have shown that unstimulated lymphocytes from patients with IBD produce more immunoglobulin than do those from controls. Exposure of the lymphocytes to PWM, a T-cell dependent B-cell mitogen resulted in a relative failure of IgA production in IBD patient but normal IgG and IgM production. These findings suggest that an underlying defect in IgA production and may be of immunopathogenic importance in some patients with IBD.

CHAPTER 9

STUDIES ON CIRRHOTIC HYPERGAMMAGLOBULINAEMIA

Hypergammaglobulinaemia is a recognised feature of chronic liver disease (Feizi, 1968). This observation together with the known increase in antibody titers to a number of gut associated antigens (Triger et al, 1972) indicate a hyperresponsive humoral immune system in these patients which is in sharp contrast to the well documented hyporesponsive cellular immune system associated with cirrhosis (see Chapter 5). Recently the development of sensitive assays has allowed in vitro immunoglobulin production to be measured. Peripheral blood mononuclear cells are the most accessible and various workers have shown that cells from patients with cirrhosis, with or without hypergammaglobulinaemia, produce greater amounts of immunoglobulin in vitro than those from controls (Mutchnick et al. 1981; Wands et al, 1981; Berger et al, 1979; James et al, 1979). Although there are many different clones of antibody producing cells in the systemic lymphoid system (Triger and Wright, 1973), it has been suggested that this system provides a useful model for the in vitro study of cirrhotic hypergammaglobulinaemia.

Two basic theories have been put forward to explain hypergammaglobulinaemia (Stobo, 1979). Firstly, reduced suppressor cell activity may fail to "switch off" the immunoglobulin production and such weak activity has been demonstrated, using proliferative assays, in chronic active hepatitis (Hodgson et al, 1978) and more recently in severe alcoholic liver disease (Kawanashi et al, 1981). Secondly, it is suggested that the decreased filtering capacity of the liver in cirrhosis allows gut related antigens to spill over into the systemic circulation resulting in non-specific B cell stimulation. In this study we compare the immunoglobulin producing capacity of peripheral mononuclear cells from cirrhotics with that of normal controls and investigate the possibility that cirrhotic serum contains antigens capable of stimulating normal cells to produce increase amounts of immunoglobulin. We have also studied the prostaglandin producing suppressor cell activity in this system as we have previously shown that increased activity of this system may account, in part, for the hyporesponsiveness of the cellular immune system (Chapter 5).

Methods

Thirteen patients with biopsy confirmed cirrhosis were studied (1 PBC, 8 CAH, 2 cryptogenic, and 2 alcoholic). Only 2 had hypergammaglobulinaemia. The age range was 15 to 74 and there were 9 females and 4 males. Thirty-one healthy laboratory and hospital staff served as control population. Twelve were male and 19 female and their age range was 22 to 58. Both patients and controls gave informed consent and the study conformed to the guidelines of the Committee on Human Experimentation of the University of Vermont. Peripheral blood mononuclear cells were obtained, and the methods for immunoglobulin synthesis (IgG only), and the addition of indomethacin, have previously been desribed in Chapter 8 (part 3). To investigate the effect of cirrhotic serum on normal cells, a large number of cells were obtained from a single healthy volunteer and aliquoted into 40 different specimens, 20 of which were exposed to serum from cirrhotic patients and 20 to serum from controls matched as far as possible for age and sex. These 20 patients consisted of the original 13 plus 4 CAH, 1 PBC and 2 alcoholics, all of whom had biopsy confirmed cirrhosis. Cells were incubated in a 50% concentration of the respective serum for 24 hours and then washed in PBS 5 times, recounted, and set up in culture for immunoglobulin production as previously described. Assay of the final washing fluid showed that there was no immunoglobulin remaining in this wash. Check samples were tested for viability by trypan blue exclusion at all stages of culture and all found to be at least 90% viable. The unpaired T test was used for statistical analysis and the results are expressed as means with standard error.

Results

In vitro IgG production by unstimulated PBMC from the cirrhotics was significantly greater than that of the controls ($p < .005$, see fig. 1). There was no difference between the cirrhotics and the controls in their response to pokeweed mitogen. The amount of unstimulated IgG production did not correlate with the circulating globulin level ($r = .09$) and did not appear to depend on the nature of the liver disease although numbers were too small for detailed analysis. There was no significant difference between the changes produced by the addition of

indomethacin in the two groups (-38%±24 for the controls and -32%±29 for the cirrhotics).

Normal cells exposed to cirrhotic serum produced significantly more IgG than those exposed to normal control serum ($p < .02$, fig. 2). Seven of the cirrhotic sera (4 CAH, 1 PBC, 1 alcoholic) resulted in IgG production greater than 2 SD from the mean of that produced by the cirrhotic serum. There was no correlation with the amount of IgG produced by the normal cells on exposure to the cirrhotic sera and the globulin levels in the specimens ($r = .1$).

Discussion

These results confirm previous reports which have shown increased immunoglogulin production by mononuclear cells from patients with cirrhosis. Of greater interest is the finding that there are substances in the sera of cirrhotic patients which are capable of stimulating normal cells to produce increased amounts of immunoglobulin. The alternative interpretation that cirrhotic sera contains (or is lacking) a substance which preferentially effects the immunoregulatory cells of the normal donor cannot be excluded but appears unlikely. The results provide definite evidence that non-specific B cell stimulation does occur in cirrhosis and might account, at least in part, for the hypergammaglobulinaemia in these patients. This finding is in contrast to the effect of cirrhotic serum on proliferative responses (see Chapter 5) in which cirrhotic serum has an inhibitory effect. The proliferative response to pokeweed mitogen has also been shown to be reduced (Young et al, 1979) suggesting that immunoglobulin synthesis is not dependent on the proliferative response of the B cells. It has previously been shown that the number of B cells is not increased in patients with cirrhotic hypergammaglobulinaemia (Berger et al, 1979). *In vitro* endotoxin is known to result in B cell stimulation and this may be one of the antigens accounting for the increased spontaneous IgG production in the cirrhotics as endotoxaemia may be common in patients with cirrhosis (see Chapter 3).

In the cirrhotic patients the amount of immunoglobulin produced in response to adding pokeweed mitogen the system was not statistically different from that of controls, and only the unstimulated levels

differed. As PWM is a T cell dependent B cell mitogen this may suggest that in these patients the regulatory system is intact and that defects of this system are of secondary importance. Such nonspecific B cell stimulation may well occur in all cirrhotics, as in clinical studies the degree of hypergammaglobulinaemia has been shown to correlate with the degree of Kupffer cell dysfunction rather than the degree of shunting (Pomier-Layragued et al, 1980). However, the observation that the greatest elevation of circulating immunoglobulins occur in autoimmune liver disease (CAH and PBC) might well be explained by the coexistence of reduced suppressor cell activity which has been shown to be present in immunoglobulin producing systems in both PBC (James et al, 1979) and CAH (Kakumu et al, 1980).

It is difficult to assess the relevance of the increased in vitro immunoglobulin production in this system to the hypergammaglobulinaemia associated with cirrhosis as the same observation has been found not only in other disease states associated with hypergammaglobulinaemia (eg) Sarcoidosis and S.L.E., but also in diseases which are not (eg) Crohn's disease (Chapter 8).

We were not able to identify increased activity of the prostaglandin producing suppressor cell system in cirrhotics using this system. This is surprising as in Crohn's disease increased activity was demonstrated both in the proliferative and in the immunoglobulin producing assay. Whether the lack of increased PgSS activity on immunoglobulin production in vitro is relevant to the development of the hypergammaglobulinaemia in cirrhosis is unclear but it is possible that the continual nonspecific B cell stimulation results in a failure to respond to this regulatory system.

In conclusion, lymphocytes from cirrhotics produce significantly more spontaneous IgG production than controls. Exposure of normal cells to cirrhotic serum results in increased IgG production supporting the theory that non-specific B cell stimulation is important in the pathogenesis of the hypergammaglobulinaemia in cirrohsis.

CHAPTER 10

SUMMARY AND CONCLUSION

1. Monocyte function has been studied in a total of 51 patients with biopsy-proven cirrhosis and 35 controls. There was significantly reduced monocyte spreading ($p<.05$), chemotaxis ($p<.02$), bacterial phagocytosis ($p<.05$) and bacterial killing ($p<.02$) in the cirrhotics compared to the controls. Monocytes from patients with cirrhosis produced significantly less of the lysosomal enzymes n-acetyl B-glucosaminidase and B-glucuronidase than those obtained from the controls ($p<.02$). There was no significant difference in the number of monocytes obtained, the number of macrophage precursors, and the nitro-blue tetrazolium reduction between the cirrhotics and the controls. The reduced function appeared to be mainly due to a circulating inhibitory factor and could be corrected by incubation of the cirrhotic cells in serum from control subjects. The response of monocytes from patients with cirrhosis did not differ from the controls in their response to added endotoxin or latex particles suggesting that they are capable of a normal response in the absence of the inhibitory factor. Paired specimens of portal and systemic serum were collected from patients with no evidence of liver disease undergoing vascular surgery. When added to normal human monocytes the portal serum caused a significant reduction in bacterial killing ($p<.02$) and chemotaxis ($p<.05$) compared to results obtained in the paired systemic serum. Mixing experiments suggests the presence of an active inhibitor in the portal serum.

The results suggest that monocyte function is reduced in cirrhosis apparently due to a serum inhibitor which may have originated from the portal vein. The abnormalities may account in part for the increased susceptibility of these patients to infection.

2. To investigate the possibility that a "spillover effect" of substances from the portal vein into the systemic circulation accounts for the abnormal monocyte function in patients with liver disease, human peripheral blood monocytes were incubated with paired specimens of portal and systemic serum from humans and rabbits. Monocytes incubated with portal serum released more of a lysosomal enzyme, N-acetyl-B-

glucosaminidase, than those incubated with systemic serum ($p < .05$). In order to delineate the factors responsible for this increased activity, the content of immune complexes, immunoglobulins and endotoxin in the paired specimens of portal and systemic serum was measured. Endotoxin was found in none of 16 human specimens of portal blood and five of the 16 rabbit specimens. Modestly increased levels of immune complexes, particularly of the IgG class, were found in the portal serum compared to the systemic serum ($p < .01$). Corresponding studies on immunoglobulins showed a higher concentration of IgA in portal serum ($p < .05$), but no difference in other classes of immunoglobulins or complement levels. There was good correltaion between the enzyme production by monocytes and the concentration of immune complexes, particularly IgA ($r = .687$, $p < .001$), but there was no relationship to the presence or absence of endotoxin.

This evidence suggests that portal serum contains substances, particularly immune complexes, which normally under circumstances are sequestered by the liver. These are capable of "activating" monocytes in vitro and may play a role in the pathogenesis of certain types of liver disease. These studies suggest that the effects of portal serum are different depending on which aspect of monocyte function is assessed. There is no ready explanation for this but it is possible that the short term effects are different from the long term ones.

3. Studies were undertaken in 32 patients with hepatitis B-negative or B-positive chronic active hepatitis (CAH) or chronic persistent hepatitis (CPH) to define the relationship between immunoregulatory activity and the HLA B8 and B12 phenotypes. Suppressor T-cell activity measured by a concanavalin A-induced suppressor system using allogeneic responder cells was dependent on which individual was selected as a source of responder cells. No differences were noted using isogeneic cells as responders. Suppressor T-cell activity measured by the effect of a non-induced suppressor cell on a mixed leukocyte culture was not different from controls. Increased prostaglandin-producing suppressor cell activity was found in patients with hepatitis B-negative ($p < .005$) and hepatitis B-positive ($p < .05$) CAH. When results of the suppressor activities were compared among patients with chronic hepatitis dependent

on the presence of HLA B8, B12 or neither of these phenotypes, no significant differences were present. These results provide further evidence of altered immunoregulatory function in patients with CAH, which may reflect increased suppression by a population of prostaglandin-producing suppressor cells. The results do not, however, suggest that a gene coding for altered immune regulation is linked to HLA B8 or B12.

4. The possibility that monocyte suppressor cells play a role in the lymphocyte hyporesponsiveness seen in chronic liver disease was investigated. We studied 46 patients with chronic liver disease and 46 controls using 2 assays of monocyte-mediated suppressor activity: the prostaglandin-producing suppressor cell system (PgSS) and the adherent cell suppressor system (ACSS). There was a significantly increased PgSS activity in patients with cirrhosis compared to controls ($p < .001$). The increase was seen in cirrhosis regardless of aetiology but was not found in patients with chronic active hepatitis who had not developed cirrhosis nor in patients with chronic persistent hepatitis. The suppression was abolished by monocyte depletion and was greater in autologous serum than in pooled AB serum ($p < .02$). Monocyte depletion in cirrhotic patients significantly increased the lymphocyte response to the lectin PHA-P ($p < .005$) but made no significant difference in controls. There was a significant correlation between the PgSS and the change in lymphocyte response to PHA-P on monocyte depletion ($r = .6583$, $p < .01$). ACSS activity was also increased in cirrhosis compared to controls ($p < .025$). Our initial results led us to study the mode of action of the inhibitory effect of cirrhotic serum on lymphocyte response to PHA-P. Cirrhotic serum significantly reduced the response of normal lymphocytes compared to control serum ($p < .02$), but the difference was abolished by adding indomethacin and by monocyte depletion. These results suggest that monocyte suppressor cells play an important role in the cellular hyporesponsiveness in cirrhosis. The inhibitory effect of cirrhotic serum appears to be monocyte-mediated and prostaglandin-dependent.

5. Clinical observations on differences in the sexual incidence of diseases associated with defects of immune regulation, and of the

occasional beneficial effects of pregnancy on disease course suggest that endocrine mechanisms may be important in the immunopathogenesis of these disorders. To investigate this possibility the in vitro effects of testosterone, oestradiol and progesterone on selected aspects of immune regulation were studied in normal adults. We observed the effects of these hormones on a mitogen-induced suppressor T cell system and a monocyte mediated prostaglandin-producing suppressor cell system. The addition of progesterone but not oestradiol or testosterone to the Con-A generation of T lymphocyte suppressor cells produced significantly increased suppressor cell activty ($p<.005$). Preincubation of lymphocytes with testosterone but not oestradiol or progesterone in the absence of Con A resulted in the generation of modest, but highly significant suppressor cell activity ($p<.005$). No effect on the prostaglandin-producing suppressor cell activity was observed. These findings suggest that certain endocrine changes may alter immunoregulatory function and account for some of the clinical observations previously noted in diseases associated with defects of immune regulation. These findings suggest that at least some of the immunoregulatory abnormalities in patients with cirrhosis may be related to the endocrine changes which are well documented in these patients and which occur secondary to the cirrhosis.

6. Three aspects of suppressor cell activity have been studied in 15 patients with progressive systemic sclerosis and 19 healthy controls. There was significantly increased prostaglandin-producing suppressor cell activity in the patients with progressive systemic sclerosis ($p<.01$). No significant differences were found in the concanavalin A-induced or in non-induced suppressor T cell activity. Although these results indicate an alteration in immunoregulatory function, the findings may represent a compensatory reaction to a defect in the regulation of fibrogenesis. These findings suggest that the prostaglandin-producing suppressor cell activity demonstrated in cirrhosis is non-specific and may be related to abnormal fibrogenesis.

7. Part A. Suppressor T-cell activity was measured in 18 patients with Crohn's disease and 20 controls using two different functional

assays. The effects of sulfasalazine and its metabolites on in vitro suppressor cell activity were also studied. Contrary to previous reports, the activity of a Con A-induced suppressor cell system in patients with Crohn's disease did not differ from that of controls. Furthermore, activity of a non-induced suppressor T-cell system was also normal in these patients. There was no evidence either in vivo or in vitro to suggest that sulfasalazine, a drug of known benefit in inflammatory bowel disease, exerts its beneficial action by an effect on this aspect of immunoregulation.

Part B. The in vitro effect of indomethacin (IND), hydrocortisone (HC), sulphasalazine (SS) and its metabolites sulphapyridine (SP) and 5-amino salicylic acid (5-ASA) on peripheral blood monocuclear cells (PBMC) from 49 patients with inflammatory bowel disease (IBD) and 34 controls were studied. Indomethacin caused a highly significant increase in the PBMC response to the mitogen PHA-P compared to controls ($p < .01$), indicating increased activity of a prostaglandin-producing suppressor cell system. On the contrary, SS resulted in a reduced response which was significantly greater for the IBD group than the control group ($p < .05$). This reduction was also produced by 5-ASA ($p < .05$) but not by SP. Addition of IND to PBMC incubated with SS significantly reduced the effect of SS ($p < .001$). HC resulted in a reduced response which was similar to that of controls and was not altered by the addition of IND. The response to IND, HC, SS, SP and 5-ASA was not dependent on the HLA type of the patients, disease activity, or drug therapy.

The results suggest that increased suppression by a population of prostaglandin-producing suppressor cells plays a role in the immunopathology of IBD, but that sulphasalazine does not exert its therapeutic effect by acting on this step of the immunoregulatory system. Any trials of indomethacin therapy in IBD should take into account that, in vitro, SS and IND have opposing mechanisms of action in this system.

Part C. Immunoglobulin production by cultured lymphocytes was measured in 31 patients with inflammatory bowel disease (IBD) and 31 controls. Unstimulated lymphocytes from the pateints with active IBD produced more IgG, IgM and IgA than the controls ($p < .02$). The addition of pokeweed mitogen, a T-cell dependent B-cell mitogen, resulted in a significantly smaller increase in IgA production ($p < .05$) but similar

IgG and IgM production in IBD patients compared to controls. Incubation of pokeweed mitogen stimulated lymphocytes with indomethacin resulted in increased IgA, IgG and IgM production in IBD patients but not in controls, suggesting the presence of increased activity of a prostaglandin-dependent suppressor cell system. There was no evidence, either *in vivo* or *in vitro*, that sulphasalazine, or its metabolites, altered immunoglobulin production. These results add further support to the hypothesis that in some patients abnormal IgA function may be an underlying factor in the development of IBD.

These studies show that the immunoregulatory changes occurring in cirrhosis are non-specific and occur in other diseases. The findings question the validity of previous reports of abnormal T cell regulatory function in inflammatory bowel disease and provide evidence that sulphasalazine does not exert its beneficial effect by acting on these aspects of immunoregulation.

9. Spontaneous IgG producton by lymphocytes from patients with cirrhosis was significantly greater than that of controls. There was no significant difference when pokeweed mitogen was added to the cultures. Normal lymphocytes exposed to sera from cirrhotic patients produced significantly greater amounts of IgG than those exposed to control sera. This suggests that non-specific B-cell activation is involved in the pathogenesis of cirrhotic hypergammaglobulinaemia. No evidence of increased prostaglandin-producing suppressor cell activity was found in this system. It is suggested that the severity of the hypergammaglobulinaemia in patients with cirrhosis is likely to depend on both the degree of non-specific B-cell activation and on the competence of the immunoregulatory system. The nature of the stimulatory substances remains to be elucidated. The failure to demonstrate increased prostaglandin-producing suppressor cell activity in this system may also be important and involved in the development of the hypergammaglobulinaemia, as increased activity was observed in both the proliferative and immunoglobulin production producing assay in patients with Crohn's disease.

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