Clin. exp. Immunol. (1976) 26, 222–227.

Peripheral blood lymphocyte populations in chronic liver disease

H. C. THOMAS, M. FRENI, J. SANCHEZ-TAPIAS, D. DE VILLIERS, S. JAIN & S. SHERLOCK *Department of Medicine, The Royal Free Hospital, Pond Street, Hampstead, London*

(*Received* 24 *February* 1976)

SUMMARY

Mature T lymphocyte concentrations are reduced, null cell concentrations are increased, and Fc receptor bearing (B and K) lymphocyte concentrations are normal, in the peripheral blood of patients with chronic hepatocellular or cholestatic liver disease. Some null cells can be stimulated by either thymosin or levamisole to form rosettes with sheep erythrocytes. These changes are present in viral, alcohol associated and 'autoimmune' liver disease and are therefore probably secondary phenomena relating to liver damage.

INTRODUCTION

Many abnormalities of the immune system have been described in patients with chronic liver disease. In viral hepatitis, the severity and duration of the disease is dependent on the immune response of the host to viral antigens displayed on the surface of hepatocytes (Dudley, Fox & Sherlock, 1972). In primary biliary cirrhosis and 'lupoid' hepatitis it is assumed that there is a defect in the immune regulatory system which normally suppresses a potential response to self antigens (Allison, Denman & Barnes, 1971). Secondary changes in immune reactivity follow most types of liver damage (Thomas, MacSween & White, 1973; Thomas *et al.*, 1976). These immune phenomena, common to all patients, must be distinguished from those peculiar to a particular disease and, therefore, perhaps more closely involved in primary causation.

Lymphocytes are intimately concerned with the immune response and rosetting techniques allow their populations to be identified. T-lymphocytes have a regulatory and cytotoxic importance and are enumerated by their capacity to form rosettes with washed sheep erythrocytes. A further group which includes B and K lymphocytes, bear receptors for the Fc-piece of immunoglobulin and has the capacity to destroy antibody coated cells (MacLennan, 1972). These cells may be important in autoimmune disease states where organ-specific autoantibodies have been demonstrated (Roitt, 1974). Another population of lympocytes termed null cells remains undetected by these rosetting techniques. Some nulls cells can be induced to mature into T lymphocytes by thymosin (Scheinberg, Cathcart & Goldstein, 1975), suggesting that they are immature T cells.

The purpose of this study was to compare the various lymphocyte concentrations in the peripheral blood of patients with viral, autoimmune and alcohol-induced liver disease to determine whether there are any changes peculiar to one disease and therefore involved in the primary pathogenesis of that disease.

PATIENTS AND METHODS

Sixteen patients with alcohol induced liver disease (ALD), twenty with primary biliary cirrhosis (PBC), twenty-three with chronic active hepatitis (CAH) and cirrhosis and four asymptomatic HBsAg carriers were studied. Control data were obtained from twenty healthy laboratory workers. (Table 1).

Correspondence: Dr H. C. Thomas, Department of Medicine, The Royal Free Hospital, Pond Street, Hampstead, London N.W.3.

TABLE 1. Age range and sex distribution of patients and controls studied

	No.	Male	Female	Age range
ALD	16	9	7	31–62 (38)
CAH Positive	19	12	7	35–63 (42)
Asymptomatic carriers of HB_sAg	4	3	1	23–42 (35)
CAH Negative	4	0	4	21–55 (36)
PBC	20	0	20	41–59 (50)
Controls	20	10	10	23–58 (34)

ALD = Alcohol-related disease; CAH positive = HB_sAg positive chronic active hepatitis; CAH negative = HB_sAg negative chronic active hepatitis; PBC = primary biliary cirrhosis.

Figures in parentheses are the means.

All diagnoses were based on clinical, biochemical and histological findings. Nine patients with alcohol induced liver disease had histologically proven cirrhosis, seven had alcoholic hepatitis with central fibrosis but no cirrhosis. All twenty patients with PBC had stage 3 or 4 histological changes (Scheuer, 1967). Nineteen patients had HB_sAg positive chronic active liver disease; four had primary liver cell carcinomas. Four asymptomatic HB_sAg carriers had biopsy evidence of chronic persistent hepatitis.

All patients were examined before starting treatment with prednisolone or both prednisolone and azathioprine.

Serial studies before and after steroid treatment were done in one patient with HB_sAg negative CAH.

Lymphocytes were obtained from 20 ml of fresh heparinized blood by separation on Ficoll–Triosil density gradient (sp. gr = 1·077), washed and diluted in basal Eagle's medium to give a final concentration of 4×10^6 cells/ml.

E-rosette forming cells (*E-RFC*). Washed sheep erythrocytes (Wellcome), not more than 1 week old, were diluted with Eagle's medium containing 20% foetal calf serum to give a 1% suspension by volume.

0·1 ml of the lymphocyte suspension was mixed with 0·1 of 1% erythrocytes, left at room temperature for 5 min, centrifuged at 200 *g* for 5 min and then incubated overnight at 4°C.

EA-rosette forming cells (*EA-RFC*). Sheep erythrocytes were incubated with rabbit IgG anti-sheep erythrocyte globulin for 30 min at 37°C, washed three times and resuspended in 20% foetal calf serum in Eagle's medium to give a final suspension of 1%.

0·1 ml of the lymphocyte suspension was mixed with 0·1 ml of the antibody-coated erythrocyte suspension, left at room temperature for 5 min, centrifuged at 200 *g* for 5 min and then incubated for 20 min at 37°C.

After incubation cells were resuspended, fixed in 3% gluteraldehyde and stained with trypan blue.

The percentage of rosette-forming cells was determined after counting 200 lymphocytes by two observers who had no knowledge of the identity of the specimen.

Only lymphocytes fixing at least three red cells were scored as rosetting cells. In each experiment lymphocytes from a normal subject were used as control.

The results are expressed both as the percentage of peripheral blood lymphocytes which formed rosettes and concentration of rosetting cells per mm^3 of blood.

HB_sAg was detected by direct passive haemagglutination using turkey red blood cells sensitized with horse anti-HB_sAg. (Hepatest–Wellcome Labs).

Levamisole and thymosin treatment. 0·1 ml of lymphocyte suspension (4×10^6 ml) was incubated with either 0·1 ml of thymosin (200 μg/ml) or 0·1 ml of levamisole (0·24 μg/ml) for 30 min at 37°C. In control tubes, lymphocytes were incubated in medium alone. Rosetting techniques were done after incubation as described above.

Statistics. The significance of differences in cell populations between the groups studied was analysed by Student's *t*-test.

RESULTS

E-rosette forming cells (*E-RFC*)

The percentage and concentration of peripheral blood lymphocytes that form E-rosettes were significantly ($P<0·05$) decreased in all groups of patients with chronic liver disease when compared with controls of the same age range. (Because the patients with primary biliary cirrhosis were slightly older than the other patients studied, a separate older group of control subjects was used for statistical comparison with this group of patients. (Table 2)). This decrease was greatest in patients with alcohol-induced liver disease and smallest in patients with primary biliary cirrhosis. E-RFC in patients with primary liver

TABLE 2. Lymphocyte populations (cells/mm³ blood) in chronic liver disease

	No.	Lymphocyte concentration	E-RFC		EA-RFC		Null cells	
			Percent	Concentration cells/mm³	Percent	Concentration cells/mm³	Percent	Concentration cells/mm³
ALD	16	1724±215	37±3*	673±129*	28±3	502±107	36±4*	547±85*
CAH positive	19	2111±357	44±4*	929±161*	30±4	633±130	26±5*	548±85*
Asymptomatic carriers of HB_sAg	4	1872±128	70±6	1330±207	25±6	458±114	7±5	113±86
CAH negative	4	1963±377	47±6*	892±135*	27±4	547±154	26±4*	522±155*
PBC	20	1803±182	56±2†	985±101†	30±3	540±76	14±3†	252±102†
Controls (23–58)	20	2088±130	65±2	1380±123	31±2	599±41	6±1	141±29
Controls (35–58)	7	1889±140	62±2	1249±97	32±2	640±48	8±2	151±30

* Statistically significant difference at 5% level (using Student's *t*-test) when comparison made with control group aged 23–58 years (mean 34 years).

† Statistically significant differences at 5% level when comparison made with control group aged 35–58 years (mean 48 years).

cell carcinoma were not significantly different from other patients with HB_sAg positive chronic liver disease. Four patients with chronic persistent hepatitis had normal E-RFC concentrations.

EA-rosette forming cells (EA-RFC)

These were not significantly different from the control population in all groups studied.

Null cells

These were significantly increased in all groups of patients except in those with CPH who had normal percentage and concentration.

Serial studies in HB_sAg negative CAH (Table 3)

A 40-year-old female patient with HB_sAg negative, autoantibody positive (Smooth muscle > 1/40; anti-nuclear antibody > 1/40) CAH was studied before and after treatment with 20 mg prednisolone.

Before treatment, decreased E-RFC and increased null cells were noted. Following treatment with

TABLE 3. Serial study of lymphocyte populations in a patient with HB_sAg negative chronic hepatitis

	Before treatment			Prednisolone (20 mg/day)			
	1st week	2nd week	3rd week	4th week	5th week	6th week	7th week
Lymphocytes (%)							
E-RFC	36	40	30	30	49	49	55
EA-RFC	39	33	39	48	44	46	47
Null cells	25	27	31	22	7	5	0
Lymphocytes (concentration)							
E-RFC	831	924	706	768	941	439	653
EA-RFC	901	762	917	1229	845	412	535
Null cells	578	624	729	563	144	45	0
Total lymphocyte (concentration)	2310	2310	2352	2560	1920	896	1188

Treatment with 20 mg prednisolone/day was started at the end of the 3rd week.

20 mg prednisolone/day, the patient developed lymphopenia. This was mainly due to a fall in null cells and EA-RFC but a small reduction in E-RFC also occurred.

Response to thymosin and levamisole (*Table* 4)

Thymosin treatment of normal peripheral blood lymphocytes resulted in a small increase in E-RFC but no change in EA-RFC. Levamisole produced a small reduction in E-RFC. Treatment of lymphocytes from patients with reduced E-RFC and increased null cell concentrations with either thymosin or levamisole produced an increase in E-RFC.

TABLE 4. *In vitro* effect of thymosin and levamisole on E-rosette-forming cells

Liver disease	Pretreatment			After thymosin		After levamisole	
	E (%)	EA (%)	Null (%)	E (%)	Percentage change in E	E (%)	Percentage change in E
CAH Positive	48	27	25	60	+12	43	−5
CAH Positive	42	22	36	59	+17	64	+22
CAH Positive	60	22	18	74	+14	74	+14
CAH Negative	43	24	33	53	+10	39	−4
CAH Negative	48	22	30	54	+6	51	+3
CAH Negative	40	19	41	52	+12	57	+17
PBC	45	29	26	52	+7	53	+8
PBC	65	29	6	77	+12	67	+2
PBC	59	20	21	58	−1	56	−3
PBC	52	45	3	54	+2	53	+1
ALD	47	31	22	n.d.	n.d.	61	+14
ALD	60	20	20	n.d.	n.d.	81	+21
ALD	63	20	17	86	+23	71	+8
ALD	75	25	0	70	−5	75	0
Control (mean±s.e.)	65±2	29±3	6±1	67±2	+2±1	62±2	−3±1

The control group consisted of ten normal subjects aged 23–58 years.

DISCUSSION

This study confirms that T-lymphocyte concentrations are reduced in the peripheral blood of patients with alcohol induced liver disease (Bernstein *et al.*, 1974) and chronic active hepatitis (DeHoratius, Stickland, & Williams, 1974). In addition, concentrations are decreased in patients with primary biliary cirrhosis and are normal in chronic persistent hepatitis. The Fc-receptor-bearing lymphocytes, a population which includes B and K lymphocytes, were present in normal concentrations in all patient groups studied.

The reduced concentration of peripheral blood T lymphocytes in the presence of normal concentrations of Fc-receptor-bearing cells suggests a factor in these patients which selectively affects T cells and its presence in all types of severe chronic hepatocellular and cholestatic disease suggests that it is probably secondary to liver damage. Chisari & Edgington (1974) have demonstrated that the process of E-rosette formation is dependent on the level of membrane associated cyclic AMP, therefore any change in the concentration of this substance in severe liver disease would selectively influence E-RFC concentrations. The increase in E-RFC after treatment with the thymic hormone thymosin is of particular interest because of the suggestion that this hormone exerts its effect by an action on adenyl cyclase, the enzyme which controls the rate of production of cyclic AMP (Goldstein *et al.*, 1975). Similar changes after treatment with levamisole support the suggestion that this agent exerts its immunopotentiating effect in the same manner (Hadden *et al.*, 1975).

An alternative explanation for the response to thymosin is that patients with chronic liver disease have an increased turnover of T lymphocytes and therefore have increased numbers of immature T cells in their blood. These cells can be induced to differentiate into mature E-rosette forming cells by thymosin (Wara *et al.*, 1975). Several factors may contribute to increase the turnover of T cells. The presence of splenomegaly in most patients with chronic liver disease either as a feature of the primary disease process (MacLachlan *et al.*, 1965) or as a result of portal hypertension, makes sequestration in this organ possible. Increased homing of lymphocytes to either the spleen or lymph nodes because of increased antjgenic stimulation (Zatz & Lance, 1971) is another possiblity. Finally the presence of a lymphocytic infiltrate in the liver in all the forms of hepatocellular and cholestatic disease investigated in this study, suggests that lymphocytes migrate to this organ either because they are specifically sensitized to liver antigens or for other reasons yet unidentified.

Although these facts imply an increased turnover of T cells, the presence of increased numbers of immature T cells might also occur if the capacity of the thymus to process immature cells was reduced. The possibility of such a defect in HB_sAg negative chronic active hepatitis is supported by reports of histological changes in the thymus in the early stages of this disease (Corridan, 1963), and reports of thymic atrophy induced by viral infection (White & Boyd, 1973) raise the possibility of similar abnormalities in the virus induced forms of liver disease.

Some null cells, unidentified after treatment with thymosin or levamisole, may be related to immune complex formation. Such complexes when formed in the presence of excess antigen, adhere to the surface of T-lymphocytes and prevent rosette formation. Patients with chronic liver disease have a reduced capacity to clear such complexes from the blood (Thomas, MacSween & White 1973). In addition, lymphocytotoxins prevent rosette formation, and these have been described in sera from patients with chronic active hepatitis (Thomas *et al.*, 1975).

The normal B- and K-cell concentrations noted in this study do not necessarily exclude involvement of these cells in the disease process since an increase in production may compensate for increased utilization. The marked changes in T-lymphocyte concentrations may merely reflect the smaller capacity of the lymphoid organs to replace T cells which are normally long lived, rather than selective involvement of these cells in pathogenesis.

In HB_sAg negative CAH, prednisolone is of considerable therapeutic benefit (Cook, Mulligan & Sherlock, 1970). This treatment results in decreased Fc-receptor-bearing lymphocyte and null-cell concentrations. More detailed studies are required to determine which changes correlate best with clinical and biochemical evidence of reduced disease activity.

M. F. was supported by a fellowship from Fondazione Alberto Bonino and M. Sofia Pulejo, S.J. by the Medical Research Council and D. de V. by the Stanley Johnson Fund.

Thymosin was provided by Professor A. C. Goldstein, University of Texas, Galverston, U.S.A. Levamisole was provided by Janssen Pharmaceutical, Belgium.

REFERENCES

ALLISON, A.C., DENMAN, A.M. & BARNES, R.D. (1971) Co-operating and controlling functions of thymus-derived lymphocytes in relation to auto-immunity. *Lancet*, **ii**, 135.

BERNSTEIN, I.M., WEBSTER, K.M., WILLIAMS, R.C. & STRICKLAND, R.G. (1934) Reduction in T lymphocytes in alcoholic liver disease. *Lancet*, **ii**, 488.

CHISARI, F.V. & EDGINGTON, T.S. (1974) Human T lymphocyte 'E'-rosette function. I. A process modulated by intracellular cyclic AMP. *J. exp. Med.* **140**, 1122.

COOK, G.C., MULLIGAN, R. & SHERLOCK, S. (1971) Controlled prospective trial of corticosteroid therapy in active chronic hepatitis. *Quart. J. Med.* **40**, 159.

CORRIDAN, M. (1963) The thymus in hepatic cirrhosis. *J. clin. Path.* **16**, 445.

DEHORATIUS, R.J., STRICKLAND, R.G. & WILLIAMS, R.C. (1974) T and B lymphocytes in acute and chronic hepatitis. *Clin. Immunol. Immunopathol.* **2**, 353.

DUDLEY, F.J., FOX, R.A. & SHERLOCK, S. (1972) Cellular immunity and hepatitis-associated Australia antigen liver disease. *Lancet*, **i**, 723.

GOLDSTEIN, A.L., THURMAN, G.B., COHEN, G.H. & HOOPER, J.A. (1975) The role of thymosin and the endocrine thymus on the ontogenesis and function of T-cells. *Molecular Approaches to Immunology*, p. 243. Academic Press, New York.

HADDEN, J.W., COFFEY, R.G., HADDEN, E.M., LOPEZ-CORRALES, E. & SUNSHINE, G.H. (1975) Effect of levamisole and Imidazole on lymphocyte proliferation and cyclic nucleotide levels. *Cell. Immunol.* **20**, 98.

MACLACHLAN, M.J., RODNAN, G.P., COOPER, W.M. &

FENNELL, R.M. (1965) Chronic active ('lupoid') hepatitis. *Ann. intern. Med.* **62**, 425.

MACLENNAN, I.C.M. (1972) Antibody in the induction and inhibition of lymphocyte cytotoxicity. *Transplant. Rev.* **13**, 67.

ROITT, I.M. (1974) *Essential Immunology*, 2nd edn. p. 129. Blackwell Scientific Publications, London.

SCHEINBERG, M.A., CATHCART, E.S. & GOLDSTEIN, A.L. (1975) Thymosin-induced reduction of null cells in peripheral blood lymphocytes of patients with systemic lupus erythematosis. *Lancet*, **i**, 424.

SCHEUER, P.J. (1967) Primary biliary cirrhosis. *Proc. roy. Soc. Med.* **60**, 1257.

THOMAS, H.C., RYAN, C.J., BENJAMIN, I.S., BLUMGART, L.H. & MACSWEEN, R.N.M. (1976) The immune response in cirrhotic rats: the induction of tolerance to orally administered protein antigens. *Gastroenterology*, **71**, 114.

THOMAS, H.C., MACSWEEN, R.N.M. & WHITE, R.G. (1973) The role of the liver in controlling the immunogenicity of Commensal bacteria in the gut. *Lancet*, **i**, 1288.

THOMAS, H.C., SANCHEZ-TAPIAS, J., ROSS, M. & VERRIER-JONES, J. (1975) Measles, rubella and lymphocyte antibodies in chronic active hepatitis. *Clin. Sci.* **49**, 27p.

WARA, D.W., GOLDSTEIN, A.L., DOYLE, N.E., AMMANAA, A.J. (1975) Thymosin activity in patients with cellular immunodeficiency. *New Engl J. Med.* **212**, 70.

WHITE, R.G. & BOYD, J.F. (1973) The effects of measles on the thymus and other lymphoid tissues. *Clin. exp. Immunol.* **13**, 343.

ZATZ, M. & LANCE, E.M. (1971) The distribution of ^{51}Cr-labelled lymphocytes into Ag-stimulated mice: lymphocyte trapping. *J. exp. Med.* **134**, 224.