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Lymphocytotoxins in acute and chronic hepatitis

CHARACTERIZATION AND RELATIONSHIP TO CHANGES IN CIRCULATING T LYMPHOCYTES

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SUMMARY

Serum lymphocytotoxicity (LCT) was detected in 49% of fifty-one patients with acute viral hepatitis and 72% of twenty-nine patients with chronic hepatitis. LCT was not detected in ten chronic carriers of hepatitis B surface antigen. Characterization of LCT revealed it to be active at physiologic temperatures and to be reactive against both T and B lymphocytes. The occurrence of LCT was transient in acute hepatitis and intermittent in chronic hepatitis. There was a significant inverse relationship between the percentage change in LCT over time and peripheral blood T-cell proportions amongst the patients studied. These findings indicate the importance of liver damage in the appearance of LCT and suggest that LCT may contribute to depressed lymphocyte function in liver disease.

INTRODUCTION

Enumeration of circulating lymphocyte subpopulations in acute and chronic hepatitis have shown divergent results. Some studies have revealed decreased peripheral blood thymus-derived (T) lymphocytes in these disorders (DeHoratius, Strickland & Williams, 1974; Edgington & Chisari, 1975; Thomas *et al.*, 1975) whilst others have reported normal percentages and numbers of these cells (Wicks, Kohler & Singleton, 1975; Galili *et al.*, 1975).

Preliminary data from our laboratory (DeHoratius *et al.*, 1974) demonstrated the presence of serum lymphocytotoxins (LCT) in both acute and chronic hepatitis. Mottironi & Terasaki (1970) also found lymphocytotoxins in the sera of patients with acute hepatitis as well as in other acute viral illnesses. Thomas *et al.* (1975) recently confirmed the presence of LCT in patients with chronic hepatitis.

Specificity of the hepatitis LCT for lymphocyte subpopulations has not previously been reported. Investigations of this feature in other disorders associated with LCT such as systemic lupus erythematosus and inflammatory bowel disease have revealed specificity for both T- and B-cell surface determinants (Lies, Messner & Williams, 1973; Wernet & Kunkel, 1973; Winfield *et al.*, 1975b; Strickland *et al.*, 1975). Furthermore, a correlation between LCT and lymphopenia has been described in systemic lupus erythematosus (Butler *et al.*, 1972; Winfield, Winchester & Kunkel, 1975a).

The present study was undertaken to further characterize the lymphocytotoxins in hepatitis serum and to determine the relationship if any between LCT and T-cell depression in patients with acute or chronic hepatitis.

MATERIALS AND METHODS

Patients. Ninety patients were studied. Fifty-one had acute viral hepatitis, forty-three of whom were studied from 1 to 4 weeks from the onset of symptoms. Six had prolonged jaundice of 12–16 weeks duration whilst one had fatal fulminant hepatitis. Twenty demonstrated hepatitis-B-surface antigen (HB_sAg) as measured by counter immunoelectrophoresis

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(Gocke & Howe, 1970). Of these fifty-one patients, twenty-one were followed serially with T lymphocyte and LCT determinations. The mean age was 33 years, range (13–51 years).

Twenty-nine patients had biopsy-confirmed chronic hepatitis and were classified by clinical course and liver biopsy (DeGroote *et al.*, 1968). Eleven had chronic persistent hepatitis of whom five had HB_sAg, while eighteen had chronic aggressive hepatitis, all with established cirrhosis and four had HB_sAg. Fourteen patients with chronic hepatitis were studied serially. The mean age of this group was 43 years, range (27–54 years).

Ten asymptomatic carriers of HB_sAg with no previous history of icteric hepatitis were also studied. All had normal bilirubin, serum glutamic oxalacetic transaminase, alkaline phosphatase, and bromsulfalein (BSP) retention. The mean age was 23 years, range (18–52 years).

The control group consisted of seventy-five normal subjects of similar age distribution to the patients, mean age 35 years, range (12–55 years). None of these subjects had a previous history of an icteric illness.

Microcytotoxicity assay. The microdroplet assay of Terasaki & McClelland (1964) was used to determine the presence of lymphocytotoxic activity. Sera from the patients and control populations were stored at −20°C prior to assay. Three-hour incubations were used at 15°C with rabbit serum as source of complement (Grand Island Biological Company, California, U.S.A.) (lot A830124).

Cytotoxicity was determined in control sera and sera from the patients with hepatitis using panels of unfractionated, T cell-enriched or B cell-enriched peripheral blood lymphocyte preparations from normal donors. Cytotoxicity was regarded as positive against individual donors if $\geq 20\%$ of the target cells were killed. A test serum was regarded as positive if $\geq 50\%$ of the donor panel showed significant cytotoxicity. In selected sera, cytotoxicity was tested over a range of incubation temperatures (4, 15, 25 and 37°C).

Target lymphocytes. Initially, the presence or absence of lymphocytotoxicity was established for individual sera using panels of eighteen to thirty normal donors of varying HL-A phenotype. Subsequently, a smaller number of donors were used to study specificity in cytotoxicity testing. Peripheral blood lymphocytes from six healthy adult donors of differing HL-A phenotype constituted the source of normal unfractionated lymphocytes. Venous blood was collected in heparinized syringes and lymphocytes separated by Ficoll–Hypaque flotation using phosphate-buffered saline (PBS), pH 7·4, supplemented with 5–10% heat-inactivated foetal calf serum (FCS).

T cells. Using lymphocytes from the same six normal donors T cell-enriched preparations were prepared by a method of B-cell depletion using columns of Degalan V_{26} coated with anti-immunoglobulin (Wigzell, Sundquist & Yoshida, 1972), as previously described in detail (Strickland *et al.*, 1975). The percentage of B cells in the eluates as determined by the presence of cells bearing immunoglobulin (Pernis, Forni & Amante, 1970; Unanue *et al.*, 1971) ranged from 0 to 2% in the six donors. This compared with a range of 14–33% prior to passage over Degalan columns. T-cell proportions of the final preparations ranged from 80 to 90% by the sheep red blood cell rosette technique (Fröland, 1972; Jöndal, Holm & Wigzell, 1972; Wybran, Carr & Fudenberg, 1972). Viability of the final cell preparations was in excess of 95% as determined by trypan blue dye exclusion.

B cells. B cell-enriched target lymphocytes were prepared by a method of T-cell depletion using density gradient separation of SRBC binding lymphocytes (Greaves & Brown, 1974) as previously described (Strickland *et al.*, 1975). Cells at the interface between the two layers on a Ficoll–Hypaque gradient were harvested and found to contain 5–20% T cells by SRBC rosette formation in the six normal donors and an average of 78–90% B cells as identified by surface immunoglobulins. Viability as determined by trypan blue dye exclusion was in excess of 90%.

Absorption experiments. Six sera which were demonstrated to have strong lymphocytotoxic activity were used for absorption studies. Each was absorbed with either T cell-enriched or B cell-enriched lymphocyte preparations from normal donors by incubation at 15°C for 1 hr. Cytotoxicity was then retested using these absorbed sera against unfractionated normal donor target cells from six individuals.

Peripheral blood T- and B-cell enumeration. T and B cells were enumerated as previously described (DeHoratius *et al.*, 1974). T cells were determined by spontaneous rosette formation between lymphocytes and sheep erythrocytes (Wybran *et al.*, 1972; Jöndal, Holm & Wigzell, 1972). B cells were enumerated by the presence of cell surface immunoglobulins using FITC-conjugated moncspecific antisera to IgA, IgG and IgM (Williams *et al.*, 1973).

Statistics. Wilcoxan's rank test (Bradley, 1968) was used to determine significance of T- and B-cell alterations. Spearman's rank order correlation was used to discern the relationship of LCT to changes in T cells (Bradley, 1968).

RESULTS

Frequency of LCT and degree of cytotoxicity in groups studied (Table 1)

Twenty-nine of fifty-one sera from patients with acute hepatitis (49%) had LCT against unfractionated lymphocyte target cells. Twenty-one of twenty-nine sera from patients with chronic hepatitis (72%) had LCT. By contrast, none of the ten chronic carriers of HB_sAg had LCT and only three (4%) sera from seventy-five healthy controls of similar age and sex distribution showed LCT. Many of the LCT positive sera were highly cytotoxic (Table 1). Results of serial serum dilution on average cytotoxicity in one patient with acute hepatitis are shown in Table 2. This serum when diluted showed a progressive

TABLE 1. Distribution of lymphocytotoxicity (LCT) of sera against normal peripheral blood lymphocytes

	No.	Positive sera (%)	No. of sera with cytotoxic activity against normal peripheral blood lymphocytes					
			0–19*	20–39	40–49	50–59	60–79	80–100
Control	75	4	53	23	6	2†	1	0
Acute hepatitis	51	49	8	10	4	4	11	14
Chronic hepatitis	29	72	4	2	2	4	7	10
Chronic carriers of HB_sAg	10	0	4	3	3	0	0	0

* Percentage of donor lymphocyte panel killed by sera.
† Positive cytotoxicity defined as $\geq 50\%$ of the donor panel killed.

TABLE 2. Effect of serial dilution of hepatitis sera on LCT in patient J.S.

	Unseparated PBL	T-enriched PBL
J.S. Neat	100*	100*
1:2	100	100
1:4	40	n.t.
1:8	80	30
1:16	40	40
1:32	50	50

n.t. = Not tested.
* Average cytotoxicity against PBL from six normal donors.

decrease in cytotoxicity against both unfractionated and T cell-enriched target lymphocytes but was still clearly detectable at a 1:32 dilution.

Specificity of LCT for peripheral blood lymphocyte (PBL) subpopulations

Fourteen sera from patients with acute hepatitis showing strong lymphocytotoxic activity against unfractionated PBL were tested. Three of these sera reacted with T cell-enriched target lymphocytes and three reacted with B cell-enriched target lymphocytes. Eight sera reacted against both T- and B-cell preparations. Of four cytotoxic sera from patients with chronic hepatitis tested, three reacted against both T and B cells whilst one reacted against B cells alone. Two of fifty control sera had lymphocytotoxic activity. One reacted with T cells alone and one reacted with both T- and B-cell preparations. Sera from six patients with hepatitis were tested against PBL from six normal donors following absorption of the serum with either T or B cell-enriched lymphocyte preparations. Although both T-cell absorption and B-cell absorption decreased the average cytotoxicity from that observed in the unabsorbed sera, there was no clear preferential decrease in cytotoxicity using T- or B-cell absorption.

Temperature dependence of LCT in hepatitis sera

Sera from five patients with hepatitis were tested against PBL from six normal donors at varying incubation temperatures (Table 3). Two of five sera were positive at both 4 and 25°C. However maximal cytotoxicity was observed at both 15°C (5/5) and 37°C (4/5).

TABLE 3. Serum lymphocytotoxicity at different incubation temperatures in six patients with hepatitis

Patient	Average percentage lymphocytotoxicity at: 4°C	15°C	25°C	37°C
LB	22*	50	9	29
HD	11	41	16	60
MH	9	45	33	85
WS	5	28	4	16
MX	100	100	100	100

* Average cytotoxicity against unfractionated lymphocytes from six normal donors.

TABLE 4. Percentage and number of peripheral blood T and B cells in hepatitis, chronic carriers of HB_sAg and normal controls

Patient group	No. tested	Percentage T cells*	No. tested	No. T cells	No. tested	Percentage B cells†	No. tested	No. B cells
Acute hepatitis	51	48±3‡ $P<0.001$§	46	1318±120 $P<0.005$	51	28±1 n.s.¶	51	538±32 n.s.
Chronic hepatitis	29	54±19 $P<0.001$	26	1169±136 $P<0.001$	29	25±2 n.s.	29	585±45 n.s.
Chronic HB_sAg carriers	10	58±4 n.s.	10	1703±242 n.s.	10	22±2 n.s.	10	648±32 n.s.
Normal controls	75	65±2	75	1787±223	75	22±1	75	648±16

* T cells determined by spontaneous sheep red blood cell rosettes.
† B cells determined by surface-bound immunoglobulins.
‡ Mean±1 s.e.m.
§ *P* value when compared to normal control.
¶ n.s.=Not significant.

T- and B-cell determinations

The percentage and number of both T and B cells in the patient groups studied are shown in Table 4. Amongst the patients with acute hepatitis, twenty-six of fifty-one showed T-cell percentages below 1SD from the mean in control subjects and twenty-nine of forty-six showed decreased T-cell numbers using the same criterion. Ten of twenty-nine and seventeen of twenty-six patients with chronic hepatitis showed decreased T cell percentages or numbers respectively. Two of ten chronic carriers of HB_sAg had depressed proportions and absolute numbers of T cells. Compared to normal control values, mean T-cell percentages and numbers were significantly decreased in both acute and chronic hepatitis, but did not differ significantly in healthy HB_sAg carriers (Table 4). B-cell percentages and numbers were not significantly altered in any of the groups studied. As was observed in an earlier study (DeHoratius *et al.*, 1974) there was no relationship between the presence of HB_sAg and the occurrence of T-cell depression in either acute or chronic hepatitis.

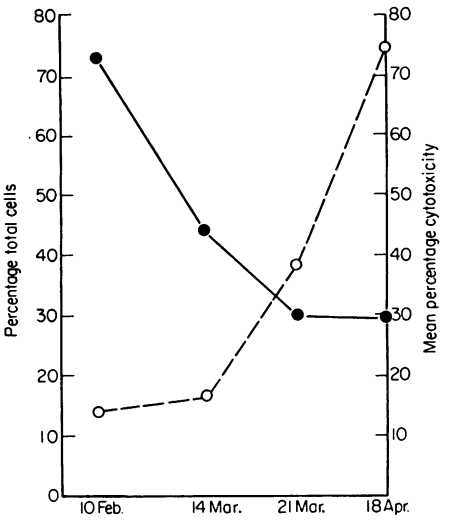

FIG. 1

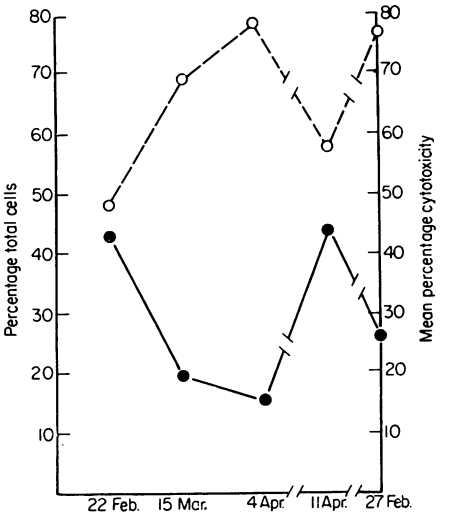

FIG. 2

FIG. 1. Changes observed in patient MH with acute hepatitis. (O- - -O) T lymphocytes; (●———●) mean percentage of lymphocytotoxicity.

FIG. 2. Changes observed in patient PM with chronic hepatitis. (O- - -O) T lymphocytes; (●———●) mean percentage of lymphocytotoxicity.

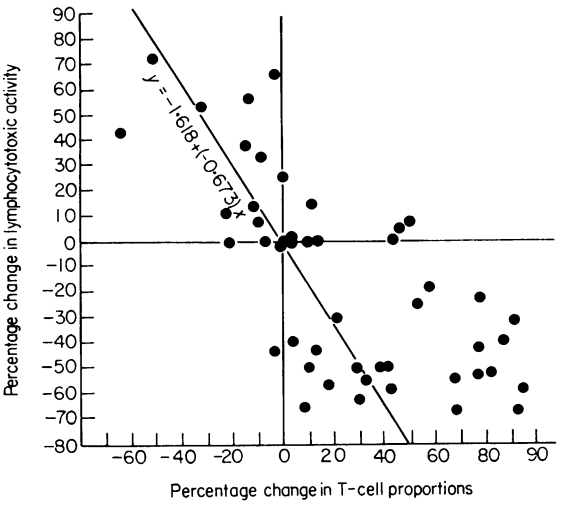

FIG. 3. Correlation between the percentage change in T-cell proportions and the percentage change in lymphocytotoxicity. $n = 49$; $r = -0{\cdot}65$.

Relationship of LCT to circulating T-cell proportions

Serial determinations of T- and B-cell proportions and LCT were carried out in twenty-one patients with acute hepatitis and fourteen patients with chronic hepatitis. A representative example of the changes observed in acute hepatitis is shown in Fig. 1. It can be seen that as T-cell proportions return to normal, LCT, depicted as average lymphocytotoxicity, decreases. Similar changes were observed in the patient with chronic hepatitis shown in Fig. 2. In this patient, there is an inverse relationship between T-cell percentages and the level of serum lymphocytotoxic activity. In order to relate lymphocytotoxic activity

to T-cell proportions in the thirty-five patients with acute or chronic hepatitis studied serially, the ninety-eight individual values in these patients were converted to percentage change. Forty-nine simultaneous determinations of percentage change in the two parameters therefore resulted. As shown in Fig. 3, there is a significant negative correlation between the percentage change in T cell proportions and the percentage change in lymphocytotoxicity ($r = 0{\cdot}65$; $P < 0{\cdot}001$).

DISCUSSION

The present study confirms our earlier findings (DeHoratius *et al.*, 1974) and those of other workers (Edgington & Chisari, 1975; Thomas *et al.*, 1975) that circulating T cells are often reduced in patients with acute or chronic hepatitis. The discrepancy between these findings and those of other authors (Wicks *et al.*, 1975; Galili *et al.*, 1975) is not readily apparent. Certainly the number of patients studied in the latter two investigations were small, and the time of blood collection in relation to the onset of hepatitis was not specified. Such details are important in view of the transient nature of the defect in acute hepatitis and the fluctuating values for peripheral blood T cells observed in chronic hepatitis over time. The finding of normal peripheral blood lymphocyte distributions in healthy HB_sAg carriers together with previous observations of normal *in vitro* lymphocyte responses to phytohaemagglutinin (Sutnick *et al.*, 1973; Nielsen *et al.*, 1973; Tong *et al.*, 1975) in such patients, indicate that in the absence of significant liver damage there is no general impairment of cell-mediated immunity, despite persistence of hepatitis B surface antigen.

The alterations in circulating T cells in acute and chronic hepatitis are accompanied by the occurrence of lymphocytotoxins in the serum. Asymptomatic HB_sAg carriers did not display this phenomenon, suggesting again a relationship of LCT to liver damage. Alternatively, it may reflect an abnormality of the host response as it has been shown that despite persistence of the antigen, such patients fail to develop HB_s antibody (Hoofnagle, Gerety & Barker, 1973). The significant correlation observed between changes in circulating T cells and changes in LCT in both acute and chronic hepatitis raises the possibility that LCT may be responsible in part for the T-cell depression in these disorders. The lymphocytotoxin appears to be active at physiological temperatures and thus could function *in vivo*. However, the present study has also shown that the hepatitis LCT is reactive *in vitro* against cell surface determinants on both T and B cells. These findings are reminiscent of those occurring in systemic lupus erythematosus where a correlation between lymphocytopenia and circulating LCT is observed (Winfield *et al.*, 1975a), variable peripheral blood T-cell depression has been reported (Messner, Lindström & Williams, 1973), and yet *in vitro* reactivity of LCT to T and B cells is generally found (Wernet & Kunkel, 1973; Winfield *et al.*, 1975b).

The possibility that additional factors may be involved in the lymphocyte abnormalities in hepatitis is suggested by a number of recent studies. Chisari & Edgington (1975) have described a lipoprotein component in hepatitis serum which inhibits spontaneous sheep erythrocyte rosettes *in vitro* and which was present in 40% of patients who showed apparent T-cell reductions in the peripheral blood. The relationship between this rosette inhibitory factor and LCT is not yet clear. However, it is likely that they are separate components, as LCT in other disorders appear to be antibodies of IgM or IgG type (Winfield *et al.*, 1975b). Another serum factor which might contribute to lymphocyte defects in hepatitis has been recently described by Wands *et al.* (1975). They found that serum from patients with acute or chronic hepatitis contained material which inhibited Con-A-stimulated lymphocyte blast transformation and cytotoxicity. The chemical nature of this inhibitory factor and its relationship, if any, to LCT have not yet been defined. Finally, a recent study of hepatic tissue lymphocyte subpopulations in a variety of liver diseases demonstrated striking infiltration of the hepatic parenchyma with T cells (Husby *et al.*, 1975). Sequestration of a significant portion of the circulating pool of T cells in the liver could therefore contribute to a relative deficiency of these cells in the peripheral blood.

The biological significance of the T-cell abnormalities and the various related serum factors including LCT in hepatitis remains unclear. Whilst there is mounting evidence that immune mechanisms are involved in the liver cell damage of either acute or chronic hepatitis (Eddleston & Williams, 1974;

Edgington & Chisari, 1975), the nature of these mechanisms is still obscure. There is no evidence that the presently described immunological abnormalities in hepatitis are of pathogenic significance. Both liver cell damage and/or persistent viral infection could account for the occurrence of these phenomena. The present studies suggest that liver damage *per se* is of greater importance.

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