Journal of Korean Medical Science
Vol. 1, No. 1, P 15-23. Sep. 1986

Immunological Aspects in Patients with Chronic Active Hepatitis
—Cellular Immune Responses—

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We studied host immune parameters which might be related to the activity and the pathogenetic mechanism of chronic active hepatitis. The subjects consisted of 45 cases with hepatitis B virus surface antigen (HBsAg)-positive chronic active hepatitis (CAH), 44 HBsAg-negative CAH, 22 with inactive chronic hepatitis, and 45 cases of normal persons, hepatitis B virus (HBV) carriers, or the patients with acute myocardial infarction. The in vitro assay for the in vivo activated lymphocytes was performed by measuring spontaneous thymidine uptake (SLT) of lymphocytes isolated from peripheral blood. SLT was significantly ($p < 0.001$) elevated in cases with HBsAg-positive (1227±806 cpm) and-negative CAH (1017±559 cpm) compared to the patients with inactive chronic hepatitis (347±79 cpm) and to the control group (320±106 cpm). SLT values observed in 7 cases with active disease (group I and II), in which remission and relapsing phase could be assessable, were elevated from 648±121 cpm in remission phase to 1548±606 cpm one to two weeks before the appearance of biochemical evidence (SGPT) of relapse. This pattern of SLT elevation, however, was not observed in patients with inactive hepatitis. Neither the abnormal distribution of T-celi subsets nor the presence of conventional HBV markers were related to the elevated SLT value. Our findings may therefore indicate that SLT might be useful in assessment of the disease activity in patients with CAH. Moreover, since the elevated SLT may be an in vitro measurement of the level of proliferating lymphocytes generated in vivo by the presence of a yet unknown antigen against which host lymphocytes are reacting, we speculate that the elevation of SLT may be the result of a clonal expansion of activated lymphocytes which could be further differentiated in vivo into effector cells which participate in the hepatocyte-lymphocyte interaction in the area of piece-meal necrosis observed in CAH.

Key Words: *Chronic active hepatitis, spontaneous lymphocyte transformation (SLT), T-cell subsets, piecemeal necrosis.*

INTRODUCTION

Chronic active hepatitis (CAH) is a progressive inflammatory liver disease of diverse etiology such as viruses, drugs, and other largely unknown factors. The cardinal histologic feature of all the different etiological

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subgroups of this disease is the presence in the portal and periportal liver tissue of a prominent mononuclear cell infiltration which leads to hepatocellular destruction known as piece-meal necrosis. (Groote et al., 1968).

Since this type of aggressive inflammation seems to be mediated by hepatocyte-lymphocyte interaction with subsequent hepatocellular degeneration, the important role of the host immune reaction against autologous hepatocytes has been stressed as a possible pathogenetic mechanism of CAH. Considerable attension has been paid to the functional integrity of cellular and humoral immunity in order to assess the type of effector immune system and the nature of target antigens against which effector cells are reacting. On the basis of abnormal functions observed in humoral (Kurki et al., 1980; Nadiello et al., 1982; Pederson et al., 1982) and cellular (Martini et al., 1970; Giustino et al., 1972; Feighery et al., 1980) immune system, it has been claimed that the humoral immune reactions directed against liver membrane antigen (LM-Ag) and liver specific membrane lipoprotein (LSP), in co-operation with cytotoxic lymphocytes, play a role in the ongoing liver cell injury in both HBsAg-positive and -negative CAH (Zum Buschenfelde et al., 1972; Miller et al., 1972; Thompson et al., 1974; Facchini et al., 1978; Tage-Jensen et al., 1977; Jensen et al., 1978). This, so-called,autoimmune hypothesis seemed to be further supported by recent observations of defective immunoregulatory suppressor cell functions in CAH (Eddleston et al., 1974; Hodgson et al., 1978; Kakumu et al., 1980; Galocsy et al., 1981; Nonomura et al., 1982).

Although all those findings showed frequent association with the abnormal immune parameters in this disease, their precise role in the pathogenesis is not clear. Since the appropriate normal control groups requiring MHC-restriction (Zinkernagel et al., 1979) for T cell-mediated immune responses were not possible in those cases, these observations, although highly provocative, are of uncertain significance for the understanding of pathogenetic mechanism of CAH. Many difficulties also existed in interpretation of the results obtained from antibody-dependent cell-mediated cytotoxidity (ADCC) (Lee et al., 1975; Alberti et al., 1977; Vergani et al., 1981; Hutteroth et al., 1982) because the immunoglobulins coating the liver cells of CAH are neither anti-HBs antibody nor anti-LSP antibody, but are anti-HBc antibody (Hersh et al., 1971). Moreover, since most of these tests are difficult to use in routine clinical laboratory, they are rather difficult to use as an immune monitor to assess the activity and prognosis of this disease.

In order to obtain more information on immune parameters which might be helpful for the understanding of pathogenetic mechanism and for better assessment of the activity of CAH, we studied the spontaneous lymphocyte transformation test (SLT) which has been used as an immune monitor in patients with organ transplantation (Hersh et al., 1971; Mcdonald and Lee, 1974; Thomas et al., 1983) and CAH (Maerker-Alzer et al., 1974). We report the results obtained from the patients with CAH and compared with the controls.

METHODS

Study Groups

111 patients with chronic hepatitis were studied. Three groups consisted of 45 cases with HBsAg-positive CAH (group 1), 44 HBsAg-negative CAH (group II) in which 23 cases were HBc antibody-positive, and 22 cases with inactive chronic hepatitis (group III) in which the levels of serum transaminases were normal during observation. The cases of this group were initially diagnosed as CAH by histologic criteria, but during the period of this study there was no relapse. The patients in group III were as well HBsAg-positive (12 cases) as HBsAg-negative (10 cases).

The diagnosis was established in each case on the basis of clinical (Sherlock, 1981) and histological observation according to the criteria of De Groote et al. (1968). The levels of serum transaminases in group I and II were over 25 IU/L and less than 25 IU/L in group III. None of these patients had anti-HBs antibody nor autoantibodies such as anti-LSP antibody, anti-LMAg antibody, and anti-nuclear antibody as tested with immunofluorescence technique. The histological pictures observed in group I and II, except for 3 cases (group II) who showed severe CAH, were regarded as moderately active chronic hepatitis in which the main morphologic features were the presence of piecemeal necrosis with marked widening of portal tract and with irregular fibrosis leading to distortion of lobular architecture. Bridging necrosis, collapse, and nodule formation were not observed in those cases. The patients with alcoholic liver diseases, viral infection other than HBV, organ transplantation, or with known lymphoproliferative disease were excluded from this study.

The control group (group IV) consisted of 45 cases in which 19 were normal persons, 14 were HBV carriers without clinical evidence of liver diseases, and 12 cases suffered from recent myocardial infarction. All these control control cases, except for 14 HBV car-

rier, were negative for HBV markers as tested by radioimmuno-assay.

Isolation of Peripheral Blood Lymphocytes (PBL)

Heparinized samples of peripheral venous blood were obtained from all subjects. PBL were isolated on Ficoll-Isopaque (Lymphoprep™, Nyegaard and Co. A/S, Oslo, Norway) gradient according to a modification of the technique of Boyum (1968). They were washed three times in cold MEM (Eagle's minimal essential media, Flow Lab.) before being suspended at a cell density of 10^6/ml in MEM containing glutamine (2 mM) 3% $NaHCO^3$, penicilline (100 IU/ml), streptomycin (0.1 mg/ml), and 20% heat-inactivated (at 56°C for 30 min) pooled human AB plasma taken from healthy blood donors. The viable cells were counted by using 0.16% trypan blue (Gibco) in physiological solution. Generally about 95% viability and 87 to 92% lymphocytes were obtained.

Spontaneous Lymphocyte Transformation (SLT)

SLT assay of PBL was carried out according to the method described by Crowther et al. (1969) and Vessella et al. (1977) with minor modifications. Four × 10^5 PBL in 0.25 ml of MEM supplemented with glutamine and 20% heat-inactivated pooled human AB plasma were cultured in a microtitration plate (Flat-bottomed, 96 wells, Greiner, Germany) at 37°C in humidified atmosphere containing 5% CO_2. At the start of cultivation, the cells were labelled with 1 uCi/well of ^{3}H-thymidine (Tritiated thymidine, SA: 20 Ci/mM, The Radiochemical centre, Amersham) and harvested 20 hr later using a semi-automatic harvestor (MASH). The cell pellets on the filter paper were dissolved at room temperature in 0.1 ml of Lumasolve (Lumac). After 4 hr, 5 ml of Lipoluma (Lumac) were added. The radioactivity was counted by a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3375. All cultures were carried out in triplicate under sterile condition.

Phytohemagglutinin (PHA) Stimulation Test

Stimulation test with PHA (Wellcome reagent grade, 5 ml dried, Beckenham, UK) was performed as described elsewhere (Park et al., 1979). Four × 10^5 PBL suspended in 0.25 ml of MEM supplemented with glutamine and 20% heat-inactivated pooled human AB serum, were incubated with 2 ul/well of PHA. After 72 hr the cells were labelled with ^{3}H-thymidine and harvested as described above.

Mixed Lymphocyte Culture (MLC)

One-way stimulation of MLC was performed according to a modification of the method described by

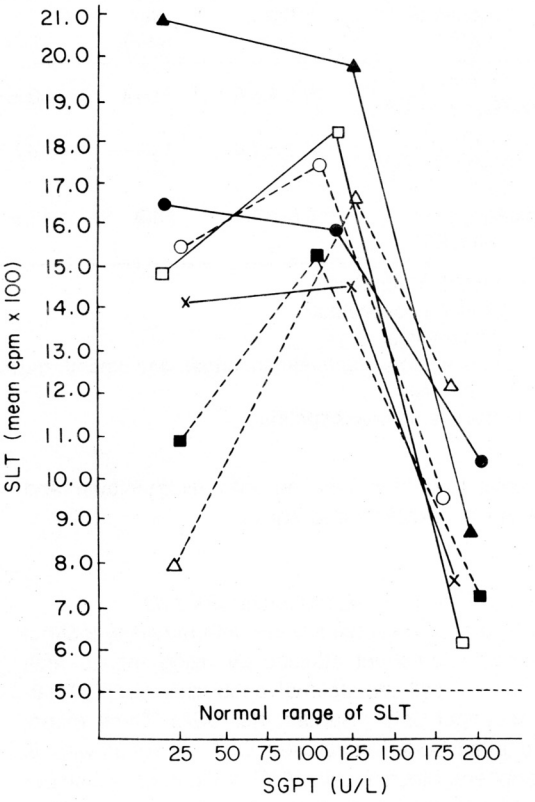

Fig. 1. Changes in levels of SLT in 7 aptients (———: group I, ---; group II) who showed during 18 months of observation, a temporal remission followed by relapsing phase. There was no linear relation between the levels of SLT and SGPT checked simultaneously from remission to relapse. Note that the elevated level of SLT in the beginning of relase was observed one to two week before the elevation of SGPT. There is also a tendency that the level of SLT is declined when the level of SGPT is reached to the maximum peak.

Bach and Voynow (1966). The MLC consisted of 2 × 10^5 PBL from patients as responder cells and 2 × 10^5 PBL from normal allogeneic persons as stimulator cells in 0.2 ml of MEM. The stimulator cells were treated with Mitomycin (Mitomycin C, 2 mg, Serva). Five × 10^6 PBL in 0.1 ml of MEM containing 10% heat-inactivated pooled human AB serum were incubated with 75 ug of Mytomycin at 37°C for 20 min in a CO_2 incubator. After three washes with cold MEM the viable cells were counted and resuspended to obtain 2 × 10^5 cells/0.1 ml of MEM. Each stimulator-responder combination was incubated for

Table 1. Clinical and Laboratory Findings in Patient Groups

Groups of patients	Age (yr)	Sex (M/F)	SGOT (U/L) (5-24)*	SGPT (U/L) (2-19)*	AP (U/L)# (40-120)*	Gamma globulin (0.78-1.52)*	Ab**
I (n = 45) HBSAg (+) – CAH	49.3 ± 4.6	31/14	74.8 ± 53.9	51.2 ± 45.8	132.2 ± 67.5	2.06 ± 0.59	–
II (n = 44) HBsAg (–) – CAH	52.8 ± 3.8	13/31	77.3 ± 47.3	63.9 ± 32.6	174.8 ± 64.8	2.43 ± 0.52	–
III (n = 22) HBsAg (+) or (–) – ICH	56.0 ± 4.2	13/9	16.7 ± 5.2	15.2 ± 4.9	99.8 ± 42.2	1.74 ± 0.66	–

n : number of patients
: Alkaline phosphatase
* : normal value
**: antoantibodies (anti-mitochondrial, anti-amooth muscle, anti-nuclear, anti-LSP, and anti-LMAg antibodies)
– : not present
ICH: inactive chronic hepatitis

5 days and then labelled with ^{3}H-thymidine and harvested as described above.

Determination of T-Lymphocyte Subsets

T-lymphocyte subsets were determined by indirect immunofluorescent staining by using mouse anti-human T cell monoclonal antibodies (Ortho Pharmaceutical Corp., Raritan, NJ, USA). Three monoclonal antibodies were used; OKT 3 reacting with all peripheral blood T cells, OKT 4 directed to T-helper cells, and OKT 8 identifying T cell subset with both surpressor and cytotoxic functions (Reinherz et al., 1979a; 1979b; 1979c; 1980). A volume of 0.1 ml of PBL suspension (2×10^{6}/ml) was mixed with 5 ul of each reconstituted monoclonal antibody (50 ug/ml) and incubated at 4°C for 30 min. After incubation the cells were washed three times in cold PBS (Phosphate ruffered saline, without Ca and Mg^{++} Flow Lab.) and resuspended in 0.1 ml PBS. They were stained at 4°C for 30 min with fluorescein-conjugated goat anti-mouse IgG (GAM/IgG-Fc/FITC, Nordic Immunological Lab., B.V., Nordic Pharmaceuticals, The Netherlands) and then washed three times in cold PBS and resuspended in a drop of PBS. Membrane immunofluorescence was evaluated with a Leitz Orthoplin microscope equipped with an I_2 excitation filter, a barrier filter and a Ploem incident illumination. Three hundred cells were counted for the calculation of percentage of the positively stained cells.

RESULTS

SLT

The results of SLT observed in the different groups

Table 2. Multiple Comparison of SLT

Groups and number of cases		I (n = 45)	II (n = 44)	III (n = 22)	IV (n = 45)
		SLT (cpm ± SD)			
		1227 ± 806	1017 ± 559	347 ± 79	320 ± 106
I	(n = 45)	–	ns	880***	907***
II	(n = 44)	ns	–	670***	697***
III	(n = 22)	– 880***	– 907***	–	ns
IV	(n = 45)	– 670***	– 697***	ns	–

n : number of cases
cpm ± SD : mean counts per minute ± standard deveation
*** : significant at $p < 0.001$ by using Scheffe's multiple comparison method
ns : not significant
– : not compared

of patients are summarized in Table 2. A significant elevation ($p < 0.001$) of SLT in group I (1227 ± 806 cpm) and in group II (1017 ± 559 cpm) were observed as compared with the group of inactive chronic hepatitis (group III, 347 ± 79 cpm) as well as with the control (320 ± 106 cpm). In p tients with inactive chronic hepatitis, the man SLT level was statistically not different from the control group. By Scheffe's multiple comparison, the SLT values observed in patients with HBsAg-positive CAH were not different from those observed in the HBsAg-negative CAH patients. Among the patients in group III, the mean SLT in 12 HBsAg-positive cases was also not different from the one in the HBsAg-negative cases.

In the control group, the SLT values in normal persons were not different from those of healthy carriers of HBV and of the patients with acute myocardial infarction.

Relation between the Values of SLT and Serum Transaminase

Four patients in group I and 3 in group II, in which remission and relapsing phase of the disease activity could be assessable, were serially checked for their SLT and SGPT. As indicated in Fig. 1, the results observed in those cases are; 1) there is no linear relation between the values of SLT and SGPT, 2) SLT levels in patients with active disease (group I and II), even in brief remission period, were not returned to the normal value (below 500 cpm), 3) in the beginning of relapse, the value of SLT were elevated from 648 ± 121 cpm to 1548 ± 606 cpm one to two weeks before the elevation of SGPT.

PHA Stimulation Test

The results of PHA stimulation tests, statistically analysed with multiple comparison method in each group, are summarized in Table 3. The patients with HBsAg-positive and-negative CAH as well as the cases with inactive chronic hepatitis show neither suppressed PHA test nor increased values in PHA test as compared with the control group.

MLC

The MLC responses of peripheral blood lymphocytes from the patients of group I and II were compared to the normal persons. The reactivity of lymphocytes taken from CAH patients were significantly increased in co-culture with syngeneic cells ($p < 0.001$) and with allogeneic stimulator cells ($p < 0.05$) (Table 4).

Determination of T-Lymphocyte Subsets

The determination of T-lymphocyte subsets using OKT monoclonal antibodies was carried out simultaneously with SLT in each case. As summarized in Table 5 and 6, the distribution of T-lymphocyte subsets in CAH patients (group I and II) with an increased SLT value was not different from the cases in group III and control subjects. Neither HBsAg-positive nor HBsAg-negative CAH patients showed an increase in OKT 4-defined cells or a ecrease in OKT 8-defined cells as compared to the patients in group III and to the control group.

DISCUSSION

The SLT measures an in vitro blastogenic property of the circulating lymphocytes without addition of mitogens and/or antigens and represent therefore a measure of the blastogenic activity of lymphocytes which were already stimulated in vivo (Vessella et al., 1977). It may be presumed that the values of SLT are modulated by the presence of antigen (s) which stimulate the circulating lymphocytes leading to proliferation of these cells. This test has recently been used as an immune monitor in patients with renal transplantation (Hersh et al., 1971; Mcdonald and Lee, 1974; Thomas et al.,1983), Hodgkin's disease (Pauw

Table 3. Results of PHA Stimulation and Multiple Comparison*

Groups and number of cases		^{3}H-thymidine incorporation	
		No Stimulant	PHA Stimulation
I	(n = 45)	*753.3 ± 345.0	121290 ± 36749
II	(n = 44)	711.1 ± 382.8	120210 ± 47834
III	(n = 22)	731.3 ± 364.9	116040 ± 40572
IV	(n = 45)	761.8 ± 365.9	126040 ± 42352

* : Using Scheffe's multiple comparison method, no significant difference was obtained between every two groups neither for PHA stimulation nor the group without stimulation.

Table 4. Results of MLC and Multiple Comparison

Groups and number of cases	^{3}H-thymidine incorporation	
	$R + R^m$	$R + S^m$
	mean counts per minute ± SD	
I and II (n = 22)	837.3 ± 362.7*	35880 ± 20703**
IV (n = 30)	500.9 ± 348.8*	24430 ± 18958**

R : Responder cells
R^m : mitomycin-treated syngeneic responder cells
S^m : mitomycin-treated allogeneic stimulator cells
* : Scheffe's multiple comparison yielded a significant difference at $p < 0.001$
** : significant at $p < 0.05$

Table 5. Results of SLT AND T Cell Subsets in patient groups and control

Tests	Groups and Number of Cases			
	I (n = 24)	II (n = 25)	III (n = 12)	IV (n = 20)
SLT (cpm)	1037 ± 456	1012 ± 485	325 ± 79	324 ± 90
OKT-3 (%)	67.1 ± 9.9	68.6 ± 9.1	66.5 ± 12.0	70.0 ± 8.4
OKT-4 (%)	59.1 ± 13.0	55.2 ± 14.9	48.2 ± 17.1	55.1 ± 8.8
OKT-8 (%)	31.0 ± 11.2	35.0 ± 14.1	37.4 ± 14.1	32.5 ± 8.8

et al., 1980), and in certain viral infection (Gavosto et al., 1959). This test has also been used in patients with CAH (Maerker-Alzer et al., 1974) in which the persistent high level of SLT were frequently observed in patients with poor prognosis.

The data observed in the present study show that the values of SLT and MLC are persistently elevated in patients with CAH (group I and II), but not in cases with inactive chronic hepatitis (group III) and the control group. Since none of the patients involved in the present study had a viral infections other than HBV, nor organ transplantation or malignant lymphoproliferative disorders, the increased level of SLT and MLC might be an indication that the circulating lymphocytes in those patients are being stimulated by unknown antigen (s) which is related to the chronic liver inflammation.

in CAH with elevated serum transaminases and in

One may speculate that the elevated level of SLT observed in patients with CAH may not be a pathogenetic determinant of this disease but merely a secondary phenomenon caused by liver cell necrosis. This possibility, however, is unlikely in the present study because the following findings are not in support of

Table 6. Results of Multiple Comparisons of SLT and T Cell Subsets

Group Comparisons	Differences between group mean and significance of Scheffe's t-value			
	SLT	OKT-3	OKT-4	OKT-8
I-II	ns	ns	ns	ns
I-III	9.77***	ns	ns	ns
I-IV	13.35***	ns	ns	ns
II-III	9.22***	ns	ns	ns
II-IV	12.67***	ns	ns	ns
III-IV	ns	ns	ns	ns

ns : not significant
***: significant at $p < 0.001$

this speculation. Firstly, the tissue necrosis caused by recent myocardial infarction with the elevated level of serum transaminases, LDH, and CPK did not show an increased level of SLT. Secondly, the individual values of SLT measured serially from remission to relapsing phase in which the levels of serum transaminases were temporarily normal. All these findings might be an indication that the increased SLT in those patients is not a secondary epiphenomenon caused by liver cell necrosis but might be a primary pathogenetic determinant related to chronic liver inflammation.

In order to assess the possibility that the disturbed T cell functions or altered distribution of T cell subsets in peripheral blood may be related to the high SLT, we have simultaneously investigated other immune parameters. As indicated in the results, we could not observe a generalized T cell hyporesponsiveness to PHA and allogeneic major histocompatibility antigens but show rather an enhanced stimulation in MLC. We have also failed to find a relation between the level of SLT and the percentage as well as absolute number of T-helper and T-suppressor cells which were determined by OKT monoclonal antibodies.

The results obtained in this study are, at first sight, not in agreement with the results of other investigators (Martini et al., 1970; Giustino, 1972, Feighery, 1980). It is, however, possible that the differences in patient populations studied may be responsible for this dissimilarity. Indeed our cases consisted of moderately active chronic hepatitis in which neither nutritional problems which may influence the host immune system nor the presence of autoantibodies were observed.

Since we could not observed a positive relation between the high SLT and the distribution of T cell subsets characterized by OKT monoclonal antibodies, we do not know the precise nature of cell type which is responsible for the high SLT. It is, however, conceivable that this findings might be explained by disturbed immunoregulation caused by a selective clonal expansion of activated T cells which could be determined with surface markers other than OKT monoclonal antibodies. Indeed, our preliminary findings observed recently show that the number in peripheral blood of T cells which express HLA-DR and Tac antigens on their cell surface in patients with CAH were significantly higher than those of chronic persistent hepatitis and of liver cirrhosis. These results in conjunction with high SLT value may lead to a speculation to suggest that the high SLT observed in the present study might be caused by a selective clonal expansion of activated T cells expressing HLA-DR and/or Tac antigens. Although underlying mechanisms involving these findings may be triggered by defective suppressor cell function, it is, for the time being, difficult to assess since assay for specific function of human suppressor T cells has not yet clearly defined.

The identity of the target antigen (s) responsible for the high SLT in our patients is not known. The liver specific protein (LSP) and LMAg are not likely to be the target antigen (s) in the present study. As described in the method, none of the sera taken from our patients involved in the present study do not have an autoantibody which is reactive with normal antigen (s) expressed on the liver cells in our in vitro system. The presence of HBsAg itself is also not likely to be the cause. In fact, HBsAg-positive patients in group III and those of healthy carrier in control group did not show an increased SLT and there was also no difference between the groups with an active disease with or without HBsAg. These findings are further supported by the facts that the amounts of HBsAg in the liver tissue (Ray et al., 1979a, 1979b) and the distribution of HBV markers such as HBsAg (Wright, 1980) and HBcAg (Blum et al.,1984) are not directly related to the actual inflammatory process in the liver. Although the persistence of HBV with or without presence of HBsAg and/or Delta agent (Brechot et al., 1985) is linked to the chronicity of hepatitis, their exact role in this pathological process is still unknown. However, it might be considered that although the host immune system could not mount sufficient responses to eliminate the virus, it continues in this circumstances to damage the liver by the presence of a yet unidentified viral neoantigen (s) which stimulates the circulating lymphocytes leading to the high SLT.

Whatever the precise chemical nature of the target antigen (s) responsible for our findings, the high SLT observed in patients with CAH might be a pathogenetic determinant which is related with the activity and prognosis of chronic hepatitis. Indeed the increased level of SLT in relapsing cases were observed before elevation of serum transaminases. Since the predominant cells infiltrated in the area of piecemeal necrosis seems to be T-suppressor/cytotoxic cells (Thomas et al., 1982), it is tempting to speculate that the high SLT in our cases with moderately active chronic hepatitis may be an in vitro measurement of the level of activated T cells generated in vivo by the presence of neoantigen (s) against which host lymphocytes are reacting. These activated lymphocytes can be recirculated and further differentiated into effector cells which are infiltrated in the area of piecemeal necrosis leading to hepatocalluar degeneration.

ACKNOWLEDGEMENT

We are grateful to Professor Meyer Zum Buschenfelde and Dr. Manns who kindly provided anti-LMAg antiserum which was used as positive control serum for LMA assay.

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