Rosette inhibition test in chronic liver disease

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SYNOPSIS A role has recently been assigned to cell-mediated immunity in chronic liver diseases in addition to the well-known alterations of humoral immunity. We now report the results of the rosette inhibition test for the evaluation of T-lymphocyte 'sensitization' in patients with different chronic liver disorders. A cell-mediated immune reaction was found in 81% of patients with chronic active hepatitis and in 71% with primary biliary cirrhosis, whereas patients with chronic persistent hepatitis showed no reaction. The correlation with the incidence of hepatitis B antigen showed that T-lymphocyte sensitization was more frequent in the group of HB-positive patients. Finally, improvement of the test was observed in four out of nine patients given immunosuppressive treatment.

Changes of cell-mediated immunity have recently been proposed in the pathogenesis of some chronic liver diseases (Dudley *et al*, 1972b; Popper and Mackay, 1972). On the basis of knowledge of the different populations of peripheral lymphocytes we attempted to demonstrate a specific sensitization of T-cells in chronic liver disease by means of the rosette inhibition test (RIT). T-lymphocytes are able to form spontaneous rosettes with sheep red blood cells *in vitro*, and this property is a sensitive physiological marker of this subpopulation (Jondal *et al*, 1972). Bach and Antoine (1968) have reported that antilymphocyte serum markedly reduces spontaneous rosette formation: on this property is based the RIT (Bach *et al*, 1969). In the beginning the RIT was used to evaluate the degree of immunosuppression obtained with various cytotoxic drugs in patients with renal or lung allografts (Munro *et al*, 1971; Cullum *et al*, 1972). Recently it was reported that the RIT might also be useful in assessing the degree of T-lymphocyte sensitization in subjects with autoimmune diseases (Farid *et al*, 1973).

In the present study the RIT was used in patients with chronic liver diseases to investigate T-lymphocyte sensitization and to evaluate the effect of immunosuppressive therapy. The distribution of T and B cells in the peripheral blood, serum auto-antibodies, and hepatitis B antigen (HB_sAg) has also been evaluated.

Patients and methods

The group of 64 cases of chronic liver disease consisted of 12 patients with chronic persistent hepatitis (CPH), 37 with chronic active hepatitis (CAH), 8 with primary biliary cirrhosis (PBC), and 8 with alcoholic cirrhosis (AC). The diagnosis based on clinical and biochemical criteria were confirmed in all cases by histological examination of liver biopsies. Patients were not previously treated with corticosteroid or immunosuppressive drugs. Twenty-four healthy subjects were tested as a control group. Nine patients (6 CAH and 3 PBC) were followed for periods ranging from 3 to 12 months after starting the immunosuppressive therapy. In these patients the treatment began with 25 mg of prednisone per day, the dose being tapered gradually; in two cases azathioprine, 100 mg daily, was added.

ROSETTE INHIBITION TEST

The method used was that described by Bach *et al* (1969) as adapted by Munro *et al* (1971) with the following modifications: lymphocytes were separated from peripheral blood, with the addition of preservative-free heparin, by a technique of sedimentation in Ficoll-Isopaque density gradients (Jondal *et al*, 1972). The cells were washed three times in Hank's BSS and adjusted to 4×10^6 /ml. 0.1 ml of this suspension was incubated with 0.25 ml of serial dilutions of antilymphocyte globulin (ALG)¹ for 90 min at 37°C. The dilutions of ALG ranged from 1:4000 to 1:128 000. A tube in which Hank's BSS was substituted for ALG was also set up as a control. Sheep

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¹Equine gamma globulin against human lymphocytes (50 mg/ml).

Fig 1 Rosette inhibition test. Per cent rosette formation compared to the control was plotted against ALG concentration. The concentration at which 75% rosettes was observed was termed the MIC.

Fig 2 Distribution of values of MIC in different groups of chronic liver diseases.

red blood cells (SRBC)² (0.1 ml, $120 \times 10^6/\text{ml}$) were subsequently added. Cells were incubated at 37°C for 5 min, centrifuged at 200 *g* for 5 min, incubated on ice for 90 min, and gently resuspended. The percentage of rosette-forming cells was calculated, counting 500 lymphocytes in a Fuchs-Rosenthal chamber. The number of rosettes in the control tube gives the percentage of T-lymphocytes. The dilution of ALG necessary to reduce the rosette formation to 75% of the control was taken as the minimal inhibitory concentration (MIC) (fig 1).

Two different methods were used to identify B-lymphocytes—the EAC rosette test described by Stjernswärd *et al* (1972) and the immunofluorescence test for surface immunoglobulins (Pernis *et al*, 1970). ANA, AMA, and SMA were detected by means of indirect immunofluorescence (Coons and Kaplan, 1958), according to Roitt and Doniach (1969). HB_sAg was detected in serum by counter-immunoelectrophoresis (Gocke and Howe, 1970), and controlled using the solid-phase Abbott radioimmunoassay.

Results

The percentages of T and B lymphocytes in the different groups of liver diseases are in the same range as in normal subjects (T-cells = 47.2 ± 8.4 , B-cells = 21.0 ± 3.2). The results of RIT are shown in figure 2. The MIC of the normal subjects ranged from 1:16 000 to 1:48 000 dilutions of ALG and the mean value was 1:25 000. The MIC values of the patients with CPH usually fell within the normal range; increased resistance of the lymphocytes to ALG was demonstrated in 30 patients with CAH (81% of cases) and in five with PBC (71%). The

subjects with AC showed variable results: in five cases the MIC fell within the normal range, in two it was higher than normal, and in one lower (1:64 000). The differences between the mean values of MIC in normal subjects and in CAH patients ($t = 7.13$, $P < 0.001$) and PBC patients ($t = 2.1$, $P < 0.05$) were statistically significant.

In patients with CAH, smooth muscle auto-antibodies were more frequently detected in patients with a high value of MIC (36%) than in those with a normal value (15%). In fig 3 the RIT is related

Fig 3 Correlation between MIC values and incidence of HB_sAg in patients with chronic active hepatitis.

* Fresh SRBC were obtained weekly.

Fig 4 MIC titres in patients with chronic liver diseases during immunosuppressive treatment.

to the incidence of HB_sAg in CAH: the MIC values are abnormal in all HB_sAg-positive patients and in 61% of those HB_sAg-negative. Therefore all patients with a normal value of MIC belong to the HB_sAg-negative group. The difference between the two groups is significant ($\chi^2 = 6.754$, $P < 0.01$).

Figure 4 shows the MIC values in nine patients submitted to immunosuppressive treatment: four patients were followed for about one year of therapy, one for six months, and four began the treatment only three months before the last examination. During the treatment the total lymphocyte count decreased slightly in all patients, but the ratio between T and B cells remained unchanged. The MIC value became normal in three patients after a few months of therapy, and in one (PBC) after one year. The other patients showed minimal or no change in the value of MIC.

Discussion

The suggestion that cellular immunity plays an important role in the pathogenesis of chronic liver diseases, such as CAH and PBC, stems from various observations. The predominant infiltration of mononuclear cells in portal and periportal spaces has been frequently recognized as similar to that of liver homograft rejection or of immune reaction in other tissues (Paronetto *et al*, 1964; Doniach *et al*, 1966; Popper and Mackay, 1972). The role of circulating lymphocytes in the production of liver damage has been demonstrated in experimental hepatitis induced in normal animals by intravenous injection of lymphocytes sensitized to liver extracts (Warnatz *et al*, 1967; Warnatz, 1969). Moreover

the reactivity of lymphocytes to liver antigens has been shown to occur in human chronic liver diseases by means of lymphocyte transformation and leucocyte migration tests (Tobias *et al*, 1967; Miller *et al*, 1972).

We have used the RIT to investigate the immune reactivity of T-lymphocytes in patients with chronic liver diseases. Although the immunological basis of this test is not clear, recent results obtained in cases of immunosuppression (Morton *et al*, 1974) support the value of the RIT as a measure of T-lymphocyte function. The marked increase in MIC values which was found in 81% of patients with CAH and in 71% of patients with PBC, compared with the normal MIC found in all patients with CPH, supports the view that cellular immunity is a factor in the activity of the liver disease. The same conclusion had been reached by means of the leucocyte migration test, which demonstrated a sensitization of lymphocytes to a liver lipoprotein in patients with CAH (Miller *et al*, 1972) and to a protein fraction from bile in patients with PBC (Eddleston *et al*, 1973).

Despite the increase of MIC observed in many patients with chronic liver diseases, an increase in T-lymphocyte count was not found, as reported in other autoimmune disorders. The most probable interpretation is that in these patients only a small subpopulation of T-cells was stimulated, too few to affect the total number but numerous enough to indicate a degree of sensitization.

Concerning the humoral immunity, it was found that smooth muscle autoantibodies are more frequent in patients with abnormal values of MIC than in those with normal values, but the difference between the two groups is not significant. More important is the correlation between the incidence of HB_sAg and the change in MIC in the group of CAH patients: subjects with positive HB_sAg showed a higher frequency of T-lymphocyte sensitization (100%) than those with negative HB_sAg (61%). This finding suggests that HB_sAg is associated with an activation of T-lymphocytes: the alteration of cellular immunity in chronic liver diseases could be related to the presence of HB_sAg, as already proposed (Dudley *et al*, 1972a; Popper and Mackay, 1972).

Regarding the effect of immunosuppressive treatment, a return to normal of the MIC was observed in four out of nine cases studied. Therefore patients with chronic liver disease show a different sensitivity of T-cells to immunosuppressive therapy.

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