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Serum antibodies against alcohol-treated rabbit hepatocytes in patients with alcoholic liver disease

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SUMMARY

The existence of antibodies against alcohol-treated rabbit hepatocytes in sera of patients with alcoholic liver disease was investigated utilizing a ^{125}I-labelled protein A assay. The sera, after two absorptions with normal rabbit hepatocytes, were incubated with hepatocytes isolated from rabbits which had been treated for 4 days with a daily dose of ethanol (1 g/kg body weight) intravenously.

Serum antibodies against alcohol-treated hepatocytes were detected in 21 of 55 patients with alcoholic liver disease; this was associated with a distinct, mixed granular and linear fluorescence staining the surface of alcohol-treated hepatocytes. By contrast, none of 23 patients with other liver diseases who had no history of excessive alcohol intake showed the antibodies in their sera. The values of the antibodies of patients with alcoholic active cirrhosis and alcoholic hepatitis were significantly higher than those of other types of alcoholic liver disease. These results showed that antibodies against alcohol-treated hepatocytes are present in alcoholic liver disease with inflammation. The role of these antibodies on liver cell damage in alcoholic liver disease remains to be clarified.

Keywords antibodies against alcohol-treated hepatocytes alcoholic liver disease alcoholic hepatitis liver membrane antibody protein A

INTRODUCTION

Participation of immunological mechanism in liver-cell damage has been postulated in alcoholic liver disease (Paronetto, 1981; Zetterman & Sorrell, 1981). Indeed, peripheral blood lymphocytes have been shown to have cytotoxicity to cultured hepatocytes in both alcohol-treated baboons (Paronetto & Lieber, 1976; Lue, Paronetto & Lieber, 1981) and humans with alcoholic liver disease (Izumi, Hasumura & Takeuchi, 1983; Actis *et al.*, 1983). From these results, antibody-dependent cell-mediated cytotoxicity has been suggested to be involved in liver-cell damage. Despite extensive studies, however, the target antigens have not yet been identified.

Recently MacSween and colleagues (MacSween, Anthony & Farquharson, 1981; Anthony, Farquharson & McSween, 1983) showed the presence of antibodies against alcohol-altered hepatocytes in the sera of patients with alcoholic liver disease. However, Krogsgaard, Tage-Jensen & Gluud (1982) could not confirm this observation. One possible reason for this discrepancy is that in both experiments the immunofluorescent method was used to detect the liver membrane antibody: by this method, it is difficult to quantitate the observed results.

In the present study, a quantitative method using ^{125}I-labelled protein A was utilized for the detection of the antibodies.

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MATERIALS AND METHODS

Patients. Fifty-five patients with alcoholic liver disease and 23 with other liver diseases were included in this study. Patients with alcoholic liver disease had a history of drinking more than 80 g of ethanol per day for more than 10 years, and none of them had HBsAg, nor had a history of blood transfusion. Needle biopsy of the liver was performed on all of them. According to the histological findings, the patients were classified into five groups: 11 cases with active cirrhosis, 9 with inactive cirrhosis, 8 with alcoholic hepatitis, 15 with hepatic fibrosis, and 12 with fatty liver. Active cirrhosis was differentiated from inactive cirrhosis by the presence of piecemeal necrosis associated with extensive inflammation. The diagnosis of alcoholic hepatitis was in accord with the Fogarty nomenclature (Leevy & Tygstrup, 1976). The diagnosis of hepatic fibrosis was made by the presence of the following three abnormalities (Takada, Nei & Matsuda, 1982), (a) pericellular creeping fibrosis, mainly in the centrolobular area and sometimes in the portal area, (b) relatively scanty cellular inflammation in the portal triads, with or without fatty metamorphosis, and (c) absence of the features of alcoholic hepatitis, chronic hepatitis and cirrhosis.

Patients with other liver diseases included five cases with HBsAg positive chronic active hepatitis, seven with non-B chronic active hepatitis, four with cryptogenic liver cirrhosis, two with acute type A hepatitis, one with acute type B hepatitis, two with autoimmune chronic active hepatitis, one with primary biliary cirrhosis and one with drug-induced liver disease. None of them had a history of excessive alcohol intake.

Twenty-four normal healthy people and nine alcoholics who showed no abnormality of liver function (alcoholic controls) served as normal controls.

All sera were stored at $-70°C$ before use. The sera were taken within 1 week of admission. Test and control serum samples were heat-inactivated at 56°C for 30 min.

Target cells. Male New Zealand white rabbits (body weight 2 kg) were pretreated (alcohol-treated rabbits) intravenously for 4 days with ethanol (1 g/kg of body weight per day) diluted with 20 ml saline. After intravenous infusion, the ethanol concentration in the peripheral blood determined by gas-liquid chromatography was approximately 10 mM. Histologically, the ethanol caused no apparent changes in hepatocytes such as necrosis or fatty degeneration. On the fourth day, hepatocytes were isolated 2 h after the termination of the intravenous administration of ethanol. In the corresponding controls, saline alone was infused for 4 days (non-treated rabbits).

Alcohol-treated and non-treated hepatocytes were isolated from alcohol-treated and non-treated rabbit livers, respectively, by the method of Berry & Friend (1969) with following modifications: the livers were perfused with Ca^{++}-free Hanks' balanced salt solution (HBSS) and then perfused with HBSS containing 0·05% collagenase (Sigma type 1, Saint Louis) and 2 mM Ca^{++}. The cell suspension was filtered through four layers of gauze and was centrifuged at 50*g* for 1 min. After washing three times with phosphate-buffered saline (PBS, pH 7·2) containing 1% bovine serum albumin (BSA), the isolated hepatocytes were resuspended in RPMI 1640 (Flow laboratories, McLean) including 10% fetal calf serum (FCS) (Reheis Chemical Co., Phoenix) to a final concentration of 4×10^5/ml. Cell viability determined by the trypan blue exclusion test was $81{\cdot}0 \pm 7{\cdot}4\%$ (mean ± s.d.).

Antibody assay using ^{125}I-labelled protein A. Staphylococcal protein A was used to detect immunoglobulins attached to hepatocytes, since protein A has a characteristic property to bind specifically to the Fc portion of IgG (Foesgren & Sjoquist, 1966), and since non-specific binding of protein A to cell membrane is small (Brown, Klitzman & Hellström, 1977).

In each trial, 100 μl of the hepatocyte suspension were delivered into plastic tubes (12·5 × 77 mm) and each suspension was exposed to serial dilutions of 100 μl of test or control sera for 60 min at 37°C under 95% O_2 and 5% CO_2. The hepatocytes were then washed three times with PBS containing 1% BSA. Following the addition of ^{125}I-labelled protein A (Amersham Int., Amersham), which was adequately diluted with RPMI 1640 containing 10% FCS, the hepatocytes were again incubated for 60 min at 37°C under an atmosphere of 95% O_2 and 5% CO_2. After washing three times with PBS containing 1% BSA, 100 μl of 2 M NaOH was added and radioactivity of the hepatocytes, was counted in gammacounter.

Each serum of tests and controls was measured in duplicate. Non-specific binding of protein A

to collagenase-isolated, alcohol-treated hepatocytes was less than 0·1% of the activity of protein A used; a suitable correction for the unspecific binding was made when each value of radioactivity was calculated.

Absorption of serum. In the preliminary experiments, antibodies against non-treated isolated hepatocytes were studied using ^{125}I-labelled protein A in sera from 21 patients with alcoholic liver disease and 10 with other liver diseases. Among the twenty-one sera from patients with alcoholic liver disease, eight were found to be positive for antibodies, while in sera from those with other liver diseases, five were positive.

In order to absorb the antibodies to react with non-treated rabbit hepatocytes, all the test and control sera (100 μl) were incubated with an equal volume of non-treated, isolated rabbit hepatocytes for 60 min at 37°C, and after removing the non-treated hepatocytes by centrifugation, the supernatant was utilized for the testing of antibody against alcohol-treated hepatocytes. This absorption procedure with non-treated hepatocytes (repeated two times) was found to be sufficient to remove the antibodies to non-treated, normal rabbit hepatocytes, by measuring the antibodies in three sera from non-B chronic active hepatitis and two from autoimmune chronic active hepatitis, since no further decrease of binding activities to non-treated hepatocytes was observed after more than three absorptions (Fig. 1).

Normalization of antibody titre. Because the titre of antibodies was affected by many factors such as the activity of the ^{125}I-labelled protein A, preparation of hepatocytes or room temperature, normalization was done using two standard reference sera, according to the method described by Frazer, Kronberg & Mackay (1983). Since one of the sera from alcoholic active cirrhosis showed a high binding activity to alcohol-treated hepatocytes, this was assigned a binding value of 100% at a serum dilution of 1:20. Another serum from normal control showed little binding at 1:20 dilution, so this was assigned a binding value of 0%. Binding curves thus obtained from these two reference sera with different serum dilutions are shown in Fig. 2. In the following experiments, the radioactivities of reference sera with a dilution of 1:20 were always tested along with the measurement of those of test samples, and the binding value to the reference sera was calculated.

Indirect immunofluorescence. The isolated hepatocytes from alcohol-treated rabbits were

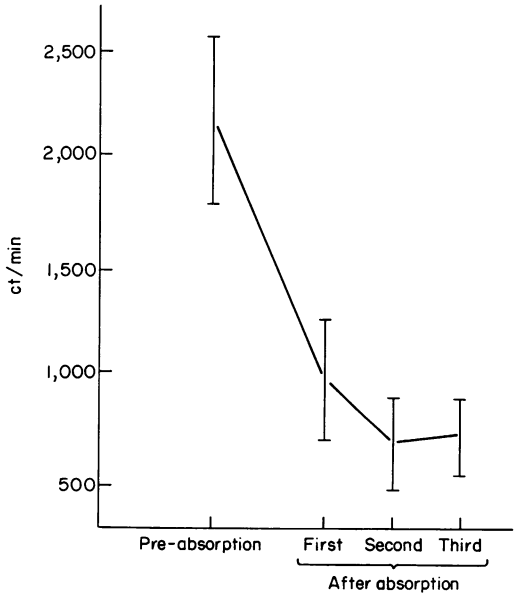

Fig. 1. The binding activities to normal rabbit hepatocytes before and after one to three absorptions of patients' sera with non-treated, normal rabbit hepatocytes. Data indicate mean (±s.d.) of three sera from non-B chronic active hepatitis and two from autoimmune chronic active hepatitis.

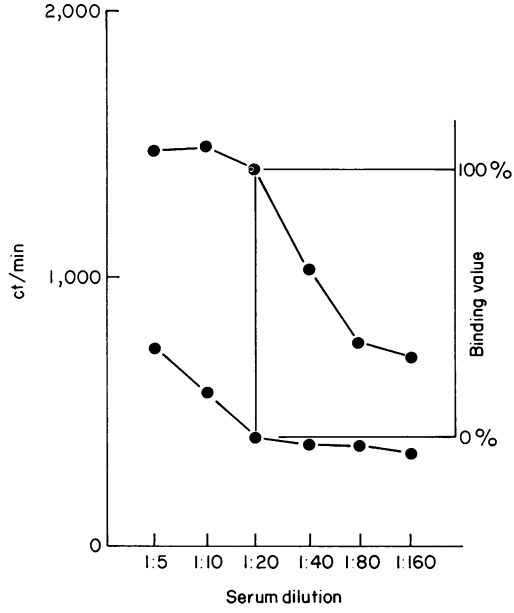

Fig. 2. Binding curves obtained from two reference sera for antibodies against alcohol-treated rabbit hepatocytes using [^{125}I]protein A. Since one of the sera from alcoholic active cirrhosis showed a high binding activity to alcohol-treated rabbit hepatocytes, this was assigned a binding value of 100% at 1:20 dilution; another serum from normal control showed little binding at a serum dilution of 1:20, so this was assigned a binding value of 0%. The antibody titre of each serum was shown as percentage binding to the reference sera at a dilution of 1:20 in subsequent experiments.

suspended in RPMI 1640 including 10% FCS to final concentration of 2×10^6/ml. 100 μl of the cell suspension were incubated for 60 min at 37°C under 95% O_2 and 5% CO_2 with sera from 20 patients with alcoholic liver disease and 5 normal controls; each serum was diluted 1:20 with RPMI 1640 including 10% FCS before use. After washing three times with PBS including 1% BSA, the hepatocytes were then incubated with anti-human immunoglobulin conjugated with fluorescein (Behringwerke AG, Marburg) diluted 1:10 with PBS for 30 min at 37°C under an atmosphere of 95% O_2 and 5% CO_2. After washing three times with PBS including 1% BSA, the hepatocytes were immediately observed under a fluorescein microscope.

Statistical analysis. Statistical significance was assessed by the non-parametric analysis using Sheffé's multiple comparison (Sakuma, 1977).

RESULTS

Serum antibodies against alcohol-treated rabbit hepatocytes

The intra- and inter-variation of the assay were 4·3±2·3% and 9·3±4·4% (mean±s.d.), respectively.

The value of normal control sera after two absorptions with non-treated rabbit hepatocytes was 11·1±7·8% (mean±s.d.); thus, the value of 26·7%, obtained by calculating the mean + 2 s.d., was determined the upper limit of normal range. Serum antibodies against alcohol-treated rabbit hepatocytes were found to be present in 21 of 55 patients with alcoholic liver disease (Fig. 3). On the other hand, none of 23 patients with other liver diseases, including two cases of autoimmune chronic active hepatitis, and none of 9 alcoholic controls showed any existence of the antibodies in their sera. In alcoholic liver disease, the antibodies were positive in 9 of 11 with active cirrhosis, 3 of 9 with inactive cirrhosis, 5 of 8 with alcoholic hepatitis, 3 of 15 with hepatic fibrosis, and 1 of 12 with fatty liver (Fig. 3).

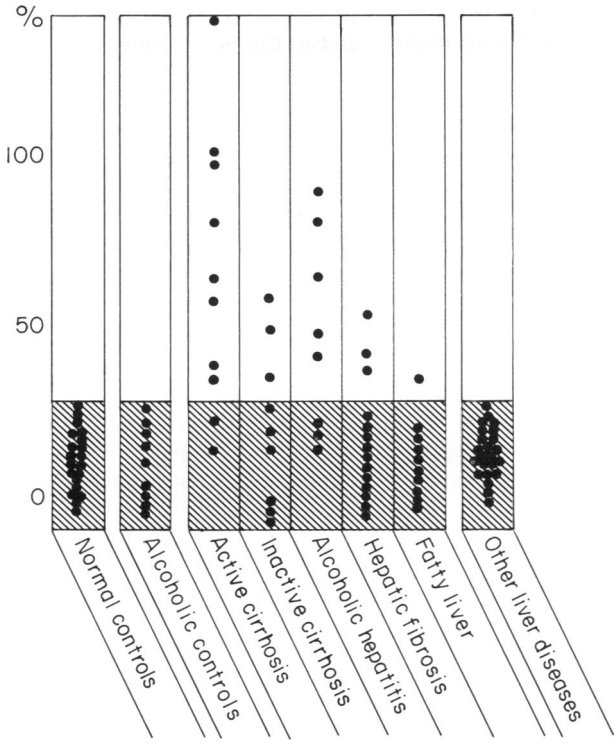

Fig. 3. Serum antibody titre against alcohol-treated rabbit hepatocytes at a serum dilution of 1:20 after two absorptions with non-treated hepatocytes. The value of normal control sera was $11{\cdot}1 \pm 7{\cdot}8\%$ and the value of $26{\cdot}7\%$ obtained by the mean ± 2 s.d., was determined the upper limit of normal range.

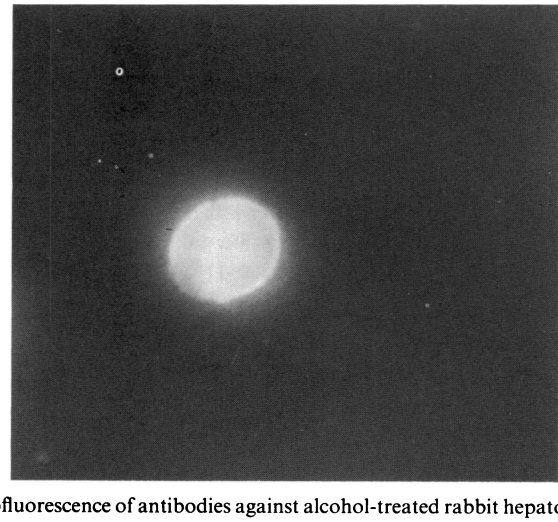

Fig. 4. Indirect immunofluorescence of antibodies against alcohol-treated rabbit hepatocytes. After absorptions of patients' serum with non-treated hepatocytes, weak, but distinct mixed granular and linear fluorescence was observed along the alcohol-treated hepatocytes.

The mean values of percentage binding activities in alcoholic active cirrhosis and alcoholic hepatitis (54·9%) were significantly higher than those in other types of alcoholic liver disease, i.e. inactive cirrhosis, hepatic fibrosis, and fatty liver (16·9%, $P<0·1$), or non-alcoholic liver diseases (11·9%, $P<0·01$).

As expected, the sera that were positive against alcohol-treated rabbit hepatocytes did not react with non-treated rabbit hepatocytes; this indicated that two absorptions with non-treated rabbit hepatocytes were sufficient to remove antibodies against normal hepatocytes. In addition, two absorptions of these sera with alcohol-treated rabbit hepatocytes provided no significant binding to alcohol-treated hepatocytes.

Indirect immunofluorescence

Twenty sera from patients with alcoholic liver disease were tested by indirect immunofluorescent assay for antibodies against alcohol-treated rabbit hepatocytes after two absorptions with non-treated rabbit hepatocytes. Only three of six sera that were positive for antibodies against alcohol-treated hepatocytes using ^{125}I-labelled protein A assay showed mixed granular and linear fluorescence along the surface of alcohol-treated rabbit hepatocytes (Fig. 4): none of 14 sera that were negative for the antibodies by ^{125}I-labelled protein A assay showed any immunofluorescent staining.

DISCUSSION

In the present study, the antibodies reacting with alcohol-treated rabbit hepatocytes were detected only in sera of patients with alcoholic liver disease. No such antibodies were demonstrated in sera of patients with non-alcoholic liver disease.

The antibodies against alcohol-treated hepatocytes were first described by MacSween, Anthony & Farquharson (1981), using indirect immunofluorescence. Furthermore, Trevisan *et al.* (1983) observed the existence of immunoglobulins G and A on the cell membrane of hepatocytes from biopsied samples of patients with alcoholic liver disease; they also showed that the membrane-bound IgG was not correlated with the presence of serum antibodies to liver membrane antigen (Trevisan *et al.*, 1981). From these results, it has been suggested that alcohol could be responsible for antigenic modification of hepatocyte membrane, triggering a humoral immune response that appears to be distinct from liver-specific autoantibodies (Trevisan *et al.*, 1981, 1983).

In another study, however, the presence of antibody to alcohol-treated hepatocytes was denied in alcoholic liver disease (Krogsgaard, Tage-Jensen & Gluud, 1982). The reason of this discrepancy is not clear, but it could be dependent upon the immunofluorescent method used in these two experiments; indeed, it is well-known that the staining method is less sensitive and quantitative. Thus in the present study, we employed a quantitative method using ^{125}I-labelled protein A in order to detect antibodies against alcohol-treated rabbit hepatocytes. It has been reported that ^{125}I-labelled protein A assay is useful to detect antibodies to viable cell membrane, because non-specific binding of protein A to cell membrane is little compared with the radio-labelled IgG method (Brown, Klitzman & Hellström, 1977). ^{125}I-labelled protein A was therefore utilized to detect liver membrane autoantibodies (Frazer, Kronberg & McKay, 1983). Using this technique, the existence of serum antibodies against alcohol-treated rabbit hepatocytes was confirmed as it was in the results reported by MacSween, Anthony & Farquharson (1981), Anthony, Farquharson & McSween (1983), and Neuberger *et al.* (1984). In their reports, however, the antibodies against alcohol-treated hepatocytes were also detected in the sera of some patients with fatty liver (Anthony, Farquharson & McSween, 1983; Neuberger *et al.*, 1984). In the present study, the antibodies were positive only in one case with fatty liver. This discrepancy may be caused by the degree of fatty liver, since in the present study, fatty infiltration in the liver was mild to moderate in most fatty liver cases. Another cause of difference may depend on the difference of the methodology: Anthony, Farquharson & McSween (1983) and Neuberger *et al.* (1984) have utilized anti-human immunoglobulin as the second antibody, which was reported to have higher non-specific binding to cell membrane than protein A (Brown, Klitzman & Helström, 1977). Secondly, both of them have

isolated hepatocytes without using collagenase in indirect immunofluorescent assay. Hepatocytes isolated with collagenase are reported not to have Fc or C3 receptors on cell membrane, while on the cell membrane of mechanically isolated hepatocytes, Fc and C3 receptors are demonstrated (Ramadori *et al.*, 1983). Because of low non-specific binding of immunoglobulin, we chose hepatocytes isolated with collagenase as target cells for the detection of liver-cell membrane antibody.

When immunofluorescent assay was performed, we could detect the serum antibodies against alcohol-treated rabbit hepatocytes in only three of six sera in which antibodies against alcohol-treated rabbit hepatocytes were positive by ^{125}I-labelled protein A assay. As expected, in the sera that were negative for the antibodies by ^{125}I-labelled protein A assay, none showed positive immunofluorescence on alcohol-treated rabbit hepatocytes. This indeed depends upon the difference of relative sensitivity between the techniques. Of particular importance is, however, the finding in the present ^{125}I-labelled protein A assay that the sera which had bound to alcohol-treated rabbit hepatocytes became non-reactive with alcohol-untreated, normal hepatocytes after two absorptions with the latter. It is likely, therefore, that these sera bind new or altered hepatocyte antigens induced by alcohol.

The pathogenic significance of the antibodies against alcohol-treated rabbit hepatocytes can not be clarified in the present study. However, the serum antibodies were detected more frequently in the patients with histological features of active inflammation, namely, active cirrhosis and alcoholic hepatitis. In addition, the antibody values were significantly higher in the cases of active cirrhosis and alcoholic hepatitis than in the cases of other types of alcoholic liver disease and in the cases of other liver diseases. It is conceivable, therefore, that the serum antibodies against alcohol-treated rabbit hepatocytes play, at least in part, a role in liver-cell damage in alcoholic liver disease; the precise mechanism involving these antibodies in immunologically induced liver injury remains to be elucidated.

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