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Antibodies to liver membrane antigens in chronic active hepatitis (CAH). II. Specificity for autoimmune CAH

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SUMMARY

An immunoradiometric assay for IgG class autoantibody to liver membrane antigens, based on serum binding to glutaraldehyde treated monkey hepatocytes, was used to examine sera from patients with chronic active hepatitis (CAH) and other acute and chronic liver diseases. All sera from normals and patients showed binding, up to a titre of 1/2,048. For comparison of assays, results were normalized by selecting two reference sera, one with a high degree of binding, and one from a healthy subject with a low degree of binding: at a dilution of 1/2,048, these sera were given binding values of 100% and 0%. The values for the binding of unknown sera at the same dilution were calculated from these two reference values. For 26 patients with autoimmune CAH, the mean (±s.d.) percentage binding value (70±33%) was significantly higher than the mean value for 26 healthy subjects (10±15%), and high binding values were significantly associated with biochemically active hepatitis. The mean percentage binding value was moderately increased for eight patients with HBsAg associated CAH (42±12%), 13 patients with alcoholic hepatitis with cirrhosis (37±25%) and 45 patients with acute viral hepatitis A (40±27%) or B (52±37%). At a cut-off binding value of 65%, the assay as a single diagnostic procedure was shown to have a 70% sensitivity and a 95% specificity for the diagnosis of autoimmune CAH. Better understanding of the pathogenetic significance of antibodies to liver membrane antigens in CAH and other liver diseases will depend upon biochemical analysis of the presumably multiple antigenic determinants on the hepatocyte membrane.

Keywords chronic active hepatitis autoimmunity liver membrane radioimmunoassay hepatocytes

INTRODUCTION

An immune response to an organ specific antigen is one of the classical markers of organ specific autoimmune disease. The first liver membrane antigen to be described was that identified by Meyer zum Büschenfelde & Miescher (1972), and is now known as liver specific lipoprotein (LSP) (McFarlane *et al.*, 1977). A positive *in vitro* lymphokine release assay, indicative of T cell-mediated immunity to LSP, was held to be specifically associated with the autoimmune type of chronic active hepatitis (CAH) (Meyer zum Büschenfelde, Knolle & Berger, 1974). Subsequently radioimmunoassays for serum antibody to LSP proved to be positive in patients with various acute and chronic liver diseases (Jensen et al., 1978; Mieli Vergani & Eddleston, 1981). The binding of serum antibody to

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liver membrane antigen(s) (LMAg) other than LSP was first demonstrated by the use of immunofluorescence and intact rabbit hepatocytes (Hopf, Meyer zum Büschenfelde & Arnold, 1976), and anti-LMAg was claimed to be a more specific marker than anti-LSP for autoimmune CAH (Meyer zum Büschenfelde *et al.*, 1979).

Definition of the biochemical nature, specificity for liver, and cellular location of LMAg and LSP is only partial. Both LMAg and LSP are heterogenous mixtures of membrane associated proteins, but with different antigenic constituents (Meyer zum Büschenfelde *et al.*, 1979). We sought to develop a method that would allow recognition of antibodies to any liver membrane antigens and, as immunofluorescence is not readily standardized or quantitated, an immunoradiometric assay was chosen to measure the binding of serum gamma-globulin to liver membrane antigens on intact hepatocytes. Such reactivity might include antibody with specificity for some or all of the antigenic determinants of LSP or LMAg.

MATERIALS AND METHODS

Diagnostic criteria and sera tested. Sera were studied from patients with the clinical and histological features of CAH (Leevy, Popper & Sherlock, 1976; International Group, 1977). Such patients were further classified into those having (i) autoimmune CAH (CAH-A) if the serum was negative for HBsAg and three of four criteria were met, namely, female sex, met by 80% for the present cases, the HLA phenotype B8-DR3, met by 72%, seropositivity to a titre of 1/80 for anti-nuclear antibody (ANA), met by 56%, and seropositivity to a titre of 1/80 for anti-smooth muscle autoantibody (ASMA), met by 64%; (ii) hepatitis B virus (HBV) associated CAH (CAH-B) if the serum was positive for hepatitis B surface antigen (HBsAg) or (iii) cryptogenic CAH (CAH-C) if the serum was negative for HBsAg and criteria for CAH-A were not met. CAH was held to be active if the serum levels of two transaminase enzymes, alanine aminotransferase and aspartate aminotransferase, were twice the upper limit of the normal range at the time of assay, and at least once during the preceding three months.

It was not part of the design of this study to assess independently the effect of stage of disease or the influence of treatment on titres of antibody to liver membrane antigens although 'activity' of disease was assessed. Many patients, and most with CAH-A, were receiving, or had received, one or more prolonged courses of immunosuppressive treatment, prednisolone with or without azathioprine.

Sera were also obtained from patients with acute hepatitis A diagnosed by the presence of IgM antibody to hepatitis A virus (HAV), acute hepatitis B diagnosed by the presence in serum of HBsAg, alcoholic hepatitis with cirrhosis diagnosed by a history of high alcohol consumption, over 120g per day, and consistent liver biopsy appearances (Leevy *et al.*, 1976), sclerosing cholangitis, primary biliary cirrhosis, hemochromatosis, systemic lupus erythematosus (SLE) and multiple myeloma of IgG isotype. Sera from 26 health laboratory staff comparable for age with the CAH-A group were used as a normal panel.

Measurement of antibody binding to liver membrane antigens. An immunoradiometric assay as previously described (Kronborg, Frazer & Mackay, 1982) was used to determine the binding of serum IgG to LMAg. This assay uses monkey hepatocytes treated with 2% glutaraldehyde to stabilize membrane antigens and to preserve the cells. A suspension of 10^5 cells was exposed for 1 h at room temperature in microtitre trays to serial dilutions of 50 μl of test or control sera, washed, and exposed to ^{125}I-labelled staphylococcal protein A for 30 min. The cells were washed and the bound ^{125}I-protein A quantitated by gamma spectrometry. Assays were performed in triplicate.

In the development of the assay, two problems became evident. First, whereas studies using immunofluorescence and isolated hepatocytes by others (Hopf *et al.*, 1976), and in our laboratory (unpublished observations), allowed discrimination of positive and negative sera, the radiometric assay showed binding to hepatocytes at serum dilutions of up to 1/2,048 of almost all sera from normal subjects and patients. Second, there were substantial differences in the crude results of binding in ct/min for assays performed with the same serum on different days, attributable to differences in the specific activity of the ^{125}I-protein A, the reactivity of different batches of

hepatocytes, room temperature and other undefined variables. Intra-assay duplicate determinations of the binding of control sera to hepatocytes were in contrast reproducible, the scatter of results ranging over 6% of the mean (data not shown). Hence, to compare results of assays on different occasions, a normalization procedure was adopted, based on reference standards. Thus, from preliminary experiments, there were selected two standard reference sera, one which had a high degree of binding to hepatocytes at a serum dilution of 1/2,048 and was assigned a binding value of 100% at that dilution, and another which showed little binding at a serum dilution of 1/2,048 and was assigned a binding value of 0%. Binding curves obtained with these reference sera were used to establish percentage binding values at a dilution of 1/2,048 of all sera in subsequent experiments, as shown in Fig. 1. In any experiment, if the binding value thus defined of two further reference sera differed from previously determined 'standard' values of 30% and 65% by more than a mean for replicates of 5%, all results for that run were discarded. Duplicate samples of a test serum gave binding values reproducible to ±3% within batches and ±5% between batches. Each test run contained a random mixture of sera from different diagnostic groups.

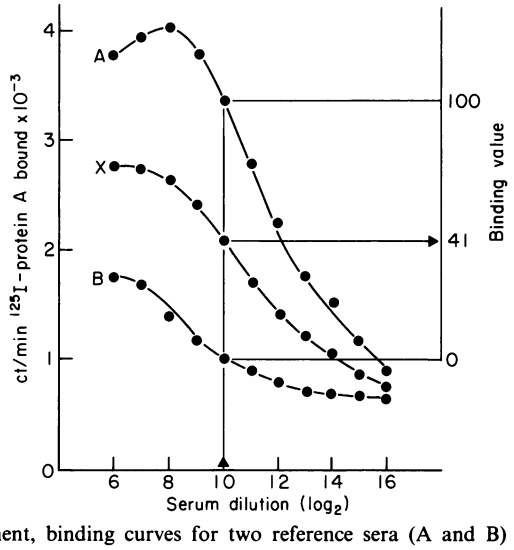

Fig. 1. For each experiment, binding curves for two reference sera (A and B) were constructed and an appropriate serum dilution (2,048) selected at which binding values for the unknown sera were determined. Thus, serum X is determined to have a binding value of 41%.

Other assays. Serum IgG concentrations were measured either by nephelometry or by radial immunodiffusion on tripartigen plates (Behring). Anti-smooth muscle antibody and anti-nuclear antibody were measured by indirect immunofluorescence (Whittingham, 1972) using as substrates sections of rat stomach and rat liver respectively. HLA typing for A, B and DR locus antigens was performed on peripheral blood lymphocytes by microcytotoxicity (Tait *et al.*, 1980). HBsAg was measured by radioimmunoassay using the AUSRIA kit (Abbot).

Statistics. Student's *t*-test was used to compare means of groups, and correlation co-efficients were derived by the least squares method, using the HP41C calculator statistics pack provided by Hewlett-Packard (California).

RESULTS

Antibody to liver membrane antigens in autoimmune CAH

The 26 patients with CAH-A gave a mean percentage binding value of antibody (±s.d.) of 70±33% (Fig. 2), whereas the value for 26 healthy subjects was 10±15%. The 22 females with CAH-A had a significantly higher mean binding value (77±27%) then the four males (37±32%) ($P < 0{\cdot}05$), and

the 14 females with onset of disease at an age less than 30 years had a significantly higher mean binding value (82 ± 22%) than the eight females with later onset of disease (57 ± 30%) ($P < 0.05$). An effect of age could not be assessed for the four males with CAH-A. Among healthy subjects, there were no significant differences in the mean percentage binding values between the 13 males (7 ± 15%) and the 13 females (12 ± 15%), nor did binding values differ significantly according to age.

The eight patients with CAH-A with biochemically active disease had significantly higher mean binding values (95 ± 22%) than 16 patients with inactive disease (60 ± 30%) ($P < 0.01$); for two patients insufficient data were available to assess disease activity. Patients receiving no therapy, those receiving prednisolone, and those receiving prednisolone and azathioprine had binding values of 58 ± 25%, 70 ± 30% and 77 ± 22%, respectively, but these differences were not significant. The 19 patients with the HLA phenotype A1 B8 DR3 had a non-significantly higher mean binding value (72 ± 32%) than the seven which lacked this phenotype (62 ± 40%).

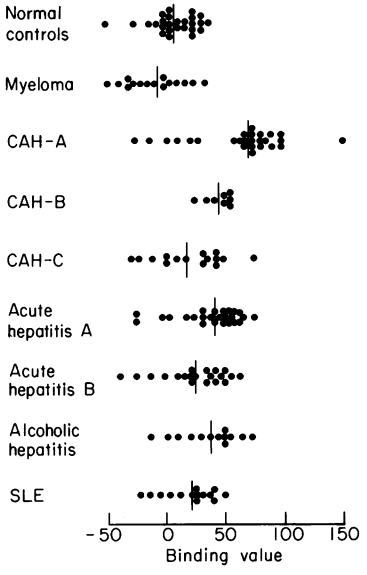

Fig. 2. Antibody to LMA in various liver and autoimmune diseases. Each point represents a determination on a single serum, and the bar the mean value for the group.

We have previously demonstrated for this immunoradiometric assay that the binding of IgG to hepatocytes is by the antigen specific F(ab)′ region of IgG (Kronborg *et al.*, 1982). Since elevated serum IgG concentrations are characteristic of CAH-A, we sought to establish whether higher binding values were due to increased non-specific binding of IgG to hepatocytes. Hence serum IgG concentrations were compared with binding values in 14 patients, but no significant correlation was found, in that the mean serum IgG was 19·0 ± 7·6 g/l and the correlation coefficient (r^2) for the serum IgG concentration with the binding value was 0·05.

Antibody to liver membrane antigens in other diseases

The binding values for various liver diseases are shown in Fig. 2. These were significantly elevated, compared to healthy subjects, in 25 patients with acute virus A hepatitis (40 ± 27%, $P < 0.01$), 20 with acute virus B hepatitis (52 ± 37%, $P < 0.01$), 13 with alcoholic hepatitis with cirrhosis (37 ± 25%, $P < 0.01$), eight with CAH-B (42 ± 12%, $P < 0.01$), and values were also above those for controls for the small numbers of cases of sclerosing cholangitis and primary biliary cirrhosis. The mean binding values for 15 patients with CAH-C, three with hemochromatosis and 14 with SLE did not differ significantly from that for healthy subjects, and the value for 14 patients with IgG myeloma was significantly lower (−8 ± 25%, $P < 0.01$) than that for healthy subjects.

Diagnostic value of antibody to liver membrane antigens

If the assay were to be used as a diagnostic test for CAH-A, a percentage binding value of 65% could be selected. This would give a 95% specificity for CAH-A, since five of 94 patients with chronic liver disease other than CAH-A had binding values above this level, and binding values greater than 65% were found in 18 of 26 (70%) patients with CAH-A. Thus a 65% binding value would provide a 70% sensitivity with a 95% specificity for the diagnosis.

DISCUSSION

Autoantibody of IgG isotype to liver cell membrane antigens (LMAg) was shown to be measurable in serum by means of an immunoradiometric assay. This reactivity is presumed to be similar to that previously demonstrable by immunofluorescence using isolated rabbit liver cells (Hopf *et al.*, 1976) and called liver membrane antibody (LMA). This was claimed to have a high degree of specificity for autoimmune CAH whether assessed by immunofluorescence or by the immunoradiometric assay (Mackay *et al.*, 1982). With further technical modifications and experience with the assay, it appears that reactivity with liver cell membranes is present in the serum of patients with acute or chronic hepatitis and in normal subjects as a continuous variable.

There was a wide range of binding values of antibody to LMAg among normal subjects. This observation, and the lower binding values found for patients with myeloma in whom IgG levels are low, suggest that the binding of human serum to monkey hepatocytes may depend in part upon 'background' levels of IgG autoantibodies to LMAg and naturally occurring antibodies to xenoantigens, although such natural antibodies are said to be more frequently IgM than IgG (Daar & Fabre, 1981). It is uncertain whether the IgG antibodies to liver membrane antigens in normal serum, or the sera of patients with miscellaneous acute or chronic liver diseases, are directed against the same membrane antigens as the antibodies present in high titre sera from patients with CAH, but this is currently being investigated. Presumably there is a 'background' level of natural antibody to liver membrane antigens in all sera and, over and above this, reactivity with liver membrane antigens develops in various inflammatory liver diseases, with the possibility of a reactivity unique to the disease occurring in autoimmune CAH.

In assessing the pathogenicity of this presumed disease specific membrane autoantibody, it would be important to determine whether it had special functional capabilities, for example, ability to fix complement or bind a cytotoxic effector cell, equivalent to those of potentially damaging antibodies to pancreatic islet cells found in early cases of insulin-dependent diabetes mellitus (Lernmark & Baekkesov, 1981). Pending biochemical characterization of liver specific antigens, we must concede the alternative explanation for the observed results that patients with CAH and other liver diseases develop antibody to LMAg as a consequence of continuing hepatocyte injury produced by as yet undefined mechanisms, and the higher titres found in patients with autoimmune CAH are simply due to the sex and age of this group. Supporting this is the great difficulty encountered in producing CAH in rodents immunized with liver membranes despite their development of high titres of serum antibody to hepatocyte antigens (Feighery *et al.*, 1981; Bartholomaeus *et al.*, 1981). Although the patients with CAH-A had a significantly higher antibody to LMAg than did patients with CAH-B, six of the eight patients with CAH-B were male, and the mean binding value of 42% for this group was similar to the mean value of 37% for the four males with CAH-A.

The definition of CAH and its subtypes is hindered by the lack of a specific serological test for the disease. Immune reactivity to LSP, initially held to be a specific marker of CAH (Meyer zum Büschenfelde *et al.*, 1974), has subsequently been associated with many liver diseases including acute viral hepatitis (Jensen *et al.*, 1978; Kakuma *et al.*, 1979) and drug-induced damage to the liver (Meliconi *et al.*, 1982). Serum antibody to LSP cannot therefore be used as a serological marker for CAH, although the titre of antibody to LSP correlates with the degree of periportal piecemeal necrosis (Jensen *et al.*, 1978). Antibodies to LMAg also have been held to be specific for autoimmune CAH (Meyer zum Büschenfelde *et al.*, 1979), but this was not borne out in the present study. However, despite the presence of low levels of binding activity of serum in many acute and chronic liver diseases, the assay may have diagnostic usefulness, since a cut-off value of 65% binding

activity in the assay would have achieved a 70% sensitivity and a 95% specificity for autoimmune CAH (active and inactive cases) amongst all the patients in our study with acute or chronic liver disease.

In conclusion, although high binding values for antibody to LMAg are found in CAH-A the claimed specificity for CAH-A of antibody to LMAg determined by immunofluorescence (Hopf *et al.*, 1976) is not found with an immunoradiometric assay; perhaps this is due to the relative insensitivity of the immunofluorescence technique. The binding of normal and various disease sera to the membrane of liver cells suggests that there are multiple potential antigenic determinants on the liver cell membrane and characterization of these is required before assessment can be made of the pathogenetic significance of antibodies to liver membrane antigens.

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