PAPER AND ORIGINALS

Cell-mediated Immunity to Human Tamm-Horsfall Glycoprotein in Autoimmune Liver Disease with Renal Tubular Acidosis

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British Medical Journal, 1974, 4, 491-494

Summary

Cell-mediated immune responses to Tamm-Horsfall glycoprotein isolated from human urine were investigated using the leucocyte migration test. Abnormal responses were found in 91% of patients with active chronic hepatitis or primary biliary cirrhosis with an associated renal tubular acidosis (R.T.A.) but in only 19% of those without R.T.A. In nearly all of a group of patients without autoimmune liver disease and in a control group of normal subjects results were within normal limits. In addition, using an immunofluorescent technique with rabbit antibody to human Tamm-Horsfall glycoprotein, it was possible to show the presence in human liver cell membrane of material reacting immunologically as Tamm-Horsfall. These findings suggest that the development of an immune response to this glycoprotein, initiated by release of cross-reacting antigens from damaged hepatocytes, could be the mechanism underlying the occurrence of R.T.A. in some patients with autoimmune liver disease.

Introduction

A number of systemic manifestations may occur in both active chronic hepatitis and primary biliary cirrhosis and it has been postulated that these, like the underlying liver disease, are based on the development of abnormal immune reactions. The most striking association is the high incidence of renal tubular acidosis (R.T.A.) in these diseases. Golding

et al. (1973) in a detailed study of 228 patients from this unit with active chronic hepatitis, primary biliary cirrhosis, or cryptogenic cirrhosis found R.T.A. in 32%. In the few patients in whom renal tissue was available for histological examination peritubular lymphocyte infiltration was found, suggesting the presence of a cell-mediated immune response. In addition, using the leucocyte migration test with a human kidney homogenate as antigen, evidence of cell-mediated hypersensitivity was found in 59% of patients with autoimmune liver disease and R.T.A. (Smith *et al.*, 1972). Kidney homogenates, however, contain many proteins and identification of the specific antigens to which sensitization is directed would be an important step forward.

In this context the immunological responses to proteins specifically related to tubular cells are clearly most relevant. The glycoprotein first isolated from the urine by Tamm and Horsfall (1950) seems to be produced in the kidney (Cornelius *et al.*, 1965) and, as shown by immunofluorescent studies, is present particularly in the cells of the ascending limb of the loop of Henle and the distal tubule (McKenzie and McQueen, 1969), regions known to be most important for the acidification process (Rhodin, 1967). Examination by immunodiffusion of the kidney homogenate used by Smith *et al.* (1972) showed it to contain the Tamm-Horsfall glycoprotein, a fact that prompted us to carry out the further studies reported here, in which the possibility that renal tubular acidosis is the consequence of a cell-mediated immune response directed against this antigen was investigated.

Patients and Methods

Diagnoses in the 24 patients with active chronic hepatitis and the 24 with primary biliary cirrhosis were based on current clinical, biochemical, and histological criteria. Twenty-three patients with various liver diseases not attributable to autoimmunity were also investigated. These included alcoholic cirrhosis (6 cases), liver damage from paracetamol overdose (4), idiopathic haemochromatosis (3), halothane hepatitis (2), primary hepatocellular carcinoma (2), extrahepatic ob-

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structive jaundice (2), carbon tetrachloride liver injury (1), severe liver damage from *Amanita phalloides* (2), and hepatic hydatid disease (1). The control group consisted of 16 healthy laboratory workers.

None of the patients had overt R.T.A.—that is, a urinary pH above 5.2 together with a spontaneous metabolic acidosis—but the incomplete type was present in seven of the 24 patients with active chronic hepatitis (29%) and in four of the 24 with primary biliary cirrhosis (17%). None of the group with other liver diseases showed any impairment in renal acidification. In the detection of incomplete R.T.A. calcium chloride (50 mEq three times a day for three days) was used as the acid load to avoid the administration of ammonium chloride to patients with liver diseases. Blood pH, bicarbonate, and electrolytes were measured before and on the third day of the acid load. On that day also all urine was collected and the pH, titratable acidity, and ammonia were measured. Later in the study a screening test was used in which urinary pH was measured at intervals after a single dose of calcium chloride (75 mEq). Renal acidification in those patients with an abnormal screening test result was then evaluated in more detail, using the full three-day acid load.

PREPARATION OF ANTIGENS AND IMMUNOLOGICAL TECHNIQUES

Tamm-Horsfall glycoprotein was prepared from normal human urine by precipitation with NaCl at 0.58-M concentration as described by Fletcher *et al.* (1970). Antibodies to a freeze-dried preparation were then raised in rabbits as described by Marr *et al.* (1971).

A macromolecular kidney protein fraction which did not contain Tamm-Horsfall glycoprotein was prepared from human kidney obtained within a few hours of death by the method used for preparation of a liver-specific lipoprotein (Eddleston *et al.*, 1973). After centrifugation of a homogenate at 105,000 *g* the supernatant was subjected to sequential gel filtration on Sephadex G100 and Sepharose 6B. The fraction appearing in the excluded volume from Sepharose 6B (molecular weight 1×10^6) was used in the leucocyte migration test.

The technique used for the leucocyte migration test was as described by Mitchell *et al.* (1972). The optimal concentration of Tamm-Horsfall glycoprotein was found to be 20 $\mu\text{g}/\text{ml}$ of medium. Measurements of migration indices in the normal subjects gave a mean of 0.94 with lower and upper limits (mean ± 2 S.D.) of 0.79 and 1.09 respectively. The optimal concentration for the macromolecular kidney protein fraction was found to be 30 $\mu\text{g}/\text{ml}$, which gave lower and upper limits in normal subjects of 0.71 and 1.03 respectively, with a mean of 0.87.

Immunofluorescent Technique.—Normal human liver obtained within 15 minutes of death was used as substrate. Blocks of tissue were snap-frozen in liquid nitrogen, and 5- μm sections were cut in a cryostat at -20°C . Unfixed sections were air-dried at room temperature and stained by a standard indirect immunofluorescent technique, using the rabbit antibody to human Tamm-Horsfall glycoprotein at a dilution of 1 in 5 followed by fluorescein-labelled sheep anti-rabbit immunoglobulin (Wellcome Reagents).

Results

Of the 11 patients with either active chronic hepatitis or primary biliary cirrhosis and R.T.A. inhibition of leucocyte migration in the presence of Tamm-Horsfall glycoprotein was found in five and stimulation in five. By comparison, in the 37 patients with the same liver diseases but without R.T.A. inhibition was observed in only one and stimulation in six. Among the 23 patients with non-autoimmune liver diseases

TABLE 1—Results of Leucocyte Migration Test with Tamm-Horsfall Glycoprotein as Antigen in Patients with and without Renal Tubular Acidosis (R.T.A.)

	No. Tested	No. (%) with Inhibition	No. (%) with Stimulation	Total (%) Abnormal
Active chronic hepatitis or primary biliary cirrhosis with R.T.A.	11	5 (45.5)	5 (45.5)	10 (90.9)
Active chronic hepatitis or primary biliary cirrhosis without R.T.A.	37	1 (2.7)	6 (16.2)	7 (18.9)
Other liver diseases	23	0	2 (8.3)	2 (8.3)

there were only two who had abnormal migration indices, with values just above the upper limit or normal (table 1).

When both inhibition and stimulation were considered to indicate cellular hypersensitivity the difference in the frequency of sensitization between those with and those without R.T.A. (91% and 19% respectively) was highly significant ($P < 0.001$). When inhibition alone was taken as an abnormal response the frequencies of sensitization fell to 45.5% and 3% respectively, which again were significantly different ($P < 0.001$). There were no differences between the findings in active chronic hepatitis patients and those in primary biliary cirrhosis patients when analysed separately.

The association between sensitization to Tamm-Horsfall glycoprotein and failure of urinary acidification is shown in fig. 1, where urinary pH is compared with the result of the leucocyte migration test. Thus, the mean urinary pH ($\pm$ S.D.) in those showing an abnormal result (including both stimulation and inhibition) was 5.4 ± 0.56 , while that found in those without sensitization to Tamm-Horsfall glycoprotein was 4.9 ± 0.25 —a highly significant difference ($P < 0.001$). The distribution of urinary pH in those patients with abnormal migration indices appeared continuous, and there was no evidence of polarization into two distinct subgroups with different responses to acid load.

FIG. 1—Relation between results of leucocyte migration test (L.M.T.), using Tamm-Horsfall glycoprotein as antigen, and urinary pH after acid load in patients examined by three-day test.

When the macromolecular kidney fraction was used as the antigen in the leucocyte migration test no correlation could be detected between the frequency of abnormal migration indices and either the occurrence of R.T.A. (table II) or the urinary pH (fig. 2).

Localization of Cross-reacting Antigens in Liver.—Sections of human liver exposed to antibody to Tamm-Horsfall glycoprotein followed by a fluoresceinated anti-immunoglobulin repeatedly showed linear polygonal staining outlining the liver cell membranes when examined by fluorescence microscopy

TABLE II—Results of Leucocyte Migration Test with Macromolecular Kidney Fraction as Antigen in Patients with and without Renal Tubular Acidosis (R.T.A.)

	No. Tested	No. (%) with Inhibition	No. (%) with Stimulation	Total (%) Abnormal
Active chronic hepatitis or primary biliary cirrhosis with R.T.A.	10	3 (30)	0	3 (30)
Active chronic hepatitis or primary biliary cirrhosis without R.T.A.	22	4 (18.2)	3 (13.6)	7 (31.8)

FIG. 2—Relation between results of leucocyte migration test (L.M.T.), using macromolecular kidney fraction as antigen, and urinary pH after acid load in patients examined by three-day test.

FIG. 3—Section of normal human liver examined by indirect immunofluorescence with rabbit antibodies to human Tamm-Horsfall glycoprotein. Linear polygonal staining outlines hepatocytes. Granular appearance within cytoplasm of some liver cells is due to red autofluorescence of lipofuscin. ($\times 187$.)

(fig. 3). This pattern was not evident when normal rabbit serum was used instead of serum containing antibodies to the Tamm-Horsfall glycoprotein.

Discussion

There is increasing evidence that immunological mechanisms, mainly of the cell-mediated type, are involved in the production of the liver damage in active chronic hepatitis and primary biliary cirrhosis. For instance, we have shown cell-

mediated immunity to a human liver-specific antigen in patients with active chronic hepatitis (Miller *et al.*, 1972), and immunization of rabbits with this antigen produced liver lesions very similar to those seen in man (Meyer zum Büschenfelde, 1972). In addition, we have recently shown that lymphocytes from patients with active chronic hepatitis are directly cytotoxic to isolated hepatocytes, and the antigen involved in this cytotoxicity reaction appears to be the liver-specific protein (Thomson *et al.*, 1974). In patients with primary biliary cirrhosis cell-mediated immune responses to a protein fraction of human bile have also been demonstrated, and it has been suggested that these might be associated with the initial bile duct lesion in this condition (Eddleston *et al.*, 1973).

Similar mechanisms could be at work in the development of the renal tubular acidosis seen in both of these liver diseases. The finding of immune responses to the Tamm-Horsfall glycoprotein which is present in the renal tubular cells at the principal sites of the acidification process suggests the possibility that an autoimmune reaction directed against this protein could damage the renal tubule. The specificity of this immune response is shown by the completely different findings with the macromolecular kidney fraction and argues against the possibility that the immune reactions shown are secondary to kidney damage.

We have considered the finding of stimulation of migration in the leucocyte migration test as indicative of weak sensitization, as initially proposed by Søborg (1968). Though such an interpretation has been questioned by other workers, in three out of four patients with stimulation of migration lymphocyte transformation with Tamm-Horsfall glycoprotein was also demonstrable.

The findings on immunofluorescence suggest that antigenic material reacting with antibody to Tamm-Horsfall glycoprotein is also present in the liver cell membrane. Release of this material after damage to hepatocytes could initiate an immune response which, because of the cross-reaction, is directed at both the kidney and liver antigens. It is easy to imagine such a sequence of events in active chronic hepatitis where hepatocellular damage is a prominent histological feature, but less so in primary biliary cirrhosis, where the bile ducts seem to be the focal point, at least initially, of the inflammatory reaction. In many cases, however, inflammation extends into the lobule and piecemeal necrosis of hepatocytes may also be observed. Furthermore, some patients with primary biliary cirrhosis show sensitization to the liver-specific protein (Miller *et al.*, 1972), and it is in this group that an immune response to Tamm-Horsfall glycoprotein might be expected to occur. Indeed, such a relationship could be shown in the 16 patients in whom cell-mediated immunity to both antigens was tested. Eight were found to be sensitized to liver-specific protein and in six of these sensitization to Tamm-Horsfall glycoprotein was also found. In contrast, only one of the eight patients who had normal migration indices with liver-specific protein had an abnormal response to Tamm-Horsfall glycoprotein. These results are unlikely to be due to direct cross-reaction between these two antigens as antibody to Tamm-Horsfall glycoprotein does not react with liver-specific protein on immunodiffusion. A different liver cell membrane antigen must therefore be responsible for the cross-reaction. If sensitization to this component occurred as a consequence of hepatocyte damage then the development of R.T.A. in some patients with active chronic hepatitis and primary biliary cirrhosis would be explained.

We thank Drs. Anne M. S. Grant and R. D. Marshall of St. Mary's Hospital Medical School for their generous donation of purified Tamm-Horsfall glycoprotein and antisera and Dr. A. M. G. Cochrane who kindly performed the lymphocyte transformation studies. The generous support of the Wellcome Trust is also gratefully acknowledged.

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Cell-mediated Immunity to Intrinsic Factor in Autoimmune Disorders

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British Medical Journal, 1974, 4, 494-496

Summary

Evidence of cell-mediated immunity to gastric intrinsic factor was present in 86% of patients with pernicious anaemia and in at least 13% of patients with hyperthyroidism, 21% of patients with atrophic gastritis, and four out of nine (46%) patients with hypogammaglobulinaemia. Controls gave negative results. The four patients with hypogammaglobulinaemia and cell-mediated immunity to intrinsic factor had evidence of impaired gastric function.

Introduction

Pernicious anaemia is common in some disorders in which autoantibodies appear. Thus some 10% of patients with primary hypothyroidism, 2% of patients with Graves's disease, and about 4% of insulin-dependent diabetics have pernicious anaemia. Sera from these groups of patients often contain gastric antibodies, particularly parietal-cell antibodies, which are generally demonstrable in about 30% of such samples. There is also a high incidence of pernicious anaemia among patients with acquired hypogammaglobulinaemia. Intrinsic-factor antibodies are less common in diseases other than pernicious anaemia, but they occur in 2% of patients with Graves's disease and less frequently in the other groups as well as in relatives of patients with pernicious anaemia.

The association of pernicious anaemia with hypogammaglobulinaemia is particularly interesting because patients with hypogammaglobulinaemia may lack normal B-lymphocyte function responsible for producing humoral antibodies while they often retain T lymphocytes responsible for cell-mediated immunity. This suggests that at least in these patients, and

probably in other patients, cell-mediated immunity may be more important in producing pernicious anaemia than humoral antibodies. This view is reinforced by the failure to show humoral intrinsic-factor antibody in either the serum or gastric juice of almost one-third of patients with pernicious anaemia.

This study was undertaken to determine the frequency of cell-mediated immunity to intrinsic factor in these susceptible groups and, in the longer term, whether cell-mediated immunity to intrinsic factor would prove a better indication of the risk of the patient developing pernicious anaemia than tests for humoral antibodies.

Patients and Methods

Observations were made on 51 patients with proven pernicious anaemia, 14 with histologically-proven atrophic gastritis without pernicious anaemia, eight with acquired hypogammaglobulinaemia and one with an isolated IgA deficiency, 24 with insulin-dependent diabetes, 14 with primary hypothyroidism, 22 with Graves's disease, and eight patients with toxic nodular goitre. Observations were made also on 105 controls, 51 of whom corresponded in sex and age to the pernicious-anaemia group and 54 of whom were matched to patients in the other groups.

Crude hog intrinsic factor (Armour Pharmaceutical Company) was concentrated by the method of Ellenbogen and Highley (1967) so that 1 mg dried powder bound 45 ng vitamin B₁₂.

Human intrinsic factor was contained in gastric juice collected after pentagastrin stimulation. Bile-stained samples were discarded. The gastric juice was concentrated by ultrafiltration through an Amicon filter so that the intrinsic factor content was 600 U/ml. It was used at an intrinsic factor concentration of 150 intrinsic factor U/ml.

The *leucocyte migration inhibition (M.I.F.) test* was set up as described by Rose et al. (1969) using disposable migration chambers (Sterilin Ltd.). Leucocytes were harvested from 25 ml of heparinized blood. Tubes were set up in quintuplicate, with control tubes from which antigen was omitted. The fluid in the chamber contained either hog intrinsic factor (5 mg) diluted in 1 ml TC-199 (Burroughs Wellcome) followed by the addition of 10% fetal calf serum (Burroughs Wellcome) or human gastric juice (600 intrinsic factor U/ml) diluted with three parts TC-199 followed by the addition of 10% fetal calf serum. Intrinsic factor was replaced by additional TC-199 in controls. Migration zones were traced on paper through a photographic enlarger ($\times 15$) and areas read with a planimeter. Migration zones from the control group were within a range of 80%-120% of the mean value (see fig. 1). Inhibition of migration

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