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HEPATITIS "C" ANTIGEN IN NON-A, NON-B POST-TRANSFUSION HEPATITIS

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Summary Evidence for a new hepatitis-specific antigen has been obtained from double immunodiffusion assays between acute and convalescent sera obtained from patients with non-A, non-B post-transfusion hepatitis. The designation hepatitis C (HC) antigen is proposed. HC was found in the acute-phase sera of all 13 non-A, non-B post-transfusion hepatitis patients with longer incubation and duration periods (type 2) tested, but only transiently in 4 out of 10 acute-phase sera obtained from patients with type 1 non-A, non-B hepatitis, with shorter incubation and duration periods. The antigen was also detected in 2 out of 16 single specimens obtained during the acute phase from acute hepatitis patients who had not received a blood-transfusion. This suggests presence of a carrier state. No patients with alcoholic hepatitis and no healthy blood-donor carried HC antigen. The antigen seems distinct from those of hepatitis A and B (surface and core). It migrated in the serum β-globulin region and had a buoyant density of 1·30 and a molecular weight between 100 000 and 300 000. Antibodies against HC antigen were found in only 30% of the type-2 non-A, non-B post-transfusion hepatitis patients and did not persist for long. However, these antibodies were directed specifically against HC antigen and moved in a manner similar to 7*S* globulin on rate-zonal centrifugation.

Introduction

ONCE the association between hepatitis-B surface antigen (HB_sAg) and type-B hepatitis had been recognised it was expected that screening blood-donors for HB_sAg would eliminate post-transfusion hepatitis. However, even with screening for HB_sAg a 10% frequency of post-transfusion hepatitis has persisted,[1–5] and 90–95% of these cases have been serologically unrelated to hepatitis A or B or cytomegalovirus.[6–8] This suggested the existence of at least one more agent ætiologically responsible for human viral hepatitis. Evidence for a transmissible agent was demonstrated by inoculating blood from patients with non-A, non-B hepatitis patients into chimpanzees.[9 10] However, no serological marker was defined in these studies and no virus particle was observed. Our study was designed to detect a serological marker for this disease.

Material and Methods

Sera

In 1970–77 there were 156 post-transfusion hepatitis patients at the surgical clinic of Sendai National Hospital. 116 were negative for HB_sAg and anti-HB_s antibody (non-B post-transfusion hepatitis), and these could be further divided into two groups, the pattern of serum-glutamic-pyruvic-transaminase (S.G.P.T.) values being monophasic or biphasic[2] (fig. 1). In the monophasic group (type-1 non-B hepatitis) S.G.P.T. values rise rapidly and then fall sharply. The average incubation period was 5·7 weeks and raised S.G.P.T.s persisted for 5·8 weeks.[2] Type-2 non-B hepatitis is characterised by a rapid increase and decrease in S.G.P.T. similar to that of the monopha-

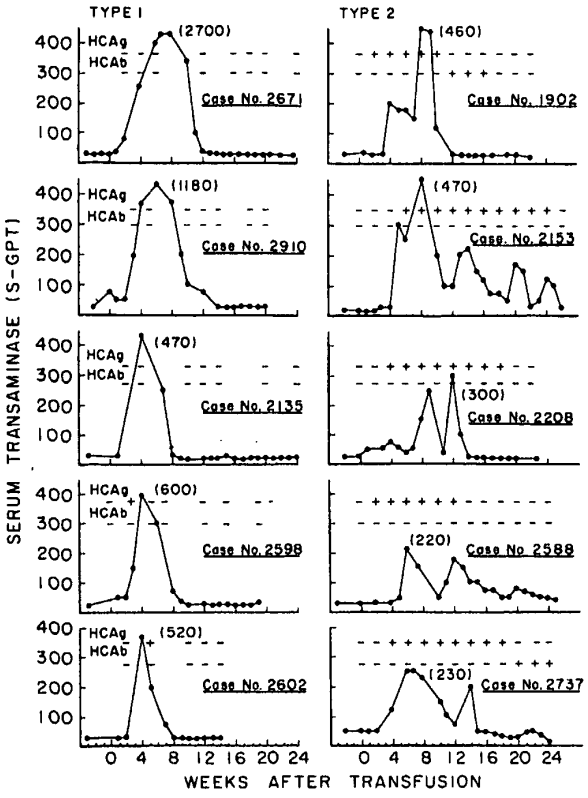

Fig. 1—Clinical courses of type-1 (short incubation and short duration) and type-2 (long incubation and long duration) non-A, non-B hepatitis observed after blood-transfusion.

sic group but followed by another increase and then a gradual decrease. The average incubation period was 7·2 weeks and increased S.G.P.T. values persisted for up to 17·5 weeks.

52 patients were classified as type 1, 30 as type 2, and the remaining 34 were unclassified (plateau type or type 3, see Discussion). Screening for a non-A, non-B hepatitis antigen (tentatively designated hepatitis C, HC) was achieved by examining 268 serum samples from 13 type-2 non-B post-transfusion hepatitis patients and 80 samples obtained from 10 type-1 non-B hepatitis patients. The sera from type-2 patients were acute phase (176) or convalescent (92). These two groups were tested against each other by immunodiffusion. Other acute hepatitis sera and sera from patients with other liver diseases such as alcoholic hepatitis and hepatoma, were obtained from Prof. F. Ichida, School of Medicine, Niigata University.

Immunodiffusion

We used Ouchterlony's double immunodiffusion technique with antiserum in a central well. Two of the surrounding wells opposite each other contained standard HC antigen and the specimens under examination were put in the remaining four wells. The gel consisted of 0·9% (w/v) agarose (Nakarai Chemical), 1% polyethyleneglycol 6000, and 1% dextran 500 (Wako Chemical) dissolved in 0·15 mol/l phosphate-buffered saline (P.B.S.), pH 7·6. The plates were incubated in a humid chamber at room temperature for 48 h.

Immunoelectrophoresis

HC antigen was identified by immunoelectrophoresis.[11] The gel contained 1% agarose plus 1% dextran 500 dissolved in 0·15 mol/l P.B.S., pH 7·6. A 3 mm diameter well was filled with HC-antigen-positive serum and electrophoresis was performed at 100 V for 60 min. Troughs 2 mm in width were filled with anti-HC-positive serum and the slides incubated in a humid chamber at room temperature for 48 h.

Gel Chromatography

The HC antigen was purified by column chromatography on 'Sepharose 6B' (Pharmacia Chemicals) and eluted with 0·01 mol/l P.B.S., pH 7·2, at a constant flow-rate of 40 ml/h at 4°C. The eluate fractions were collected in 7 ml volumes and concentrated in cellulose tubing (size 20/32, Visking) immersed in 50% polyethyleneglycol 200 000.

Rate-zonal Centrifugation of Serum Containing HC Antigen

HC antigen was precipitated from 50 ml of serum with 13% polyethyleneglycol 6000 (Wako Pure Chemicals) dissolved in 10 mmol/l P.B.S., pH 7·2. The precipitate was dissolved in 5 ml of the same buffer, then layered onto 60 ml preformed sucrose gradients (10–50% in P.B.S.) and subjected to rate-zonal centrifugation in a SW 25-2 rotor (Hitachi Manufacturing) at 24 000 r.p.m. at 4°C for 16 h. A 3 ml fraction was collected from the bottom of each tube and assayed for HC antigen by immunodiffusion. The HC-antigen-containing fractions were pooled and dialysed overnight at 4°C against P.B.S.

Equilibrium Density-gradient Centrifugation

The HC antigen fractions detected by rate-zonal centrifugation were combined and subjected to equilibrium density-gradient centrifugation in cæsium chloride dissolved in P.B.S. in a SW-65 rotor at 40 000 r.p.m. at 4°C for 48 h. Ten drops were collected from the bottom of each tube and assayed for HC antigen by immunodiffusion. The density of each fraction was determined with an Abbe refractometer.

Assay of Hepatitis-B Antigen and Hepatitis-A Antigen

HB_sAg was assayed by reversed passive hæmagglutination (R.P.H.A.) according to manufacturers' instructions ('Antihebscell', Green Cross). All positive results were confirmed by neutralisation with antibody to HB_sAg. Passive hæmagglutination tests for anti-HB_s were done on all samples by a microtitre system and with HB_sAg-coated sheep red blood-cells (Eisai). A titre of 1:8 or greater was considered to indicate anti-HB_s. Antibody to hepatitis-B core antigen (anti-HB_c) was measured in a pretransfusion and 3 month post-transfusion sample in all patients by R.P.H.A. inhibition with anti-HB_c-coated red blood-cells.[12] A 4-fold or greater increase in titre was considered to demonstrate the presence of anti-HB_c. E antigen and anti-e were detected by immunodiffusion. Antibodies to hepatitis-A antigen (anti-HA) were assayed by Dr Y. Moritsugu at the National Institute of Health, Tokyo, by immunoadherence hæmagglutination.

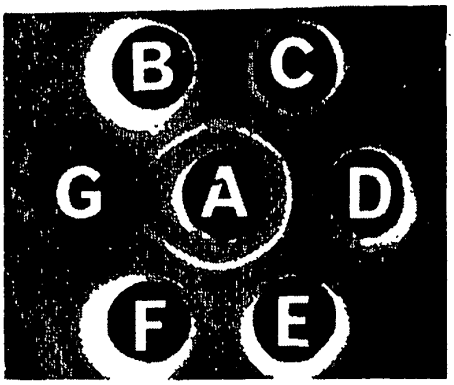

Fig. 2—Detection of HC antigen by immunodiffusion.
The centre well (A) contains human antibody to HC antigen and the peripheral wells (B-F) acute sera from type-2 non-A, non-B post-transfusion hepatitis patients. Five precipitin lines are seen showing complete identity but no precipitin line appeared between antibody (A) and the normal human serum (G).

Results

Detection of HC Antigen and Antibody by Immunodiffusion

The acute-phase sera from 13 type-2 post-transfusion hepatitis patients were used as antigens in the search for antibodies in the corresponding convalescent sera. Acute-phase sera occasionally gave precipitates with convalescent-phase sera from the same patient or from other type-2 patients (fig. 2). The precipitin line in the agar was always clear and sharp. In this way 10 HC-antigen-positive sera were selected. These sera did not react

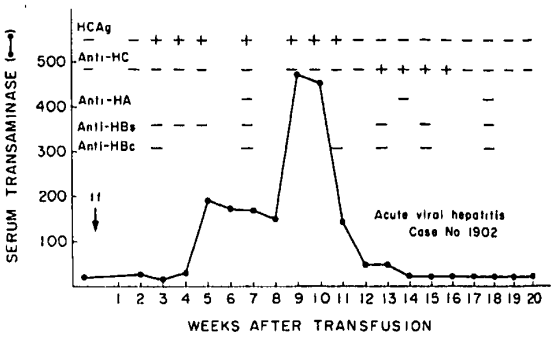

Fig. 3—Clinical course of typical type-2 post-transfusion hepatitis and detection of HC antigen and anti-HC.
HC antigen first appeared 3 weeks after blood-transfusion, and remained positive for 9 weeks. Anti-HC appeared 2 weeks after disappearance of HC antigen.

with each other or with a panel of sera containing anti-HB_s or anti-e. Negative results were also obtained when these HC sera were tested by R.P.H.A. with anti-HB_c-coated human red blood-cells.[12] The most potent HC-antigen-positive serum (no. 7555) and anti-HC positive serum (no. 269) were selected as standard reactants for later screening.

The HC antigen appeared in the sera of the 13 type-2 non-A, non-B hepatitis patients after blood-transfusion and remained for 8–24 weeks. In 11 patients HC antigen appeared 1–5 weeks after transfusion, but in 2 it was detected before transfusion (see fig. 5 and Discussion). Thus, it appears that these 2 patients were carriers of HC antigen. Fig. 3 illustrates a typical clinical course in hepatitis C. The S.G.P.T. rose rapidly 5 weeks after transfusion, followed a biphasic course, and returned to normal 7 weeks after onset. HC antigen appeared 3 weeks after transfusion and remained for 9 weeks. Anti-HC was first detected 2 weeks after the antigen's disappearance and remained for only 4 weeks. When serial sera from this patient were tested for antibody against hepatitis A (anti-HA) and B (anti-HB_s, anti-HB_c), no increases could be demonstrated. In a typical case of type-2 non-A, non-B hepatitis, HC antigens appeared during the acute phase of the illness to be superseded by anti-HC during the convalescent phase (fig. 1, cases 1902 and 2737). However, anti-HC were detected in only 4 cases out of 13, probably because the immunodiffusion test is less sensitive to antibody than to antigen.

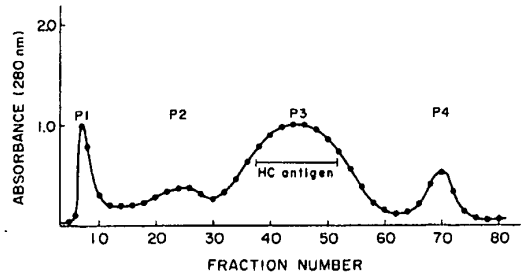

Fig. 4—Elution pattern of HC antigen by sepharose 6-B chromatography.
HC antigen is eluted between fraction 35 and fraction 55 in the third peak (p3).

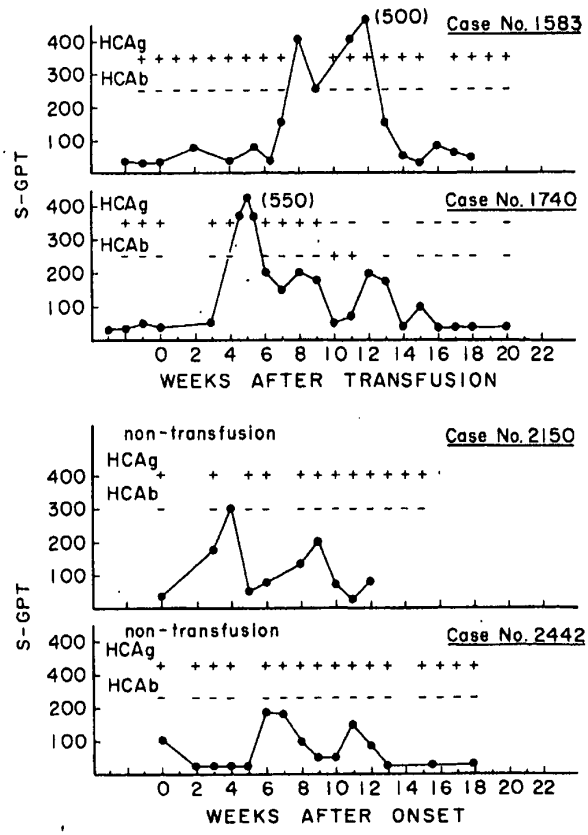

Fig. 5—Occasional detection of HC antigen in patients before blood-transfusion (upper 2 cases) and in patients without blood transfusion (lower 2 cases), indicating carrier state of HC antigen.

FREQUENCY OF HC ANTIGEN AND ITS ANTIBODY IN VARIOUS LIVER DISEASES

Disease	No.	HC+	Anti-HC+
Alcoholic hepatitis	25	0	0
Post-transfusion hepatitis			
Type-1 non-A, non-B	10	4	0
Type-2 non-A, non-B	13	13	4
Acute hepatitis			
HB_sAg+	9	2	0
HB_sAg–	7	0	0
Hepatoma	25	1	0

In 60 blood-donors no HC or anti-HC was found.

In the limited number of type-1 non-A, non-B hepatitis patients in whom HC antigen was detected (4 out of 10) the antigen appeared during the acute phase of the illness and disappeared within 1 or 2 weeks (fig. 1, cases 2598 and 2602), in contrast to the pattern in type 2. Anti-HC could not be detected in the convalescent sera of the 10 type-1 cases.

Preliminary Characterisation of HC Antigen and Antibody

Sepharose 6B chromatography of 50 ml of HC-antigen-positive sera produced four major peaks (fig. 4). HC antigen activity was recovered from fractions 35–55 only, which correspond to the IgG region. HC antigen was found in the 15–20% sucrose fraction by zonal centrifugation. Density-gradient centrifugation of a sample that had been partially purified by rate-zonal centrifugation resulted in the HC antigen being concentrated at a buoyant density of 1·30. Immunoelectrophoresis of the HC antigen showed that its precipitating activity was in the serum β-globulin region.

In the anti-HC rate-zonal centrifugation studies, the *S* values were estimated from the anti-HC antibody positive fraction in relation to the IgG and IgM peaks. The anti-HC migrated with the IgG references. Immunoelectrophoresis of anti-HC showed that it reacted with HC-antigen-positive serum in the serum γ-globulin region.

Detection-rate of HC Antigen

The distribution of the HC antigen was examined in the sera obtained from different types of hepatitis (table). HC antigen was not detected in alcoholic hepatitis. Of 10 patients with type-1 post-transfusion hepatitis only 4, transiently, had the HC antigen specificity in their sera. On the other hand, all 13 patients with type-2 post-transfusion hepatitis were positive for HC

antigen. Specimens from the remaining 17 type-2 patients have not yet been analysed because of insufficient anti-HC. Anti-HC was found in only 4 cases of type-2 hepatitis, and nowhere else in this material.

Discussion

During the period 1970–77 apparent non-B hepatitis was found in 116 out of the 1082 patients (10·7%) who received blood-transfusions at the surgical clinic of Sendai National Hospital. Successively obtained sera from 7 of these cases were examined for anti-HA rise, but none showed a significant increase. Furthermore, sero-epidemiological evidence from Japan clearly shows that hepatitis A does not occur exclusively after blood-transfusion,[1,2] so these 116 patients can be called non-A, non-B hepatitis.[4]

On clinical grounds these hepatitis patients can be divided into two groups[2] with a third type in which the S.G.P.T. was characterised by a plateau pattern, but with an incubation period (7·4 weeks) and duration (15·3 weeks) resembling type 2 rather than type 1. Type 2 can be called non-A, non-B hepatitis of longer incubation and duration.

The HC antigen was detected by immunodiffusion in 178 out of 268 serum specimens sequentially obtained throughout the incubation period and during the clinical course of 13 patients with type-2 post-transfusion hepatitis. HC antigen, once detected, persisted for 8–24 weeks. During this positive period, the qualitative immunodiffusion test revealed no striking differences in antigen concentrations; in other words, the peak period of antigen detection was difficult to find. However, the constant detection in sera obtained sequentially during the critical period of the disease suggests a close correlation between this HC antigen and type-2 post-transfusion hepatitis. In 11 cases out of 13 HC antigen was first detected 1 or 2 weeks before or coincided with the rise in S.G.P.T.

Surprisingly antibodies against HC antigen were detected in only 4 of the 13 patients, despite the availability of sequential serum specimens during the recovery phase.

HC antigen was also found in 4 out of 10 type-1 hepatitis patients. However, the antigen, first found 3 or 4 weeks after transfusion, disappeared in a week. This transience may be an additional discriminating factor between type-1 hepatitis and type-2 hepatitis. Perhaps both types have the same ætiological basis, type 1 being acute and type 2 chronic. However, this does not fully explain the ætiology of type-1 hepatitis.

Another unexpected finding was the detection of HC antigen in some patients before blood-transfusion and in patients not transfused at all (fig. 5). Although the numbers are small, these cases may indicate the presence of symptom-free carriers and lessen the ætiological significance of HC antigen as an indicator of post-transfusion hepatitis.

HC antigen migrated in the β-globulin region on immunoelectrophoresis but could easily be distinguished from HA antigen[13] and HB antigen[14,15] on the basis of its small molecular weight. HC antigen did not react with human anti-HB_s or anti-e[16] by immunodiffusion. Sera with high titres against HA and HB_c antigens did not react with three different concentrations of HC antigen.

The lower *S* value and later elution by sepharose 6B gel filtration indicate that HC is smaller than HB_sAg. Its buoyant density (1·30) suggests that it is not a lipoprotein. Thus, it differs from HB_sAg and HA antigen. Perhaps HC antigen is not a whole virus but a virus-associated substance. However, the correlation between the infectivity and antigenicity of this antigen is not yet clear.

Alter et al.[9] and Tabor et al.[10] have reported that chimpanzees inoculated with plasma or sera from acute or chronic non-A, non-B post-transfusion hepatitis patients developed the disease. However, they did not suggest the presence of a serological marker for non-A, non-B hepatitis. A serological survey of post-transfusion hepatitis with HC antigen as the marker may throw light on the ætiology of this disease.

There may be yet more agents in the ætiology of post-transfusion hepatitis; HC antigen was not detected in every patient with type-1 non-A, non-B post-transfusion hepatitis.

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CHOLERA, NON-VIBRIO CHOLERA, AND STOMACH ACID

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Summary Fasting and postprandial stomach acid production were low in 16 of 37 Bangalees convalescing from cholera or non-vibrio cholera. Gastric juice of hypochlorhydric patients did not kill cholera vibrios in vitro, whereas that from normochlorhydric patients rapidly killed vibrios in concentrations