Failure to incriminate hepatitis B, hepatitis C, and hepatitis E viruses in the aetiology of fulminant non-A non-B hepatitis

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Abstract
Sporadic non-A, non-B hepatitis is the most common indication for liver transplantation in patients presenting with fulminant and subacute liver failure. This study used serological, histological, and molecular biological techniques to examine specimens from 23 consecutive patients transplanted for sporadic non-A, non-B hepatitis. No evidence was found of hepatitis C virus, hepatitis E virus, or 'cryptic' hepatitis B virus infection.

Keywords: hepatitis, liver failure.

Liver transplantation is an established treatment for patients presenting with fulminant hepatic failure.\(^1\) Paracetamol poisoning is the most common cause of fulminant hepatic failure in patients referred to the liver unit at the Queen Elizabeth Hospital, but few of these patients are suitable candidates for liver transplantation.\(^2\) Series reported before the application of liver transplantation for fulminant hepatic failure highlighted the particularly poor prognosis of patients who were negative for both hepatitis A (HAV) and hepatitis B (HBV) serological markers.\(^3\)\(^4\) Sporadic non-A, non-B (NANB) hepatitis is the most common indication for liver transplantation in patients with fulminant hepatic failure referred to our liver unit (two of three of patients transplanted for fulminant and subacute hepatic failure). A review of the published experience\(^1\) confirms that fulminant NANB hepatitis or 'hepatitis of unknown aetiology' is the indication for liver transplantation in 50% or more of patients transplanted for fulminant hepatic failure.

The virus responsible for most cases of post-transfusion NANB hepatitis (hepatitis C virus (HCV)) and that responsible for water borne epidemic NANB hepatitis (hepatitis E virus (HEV)) have recently been characterised.\(^5\)\(^6\) Cases of fulminant sporadic NANB hepatitis can now be examined for markers of HCV and HEV infection. In addition, the application of sensitive techniques such as the polymerase chain reaction to the investigation of these patients may incriminate 'cryptic' HBV infection in the absence of conventional HBV markers.\(^7\)

We have examined acute and convalescent specimens from 23 consecutive patients with fulminant NANB hepatitis who have undergone liver transplantation in the Birmingham Liver Unit. We have sought evidence of hepatitis B, hepatitis C, and hepatitis E virus infection.

Methods

PATIENTS
One hundred and thirty one patients with fulminant hepatic failure were admitted to the liver unit during a two year period (1 September 1989-31 August 1991). More than 50% (74 of 131) were victims of paracetamol poisoning. A diagnosis of NANB hepatitis was made in 42 patients. All 42 were negative for HAV IgM, HBsAg, and IgM anti-HBc.

Twenty three (16 female) of 42 subsequently underwent liver transplantation. Median age at the time of transplantation was 47 years (range 18-60). The median interval from the onset of symptoms until the development of encephalopathy was five weeks (range 1-12 weeks). All patients were encephalopathic at the time of transplantation (five grade 2, four grade 3, and 14 grade 4 coma). Median serum bilirubin at the time of transplantation was 614 μmol/l (371-919) and median prothrombin time was 49 seconds (20-72).

SEROLOGY

Acute and convalescent (in patients surviving transplantation) serum samples were stored at \(-20^\circ\)C until examined by enzyme linked immunosorbent assay (ELISA) for anti-HCV (ELISA-2, Ortho Diagnostics). Convalescent serum samples were examined for IgG antibody to hepatitis B core antigen – anti-HBc (Abbott Diagnostics).

Convalescent serum samples were also screened using an enzyme immunoassay for the detection of antibody to HEV (under development by Abbott Diagnostics). This assay entails incubation of test and control samples with a polystyrene bead coated with recombinant HEV proteins SG3 and 8-5, representing sequences of the open reading frames 2 and 3 of the Burmese isolate of HEV. After washing, bound immunoglobulin is detected using horseradish peroxidase labelled goat antibodies to human Ig. After incubation and further washing, O-phenylenediamine solution containing hydrogen peroxidase is added. A yellow-orange colour develops in reactive wells. Those samples with an absorbance value above the cut off are designated positive.
VIRAL NUCLEIC ACID DETECTION
Acute serum samples were examined by polymerase chain reaction for HCV ribonucleic acid (RNA).

Explant liver tissue was immediately frozen in liquid nitrogen and stored at −70°C until examined for HBV DNA and HCV RNA by polymerase chain reaction.

RNA was extracted from liver using RNAzol B (Biogenesis Ltd) and chloroform, followed by isopropanol precipitation. RNA was washed in 70% ethanol and resuspended in 100 μl of sterile distilled water.

For serum RNA extraction, 100 μl of serum was added to normal (HCV negative) liver and the same extraction method was used. The addition of normal liver to serum provides carrier RNA including albumin mRNA, which can be used as an internal control for subsequent reverse transcription and polymerase chain reaction steps. Appropriate negative (normal serum or liver) and positive (anti-HCV positive haemophilic serum and liver) were included in each assay. Polymerase chain reaction contamination was also excluded by the inclusion of a water substrate control in each experiment.

Five μl of each RNA extraction was used in a reverse transcription reaction (20 μl total volume) containing 20 U RNase inhibitor and 15 U AMV reverse transcriptase (Promega) in the presence of random primers.

Five μl of cDNA was then used in each polymerase chain reaction. A nested polymerase chain reaction using primers from the highly conserved 5′-non-coding region8 produced a 286 base pair (bp) product. Intron flanking primers for albumin cDNA polymerase chain reaction provided an internal control for each specimen (cDNA product 202 bp, genomic DNA product 1788 bp, intron size 1586 bp).

A plasmid containing a DNA copy of the 5′ nucleotides of the HCV genome (pKHc8, a gift of Dr B E Clarke) was used to assess the sensitivity of detection of HCV cDNA by polymerase chain reaction. Tenfold serial dilutions of the plasmid were prepared and added to separate reactions. The least number of input copies from which amplified products could be detected was three.

DNA was extracted from liver by overnight digestion in polymerase chain reaction buffer containing 100 μg/ml Proteinase K and 0-5% Tween 20. Proteinase K was inactivated by heating at 95°C for 10 minutes. Five μl of digest was used in a 50 μl polymerase chain reaction containing 5 U Taq polymerase (Boehringer-Mannheim). HBV primers amplifying a 416 bp fragment of the HBsAg gene were chosen.7 Appropriate negative (normal liver) and positive (liver biopsy from a HBsAg, HBeAg, DNA positive carrier with positive immunostaining for HBCAg) controls were included in each assay. β Globin DNA amplification acted as an internal control for each specimen.

Polymerase chain reaction products were visualised by ethidium bromide staining after electrophoresis in agarose gel.

A plasmid containing the target HBV sequence (p300) – a gift of Dr B E Clarke was used to assess the sensitivity of the method. Tenfold serial dilutions of the plasmid were prepared, and amounts corresponding to 200–200 000 copies were added to parallel reactions. The smallest number of input copies from which amplified products could be detected was 2000. A reconstruction was also carried out by adding dilutions of the plasmid to Menghini biopsy specimens of normal liver, and extracting with proteinase K overnight. The limit of detection was exactly the same as that using the plasmid directly diluted in water.

IMMUNOHISTOLOGY
Liver biopsy was performed as part of the annual review of all patients transplanted in Birmingham. Haematoxylin and eosin stained sections were examined and all specimens were stained for both HBsAg and HBCAg.

Results
Twelve of 23 patients died within three months of liver transplantation. Eleven surviving patients have a median follow up of 42 months (range 28–43) on 31 December 1993.

Pretransplant serum had been stored at −20°C and was available for subsequent analysis in 14 of 23 patients, including eight of 12 who subsequently died.

All pretransplant serum samples were negative for both anti-HCV and HCV RNA.

Convalescent serum samples (from 11 survivors) collected three to 26 months (median 10) after transplantation were also anti-HCV negative.

Explanted liver from 22 of 23 patients was available for examination by polymerase chain reaction. Pretransplant serum, but not liver, was available for one patient. HCV RNA could not be detected by polymerase chain reaction in explanted livers. Albumin mRNA could be detected in all explanted liver tissue by reverse transcription-polymerase chain reaction.

All patients were HBsAg and IgM anti-HBC negative at the time of presentation. Convalescent serum samples were examined for IgG anti-HBC nine to 38 months (median 23) after liver transplantation. No patient has developed anti-HBC during convalescence.

HBV DNA could not be detected by polymerase chain reaction in any of 22 livers available for analysis.

All surviving patients have had at least one annual review, and 17 follow up biopsy specimens were available for examination (six patients have had two annual reviews). Histological examination confirmed the presence of mild chronic hepatitis or non-specific inflammation in most specimens.

All specimens were examined for HBCAg and HBsAg by immunostaining. None was positive.

Convalescent serum samples were examined for IgG anti-HEV 6–43 months (median 29) after liver transplantation. Antibodies to HEV were not detected.
Fulminant sporadic NANB hepatitis

Discussion

The agent(s) responsible for the syndrome of fulminant NANB hepatitis remains unknown. This syndrome is clinically indistinguishable from other fulminant viral hepatitides. The diagnosis of fulminant NANB hepatitis is dependent on the absence of serological markers of both acute hepatitis A virus infection (IgM anti-HAV) and acute hepatitis B infection (IgM anti-HBc). In addition, exposure to known hepatotoxins should be excluded. These 23 patients with sporadic fulminant hepatitis fulfilled these criteria.

The viruses responsible for post-transfusion NANB hepatitis (HCV) and epidemic NANB hepatitis (HEV) have been recently characterised.

HCV infection is responsible for nearly all cases of classic post-transfusion NANB hepatitis. HCV is also responsible for acute and chronic NANB hepatitis associated with other forms of parenteral exposure, and is frequently incriminated in sporadic community acquired (non-parental) NANB hepatitis. Acute HCV infection is commonly asymptomatic and anicteric but is characterised by a propensity to chronicity. No irrefutable case of fulminant hepatitis C infection has been published, though concurrent HCV infection may be seen in patients with fulminant hepatitis caused by other viruses.

We have examined serum and liver from 23 consecutive patients transplanted for sporadic fulminant NANB hepatitis. We failed to detect HCV RNA in serum stored at the time of presentation (14 patients), and viral RNA could not be detected in the explanted livers (22 patients). Anti-HCV was absent from serum at the time of presentation (14 patients) and could not be detected in convalescent serum of 11 survivors (serum sampled up to 26 months after liver transplantation). Prospective studies of post-transfusion hepatitis have shown that anti-HCV is commonly absent at the time of an increase in transaminase activity, and may be present for a brief period only in patients with acute resolving infection. The absence of anti-HCV in both bile and convalescent serum samples of these patients with fulminant NANB would be consistent with the pattern seen in patients with acute resolving transfusion associated HCV infection. In the studies of post-transfusion hepatitis, however, HCV RNA was detected in serum and liver at the time of an increase in transaminase activity. The absence of HCV RNA from serum samples and liver tissue of these 23 patients at the time of presentation strongly suggests that fulminant sporadic NANB hepatitis is not due to HCV infection. Our findings are consistent with the recently published findings of other groups.

Fulminant hepatitis B infection is associated with (possibly due to) an overwhelming immunological response and destruction of HBV infected hepatocytes. This response may be associated with rapid and early clearance of serum HBsAg, but IgM antibody to the HBV core antigen (IgM anti-HBc) is a consistent serological finding, and usually present in high titre. In these 23 patients with fulminant NANB hepatitis, both HBsAg and IgM anti-HBc were undetectable in serum at the time of presentation. In addition, we could not detect IgG anti-HBc in the convalescent serum of 11 long-term survivors.

HBV DNA is often undetectable by dot blot hybridisation in the serum of patients presenting with fulminant HBV. HBV sequences may, however, be detected by polymerase chain reaction in the same setting. We have used primers from a highly conserved region of the HBV genome in examining 22 explanted livers for evidence of HBV infection. HBV sequences were not detected. Wright et al recently reported 'cryptic' HBV infection (diagnosis based on the detection of HBV sequences in explanted liver by polymerase chain reaction) in patients previously labelled as fulminant NANB hepatitis. Though chronic hepatitis B infection has been shown to occur in the absence of conventional serological markers, the absence of a measurable immunological response at the time of fulminant hepatitis B infection seems in contrast with current concepts of host-virus interaction in this setting. It is unlikely that cryptic HBV infection seen in patients described by Wright et al was responsible for the development of fulminant hepatic failure. The appearance of detectable HBSAg in the serum of some of these patients after liver transplantation is probably due to the immunosuppression of patients who had very low concentrations (undetectable by routine testing) of viraemia at presentation. Indeed, the same group of investigators subsequently report the occurrence of 'cryptic' HBV infection in patients transplanted for end stage chronic liver disease. The discovery of HBV infection after transplantation (when both donor and recipient were lacking serological markers pretransplantation) may reflect a high prevalence of antecedent HBV exposure in the donor or recipient populations, or both of that transplant programme.

HBV frequently reinfects the transplanted liver, in both acute and chronic hepatitis patients may be associated with aggressive hepatitis and with a characteristic histopathological appearance known as 'fibrosing cholestatic hepatitis'. Seventeen liver biopsy specimens (from 11 patients) taken one and two years after transplant were specifically examined for evidence of HBV infection. Inflammatory infiltrate was not graded as severe in any case, and changes of fibrosing cholestatic hepatitis were not seen. Immunohistochemical staining for both HBSAg and HBcAg was negative in the follow up biopsy specimens of all 11 survivors.

In summary, we have used sensitive serological and molecular methods in the examination of 23 consecutive cases of fulminant NANB hepatitis but found no evidence of HBV infection.

The cause(s) of sporadic fulminant NANB hepatitis remains a mystery. Sallie et al have incriminated hepatitis E infection in some patients presenting to King's College Hospital with fulminant NANB. This finding was,
The authors wish and the fulminant reported histological and from severe A NANB may acute plant liver for NANB hepatitis. It seems unlikely that HEV is a principal cause of fulminant NANB hepatitis in the United Kingdom. Possible aetiological agents include both environmental toxins and infectious agents. We have not seen temporal or geographical clustering of cases, suggesting either low infectivity or some form of host predisposition (like the predisposition of pregnant women to suffer liver failure when infected with HEV).

We have previously reported an increased incidence of chronic hepatitis in the post-transplant liver biopsy specimens of patients transplanted for fulminant NANB hepatitis. Aggressive chronic hepatitis progressing to cirrhosis has been described in the native liver of a patient undergoing auxiliary liver transplantation for fulminant NANB hepatitis. These findings are consistent with a viral aetiology for this syndrome.

Fulminant NANB hepatitis is the most common cause of acute liver failure treated by liver transplantation. Recurrent disease (reinfection) may occur but is rarely the cause of acute liver failure, a situation similar to that seen in patients transplanted for HCV infection. More than 80 patients with fulminant NANB have been transplanted in Birmingham. A single case of recurrent subacute hepatitis has been seen in this cohort—a 22 year old woman developed liver failure resulting from severe hepatitis that developed in the graft after 18 months of chronic hepatitis. She died shortly after retransplantation and histological changes in the damaged allograft were similar to those seen in the native liver.

The longterm prognosis (with respect to recurrent disease) of patients transplanted for fulminant NANB hepatitis remains uncertain.

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