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The occurrence of hepatitis B and C viruses in Pakistani patients with chronic liver disease and hepatocellular carcinoma

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SUMMARY

To study the occurrence of hepatitis B virus (HBV) and hepatitis C virus (HCV) in patients with chronic liver disease (CLD) and hepatocellular carcinoma (HCC) in Pakistan, blood samples from 105 sequential patients with biopsy-proven CLD ($n = 82$) and HCC ($n = 23$) were tested for HBV and HCV markers. Of the 105, 87 (83%) had evidence of hepatitis B exposure, 58 (55%) were positive for hepatitis B surface antigen (HBsAg), 23 (22%) had hepatitis C antibodies and 25 (24%) had detectable HCV RNA. Significantly more patients with HCC had evidence of HBV exposure in the absence of HCV markers (49/82 *vs.* 20/23, odds ratio 4·49, 95% CI 1·17–25·16). The proportion of patients positive for HBsAg with no HCV markers was also significantly higher in the HCC group (34/82 *vs.* 18/23, odds ratio 5·08, 95% CI 1·59–18·96). There were more patients with only HCV markers in the CLD group than the HCC group but the difference was not statistically significant (19/82 *vs.* 1/23, odds ratio 6·63, 95% CI 0·93–288·01). A modified non-isotopic restriction fragment length polymorphism study on PCR products was used to investigate the epidemiology of HCV genotypes in Pakistan. Due to depletion of the initial samples, a second series of specimens collected one year afterwards was used. Fifteen out of 40 samples had amplifiable product and all were identified as type 3. A commercial serological typing method on the same samples also confirmed that type 3 was the predominant HCV genotype in Pakistan.

INTRODUCTION

Both hepatitis B virus (HBV) and hepatitis C virus (HCV) are causally associated with chronic liver disease (CLD) and hepatocellular carcinoma (HCC) [1, 2]. They are both blood borne viruses although HBV is also efficiently transmitted via sexual and vertical routes [3]. In many parts of the world, it is not uncommon to find individuals at risk for both HBV and HCV infections.

In Pakistan, up to 10% of adults are carriers of hepatitis B surface antigen (HBsAg) [4]. In northern areas, 10·7% of army recruits were found to be HBsAg positive and 33·2% had evidence of past exposure [5]. The carrier state was most common in older age groups (41–50 years) suggesting that in addition to perinatal and childhood transmission, the infection is also transmitted among adults, probably through contaminated needles, blood products or sexual routes [6]. The prevalence of HCV in Pakistan is not certain. A preliminary study on 100 individuals with chronic liver disease using a first generation ELISA assay for antibodies to HCV (anti-HCV) suggested that up to 43% of patients with chronic hepatitis had antibody to HCV [7]. However, no studies of HCV antibody prevalence in the general population in Pakistan have been reported.

To clarify the occurrence of hepatitis B and hepatitis

C in CLD and HCC in Pakistan, we conducted a study using stored blood samples from Pakistani patients and tested them for both HBV and HCV markers. HCV genotyping was attempted on further samples in a later phase of the study.

MATERIALS AND METHODS

Patients and samples

Serum samples from 105 sequential patients with a liver biopsy done in the Military Hospital, Rawalpindi, Pakistan and reported as having chronic hepatitis ($n = 82$) or HCC ($n = 23$) by the Armed Forces Institute of Pathology between July 1987 and September 1992 were available for study. The sera were stored at -20 °C initially and were subsequently transported to Liverpool, UK for analysis of hepatitis B and C markers. A second set of 40 stored sera, from consecutive specimens tested positive for anti-HCV by the same institute in Rawalpindi during the first quarter of 1993 were used for HCV genotype studies. This set of samples had hepatitis markers tested initially in Pakistan and had undergone several freeze and thaw cycles before arrival in Liverpool for further studies.

HBV and HCV markers

All samples were tested for hepatitis B surface antigen (HBsAg) using an automated ELISA (VIDAS, Biomerieux, France); HBsAg negative samples were further tested for HBV core antibodies (Anti-HBc, VIDAS, Biomerieux, France). All samples were tested for anti-HCV using a commercial third generation ELISA based on recombinant baculovirus protein (Murex, UK) and for HCV RNA using a reverse-transcription nested polymerase chain reaction (RT-PCR) with primers from the highly conserved 5′ non-coding region (outer sense 5′ GCCATGGCGTTAGTAYGAGT 3′; outer antisense 5′ TTTCGCRACCCAACRCTACT 3′; inner sense 5′ AGTGTCRTRCAGCCTCCAGG 3′; inner antisense 5′ ACCCAACRCTACTMGGCTAG 3′) according to a method described elsewhere [8]. A positive result was indicated by the presence of a 171 base pair product in 3% agarose gel electrophoresis stained by ethidium bromide. Contamination was strictly controlled by standard procedures [9]. Samples with discrepant ELISA and RT-PCR results were further tested by a commercial western blot assay which recognized bands of baculovirus recombinant core, NS3, NS4 and NS5 proteins (Murex, UK). All commercial assays were used and interpreted according to manufacturers' recommendations.

HCV genotype

To study the prevalence of HCV genotypes in Pakistan, a second study was performed. Because no material was left for further examination after the first study, a second series of samples were tested. This consisted of 40 consecutive sera that were tested anti-HCV positive in Pakistan by a second generation ELISA assay (Abbott, USA). The same RT-PCR method as above was used for gene amplification. The resultant 171 bp amplicon was a subset of a fragment previously described for the genotyping of HCV using the method of restriction fragment length polymorphism (RFLP) [10]. The amplicons were digested by two separate mixtures of restriction enzymes, MvaI/HinfI and ScrfI/HinfI (Boehringer Mannheim, Germany). A non-isotopic method was used similar to that described by Majid and colleagues [11] and the digestion pattern was observed in 6% Nusieve GTG gel (Flowgen, USA) stained by ethidium bromide. The pattern observed was then compared to the expected patterns deduced from published data [10]. To verify the results of this modified RFLP typing method, 23 of these 40 samples, including all those that were RT-PCR positive, were further tested by a commercial serotyping kit (Murex Diagnostic, UK) which is based on type specific antibody response to synthetic peptides from the NS4 region of HCV [12]. The typing system used by both methods agreed with a recently proposed unified format of HCV genotype nomenclature [13].

Statistical analysis

Statistical analysis was performed with the help of STATCALC facility of the EPI INFO version 5.01b software (Center for Disease Control, Atlanta, USA) using Student's t-test, chi square test with Yates correction (χ^2) or Fisher's exact test as appropriate. Odds ratio (OR), 95% confidence interval (CI) and P value were calculated to determine significance.

RESULTS

Patients

The median age of patients in the study was 48 years (range 2–81) with 87 males and 18 females. Most of

Table 1. *HBV and HCV markers in patients with chronic liver disease (CLD) and hepatocellular carcinoma (HCC). Rawalpindi, Pakistan (1987–92)*

	CLD ($n = 82$)	HCC ($n = 23$)	P value	OR	95% CI
Mean age (range)	40 (2–81)	47 (2–76)	> 0·1*	—	—
HBsAg positive alone	34 (42%)	18 (78%)	0·0039†	5·08	1·59–18·96
HBsAg or anti-HBc positive alone	49 (60%)	20 (87%)	0·0293†	4·49	1·17–25·16
Anti-HCV or HCV RNA positive alone§	19 (23%)	1 (4%)	0·0327‡	6·63	0·93–288·01
Both HBsAg and HCV positive	5 (6%)	1 (4%)	0·6075‡	1·43	0·15–70·6
Both HBsAg and HCV negative‖	24 (29%)	3 (13%)	0·1924†	2·76	0·71–15·70

* Student's t-test.
† χ^2 test with Yates correction.
‡ Fisher's exact test.
§ 13 anti-HBc positive (CLD = 12, HCC = 1).
‖ 16 anti-HBc positive (CLD = 14, HCC = 2+1 equivocal).

the patients attending the hospital were male army recruits. Eighty-two (65 male, 17 female) patients had chronic hepatitis and cirrhosis of various grades (CLD group) and 23 patients (22 male, 1 female) had HCC. The mean age of patients in the HCC group was 47 (median 51) and was slightly higher than those in the CLD group (mean age 40, median age 45), but this difference was not statistically significant ($P > 0·1$, Table 1). No data were available on the transfusion history of the patients.

HBV markers

Fifty-eight (55%) of 105 samples were positive for HBsAg of which 39/82 (48%) were from patients with CLD and 19/23 (83%) with HCC. Fifty-two (90%) of these 58 patients had HBsAg alone with no HCV markers. The proportion of patients who were HBsAg positive only with no HCV markers were significantly higher in the HCC group than in the CLD group ($P = 0·0039$, Table 1). Of the remaining samples, 29/47 (62%) were anti-HBc positive. Thus, overall 87/105 (83%) patients had evidence of HBV exposure. When CLD and HCC were considered separately, 65/82 (79%) and 22/23 (96%) respectively had evidence of current or previous HBV infection.

HCV markers

Twenty-three (22%) of 105 samples had detectable anti-HCV by third generation ELISA and 25/105 (24%) samples were positive for HCV RNA by RT-PCR. Discrepant analysis showed that four samples were ELISA negative but RT-PCR positive and two were ELISA positive but RT-PCR negative. Western blot analysis of the discrepant samples showed that of the two ELISA positive RT-PCR negative samples, one was confirmed as anti-HCV positive but the other gave indeterminate serological results with reactivity against core antigen only. Of the ELISA negative RT-PCR positive samples, three were truly antibody negative but one had weak reactivity against core, NS4 and NS5 antigens by western blot and was thus a false negative ELISA. Hence, 23/105 (22%) were confirmed to have HCV antibodies of which 22 were RT-PCR positive; 25/105 (24%) were RT-PCR positive, of which 22 were anti-HCV positive. All patients with confirmed discrepant anti-HCV and RT-PCR results had cirrhosis on histological examination. The patient who was anti-HCV positive without HCV RNA had evidence of past HBV infection (anti-HBc positive, HBsAg negative). Of the three patients who were anti-HCV negative with detectable HCV RNA, two had no HBV markers and one was HBsAg negative, anti-HBc positive. Overall, 26/105 (25%) patients had evidence of HCV infection and 20 of these 26 patients (77%) had HCV infection in the absence of HBV markers. The proportion of patients with HCV infection only was higher in the CLD group than in the HCC group ($P = 0·0327$, OR 6·62, 95% CI 0·93–288·01, Table 1). Although the P value was less than 0·05, statistical significance was not established as the exact 95% CI contained zero and the range too wide.

HBV and HCV co-infection

Nineteen out of 105 patients (18%) had evidence of both HBV and HCV exposure. Six patients were

positive for both HBsAg and HCV RNA, 5 of whom had CLD and 1 had HCC. Twenty-seven patients were negative for both HBsAg and HCV markers, of whom 24 had CLD and 3 had HCC. However, 16 (14 CLD, 2 HCC) of these 27 had evidence of previous HBV exposure as anti-HBc was present. Overall only 10/105 (10%) patients had no evidence of exposure to either virus.

CLD and HCC

Patients in the HCC group had a significantly higher proportion of HBsAg positivity and more patients in this group had positive HBV markers but negative HCV markers than the CLD group ($P = 0{\cdot}0293$, Table 1). Only two HCC patients had any HCV markers (both anti-HCV and HCV RNA positive) but both had HBV markers as well. One HCC patient had no HBV or HCV markers, but in this case, the anti-HBc result was close to cut-off and should be classified as equivocal. There was an insufficient sample left to repeat or to verify with further tests. Seven patients, all with CLD, had only HCV markers but no HBV markers.

HCV genotype

Of the 40 samples available for the genotyping study, 15 (38%) were positive by RT-PCR. Using the modified method of RFLP, all of the 15 amplifiable samples showed a pattern corresponding to that of genotype 3 upon digestion. All 15 samples were subjected to the serotyping assay, and 12 were type 3, 1 type 1 and 2 were untypable. Eight RT-PCR negative samples were also serotyped, of which 4 were type 3 and 4 were untypable. The results of the serotyping assay largely corresponded to that of RFLP. Neither of these two assays, however, allowed further subtyping into 3a or 3b.

DISCUSSION

This study provided some information about the occurrence of HBV and HCV in patients with CLD and HCC in Pakistan. However, it was limited by the absence of a control group without liver disease. The prevalence of hepatitis C in normal Pakistani population and the risk factors for each patient with regard to HBV and HCV exposure were not known. Hence, the assumption that patients with CLD and HCC came from a similar population has to be made during statistical analysis. Despite these shortcomings, our results suggest that 90% of the studied patients were infected with either HBV or HCV at some time and up to 18% were infected by both viruses. HBV appeared to be an important cause of both CLD and HCC in Pakistan as more than 55% of patients were HBsAg positive and 87% had evidence of exposure. The role played by HCV was probably less than that of HBV as only 25% of patients had HCV markers and most HCV infected patients had CLD rather than HCC. HBV had a greater association with HCC than HCV as a significantly higher proportion of HCC patients in this series were HBsAg positive or had evidence of previous HBV exposure. In contrast, there were more patients with HCV markers alone in the CLD group than HCC. Statistical significance, however, was not established probably because the number of patients studied was too small. This association should be clarified with a larger study.

About 10% of patients had neither HBV nor HCV markers. Because we did not use molecular methods for HBV detection, it was possible that some of these could have had HBV infection without serological markers [14]. Alcohol consumption is not a significant problem in Pakistan but chemicals such as aflatoxin in the environment may be the cause of CLD or HCC in a few cases. The role of new hepatitis agents such as hepatitis G is not certain and should also be considered [15].

The effect of the interaction between HBV and HCV in patients infected with both viruses is controversial. Studies from Taiwan suggested that hepatitis C infections displaced hepatitis B virus and became the dominant cause of chronic liver disease [16, 17]. Other studies have reported conflicting results and suggested that hepatitis C viraemia is less common in the presence of hepatitis B infection [18–20]. The age at which each infection is acquired, the sequence of infection and the predominant viral genotype may be important in determining the pattern of interaction. It is possible that different geographical areas have a different pattern of interaction dependent on the local epidemiology of the two viruses. To investigate this, it is necessary to use molecular methods that detect viral genome in addition to conventional serological methods. The discrepancy between anti-HCV and HCV RNA in this study indicates that it is possible to detect nucleic acid in the absence of antibody despite using a third generation immunoassay. Similarly, it is possible that some of the anti-HBc positive, HBsAg negative patients in this

series were HBV DNA positive in the absence of detectable antigen [14].

Strong evidence for a causative role of HCV in HCC was reported from South Africa [21] and Japan [22] but the association is less clear in countries such as Southern China [23]. The geographical variation in prevalence of HCV in association with HCC may in part be due to the different assay systems used [24], the locally prevalent genotype of virus [25] or viral load [26]. Since there were insufficient data on the prevalent genotypes of HCV in Pakistan, the second phase of this study was carried out as an attempt to address this. The number of RT-PCR positive samples (15/40) in the second phase of the study was lower than expected and this was probably the result of repeated freeze and thaw of samples during storage and transport. Also, the ELISA results were not further verified by immunoblot assay and some may represent false positive results. The first set of samples in this study was collected over a 5-year period and the samples used for typing were collected 1 year afterwards. This raised concern on the validity of the genotyping results as the samples used for genotyping did not come from the same groups of patients with CLD or HCC and the predominant genotype may change with time. However, phylogenetic studies by others have shown that although changes in sequence is common, the evolution of major genotypes develop over 100–120 years [27]. Introduction of new genotypes should be considered, but the chronicity of the illness and the almost uniform detection of a single genotype did not support this suggestion. The modified RFLP method used is much simpler than the original method and did not require radio-isotopes [10, 11]. The result was largely in agreement with the commercial serotyping assay and this is similar to the experience of others comparing different genotyping and serotyping methods [28, 29]. Since the RFLP method only typed the predominant strain whereas the serological method tested for the presence of type-specific antibodies, discrepancy is possible if a patient has had infections by multiple genotypes. This may explain the single discrepancy in this series.

HCV Type 3 is known to cause less severe disease than type 1, especially 1b [27, 30]. This may account for the less dominant role of HCV in HCC compared to HBV found in this study. However, the design of this study did not allow the study of HBV and HCV interaction directly. If the predominant type of HCV is an important factor in determining the interaction, the results from other geographical areas which have different HCV genotypes may not apply to Pakistan [16, 18]. It will be interesting to study HBV and HCV interaction and their relative associations with HCC in neighbouring countries such as South India where HCV genotype 1 rather than type 3 is the predominant subtype [31].

HBsAg prevalence studies suggest that HBV is acquired in both childhood and adulthood in Pakistan [6]. Perinatal and horizontal transmission of hepatitis B in early childhood probably account for much hepatitis B carriage, and transmission at this age is less likely to be associated with hepatitis C transmission [32]. However, there is a strong cultural pressure in Pakistan to request injections for many ailments, and we speculate that this accounts for a proportion of cases who acquire hepatitis B in adulthood, as in South India [33]. Hepatitis C could also be acquired by this route, and both viruses may be transmitted by unscreened blood transfusions. Unfortunately, data were not available on the transfusion and injection histories of our patients. The role of sexual transmission of hepatitis B in Pakistan has not been defined and this needs to be explored as well.

Further studies are needed to clarify the epidemiological importance of iatrogenic spread of HBV and HCV and their interaction in the Indian subcontinent. It is necessary to conduct surveys to obtain basic data on the prevalence of HBV and HCV in the general population. Future studies should be prospective and include a larger number of patients and a control group without liver disease. Patients dually infected with HBV and HCV should be enrolled from Pakistan as well as from neighbouring countries to study the epidemiology of different infecting genotypes of hepatitis C in relation to concurrent hepatitis B infection and to correlate with clinical severity. Additional molecular methods such as HBV DNA assay or HBV PCR in conjunction with quantitation of HBV and HCV viral load should be used.

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