Serum and tissue positivity for hepatitis B virus markers in histopathologically proven glomerulonephropathies

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Summary. To assess the pathogenic significance of hepatitis B virus (HBV) in glomerulonephritis (GN), 98 patients with histopathologically proven glomerulonephropathies were screened for HBV markers, complement components and levels of circulating immune complexes (CICs); and renal biopsies from 31 of them were examined for the presence of hepatitis B surface antigen (HBsAg), and its location, by immunoperoxidase staining. The HBsAg positive rate in the patients (who came from a population with 10% HBsAg positivity) ranged from 51.9% in minimum change nephrotic syndrome (MCNS) to 81.8% in patients with proliferative glomerulonephritis (PGN). Whereas 24.5% of the cases were positive for HBsAg only, 10.2% had anti-HBcIgM with HBsAg, 13.3% had HBeAg with HBsAg and 9.2% had HBsAg, HBeAg and anti-HBcIgM. Complement component C3 levels were decreased in all groups of GN studied, but C4 levels varied. CIC levels were significantly increased ($p < 0.01$) only in HBsAg-positive MCNS, focal glomerulosclerosis (FGS) and membranous glomerulonephritis (MGN). Of the 31 renal biopsies examined for the deposition of HBsAg, 4 (12.9%) were found to be positive for HBsAg *in situ*; 64.5% of biopsied patients were seropositive for HBsAg and 77.4% had CICs. All the four in-situ HBsAg-positive cases were seropositive for HBsAg, HBeAg and anti-HBcIgM with significantly high CIC levels ($p < 0.01$). HBsAg deposition was intracytoplasmic in the mesangial cells of the glomeruli, in the glomerular basement membrane or in the tubules, or in a combination of these sites.

Introduction

The association between hepatitis B virus (HBV) infection and membranous glomerulonephropathy was first reported by Combes *et al.* (1971). Since then there have been several reports (Takekoshi *et al.*, 1978; Slusarczyk *et al.*, 1980; Levy *et al.*, 1982; Hsu *et al.*, 1983); all, except our own (Thyagarajan *et al.*, 1985), were from countries other than India. To define more precisely the extent of the association of HBV with glomerulonephropathies (GN) and the pathogenic significance of such association, we examined 98 histopathologically proven cases of GN for HBV markers (HBsAg, HBeAg, anti-HBcIgM, anti-HBs and anti-HBe), complement components and circulating immune complexes (CICs) in the serum and in some of our patients for in-situ HBsAg in renal biopsies.

Materials and methods

Patients

A total of 98 patients attending the nephrology departments of the Institute of Child Health and Hospital for Children, Madras and Government Stanley Hospital, Madras was studied. As well as clinical characterisation, they were confirmed histopathologically by the procedure of Anderson and Kissane (1977) as cases of minimum change nephrotic syndrome (MCNS) 27, membranous glomerulonephritis (MGN) five, membranoproliferative glomerulonephritis (MPGN) 18, diffuse proliferative glomerulonephritis (DPGN) 15, focal glomerular sclerosis (FGS) 13, and proliferative glomerulonephritis (PGN) 11. The remaining nine cases included crescentic glomerulonephritis (CGS), transplanted kidneys with chronic rejection, and others. Also included in the study were 106 (20 children and 86 adults) apparently healthy, controls of similar age and sex distribution.

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Serum markers

Serum samples from all these patients were screened for HBV markers (HBsAg, HBeAg, anti-HBcIgM, anti-HBs and anti-HBe) by ELISA with commercial kits (Hoechst Pharmaceuticals India Ltd, and Abbott Laboratories, Chicago). Complement components C3 and C4 were estimated by the single radial immunodiffusion (SRID) method of Mancini *et al.* (1965). Circulating immune complex (CIC) levels were measured by the polyethylene glycol (PEG) precipitation technique of Haskova *et al.* (1978); CICs were precipitated with PEG 4·16% (g/100 ml of distilled water) and measured spectrophotometrically; the OD readings gave a PEG index which was multiplied by 100 to give a PEG percentage measurement of CIC levels in comparison with the levels in normal controls (100 PEG%).

Biopsies

As the original renal biopsy blocks from 67 of the patients were not available, only 31 of them (MCNS seven, MGN four, DPGN eight, MPGN eight, FGS two and transplant kidney with chronic rejection two) could be examined for the presence of HBsAg *in situ* by the immunoperoxidase method, with the universal immunoperoxidase staining kit supplied by Ortho-diagnostics (P) Ltd, India (controls, figs. 1 and 2).

Results

Of the 98 patients, 56 (57·1%) were HBsAg seropositive. The highest proportion of HBsAg-positive patients was found amongst those with MGN (80·0%) and PGN (81·8%), whereas fewer of those with FGS (61·5%), DPGN (60·0%), MCNS (51·9%) and others were seropositive (table I). The

Fig. 1. Biopsy no. 306 (344 ×). Kidney biopsy section showing two glomeruli without immunoperoxidase positivity—negative control.

Fig. 2. Biopsy no. L.B. (215 ×). Biopsy section of hepatoma liver showing immunoperoxidase positivity for HBsAg—positive control.

Table I. Occurrence of HBsAg in renal disorders by histopathological classification

Patient group	Histopathological condition	Children		Adults		Total	
		Number tested	HBsAg positive	Number tested	HBsAg positive	Number tested	HBsAg positive
A	Minimum change nephrotic syndrome (MCNS)	13	6	14	8	27	14
B	Focal glomerulosclerosis (FGS)	8	5	5	3	13	8
C	Membranous glomerulonephritis (MGN)	1	0	4	4	5	4
D	Membranoproliferative glomerulonephritis (MPGN)	10	5	8	2	18	7
E	Diffuse proliferative glomerulonephritis (DPGN)	8	5	7	4	15	9
F	Proliferative glomerulonephritis (PGN)	8	7	3	2	11	9
G	Crescentic glomerulonephritis (CGN)	0	0	3	1	3	1
H	Miscellaneous group	0	0	6	4	6	4
Total		48	28	50	28	98	56

percentage of HBsAg-positive patients was approximately the same in children and adults. Of the 106 controls one of 20 children and 7 of 86 adults were seropositive for HBsAg only.

Testing the patients' sera for the other HBV markers showed that 24.5% had HBsAg only, 10.2% had anti-HBcIgM and HBsAg, 13.3% had HBeAg and HBsAg, and 9.2% had HBsAg, HBeAg and anti-HBcIgM. Interestingly only 4.1% of the patients had anti-HBs and 2.0% had anti-HBe as well as anti-HBs (table II).

Estimation of complement components (C3 and C4) and CIC levels revealed that the C3 level was decreased in all histopathological groups of GN; C4 was significantly decreased in HBsAg-positive PGN and FGS ($p < 0.01$) and increased in HBsAg-positive DPGN. Similarly a significant decrease of C4 was seen in HBsAg-negative MGN and DPGN, whereas in HBsAg-negative MPGN it was the same as in controls and higher than in HBsAg-positive MPGN ($p < 0.01$) (table III).

CIC levels were increased only in HBsAg-positive MCNS, FGS, MGN and CGN cases ($p < 0.01$) whereas MPGN, DPGN and PGN cases had low levels of CICs whether they were HBsAg-positive or -negative.

To study the association of HBV with GN in the kidney, and the pathogenic significance of such association, renal biopsies from 31 patients were examined for the presence of immune complexes (IIC) specific for HBsAg *in situ* by the immunoperoxidase technique with paraffin sections. Serum HBsAg status and levels of CICs are correlated with the finding of IIC in table IV. Of these 31 cases, 20 (64.5%) were HBsAg seropositive, 24 (77.4%) had increased levels of CICs and four (12.9%) had IIC specific for HBsAg. One case each of MGN, DPGN, MPGN and transplanted kidney with chronic rejection had IIC, HBsAg in serum, and higher levels of CIC than did the controls. All these four cases also had HBeAg and anti-HBcIgM

in their sera and none of them had either anti-HBs or anti-HBe (table V).

Sections stained by the immunoperoxidase method and examined by light microscopy showed, in these four cases, HBsAg in the glomeruli (probably mesangial cells), glomerular basement membrane (GBM) and in the tubules, either singly or in combination (figs. 3–6).

Discussion

In recent years the possibility of a pathogenic role for HBV in glomerulonephropathies has been reported frequently. These reports have stressed the common association of HBV infection with two types of glomerular diseases—MGN and MPGN

Fig. 3. Biopsy no. 1179 (215 ×). Renal biopsy section showing immunoperoxidase positivity, arrowed, for HBsAg in the mesangial cells of the glomeruli and tubules.

Fig. 4. Biopsy no. 1696 (215 ×). Renal biopsy section showing immunoperoxidase positivity, arrowed, for HBsAg in the glomerular basement membrane and mesangial cells of the glomeruli and tubules.

Table II. HBV markers pattern in 98 histopathologically proven cases of GN

Marker pattern	Number (percentage) positive
HBsAg alone	24 (24.5)
HBsAg and anti-HBcIgM	10 (10.2)
HBsAg and HBeAg	13 (13.3)
HBsAg, HBeAg and anti-HBcIgM	9 (9.2)
Anti-HBs	4 (4.1)
Anti-HBs and anti-HBe	2 (2.0)

Table III. Serum complement components (C3, C4) and circulating immune complex (CIC) levels in 48 children and 5 adults with histopathologically classified renal disorder

Histopathological condition and serum HBsAg status	Number tested	Mean (SD) complement component levels mg/dl		Mean (SD) CIC level (PEG %)
		C3	C4	
MCNS				
HBsAg-positive	14	36.2 (11.8) *p < 0.1	33.8 (9.6) *NS	178.7 (96.6) *p < 0.01
HBsAg-negative	13	35.8 (9.7) †NS	33.9 (21.2) †NS	111.5 (69.6) †p < 0.10
FGS				
HBsAg-positive	8	34.4 (12.6) *p < 0.01	16.5 (10.4) *p < 0.01	215.8 (68.1) *p < 0.01
HBsAg-negative	5	40.9 (5.5) †NS	24.3 (10.9) †NS	121.0 (33.8) †p < 0.01
MGN				
HBsAg-positive	4	48.9 (13.9) *p < 0.01	38.3 (12.7) *NS	298.0 (94.2) *p < 0.01
HBsAg-negative	1	40.9	21.3	127.8
MPGN				
HBsAg-positive	7	48.4 (20.9) *p < 0.01	21.9 (11.2) *NS	109.3 (72.4) *NS
HBsAg-negative	11	40.8 (2.7) †NS	32.9 (9.5) †p < 0.05	96.1 (75.8) †NS
DPGN				
HBsAg-positive	9	35.6 (13.3) *p < 0.01	38.5 (11.9) *p < 0.05	113.9 (81.1) *NS
HBsAg-negative	6	38.7 (1.3) †NS	18.8 (3.7) †p < 0.01	132.8 (53.3) †NS
PGN				
HBsAg-positive	9	40.0 (1.1) *p < 0.01	17.5 (3.7) *p < 0.01	108.7 (74.9) *NS
HBsAg-negative	2	42.7 (6.1) †NS	25.8 (6.6) †NS	136.1 (2.2) †NS
CGN				
HBsAg-positive	1	36.2	28	221.0
HBsAg-negative	2	51.1 (4.1)	28.3 (3.7)	60.4 (15.6)
Miscellaneous cases				
HBsAg-positive	4	43.6 (2.8) *p < 0.01	21.8 (11.2) *NS	243.9 (11.4) *p < 0.01
HBsAg-negative	2	30.5 (10.5) †NS	15.8 (1.9) †NS	244.2 (76.4) †NS
Matched controls				
Children				
HBsAg-positive	1	68.4	24.8	108.2
HBsAg-negative	19	72.8 (4.6)	28.2 (10.2)	114.4 (16.4)
Adults				
HBsAg-positive	9	82.6 (3.2)	26.7 (2.2)	105.3 (7.8)
HBsAg-negative	77	78.7 (2.6)	31.3 (10.8)	112.3 (8.7)

* HBsAg-positive *vs* HBsAg-positive controls.† HBsAg-positive *vs* HBsAg-negative.

NS = Not significant.

Fig. 5. Biopsy no. 1675 (860 $\times$). Renal biopsy section showing immunoperoxidase positivity, arrowed, for HBsAg in the mesangial cells of the glomeruli alone.

Fig. 6. Biopsy no. 1179 (215 $\times$). Renal biopsy section showing immunoperoxidase positivity, arrowed, for HBsAg in the tubules alone.

(Levy *et al.*, 1982). In our present series we too found the highest prevalence of HBsAg in MGN (80%). After the first report of Combes *et al.* (1971) demonstrating HBsAg in MGN, several workers reported HBV markers in 20.6–100% of MGN (Takekoshi *et al.*, 1978, 11 of 11; Slusarczyk *et al.*,

1980, 24 of 98; Hsu *et al.*, 1983, 13 of 63, Hirose *et al.*, 1984, 10 of 16).

Besides MGN, as in the present series, MPGN (Knieser *et al.*, 1974; Myers *et al.*, 1973; Slusarczyk *et al.*, 1980; Levy *et al.*, 1982; Lai *et al.*, 1987), FGS, MCNS, diffuse extra capillary and miscellaneous

Table IV. HBsAg, CIC and IIC positivity in histopathologically classified renal disorders

Histopathological condition	Number tested	Serum HBsAg positive	CIC Level		IIC positive
			Number positive	Mean (SD) PEG %	
MCNS	7	2	5	128.7 (36.5)	0
FGS	2	2	2	128.2 (62.1)	0
MGN	4	3	3	292.1 (68.2)	1
DPGN	8	8	5	183.9 (21.1)	1
MPGN	8	3	7	118.3 (27.4)	1
Chronic rejection	2	2	2	222.0 (11.4)	1
Total	31	20 (64.5%)	24 (77.4%)	178.8 (68.5)	4 (12.9%)

Table V. HBV markers pattern in IIC-positive patients

Histopathological condition	HBV marker positivity					IIC	CIC
	HBsAg	Anti-HBs	HBeAg	anti-HBe	anti-HBcIgM		
MGN	+	—	+	—	+	+	+
MPGN	+	—	+	—	+	+	+
DPGN	+	—	+	—	+	+	+
Chronic rejection	+	—	+	—	+	+	+

+ = positive; — = negative.

groups (Slusarczyk *et al.*, 1980) and IgA nephropathies (Lai *et al.*, 1987) have also been shown to be associated with HBs antigenaemia.

Animal experiments and observations on human subjects have demonstrated that HBV-containing immune complexes may be formed in the course of HBV-induced acute and chronic hepatitis (Slusarczyk *et al.*, 1980; Lesavre *et al.*, 1982). Immune complex glomerulonephritis has been reproduced in an experimental model by inoculation of baboons with human HBsAg (Gyorkey *et al.*, 1975) and immune complex glomerulonephritis has been demonstrated in patients with acute hepatitis B infection (Ozawa *et al.*, 1976).

Immune complex-mediated glomerulonephritis is held to require a continuous supply of antigen and a maintained antibody response for development and progression of the disease. Accepting this hypothesis, the finding of increased levels of CICs in our HBsAg-positive GN patients, who are drawn from a population with a high (around 10%) HBsAg carriage rate (Thyagarajan *et al.*, 1985), strongly suggests that HBsAg immune complexes contribute to the pathogenesis of glomerulonephritis in such patients; and confirms the conclusions of others (Vos *et al.*, 1973; Furuse *et al.*, 1982).

Demonstration of HBV-specific antigens within the glomeruli has been considered to be strong evidence for the aetiological association of HBV with GN. The present study showed HBsAg deposits in the renal biopsies from only 12.9% of the cases tested.

Slusarczyk *et al.* (1980) demonstrated the presence of HBsAg and HBeAg and hypothesised that only the low mol. wt components of these antigens might be involved in the formation of immune complexes. Takekoshi *et al.* (1979) and Hirose *et al.* (1984) have demonstrated the presence of HBeAg and suggested that this antigen, whose mol. wt is below 3×10^3 Kda, could easily induce the forma-

tion of subepithelial deposits, whereas the deposition of HBsAg, whose mol. wt is high, appeared unlikely. This might also explain the low rate of HBsAg positivity in renal biopsies in this series.

Most workers have reported immunofluorescence (IF) studies of the renal parenchyma (Myers *et al.*, 1973; Brzosko *et al.*, 1974; Knieser *et al.*, 1974) and HBsAg has been demonstrated in association with immunoglobulins along capillary walls or in the mesangium, or both. Glomerular deposits of immune complexes containing HBsAg or HBeAg, or both, were detected in 41, 61 and 60% of cases of membranous nephropathy, IgA nephropathy and MPGN respectively by Lai *et al.* (1987).

The pattern of immunoperoxidase positivity for HBsAg in renal biopsies, as observed in the present series, has shown HBsAg deposits in the mesangial cells (intracytoplasmic), GBM and tubules; one or all of these sites being affected. Having reviewed previous reports we find that the only report, apart from this present one, of the demonstration of HBsAg in paraffin sections by the immunoperoxidase method and light microscopy is that of Lai *et al.* (1987). Ours may be the first report to show HBsAg in tubules. Combes *et al.* (1971), Hsu *et al.* (1983), Kohler *et al.* (1974) and Blaker *et al.* (1974) have demonstrated HBsAg in the mesangial cells and GBM alone. Even though Slusarczyk *et al.* (1980) and Knieser *et al.* (1974) have employed the immunoperoxidase technique, their observations for HBsAg have been by electronmicroscopy.

On the basis of the finding of HBsAg, HBeAg and anti-HBcIgM in the IIC (HBsAg) positive cases and the intracytoplasmic localisation of HBsAg in the mesangial cells of the glomeruli, it is tempting to suggest that the kidney is a secondary site of HBV multiplication. However it requires further in-depth electronmicroscopic studies to confirm the present observations.

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