Circulating HBsAg/IgG Complexes in idiopathic Chronic Glomerulonephritis

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We measured HBsAg/IgG complexes (CX) quantitatively by ELISA from HBsAg positive serum of 35 liver disease patients, 15 patients with glomerular diseases (8 membranous glomerulonephritis (MGN) 7 membarnoproliferative glomerulonephritis (MPGN)), and 50 healthy carriers of HBsAg. HbsAg/IgG CX was detected in 26 out of 35 liver disease patients (74.3%), 5 out of 50 healthy carriers (10%), and one of both 8 MGN patients (12.5%) and 7 MPGN patients (14.3%).

HBsAg/IgG CX was big in size and wide spreaded in the liver disease groups but negligible in the healthy carrier group (41±2.6 ng/ml, n=5) and MGN (50 ng/ml, n=1) and MPGN (42 ng/ml, n=1) group. In the liver disease group, no one suffered from glomerulopathy even with a wide spectrum of HBsAg/IgG CX.

These results suggest that HBsAg/IgG CX in plasma is non causative of MGN and MPGN.

Key Words: Chronic Glomerulonephritis, Circulating HBsAg/IgG Complexes

INTRODUCTION

The suggestion that hepatitis B virus (HBV) may be involved in a pathologic process leading to the development of chronic glomerulonephritis is derived from the clinical and immunological similarities of the HBV infection to serum sickness. Since the first report[1] of the association between HBV and membranous nephropathy, there have been several other reports[2~16] strongly suggesting that HBV, through an immune response elicited by one of its antigens, is causally related to the development of glomerular changes in humans.

On renal biopsy the lesion was variable including MGN[1,4,7,8,19~21], MPGN[3,4,15], Mesangioproliferative GN[5,9,13], and IgA GN[20~22], but the exact pathogenic mechanism by which certain individuals with chronic HBV infection develop

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glomerulonephritis is unknown. In spite of so many papers[4,7,10,12,13,19,20,22] demonstrating HBsAg in the glomerular capillary wall, HBsAg has been considered unlikely to deposit subepithelial space because of its large size ($>10^6$ dalton) and anionic charge[23], but it may be deposited by a trapping mechanism in the mesangial and subendothelial area regardless of its size and charge[23]. In our preliminary study, HBsAg/IgG was positive in HBsAg positive serum of 74.3% of the B viral liver disease group and 10% of the healthy control group. The presence of HBsAg/IgG CX reflects the presence of anti HBs in circulation even if this were not detected by a conventional assay system for anti HBs. So, the quantitative measurement of the HBsAg/IgG CX may reflect the characteristics and nature of anti HBs in each case.

In this regard, it is interesting how the prevalence rate and amount of HBsAg/IgG CX will be found in the HBsAg positive serum of MGN and MPGN patients.

PATIENTS AND METHODS

1. Reagent

HBsAg was isolated from HBsAg carrier by ultracentrifusion (3,360 μg/ml). Anti human IgG and HBs labeled with peroxidase were purchased from SIGMA.

2. Study Population

The age and sex distribution of the study population are presented in Table 1. It consists of 35 liver disease patients (11 acute B hepatitis; 9 CAH; 3 CPH; 7 LC; 5 hepatoma), and 15 patients with glomerular diseases (8 MGN; 7 MPGN), and 50 healthy control group. In all cases, HBsAg was positive. The healthy control group consisted of 50 cases who visited Soonchunhyang Hospital for a general check-up and the final result was free except for the HBsAg positive finding. CAH, CPH and MGN, MPGN were confirmed histologically by biopsy, but an immunofluorescence study for HBsAg was not performed in renal biopsy.

The diagnoses of acute hepatitis, liver cirrhosis, and hepatoma were based on accepted clinical, biochemical, serological, and other conventional criteria. HBsAg assays were done with a commercial radioimmune assay kit (Aus RIA 11-125, Abbott Laboratories) (Table 1, 2).

3. Preparation of Anti HBs (IgG)

The r-globulin fraction was separated from the patient's serum during the recovery phase by Gel filtration (Sephadex-200) and passed through a Sepharose 4B column which had been coupled with HBsAg. Then the elusion was followed with 3 M KSCN in 0.5 M NH$_4$OH.

4. Preparation of HBsAg/IgG Complex Standard

The standard HBsAg/IgG CX was prepared in vitro using purified HBsAg and anti HBs. After incubation at 37℃ for 2 hrs and overnight for 4℃, the immune complexes were precipitated by adding an equal volume of 5% polyethylene glycol (PEG) in PBS, washed with 2.5% PEG, dissolved in PBS, and dialyzed and stored at −70℃ before use. The molar ratio of HBsAg and anti HBs was 1 : 6 retrospectively. The protein concentration was measured by the Lowry method[24], and HBsAg/IgG CX was expressed as the equivalents (ng/ml) of protein.

5. Pretreatment of Serum for Immune Cmplex Asaay

To avoid contamination of the free-form HBsAg or anti HBs in the samples, the serum was diluted to 1 : 10 with 0.1 M boric acid containing 0.025 M borax and 0.075 M NaCl (pH 8.4 BBS), and 1 volume of diluted serum was mixed with 1 volume of 3.5% PEG in BBS. The mixture was incubated for 5 hrs at 20℃ followed by 13 hrs at 4℃ and then the complexed form of HBsAg/anti HBs was precipitated at 1600 g for 60 mins at 4℃. The pellet was washed and dissolved in PBS.

Table 1. Age and Sex Distribution of the Cases

Clinical Diagnosis	Age (years) Mean ± SD	Sex		(Total No.)
		(Male)	(Female)	
Liver disease				
Acute hepatitis	27.2 ± 9.2	10	4	14
CAH	33.5 ± 6.5	7	2	9
CPH	31.0 ± 6.4	1	3	4
Liver cirrhosis	48.6 ± 6.5	16	2	18
Hepatoma	53.5 ± 15.0	6	0	6
				(51)
Glomerulonephritis				
Membranous GN	35.5 ± 14.5	2	6	8
MPGN	40.7 ± 12.9	1	6	7
				(15)
Healthy carrier group	38.4 ± 22.4	40	10	(50)

Table 2. Laboratory Profiles of Glomerular Disease Patients

Diagnosis	Serum Creatinine (mg/dl)	24 hour Urine Protein (mg) (range)
MGN	1.4 ± 0.4	5,423 ± 5,117 (1,584—14,400)
MPGN	1.3 ± 0.6	2,700 ± 1,568 (1,467—4,800)

6. ELISA Procedure

A 96-well micro titer plate was coated with 50 μ l of goat anti human IgG (10 μg/ml) in bicarbonate buffer (pH 9.2). Post caoating was carried out with 2% BSA in PBS for 2 hrs at room temperature. The binding of IgG as well as IgG/HBsAg CX was attained by the addition of a test sample. The HBsAg moiety of HBsAg/IgG CX was revealed by addition of horseradish peroxidase conjugated with anti HBs (HRPO-A-HBs). After a 1 hr incubation at room tempeature, enzyme activity was measured by adding 50 μl of freshly prepared substrate solution (0.4 mg of 0-phenylene diamine per ml of 0.1 M phosphate citrate buffer, pH 5.0, containing 0.06% H_2O_2). The enzyme reaction was stopped by adding 50 μ l of 4N H_2SO_4 and absorbance read at 492 nm. Each sample was tested in duplicate, and based on the standard curve from the standard complex, the levels of HBsAg/IgG CX were expressed as equivalents (ng/ml) of protein.

7. Statistics

The prevalence rate of HBsAg/IgG CX was analyzed by the confidence limit of the rate at the level of significance ($p < 0.05$).

RESULT

1. Sensitivity and Specificity of the ELISA system

Various concentrations of HBsAg/IgG CX from 24 ng/ml to 6,000 ng/ml were made by diluting a stock solution of 0.6 mg/ml HBsAg/IgG CX in PBS and used to construct a standard curve for assessing HBsAg/IgG CX in serum. The lowest detection limit of this system was 24 ng/ml. Inter assay variation, determined from standard curves constructed on results from 9 ELISA plates set up on 3 different days, showed a mean 10±3.8 (range: 4. 7~16.5) (Fig. 1).

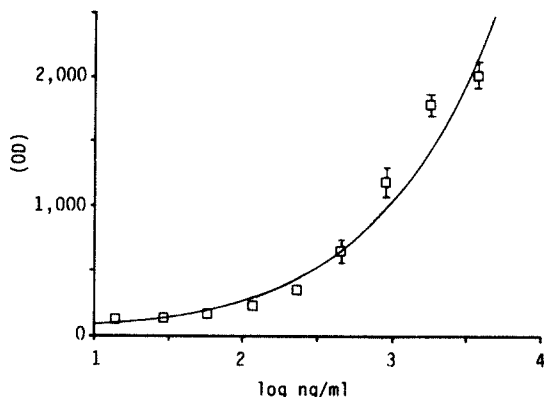$$y = 20.5451 * 10(0.5665x) \quad R=0.98 \qquad (n=9)$$

Fig. 1. *Sensitivity and reproducibility of ELISA for HBsAg/IgG complexes. The mean of coefficiency of variance from varying concentrations of standard HBsAg/IgG complexes (from 24 to 6,000 ng/ml) was 10.3±3.8 (range 4.7-16.5), with the curve constructed from 9 ELISA plates and set up on 3 different day.*
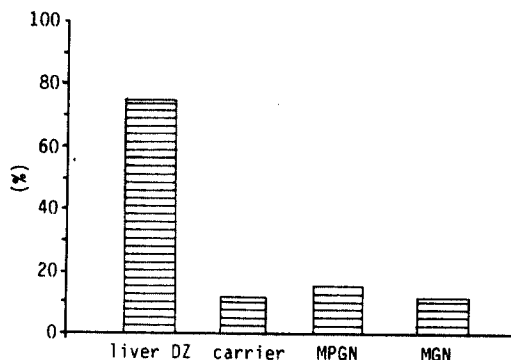

Fig. 2. *Prevalence rate of HBsAg/IgG CX in each group.*

The specificity was tested using HBsAg in PBS, human IgG, and the serum of 40 cases in which B virus related markers (HBsAg, anti HBs, anti HBc, HBeAg) were all negative.

2. The detection Rate and Mass of HBsAg/IgG CX

HBsAg/IgG CX was detected in 5 out of 50 in the healthy control group (10%), one of Both, 8 MGN patients (12.5%, 50 ng/ml) and 7 MPGN patients (14.3%, 42 ng/ml), and 7 out of 11 acute B hepatitis cases (63.6%), 8 out of 9 CAH patients (88.9%), all of 3 CPH patients (100%), 4 out of 7 liver cirrhosis patients (57.1%), and 4 out of 5
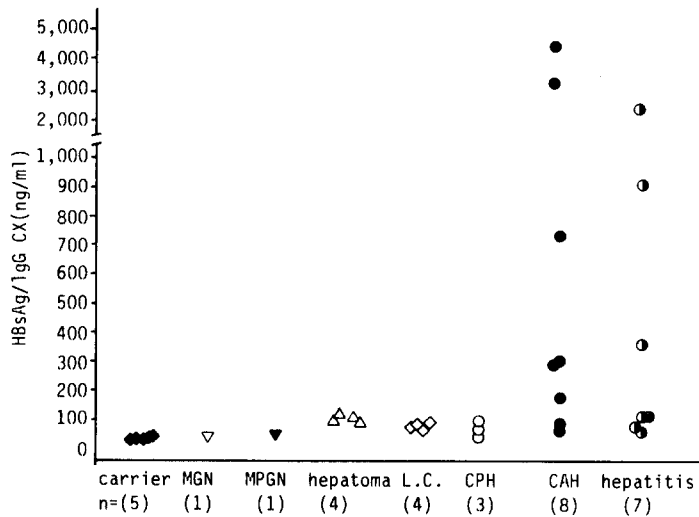
Fig. 3. *Distribution of HBsAg/IgG CX in various clinical states.*

hepatoma patients (80%).

The quantitative range of HBsAg/IgG CX was from 39 ng/ml to 45 ng/ml in the healthy control group (mean: 41 ± 2.6 ng/ml), from 62 ng/ml to 2, 588 ng/ml in the acute viral hepatitis (median: 172 ng/ml), from 66 ng/ml to 4,466 ng/ml in CAH (median: 298 ng/ml), from 64 ng/ml to 178 ng/ml in liver cirrhosis (median: 122 ng/ml). Because the distribution pattern was not normal, it could not be compared statistically, but the HBsAg/IgG CX look bigger in acute viral hepatitis and CAH than in CPH, liver cirrhosis and hepatoma patients (Fig. 2, 3).

DISCUSSION

Even though recent evidence suggests that, with few exceptions, circulating immune complex measurements do not correlate with disease activity[25], from the background of serum sickness animal models[26,27], it has been accepted that continuous formation of the immune complex is necessary to maintain the chronicity of GN.

The methods commonly used to gather the evidence that supports of the theory of HBV as a primary cause of immune complex GN were the detection of HBsAg in glomerular basement membrane in association with immune globulin and the detection of free form and/or complexed form of the HBAg and anti HBs in circulation.

Experimental studies[28] in rats indicate that

complexes with a molecular mass of 1,000,000 daltons or less precipitate in the glomerular basement membrane and subepithelial space to induce typical MGN. The 20 nm spherical HBAg particles, the most commonly demonstrated antigen, have a molecular mass of 3,000,000 daltons even without antibody[28].

In this regard, in spite of so many papers showing HBsAg deposited in the basement membrane, HBsAg seems impossible to pass through the size barrier of the basement membrane. Anyway, an immune complex containing small-sized HBsAg molecular fragments, but with the same immunoreactivity, may be responsible for the lesion[28].

The renal lesions that have been presented in literature[6,12~14] appear to span nearly the whole spectrum of renal pathology, although the MGN and MPGN have been more common. As far as the accumulated knowledge about the HBsAg/anti-HBs CX in pathologic process of glomerulonephritis is concerned, MGN may be due to an in situ HBsAg/antiHBs CX formation in the subepithelial space, and MPGN may be due to a trapping process of circulating HBsAg/anti HBs CX in the subendothelial space and mesangium.

The exact incidence of HBV-associated GN is not known, but several reports[5,12~14] suggest that, from cases of GN with immunofluorescent evidence of an immune complex lesion, HBsAg was positive in as many as 16.3~56.2% of cases.

Glomerular lesions could be influenced by

various factors including quantitiy and size of the complexes as well as by genetically-determined differences in individual host responsiveness[29].

If HBsAg is a primary cause of immune complex glomerulonephritis, the nature and characteristics of anti HBs, including avidity and/or affinity, the amount, isotype, and idotype of anti HBs should be the key of pathogenesis. Of course, the nature and characteristics of HBsAg/IgG CX should be influenced not only by the nature of anti HBs but also by the nature and amount of HBsAg. HBsAg may accumulate levels as high as 10^{14} particles or 200 μg/ml in the blood of HBV-infected individuals[30], and it will vary depending on the clinical situation. But in a chronic state of HBV infection, the concentration of HBsAg will be stable, and considering that more than 80% of the particle surface area can still be exposed even after saturation with anti HBs[31], the HBsAg surface area is not limiting the amount of antibody that can bind, the nature of anti HBs is a more important factor in HBsAg/IgG CX formation. In the liver diseases group, the range of HBsAg/IgG CX was wide, ranging from 62 ng/ml to 4466 ng/ml. Theoretically, HBsAg/IgG CX within these ranges might include various degrees of affinity and variable kinds of antibody class. The incidence of HBsAg/IgG CX was high in the B-virus related liver diseases group (57~100%), but it was 10% in the healthy carrier group and 14.3% in the MGN group and 12.5% in the MPGN group. The mass of HBsAg/IgG CX was large and variable in the liver diseases group but similar in the healthy carrier group (41±2.6 ng/ml, n=5) and in the MGN (50 ng/ml) and MPGN (42 ng/ml) groups.

With the finding that in the liver disease group, no one suffered from glomerulopathy even with various kinds of anti HBs, our result showed that the characteristics of antibody formed against HBsAg are similar to those between the healthy carrier group and the MGN and MPGN group. Circulating HBsAg and it's antibody seems to be non causative of MGN and MPGN in HBsAg positive individuals.

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