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Analysis of viral proteins in circulating immune complexes from chronic carriers of hepatitis B virus

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SUMMARY

Sera from 54 children (mean age 5·8 years) with chronic hepatitis B virus (HBV) infection were investigated for the presence of immune complexes containing HBV proteins. Clinical diagnosis was established by histology and biochemical markers and included chronic persistent (36 cases) or chronic aggressive (seven) hepatitis, liver cirrhosis (six) and HBV-mediated membranous glomerulonephritis (five). Circulating immune complexes were precipitated with 2·5% polyethylene glycol and analysed by immune blot using monoclonal antibodies against S, pre-S2 glycopeptide, pre-S1 and HBe/c epitopes. All sera, including those from 11 healthy HBV-negative blood donors contained PEG-precipitable substances, but the amount of precipitate did not correlate with the presence or amount of HBV proteins. The great majority (36 out of 40) of HBeAg-positive patients contained HBs proteins in immune complexes, but no detectable HBe protein. The immune complexes usually contained more pre-S1 than the free HBsAg particles from the same patient. The precipitates of anti-HBe-positive patients rarely contained HBV proteins (two out of 14) and, if so, in low amounts. During follow up of six patients we found that high levels of HBs-containing immune complexes may be correlated with subsequent elimination of HBV. This elimination is possibly initiated by binding of anti-pre-S1 antibodies to HBV and HBs particles.

Keywords hepatitis B virus chronic hepatitis B pre-S1 enriched immune complexes HBsAg HBeAg

INTRODUCTION

Acute and chronic hepatitis B virus (HBV) infection is usually characterized by large amounts of free HBV surface antigen (HBsAg) and e antigen (HBeAg) in the blood. Resolution of HBV infection requires elimination of these antigens and is usually followed by appearance of the corresponding antibodies, anti-HBs and anti-HBe. In chronic infections elimination of HBsAg is absent or incomplete. There are two groups of HBsAg carriers: those with and without HBeAg. HBV titres decrease dramatically in most cases if HBeAg disappears from the blood, even if HBsAg remains positive. Circulating immune complexes (CIC) were detected during the early phase of acute hepatitis B (Madalinski & Bragiel, 1979; Pernice *et al.*, 1979; Lambert *et al.*, 1980; Anh-Tuan & Novak, 1981). From follow-up studies in acute hepatitis B it was concluded that the early appearance of immune complexes (ICs) would indicate subsequent elimination of the virus from the organism (Madalinski & Bragiel, 1979; Markus *et al.*, 1986), but IgM–HBsAg complexes are considered to be a bad prognostic marker if they persist (Caredda *et al.*, 1982; Palla *et al.*, 1983; Frisch-Niggemeyer, Hofmann & Kunz, 1984). A negative role of the IgG4 subclass in HBsAg elimination was also suggested (Rath & Devey, 1988). CIC containing HBsAg have also been found in patients with chronic HBV (Brown *et al.*, 1983) and in healthy chronic HBsAg carriers (Palla *et al.*, 1983; Sansonno *et al.*, 1986). These complexes and even more ICs to HBeAg seem to play a role in IC-mediated diseases, like HBV-associated glomerulonephritis of childhood and in periarteritis nodosa (Ito *et al.*, 1981; Gupta & Kohler, 1984; Gregorek *et al.*, 1986). Until now, HBV antigens in ICs have been studied by ELISA, but the exact viral protein composition has not been analysed.

HBsAg consists of three protein domains: S, pre-S2, and pre-S1. Electrophoresis of reduced and denatured HBs proteins detects: (i) a small HBs protein pair (SHBs) of 24 or 27 kD which consists only of the S domain; (ii) a middle HBs protein pair (MHBs) of 33 or 36 kD which also contains the pre-S2 domain in a glycosylated form; and (iii) a large HBs protein (LHBs) of 39 or 42 kD which, in addition, contains the pre-S1 domain and the pre-S2 in a nonglycosylated form (Stibbe & Gerlich, 1983; Heermann *et al.*, 1984; Neurath *et al.*, 1985). HBeAg was

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characterized as a heterogeneous secreted product of the HBV core gene region of 16–25 kD (Bruss & Gerlich, 1988; Standring *et al.*, 1988; Seifer, Heermann & Gerlich, 1990). The structure of these HBV proteins has been recently reviewed by Alberti *et al.* (1990) and Gerlich *et al.* (1989).

The purpose of our study was to detect and analyse HBV proteins in the CIC from sera of children with various forms of chronic hepatitis B and HBV-mediated glomerulonephritis. CIC from sera were precipitated with 2·5% PEG and the precipitate obtained was analysed after dissociation with SDS by the technique of immunoblotting using monoclonal antibodies (MoAbs) against the sequential epitopes of the S, glycosylated pre-S2 and pre-S1 domain, and against HBe/c proteins. In selected serum samples we compared the composition of HBs proteins precipitated by PEG to those which remained in the supernatant.

We compared the occurrence and amount of viral proteins in CIC with the serological and clinical data. Our findings show a surprising link between occurrence of HBs-containing CIC and HBe antigenaemia.

MATERIALS AND METHODS

Serum samples were collected from 54 children: 49 with chronic hepatitis and five with HBV-mediated membranous glomerulonephritis (HBV-MGN). The children were under observation and treatment at the Department of Gastroenterology and Department of Nephrology, Children's Memorial Hospital, Warsaw. Children were considered to be chronically infected with HBV if they were HBsAg positive for 6 months or more. Average duration of HBs antigenaemia was 3·5 years for HBeAg positive patients, and 4·2 years for anti-HBe-positive patients. The histological diagnosis was established before entrance to our study. The mean alanine aminotransferase (ALT) levels at the time of biopsy were 149 U/l (range 21–403) for patients with chronic persistent hepatitis (CPH), 190 U/l (60–490) for chronic aggressive hepatitis (CAH), and 50 U/l (19–213) for liver cirrhosis (LC). Control serum samples from 11 blood donors were collected at the Blood Transfusion Service of the University of Göttingen.

Assays for HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc were performed with kits from Abbott Laboratories. Concentration of HBsAg in patients' sera was measured by electroimmunodiffusion (rocket electrophoresis) as described by Gerlich & Thomssen (1975). Anti-HBc IgM antibodies were tested by anti-μ capture ELISA (Gerlich *et al.*, 1986). Quantitative assay of HBV DNA was performed by dot blot hybridization as described by Zyzik *et al.* (1986).

Precipitation of ICs from sera was done according to the method of Creighton, Lambert & Miescher (1973), with modifications. Briefly, 400 μl of serum samples frozen only once, from HBsAg-positive children were centrifuged at 4°C and 8000 rev/min for 5 min in V-shaped vials. Two-hundred microlitres of 7·5% (w/w) PEG 6000 in 10 mM Tris, 130 mM NaCl, 1 mM EDTA, pH 7·4 (TNE buffer), were added to 400 μl of the clarified serum sample yielding a final concentration of 2·5%. After incubation at 4°C overnight, the obtained precipitate was spun down at 4°C and 8000 rev/min for 5 min, and washed twice with 800 μl of cold 2·5% PEG in TNE buffer. During the second wash, the amount of precipitate was estimated by spectrophotometry at 600 nm. Then the precipitate of approximately 20 μl was suspended in 40 μl of phosphate-buffered saline (PBS), pH 7·4. Six microlitres of this suspension were mixed with 6 μl of 2 × Laemmli starting buffer containing 6% SDS and 10% dithiotreitol. Thus, CIC obtained from 40 μl serum were analysed per run. SDS gel electrophoresis was performed with small 13% polyacrylamide slab gels as described by Gültekin & Heermann (1988) using the Mighty Small electrophoresis chamber (Hoefer Scientific Instruments, San Francisco, CA). Proteins were blotted to PVDF membranes (Immobilon, Millipore, Bedford, MA). Non-specific binding was blocked by 20% fetal calf serum. Purified HBsAg 20-nm particles as positive reference sample for HBs immune blots were prepared from carrier plasma as described by Gerlich & Thomssen (1975). HBcAg particles for the control of HBeAg immune blots were isolated and purified from transformed *Escherichia coli*, as described by Uy *et al.* (1986).

The protein composition of HBs particles which remained in the supernatant of 2·5% PEG precipitate was analysed by protein blotting. Particles were partially purified by the centrifugation for 1 h of 120 μl of supernatant through 50 μl of 20% (w/w) sucrose in an airfuge (Beckman) at 100 000 ***g***. The pellets were resuspended in 12 μl PBS and 6 μl were used for SDS gel electrophoresis.

For immunostaining, the following anti-HBV MoAbs were used: H166 for the S domain, kindly provided by Dr L. T. Mimms (Abbott Labs.) (Peterson *et al.*, 1984); MA18/7 for sequential epitope 29–36 of pre-S1 (Deepen *et al.*, 1990), Q19/10 for glycosylated pre-S2 (Heermann *et al.*, 1988) and MAE 1 for HBe/c protein, kindly donated by Dr E. Korec (Institute of Molecular Biology, Prague); a MoAb against complement component C3d was kindly provided by Dr O. Götze (Department of Immunology, University of Göttingen). Monoclonal antibodies were detected by rabbit anti-mouse IgG, peroxidase labelled (Dako, Glostrup, Denmark).

The staining intensity of HBs proteins from CIC was evaluated semiquantitatively by visual comparison with the reference sample of 0·6 μg HBsAg per lane. For SHBs and MHBs a score of 3 was given to CIC that contained as much SHBs or MHBs as the reference sample. A stronger staining in the CIC than in the reference sample was scored as 4, a weaker but still well detectable staining as 2, a very weak staining as 1, no staining as 0. For LHBs the reference sample was scored as 2, because it contained less LHBs than MHBs. Samples of CIC that were slightly stronger than the reference sample scored as 3, much stronger staining was scored as 4.

RESULTS

Serological data

The results of the assays performed in the 54 patients are summarized in Table 1 for 40 HBeAg-positive children and in Table 2 for 14 anti-HBe-positive children. For six patients, two serum samples were available at intervals of 7–14 months (1 year on the average). All the cases were ordered according to their serum concentration of HBsAg (in their first sample for patients with two samples), as measured by electroimmunodiffusion. The HBeAg-positive children had on average higher HBsAg concentration (median value 23·5 μg/ml) than the anti-HBe-positive children (median 8·1 μg/ml), but both groups showed a broad range of concentrations ($<0·5$–111 and $<0·5$–24 μg/ml, respectively).

Table 1. Clinical, histological, and serological data of HBeAg-positive, chronically infected children

Patient no.	Age (years)	Diagnosis	ALT (U/l)	HBsAg (μg/ml)	HBV DNA (genomes/ml)	Anti-HBc IgM	PEG precipitate (OD_{600})	Staining intensity in blots		
								SHBs	MHBs	LHBs
1	2	CPH	54	111	3×10^6	<10	0·365	1	2	2
1			54	44	8×10^7	<10	0·430	1	1	2
2	3	CPH	57	109	4×10^8	<10	0·070	4	1	2
3	6	CPH/MGN	nd	97	4×10^8	<10	0·190	1	0	1
4	2	LC	280	94	5×10^7	200	0·938	1	ND	2
5*	3	CPH	38	64	2×10^8	<10	0·092	3	ND	3
6	9	CPH	71	51	3×10^6	<10	0·204	2	4	ND
7	5	CPH	660	45	4×10^7	110	0·470	2	0	2
8	1	CPH	115	44	5×10^7	30	0·118	1	ND	2
8			169	55	3×10^8	50	0·370	2	1	2
9	6	CPH	60	43	1×10^8	<10	0·240	2	3	4
10	3	CPH	800	38	6×10^6	11	0·646	1	3	ND
11	9	CPH	84	37	5×10^6	<10	0·088	3	4	3
11			69	17	$<1\times10^5$	<10	0·060	2	4	2
12	11	CPH	28	35	3×10^6	<10	0·180	2	3	ND
13	2	CPH	112	35	9×10^6	ND	0·275	0	ND	ND
14	5	CPH	185	33	2×10^8	<10	0·125	2	1	2
15	11	LC	886	33	$<1\times10^5$	16	1·260	0	0	1
16*	6	CPH	82	31	9×10^6	73	0·990	3	2	3
17	10	CPH	67	31	3×10^7	28	0·490	2	0	2
18	5	CPH	65	26	4×10^5	9	0·410	4	4	ND
18			54	4	1×10^7	<10	0·475	3	3	3
19	7	CPH	25	25	$<1\times10^5$	<10	0·170	3	2	0
20	4	CAH	47	24	2×10^5	10	0·471	0	ND	ND
21	4	CPH	61	23	$<1\times10^5$	12	0·084	1	ND	ND
22	3	CPH	88	23	$<1\times10^5$	ND	0·164	3	3	ND
23	11	CPH	61	21	2×10^5	<10	0·096	0	ND	ND
24	5	CAH	203	20	9×10^6	138	0·170	2	2	3
25	6	CPH	161	20	1×10^7	28	0·400	2	0	1
26	2	CPH	137	19	5×10^6	12	0·106	1	ND	2
27	9	CPH	97	16	8×10^5	21	0·204	1	ND	ND
28	6	CAH	199	15	9×10^6	16	0·190	1	0	0
29	7	CPH	77	14	1×10^5	23	0·195	3	ND	ND
30	11	CPH	108	14	4×10^5	20	0·058	1	ND	3
31	2	CPH	121	12	1×10^7	29	0·400	3	1	2
32	12	LC	47	12	$<1\times10^5$	26	0·083	0	0	ND
33	3	CPH	53	11	6×10^6	10	0·600	1	0	0
34	4	CAH	511	11	8×10^5	37	0·102	3	ND	ND
34			34	78	4×10^8	13	0·120	0	0	0
35	5	CPH	99	9	$<1\times10^5$	16	0·255	2	2	ND
36	3	CPH/MGN	10	6	4×10^5	<10	0·302	1	ND	2
36				12	4×10^7	<10	0·280	3	1	1
37*	3	CPH	66	6	1×10^6	21	0·305	1	3	ND
38	6	CPH/MGN		6	2×10^6	<10	0·230	1	0	1
39	5	CPH	174	3	$<1\times10^5$	ND	0·025	4	ND	ND
40†	4	CPH	117	1	$<1\times10^5$	27	0·138	4	1	ND

* Samples containing 'truncated' HBs antigens.

† Seroconverted to anti-HBe during follow up.

ALT, alanine aminotransferase; CPH, chronic persistent hepatitis; MEN, membranous glomerulonephritis; LC, liver cirrhosis; CAH, chronic active hepatitis; ND, not done.

The $HBeAg^+$ group had often anti-HBc of IgM class (24 out of 37) in moderate titres, which is consistent with the diagnosis of chronic hepatitis B, according to the criteria of Gerlich *et al.* (1986). HBV DNA was detected by dot blot in 32 out of 40 $HBeAg^+$ patients at variable titres up to 4×10^8 genome equivalents/ml serum. The detection limit of this assay was 10^5 genomes/ml. All 14 anti-HBe^+ patients were negative in the dot blot assay. No relationship was found between the histological diagnosis or the level of ALT in serum and the viral parameters in the serum.

Table 2. Data of anti-HBe positive, chronically infected children

Patient no.	Age (years)	Diagnosis	ALT (U/l)	HBsAg (μg/ml)	Anti-HBc IgM	PEG precipitate (OD_{600})	Staining intensity in blots		
							SHBs	MHBs	LHBs
1	12	LC	17	24	88	0·080	0	ND	0
2	7	CAH	16	21	<10	0·087	0	ND	ND
3	9	CPH	20	16	<10	0·142	0	ND	ND
4	4	CAH	22	14	<10	0·084	0	ND	ND
5	5	CPH	22	13	<10	0·105	0	ND	ND
6	8	CPH/MGN	ND	11	<10	0·180	0		1
7	4	CPH	41	8	<10	0·166	1	ND	2
8	5	LC	19	8	<10	0·0708	0	ND	ND
9	4	CAH	690	7	<10	0·136	0	ND	ND
10	10	CPH/MGN	ND	7	<10	0·330	0	0	0
11	5	CPH	52	2	<10	2·300	0	ND	ND
12	6	LC	34	1	<10	0·363	0	ND	ND
13	6	CPH	15	<1	<10	0·136	0	ND	ND
14	5	CAH	19	<1	<10	0·124	0	ND	ND

See Table 1 for abbreviations

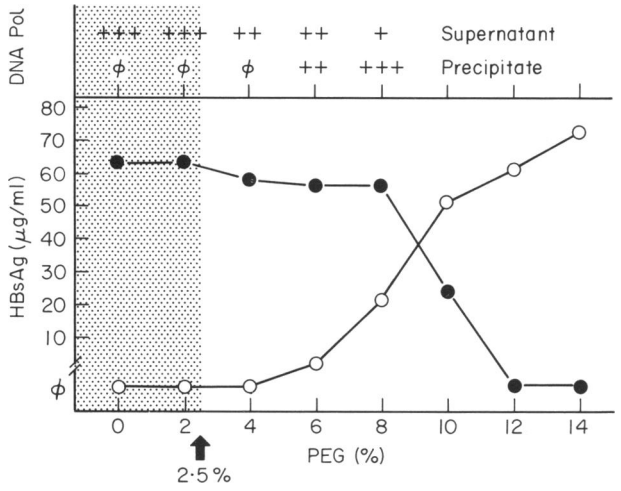

Fig. 1. Precipitation of free HBsAg particles or virion-associated DNA polymerase (DNA Pol) by PEG in the serum of a patient in the incubation period of acute hepatitis B. Serum was mixed with the same volume of a concentration series of PEG, the precipitate was spun down, washed and re-dissolved in two volumes of PBS. HBs was assayed by Laurell electrophoresis (Gerlich & Thomssen, 1975) and DNA polymerase as described by Kaplan *et al.* (1973). The scores of DNA polymerase were: +++, >3000 ct/min; ++, >1500 ct/min; +, >500 ct/min. ●, Supernatant; ○, precipitate. Shaded area, working range of PEG concentration.

Properties of the PEG precipitates

First we determined whether free HBV particles or HBs particles are precipitated by low concentrations of PEG. For this experiment we used the serum from a patient in the incubation period of acute hepatitis B who did not yet have any detectable antibody against HBcAg. This serum was likely to be free of any HBV-related CIC. Addition of 2% or 4% PEG to this serum resulted already in formation of visible precipitates. However, HBV particles—as detected by their endogenous DNA polymerase—precipitated only at 6% or more PEG, while the HBs particles required even 8% or more PEG to be precipitated (Fig. 1). Most CIC can be precipitated at 2·5% PEG (Creighton *et al.*, 1973). This concentration of PEG did not precipitate too large amounts of proteins unrelated to HBV.

In order to control HBV unrelated precipitate from occluding HBs protein, we measured in all experiments the amount of precipitate by the optical density at 600 nm (OD_{600}) (Table 3). OD_{600} was on the average 0·141 in the blood donors group. A similar average OD_{600} was observed in anti-HBe^+ sera, but the average OD value of $HBeAg^+$ sera was higher (0·296±0·258) with great individual variation (0·06–1·26). The PEG precipitate from one HBe^+ serum (case 15, Table 1) showed a very high OD (OD_{600}=1·26) and a relatively high concentration of free HBsAg (33 μg/ml), but it did not contain HBs-specific bands in the immune blot.

SDS gel electrophoresis and subsequent staining of the precipitates by Coomassie blue showed several strong protein bands of 40–70 kD, and a band of 28 kD which may correspond to the light chain of immunoglobulins. These staining patterns were very similar in HBV-infected children and in normal blood donors. In particular, no HBs- or HBe/c-specific bands could be detected by Coomassie blue staining. However, γ and μ chains were detectable in heavy precipitates (data not shown) by Coomassie blue staining and C3d by immune blot using a specific MoAb. The data show that only a minor proportion of the CIC consisted of HBV proteins and their antibodies.

Detection of HBs proteins in CIC

After gel electrophoresis and blotting of the redissolved and denatured PEG precipitates, the HBV proteins were detectable by immune staining with various HBV-specific MoAbs. The occurrence and relative quantity of HBs proteins in washed PEG precipitates are listed in Table 1. Figure 2 shows an example of an immune blot with the MoAb against the S domain. In most samples all three pairs of SHBs, MHBs, and LHBs were detected, with SHBs being the major component. However, the amount of HBs proteins in the precipitates from

Table 3. Serological markers in children with chronic HBV infection

	HBeAg$^+$ ($n=46$) (of 40 patients)	Anti-HBe$^+$ ($n=13$) (of 13 patients)
HBsAg concentration (μg/ml)	23·5	8·3
PEG precipitate mean OD_{600}	0·296 ±0·258 (s.d.) ±0·038 (s.e.m.)	0·155 ±0·091 (s.d.)* ±0·025 (s.e.m.)
Frequency		
HBV DNA$^+$	32/40 (80%)	0/14
SHBs in CIC	36/40 (90%)	1/14
MHBs in CIC	16/28 (64%)	ND
LHBs in CIC	21/25 (84%)	2/4

* Patient no. 11 from Table 2 excluded.

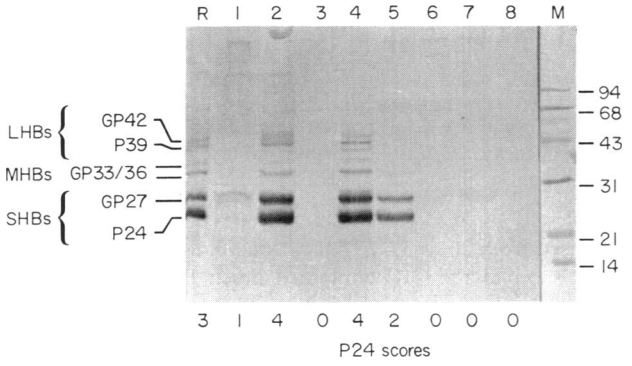

Fig. 2. Detection of SHBs, MHBs and LHBs in CIC from eight patients using immune blotting and a MoAb against the S domain. R, 0·6 μg purified HBsAg as reference serum; lanes 1–8, redissolved PEG precipitates from 200 μl patient serum; M, size markers in kD (low marker from BioRad).

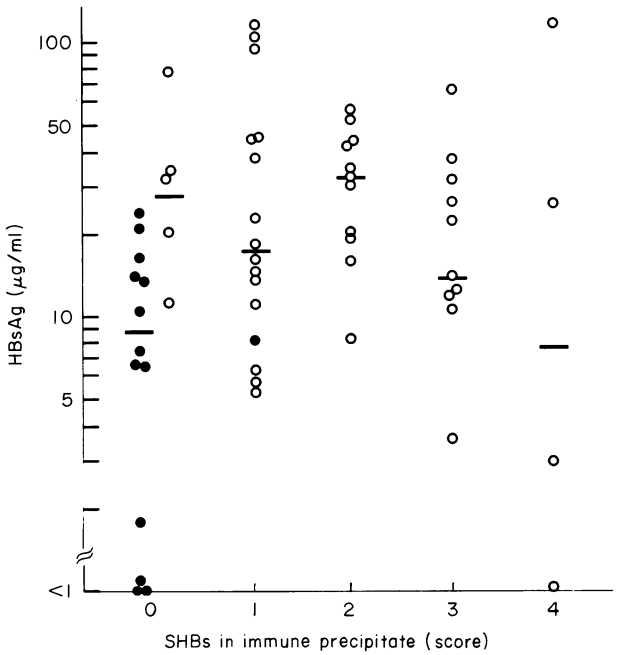

Fig. 3. Relation between the score of staining intensity for P24-SHBs in CIC and concentration of free HBsAg in the patients' sera. ●, anti-HBe positive patients; ○, HBeAg positive patients; —, median value.

patients was highly variable, as shown in Fig. 2. Some precipitates gave stronger HBs bands (lanes 2 and 4) than a reference sample containing 0·6 μg of purified HBsAg particles (lane R), others gave a weaker immune staining and some were negative (lanes 3, and 6–8). For evaluation of the immune blots, we gave scores from 0 (negative) to 4 (very strong) and considered 0·6 μg purified HBsAg as 3. The sample in lane 1 was considered 1 (borderline detectable), lane 5 was scored 2, lanes 2 and 4 as 4 (stronger than the positive control). Score 3 for HBs proteins in CIC means that the CIC contains approximately 15 μg HBs protein per ml serum.

Most HBeAg$^+$ children had HBs protein in the precipitate. Surprisingly, only two out of 14 anti-HBe$^+$ children had detectable amounts of HBs protein in CIC and these scored only as borderline detectable. In contrast, the most HBeAg$^+$ children had HBs scores of 2 or more (Table 1). The amount of HBs protein in the CIC did not correlate with the serum concentration of HBsAg (Fig. 3). The highest HBsAg concentration in a serum sample negative for CIC was 78 μg/ml. In contrast, one HBeAg$^+$ serum with $< 1·0$ μg HBsAg/ml had a HBs score of 4 in the CIC.

Ratio between LHBs and MHBs

Previously it has been published (Heermann *et al.*, 1984) that all HBV carriers have 20-nm HBsAg particles as predominant form of HBsAg in the serum, even if they have very high HBV DNA titres. Moreover, it was consistently found that MHBs was more abundant in these particles than LHBs, as seen in Fig. 2, lane R, for the reference sample. However, Fig. 2 suggests that in some CIC (e.g. lane 5) LHBs was at least as abundant as MHBs, whereas the reference sample of purified 20-nm HBsAg particles had a well-detectable GP36 of MHBs, but no detectable P39 of LHBs. Since the detection of LHBs by a MoAb against the S domain can be obscured by dimers of SHBs, we stained most CIC samples also with LHBs (Fig. 4a) and MHBs (Fig. 4b) specific MoAbs (also Tables 1 and 3). Figure 4 confirms that MHBs is better stained in the reference sample than LHBs (lanes 1). In contrast, LHBs was stronger and more often detected (six of 13 samples) in PEG precipitates than MHBs (two of 13 samples). Although MoAb MA18/7 against LHBs showed also some non-specific staining of other proteins, P39 and GP42 of

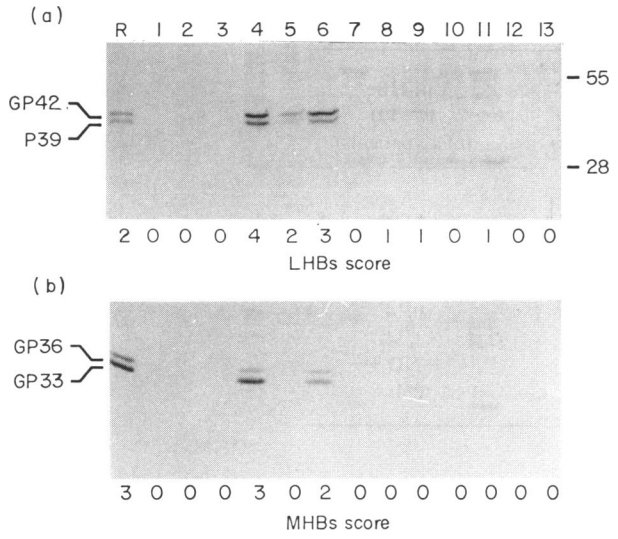

Fig. 4. Immunoblots of CIC from 13 patients stained in parallel with a MoAb against preS1, LHBs (a); or against glycosylated preS2, MHBs (b). R, reference sample of 0·6 μg purified HBsAg particles as in Fig. 1. Note the weak LHBs bands in lanes 8, 9, and 11 which were scored 1. Bands at 55 and 28 kD were rated non-specific.

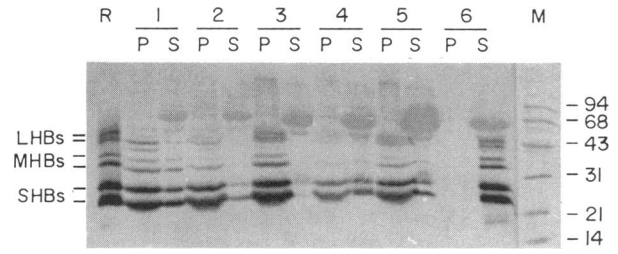

Fig. 5. Comparison of HBs protein composition (LHBs, MHBs, and SHBs) in 2·5% PEG precipitates (P, i.e. CIC) and supernatants (S) from six patients. Immune staining of the proteinblot was done with anti-S MoAb H166.

LHBs were well distinguishable and detection of LHBs was almost as sensitive as detection of SHBs (see also Table 3).

The different staining intensities of LHBs in the reference sample and in the CIC suggested that there may be an enrichment of LHBs in CIC. To substantiate this assumption further, we compared the composition of HBs particles in the PEG supernatant with that of the CIC (Fig. 5). Figure 5 confirms in a direct way the observation that no correlation exists between amount of HBs in CIC and in the free form. For example, serum no. 3 in Fig. 5 contained much more HBs protein in the precipitate than in the supernatant, while serum no. 6 had no HBs in CIC (precipitate), but very much in the supernatant. Due to these strong differences, a clear comparison was only possible in serum no. 1. Here, MHBs appeared in similar intensity in the precipitate CIC and in the supernatant, but LHBs was much weaker in the supernatant than in the corresponding CIC precipitate.

Follow up of patients

In six patients a second serum sample, obtained after 7–14 months, was available (Fig. 6). In one case (no. 1, Fig. 6) HBsAg

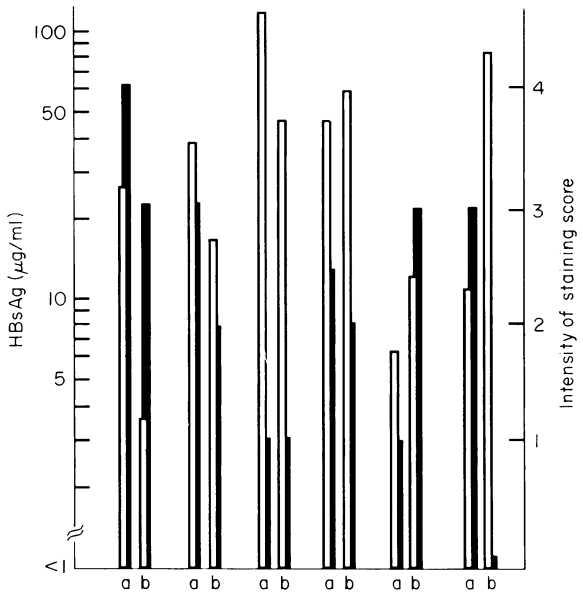

Fig. 6. Change of free HBsAg concentration (blank column) and SHBs staining intensity in CIC (solid column) during follow up of six patients. a, first serum sample; b, second serum sample, after 7–14 months.

decreased from 26 to 3·6 μg/ml within 10 months, and in both serum samples the CIC contained much HBs protein. Conversely, another HBeAg$^+$ patient with CAH (no. 6, Fig. 6) showed an increase of HBsAg concentration from 11 to 78 μg/ml. In the first sample CICs were scored 3 for HBs protein, but in the second sample after 7 months HBs had disappeared from CIC. Due to a very strong progression of CAH, this patient received corticosteroid soon after the first serum sample till the second serum sample was taken. ALT and other biochemical parameters improved to nearly normal values, but HBV replication and expression increased. The other patients with the diagnosis of CPH showed only minor alterations in free and CIC-bound HBsAg.

Detection of HBe/c proteins

CIC from 26 of the 40 HBeAg$^+$ children were analysed for HBe/c proteins using a MoAb that detects a sequential epitope present both in HBc and HBe protein (E. Korec, personal communication). Although the titres of HBV DNA and HBeAg were partly quite high, only one adult patient from another study with CPH and a low level of HBsAg (3·2 μg/ml) had a detectable amount of an 18-kD HBe protein in CIC, but none of the children did. The reference sample consisting of 0·6 μg 22-kD recombinant HBc protein produced very strong bands under these conditions (data not shown).

DISCUSSION

The standard procedure for detection of HBs-containing CIC is the binding of CIC to reagents like anti C1q or conglutinin (Brown *et al.*, 1983), anti-IgM (Palla *et al.*, 1983; Surelia & Boxall, 1990), or PEG (Sansonno *et al.*, 1986), and subsequent detection by the aid of labelled anti-HBs. This procedure can only detect HBs proteins in CIC if HBs-unrelated competing CIC are not present in large amounts, and if the CIC contain free HBs epitopes that react with the labelled anti-HBs.

Furthermore, these methods cannot distinguish the three HBs domains. Our method detects all kinds of HBs protein in PEG precipitable CIC, because the CIC are completely denatured by SDS, DTT, and heat before assay of the HBs proteins. This new approach shows, in contrast to a previous study (Brown *et al.*, 1983), that the amount of HBs proteins in CIC does not correlate at all with the concentration of free HBs and very large amounts of HBs protein may in fact coincide with very small amounts of detectable free HBsAg (e.g. cases 39 and 40 in Table 1). This is consistent with the theory that formation of ICs should support elimination of an antigen.

Immune blot analysis showed that all three well-characterized HBs proteins were present in CIC. A previous report (Gupta & Kohler, 1984) which showed 23-kD, 29-kD and 97-kD HBs proteins in CIC could not be confirmed, probably because the polyvalent anti-HBs reagent used by these investigators was not able to stain specifically denatured HBs proteins. It is known that most anti-SHBs antibodies recognize conformational epitopes. Moreover, the previous analysis did not consider the existence of MHBs or LHBs. Using the same technique as for HBs proteins, no HBe protein could be found in the PEG precipitates. This finding was unexpected and needs further investigation.

The partial enrichment of LHBs in the CIC compared to free HBsAg particles suggests that the antibodies of the CIC are predominantly directed against LHBs. Since LHBs is more abundant on HBV particles and on HBs filaments than on 20-nm HBs particles, the former two particle species would be preferentially removed from the serum or be bound in CIC. The assumption that anti-pre-S1 antibodies would contribute to the formation of HBs- and HBV-containing CIC is supported by the finding in patients with acute hepatitis who develop anti-pre-S1 first, or the so-called anti-Dane antibodies (Alberti *et al.*, 1990). The pre-S1 domain has been characterized as highly immunogenic both in experimental animals (Milich *et al.*, 1986; Heermann *et al.*, 1987) and in humans (Takai *et al.*, 1986; Deepen *et al.*, 1990). It seems conceivable that elimination of circulating HBsAg and HBV could be initiated by synthesis of anti-pre-S1.

While anti-HBs and in particular anti-pre-S1 antibodies would help to eliminate HBsAg and HBV from the circulation, they obviously cannot stop the production of HBV proteins by the infected hepatocyte. Thus, it is not surprising that many chronic HBV carriers in our study had both high levels of free HBsAg and CIC-bound HBs protein. HBV infection can only be overcome by the cytotoxic elimination of the infected hepatocytes (Mondelli *et al.*, 1982; Vento *et al.*,l 1987). Nevertheless, it is consistent with the suggested role of CIC that they were absent in a patient who showed a strong increase of HBsAg concentration (case 6 in Fig. 5) and present in high amounts in one case of elimination. More often, however, we found a steady state between CIC formation and continuous HBs synthesis. Presence of CIC may indeed be a prerequisite of HBs elimination, as postulated by Madalinski & Bragiel (1979) and Markus *et al.* (1986), but persistence of HBs containing CIC indicates persistence of HBV infection as pointed out by Carredda *et al.* (1982), Palla *et al.* (1983), and Frisch-Niggemeyer *et al.* (1984). The variable pattern of HBs-containing CIC during clinical follow up was also found by Pernice *et al.* (1979).

In our study the amount of HBs proteins in CIC quite often exceeded the amount of free HBs protein by a factor of 10 and more (Fig. 4). Laurell electrophoresis and possibly also RIA or ELISA, would preferably react with free HBsAg but not with HBs proteins in CIC. In extreme cases no HBsAg may be detectable at all, while the blood contains detectable amounts of HBsAg in CIC. Such a situation has been described for patients with HBV-associated hepatocellular carcinoma (Brown *et al.*, 1984). Therefore, it is advisable to use additional seromarkers of HBV such as anti-HBc besides HBsAg.

It was surprising to note that most $HBeAg^+$ patient had HBs-containing CIC but $anti\text{-}HBe^+$ patients did not. The absence of HBsAg in CIC from $anti\text{-}HBe^+$, non-viraemic, relatively healthy patients suggests that adult asymptomatic carriers, who were not studied here, would also have no HBsAg-containing CIC, because they are usually $anti\text{-}HBe^+$. This presumption is at variance with the report of Sansonno *et al.* (1986), but in agreement with the findings of Anh Tuan & Novak (1981). Brown *et al.* (1983) found in 40% of healthy HBsAg carriers HBs–CIC but in lower levels than in chronic HBV patients. One might speculate that the HBs particles from $anti\text{-}HB^+$ patients are devoid of pre-S epitopes, but at least as far as pre-S1 is concerned, this is not true (Deepen *et al.*, 1990). Our findings may be explained if one adopts the model of HBc/e-dependent T cell help for the induction of anti-HBs production (Milich *et al.*, 1987). Only the patients expressing and secreting HBV would show all dominant B cell epitopes of HBs and the dominant T cell epitopes of HBc/e in one particle, and only then anti-HBs-specific B cells can take up HBc/e protein and may present this after intracellular processing to HBc/e specific T helper cells. Our findings suggest that the data obtained in inbred mice may also apply to human patients.

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