Hepatitis B antigenaemia, specific immune deficiency and hepatocellular carcinoma*1

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One of the major objectives of our studies involving Hepatitis B antigen (HB Ag) is to determine whether HB antigenaemia following exposure to the HB agent is a risk factor in the development of hepatocellular carcinoma (HCC). An increased frequency of HB Ag has been demonstrated in the sera of patients with HCC[1] and those with other chronic liver diseases[2]. It has been claimed that these findings provide circumstantial evidence in support of the view that persistent HB antigenaemia (the 'carrier state') predisposes to the development of HCC[3].

There appear to be two main modes of immune responsiveness on exposure to the HB agent; development either of immunity with the production of antibody to HB Ag in the absence of detectable antigen, or of HB antigenaemia with or without specific antibody (HB Ab). In the former situation immune mechanisms appear to have been effective in eliminating the HB agent. By contrast, the carrier state of HB antigenaemia probably reflects an ineffective immune response and consequent persisting infection.

The frequency of exposure to the HB agent is represented by the combined frequencies of serum HB Ag and of HB Ab. These are best determined by the use of sensitive immunological techniques. There are three main categories of techniques in common use: —

1. Gel diffusion –immunodiffusion (ID).
   –counterimmunoelectrophoresis (CIE).
2. (a) Particle agglutination–passive haemagglutination (PHA).
   –immune adherence haemagglutination (IAHA).
   –latex particle agglutination (LPA).
   (b) Red cell haemolysis –complement fixation (CF).
3. Radioimmunoassays (RIA).

*1 Presented at the First Meeting of the International Research Group for Carcinoembryonic Proteins, Sapporo, Japan, 1973. This paper was written after the Symposium. Consideration has been given to some of the questions raised in discussion of the presentation.

The most accurate determination of frequency of exposure would be to test the sera of all subjects by RIA, and to challenge those who lack HB Ab with immunogenic but non-infectious HB Ag. Short of this, an estimate may be obtained without specific antigenic challenge.

The use of techniques in categories 1 and 2 have provided evidence that HB Ag frequencies are high in African and Asian HCC patients, and that African patients have higher titres of HB Ag than Asian Chinese [Singapore[1] ; Hong Kong[4]] or Japanese patients[3]. The frequency of HB antigenaemia in HCC patients is known to vary in different parts of the world. It is as high as 80% in Papua New Guinea (Woodfield, D. G., Personal communication, 1973). In a Caucasian group, even using a sensitive RIA, only 4% (2 of 50) HCC patients were HB Ag positive, compared to a frequency of approximately 0.1% in blood donors (Kohn, J., Personal communication, 1973). Although this frequency of 4% is low relative to HCC patients of other ethnic types, the 40-fold increase in HB Ag frequency compared to blood donors is higher than has been found in studies of similar groups elsewhere.

Based on the foregoing, it is postulated that exposure to the HB agent, a precondition for the development of HB antigenaemia, may cause HCC either directly or indirectly through one of the types of post-hepatitic pathology. Evidence relating to this proposition may be obtained by answering the following questions : —

1. Do HCC patients differ from suitable ethnic, sex and age-matched normal subjects and patients with a variety of liver diseases in HB agent exposure rate, or in the proportion of antigenaemic persons in the exposed population (HB antigenaemic rate)?
2. Do individuals who are infected by the HB agent, and in particular those who become HB Ag 'carriers', develop HCC more frequently than individuals who are not exposed?
3. Could the incidence of HCC be decreased by the introduction of measures directed towards reducing the risk of exposure and in particular of developing HB antigenaemia (eg. vaccination of the population at risk, elimination of the HB agent)?

The present status of some studies in progress in Singapore relating to the first of these questions is summarised in this paper.

The population of Singapore in 1970 was approximately 2.2 million, comprising 76% Chinese, 15% Malays and 7% Indians[5]. The age standardized incidence of HCC is higher in Chinese (31.5/100,000 persons/annum for males ; 6.9 for females) than in Malays (males 13.5 ; females 7.2) or in Indians (males 10.9 ; females 2.5)[6]. The morphological patterns of cirrhosis

also differ among Chinese and Indians. In a study based on necropsies in Singapore, Shanmugaratnam[7] found that the proportion of macronodular cirrhosis (a group he termed "post-necrotic") was relatively higher among Chinese than in Indians. On the other hand, micronodular cirrhosis (which he termed "nutritional") occurred more frequently among Indians relative to their proportion in the total necropsy population. In an analysis of 103 cases of HCC he found that 86 (83.5%) were associated with macronodular cirrhosis, 5 (4.9%) with mixed cirrhosis, 2 (1.9%) with pigmentary cirrhosis-haemochromatosis, and none with micronodular cirrhosis; 10 (9.7%) cases were not associated with any form of cirrhosis. Shanmugaratnam[7] attributed the higher frequency of HCC among Chinese to a higher frequency of macronodular ("post-necrotic") cirrhosis and suggested that viral hepatitis, which may be related to this type of cirrhosis, may play a role in the development of HCC.

Chinese male blood donors have been found to have a significantly higher HB Ag frequency than Malays or Indians [CIE–Chinese (4.2%), Malays (1.6%) and Indians (0.8%); IAHA–Chinese (8.2%), Malays (4.7%) and Indians (2.9%)][8]. Recently we have evolved a two-gel CIE procedure which has a greater HB Ag detection sensitivity than our previous CIE method and, in addition, enables semi-quantitative determination of HB Ag titre using undiluted samples. Briefly, sera of the reference hepatitis B antigen panel No. 2* which had HB Ag titres of 1 : 4–1 : 16 by CF were detectable only in 1% agarose, whereas CF titres of HB Ag greater than 1 :

Table 1. *Comparison of HB Ag frequencies detected in Singapore blood donors using agar and agarose counter-immunoelectrophoresis*

Ethnic Gronp	Single gel*1 (Agar 1.5%)		Two gel*2 (Agar 1%)		(Agarose 1%)	Total
	No. tested	No.+ve (%)	No. tested	No.+ve (%)	No.+ve (%)	No.+ve (%)
Chinese	1,632	69 (4.2)	776	39 (5.0)	11 (1.4)	50 (6.4)
Malays	445	7 (1.6)	167	1 (0.6)	2 (1.2)	3 (1.8)
Indians	248	2 (0.8)	238	0	0	0
Total	2,325	78 (3.4)*3	1,205	40 (3.3)	13 (1.1)	53 (4.4)*4

*1 Ong *et al.*, 1971.
*2 Simons *et al.*, 1973.
*3 Chinese ∨ Non-Chinese: $X^2=12.9$: $p<0.001$.
*4 Chinese ∨ Non-Chinese: $X^2=20.3$: $p<0.0001$.

* Supplied by Division of Biologics Standards, NIH, Bethesda, Maryland, U.S.A.

256 were only positive in 1% agar. Sera with HB Ag of intermediate titres produced visible bands in both gels. Table 1 shows the HB Ag frequencies of blood donors using the single gel and the two-gel procedures in two separate studies. The use of two-gels enabled an increase in the HB Ag detection rate in Chinese from 4.2% to 6.4%. The frequency differences between Chinese and non-Chinese were highly significant by both approaches.

Using the two-gel approach HB Ag was detected in 16 (34%) of 47 Singapore Chinese HCC patients. HB Ag frequencies between 34% and 42% have been consistently obtained in studies of more than 200 Singapore Chinese HCC patients using four methods [IAHA[1]; CIE, LPA and REC (Unpublished observations)]. This is 6-7 fold higher than in Singapore Chinese blood donors.

The presence of HB Ab is being investigated by REC using an HB Ag preparation of sub-type adw radiolabelled with ^{125}I[9]. To date, studies have been completed on 114 blood donors who were HB Ag negative by CIE. They comprised 56 Chinese, 26 Malays and 32 Indians. HB Ab was detected in 25 (45%) of the 56 Chinese. Twelve (46%) of the Malays and 13 (41%) of the Indians had HB Ab. These proportions of Chinese, Malays and Indians who developed immunity following exposure to the HB agent were not significantly different. It is therefore more likely that the ethnic differential in HB Ag frequencies shown in Table 1 is due to differences in host responsiveness.

The 31 of 47 Singapore Chinese HCC patients who lacked HB Ag detectable by two-gel CIE were studied for HB Ab using REC — 18 (38%) were positive. This proportion is higher than that reported by Nishioka (this symposium) for both Japanese and Kenyan patients using PHA, in whom very low HB Ab frequencies were detected. However, it is not significantly different from that in Singapore Chinese, Malay and Indian blood donors (45%, 46% and 41% respectively). This difference between the results obtained in Singapore and in Tokyo will receive attention at the forthcoming WHO-assisted workshop on HB Ag**.

The REC method is unique among RIA procedures in that the presence of HB Ab and HB Ag in the same serum may easily be determined since counter-directional movement results in spatial separation of anodally moving antigen and of cathodally moving HB Ab molecules[10]. Table 2 shows the pattern of HB Ag–^{125}I distribution when reacted in REC against dilutions of a reagent HB Ab immune sera, against 2 control sera, and

* Provided by Dr. R. H. Purcell, NIAID, NIH, Bethesda, Maryland, U.S.A.

** To be held in Tokyo, October 29–November 8.

Table 2. *Evidence for the presence of HB Ab in HB Ag-positive sera from asymptomatic blood donors. Retardation of the anodal migration of HB Ag–^{125}I*

Serum		AbWZ-2	AbWZ-1	IWZ	AgWZ	Total cpm
Reagent HB Ab	2^0	3	6	22	69	847
	2^{-2}	6	10	69	15	887
	2^{-4}	14	38	38	10	871
	2^{-6}	30	44	21	5	820
	2^{-8}	35	51	8	6	846
	2^{-10}	49	33	10	7	848
	2^{-12}	61	27	7	5	802
HB Ab-ive control		53	33	8	6	785
HB Ag+ive sera	CIE/1	47	31	12	10	846
	CIE/3	59	25	10	6	818
	CIE/4	22	32	40	6	837
	CIE/5	39	41	14	6	848
	CIE/6	30	45	18	7	855
	CIE/7	33	46	13	8	844

against 6 sera containing HB Ag detectable by CIE. From the upper section of the table it can be seen that there is a relationship between retardation of anodal migration of HB Ag–^{125}I and HB Ab titre. The radiolabel distribution in the test of HB Ag-positive serum no. 3 is indistinguishable from that of the control. Serum no. 4, by contrast, produced a marked retardation of HB Ag–^{125}I migration. Sera nos. 5, 6 and 7 all showed the same effect but to a lesser degree. Absorption of these sera with antisera to total immunoglobulin, and to gamma or epsilon chains, resulted in a loss of HB Ag–^{125}I binding activity; antisera specific to mu, alpha and delta chains had little if any effect. The method of preparation of the 'purified' HB Ag used as a radiolabelled preparation precluded the possibility of the presence of gamma-globulins. Thus, the migration-retarding effect is unlikely to be due to anti-globulin reactions which complicate other methods (eg. LPA).

This phenomenon of HB Ag–^{125}I binding was observed in 58 of 63 HB Ag positive sera in a first unpublished study, and in 29 of 39 in the present study. It is highly unlikely that sera from 'normal' blood donors contain antibodies to human serum proteins in such high proportions, even if the proteins were present as contaminants in the radiolabelled prepa-

ration and even if they did migrate anodally in an electrical field. Serum albumin (HSA) is a possible contaminant of the HB Ag–^{125}I preparation but the possibility that binding by HB Ag-positive sera was due to the presence of anti-albumin antibodies was excluded by showing that these sera did not bind HSA–^{125}I. Binding of HB Ag-^{125}I is therefore attributed to the presence of HB Ab. The presence of HB Ag in relatively high titre can be shown by CIE on the same slide in which binding by REC is determined. The coexistence of HB Ag in high titre and of HB Ab in low titre is interpreted as reflecting the presence of immune complexes which are soluble in antigen excess. Sera of 6 of the 16 HCC patients who had HB Ag also bound HB Ag–^{125}I. This proportion (38%) is significantly lower than in HB Ag-positive Chinese blood donors (74% ; $-X^2=6.7$; $p<0.01$). If the interpretation is correct that the material in HB Ag-containing serum which binds HB Ag–^{125}I is in fact specific antibody (HB Ab), then the mechanism of the HB antigenaemia in the majority of the HB Ag-positive blood donors studied is not that of a complete immunological unresponsiveness ('tolerance') to the HB agent.

Table 3. *HB agent exposure rate and HB antigenaemic rate in hepatocellular carcinoma*

Group (Singapore)	HB Ag frequency*1 (%)	HB Ab frequency*2 (%)	HB agent exposure rate (%)	HB Antigenaemic rate (%)
Indian blood donors	1.5	41	42.5	3.5
Malay blood donors	4.5	46	50.5	8.9
Chinese blood donors	8	45	53	15
Chinese HCC patients	35	35	70	50

*1 By immune adherence haemagglutination.
*2 By radioelectrocomplexing.

Table 3 is a summary of the rates of HB agent exposure and of HB antigenaemia in the three 'normal' groups and in HCC patients. It is important to note that the three normal groups differ in age and sex composition from the patients and are therefore not suitable as comparison groups. Nonetheless they do provide data relevant to an understanding of the epidemiology of the HB agent. Firstly, the HB agent exposure rates in the 4 groups are of the same order. Secondly, the HB antigenaemic rate is several fold higher in normal Chinese than in normal Malays and Indians. Thirdly, Chinese HCC patients have at least a 3-fold higher HB antigenaemic rate than normal Chinese.

These provisional findings are compatible with the view that occur-

rence of HB antigenaemia and probable immune complexaemia following exposure to the HB agent represents a deficiency of immune responsiveness. This deficiency is more common in Chinese HCC patients than in normal Chinese, and less common in normal Malays and Indians. The possibility that the ethnic difference in host immune responsiveness has an inherited genetic basis is under investigation.

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