Geographic Variation in Viral Load among Hepatitis B Carriers with Differing Risks of Hepatocellular Carcinoma

Alison A. Evans, Anna P. O’Connell, John C. Pugh, William S. Mason, Fu-min Shen, Gong-Chao Chen, Wen-Yao Lin, Amadou Dia, Souleymane M’Boup, Babacar Dramé, and W. Thomas London

Divisions of Population Science [A. A. E., A. P. O., W. T. L., J. C. P.] and Basic Science [W. S. M.] Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111; Department of Epidemiology, Shanghai Medical University. 200032 Shanghai, People’s Republic of China [F-M. S.]; Haimen City Anti-Epidemic Station, 226101 Haimen City, Jiangsu Province, People’s Republic of China [G-C. C., W-Y. L.]; and Health Service, Army of Senegal, Dakar, Senegal [A. D. S. M., B. D.]

Abstract

The risk of hepatocellular carcinoma (HCC) varies significantly among hepatitis B virus (HBV) carriers from different geographic regions. We compared serological markers of HBV infection in adult male carriers from Haimen City, China and Senegal, West Africa, where the prevalence of chronic infection is similar. HCC mortality among HBV carriers is much higher in Haimen City than it is in Senegal (age-standardized rate, 878 versus 68 per 10^5 person-years). A dramatic difference was observed when HBV DNA levels in serum were assessed among carriers by Southern blot. In the Senegalese group (n = 285), 14.5% were HBV DNA positive by Southern blot in their 20s, and this percentage declined in each subsequent decade of age to 3.3, 2.9, and 0% thereafter. In the Chinese group (n = 285), a higher prevalence of HBV DNA positivity and a less consistent reduction were seen; 29.4% were positive in their 20s, and 30.2, 23.6, and 20.6%, respectively, were positive in each subsequent decade of age. Among 102 male Asian-American HBV carriers, the prevalence of HBV DNA positivity was intermediate between the Chinese and Senegalese populations (36.8, 10.7, 3.0, and 4.6% in each subsequent decade of age). Viral titers were similar among those who were HBV DNA positive in all three populations (median value, 10^7 virions/ml range, 10^6-10^9 virions/ml). The presence of HBV DNA in serum was positively associated with serum glutathione S-transferase, a marker of liver damage. These findings suggest that the more prolonged maintenance of productive virus infection in the Chinese carriers compared with the Senegalese carriers may explain their higher risk of HCC. This profound difference in the natural history of chronic infection may be due to earlier age of infection in China or to as yet unknown environmental or genetic factors.

Introduction

Chronic infection with HBV is etiologically associated with HCC. Epidemiological studies have found that chronic carriers of HBV are 5–148 times more likely to develop HCC than are noncarriers (1). It has been suggested that the absolute lifetime risks of HCC for individual HBV carriers in all populations are similar and range from 20–25%. Such estimates have, in fact, only been calculated for a few special populations (2, 3). Other studies have estimated risk from clinic-based studies in which incidence rates may be elevated due to referral bias (4–6). Because subjects tend to enter clinic-based studies because of prior knowledge of their HBV status and/or symptoms of liver disease, HBV carriers in these studies are usually not representative of those in the general population, the majority of whom remain asymptomatic.

On the other hand, there is good evidence that the risk of HCC varies significantly among HBV carriers from different geographic regions. For example, cancer registry data from known HBV-endemic populations give age-standardized incidence rates for HCC among males 35–64 years of age as follows: (a) in Qidong City, China, 218/100,000 person-years; (b) in The Gambia, West Africa, 79/100,000 person-years; and (c) among the Miao ethnic group in New Zealand, 27/100,000 person-years (7). However, the prevalence of chronic HBV infection in these three populations is similar, ranging from 16–19% of adults (8–10). Clearly, there are many differences among these populations that may explain the differences in HCC risk. These include environmental factors such as exposures to carcinogens, diet and lifestyle factors such as alcohol consumption and cigarette smoking, genetic and ethnic differences, and differences in the age and mode of transmission of HBV. Numerous observational studies in Asia and Africa have also established a profound geographical difference between the regions in the transmission and natural history of HBV infection (11–15).

In the present investigation, we examined whether serological markers of chronic HBV infection in adults differed between Chinese and West African men. To test for differences, we used representative cross-sectional random samples of large cohorts of male adult HBV carriers in Haimen City, China (adjacent to Qidong City) and Senegal (adjacent to The Gambia) and adult male Asian-American HBV carriers living in the

1 The abbreviations used are: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBeAg, hepatitis B surface antigen; EIA, enzyme immunoassay; GST, glutathione S-transferase; HBeAg, hepatitis B e antigen; anti-HB, antibody to HBeAg.

Received 11/10/97; revised 4/20/98; accepted 4/30/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by USPHS Grants CA-40737 and CA-06927 from the NIH and by an appropriation from the Commonwealth of Pennsylvania.

2 To whom requests for reprints should be addressed, at Division of Population Science, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111. Phone: (215) 728-2497; Fax: (215) 728-3574; E-mail: A_Evans@fccc.edu.
The age-standardized HCC incidence rates in HBsAg-positive males observed in these cohorts are 877.9/100,000 person-years in Haimen, 68.3/100,000 person-years in Senegal, and 330.0/100,000 person-years in United States Asians. HBV carriers in all three cohorts were identified through routine screening rather than through clinical presentation. All serological testing was carried out in a single laboratory so that assay results would be directly comparable.

Materials and Methods

Study Populations

All subjects were HBsAg positive by EIA and were randomly selected from their respective cohorts. The age distribution of study subjects reflects the target age distribution of the cohorts, although not necessarily that of the source populations.

China. In 1992–1993, we screened 60,954 male adult residents of Haimen City, China for HBsAg. For the present study, we chose 285 HBsAg-positive adult males from Haimen City who were randomly selected from 9,620 HBsAg-positive members of that cohort. All serum samples were drawn at study entry. The mean age of the selected subjects was 41.2 years (SE, 0.54 year; range, 25–64 years). Haimen City is located in Jiangsu Province, China and is adjacent to Qidong City on the north bank of the Yangtze River. This region is in the southeastern coastal area of China, which is known to have some of the highest incidences of HCC in the world.

Senegal. In 1992–1993, we screened 13,036 male adult soldiers on active duty in the Armed Forces of Senegal for HBsAg and found 2611 HBsAg-positive members. For the present study, we randomly selected 289 HBsAg-positive samples that were drawn at study entry. The mean age of the selected soldiers was 29.9 years (SE, 0.46 year; range, 20–55 years). Routine screening for HBsAg is not otherwise performed in the Senegalese Army, and entry into the military is not prohibited to HBV carriers.

United States Asian. For the United States Asian samples, we randomly selected 102 HBsAg-positive subjects from 1757 male Asian-American HBV carriers followed longitudinally by the Liver Cancer Prevention Center of the Fox Chase Cancer Center. These carriers were identified through routine screening in doctors’ offices, churches, schools, and health fairs in the Asian-American community in and around Philadelphia. Samples were drawn in 1996. Most subjects for this study were of Vietnamese or Cambodian ethnic origin, and all but one were born outside the United States. The mean age of the selected subjects was 40.7 years (SE, 1.11 years; range, 21–61 years). Because new HBV infections in adults are rare in the three study populations, HBsAg positivity in an adult was assumed to reflect chronic HBV carriage.

The populations chosen for our study were ascertained through screening programs, and differences in these screening programs must be considered as possible explanations for our findings. The Haimen City cohort is probably the most representative of the HBV carrier population in that region, because screening was accomplished by teams traveling from village to village to collect blood from all available residents. The Senegal Army cohort is drawn from all ethnic groups and geographic regions of the country; however, like most occupational cohorts, it is likely to contain more healthy people than a survey of the general population. Nevertheless, the prevalences of HBsAg and HBV DNA found in our cohort are similar to those reported in other studies in Africa (14, 17, 18). Because no screening for HBsAg is performed on entry into the Army (usually around 20 years of age), and because most carriers in this population remain healthy and asymptomatic throughout much of their adult lives, it is unlikely that our sample seriously underrepresents HBV DNA-positive individuals. The United States Asian subjects in this study were identified through screening programs in the Asian-American community in Philadelphia. Although most carriers in our study sample were initially identified through nonclinical sources, the samples used in this analysis were obtained in the offices of their primary care physicians. Community primary care physicians participating in this study sent blood samples from Asian-American patients and their accompanying family members for free HBsAg screening through the Fox Chase Cancer Center. Although the prevalence of liver disease is low in this carrier population, and the reason for visiting a physician was usually for illnesses unrelated to the liver, the subjects were ascertained in a clinical setting; therefore, they may be less healthy than carriers in the general Asian-American population.

Serological Tests.

HBsAg was quantified using a commercial EIA (Auszyme; Abbott Laboratories, North Chicago, IL) with serial dilution and comparison with known standards. HBV DNA in serum was detected by Southern blot hybridization and quantified with the Bio-imaging Analyzer System (Fuji Medical Systems USA, Stamford, CT). The limit of sensitivity of the assay was approximately $5 \times 10^3$ virions/ml serum. PCR was used to detect levels of the virus below this limit in a random subset of serum samples and to confirm a random subset of Southern blot positives. HBV DNA was extracted from 100 μl of serum. The sample was amplified by PCR with Taq DNA polymerase for 30 cycles. The primers used were 5'-GGGTGGAGCCCTCAG-GCTCATGGCA-3' (minus strand) and 5'-GAAGATGAG-GCATAGCAGCAGGAT-3' (plus strand). PCR reaction products were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining. The sensitivity of the PCR assay was $2 \times 10^3$ virions/ml or 1000 viral particles in 50 μl of serum. Serum GST, a measure of liver damage, was detected using an EIA. This assay was chosen because it measures the presence of the enzyme protein itself, rather than enzyme activity; therefore, it can be used on stored serum samples, unlike assays for serum transaminases (19). GST is released by hepatocytes in response to injury. Its serum half-life is shorter than that of the transaminases, and it has been shown to correlate well with serum transaminase measurements in patients with chronic liver disease (20, 21). Human GST extracted from the liver was used as the immunogenic protein to prepare polyclonal antibody and was also used as the standard in protein assays (22).

All samples were processed within 24 h of collection in the field by the respective laboratories in China, Senegal, and Philadelphia. HBsAg tests were performed immediately on samples collected in Philadelphia. All other tests, including the HBsAg quantitations, were performed on stored samples at the Fox Chase Cancer Center in 1996. All serum samples were kept at −20°C from the time of collection until the time of the study assays. Both HBsAg and HBV DNA are stable in long-term storage at this temperature (23).
High-Titer Viremia Is More Prolonged in HBV Carriers from Haimen City. In all three of the populations studied, infection with HBV occurs during infancy and early childhood in the great majority of cases. Most of those who were HBV carriers in adulthood had already converted from HBV DNA positive to HBV DNA negative on Southern blot by the time they reached early adulthood (Fig. 1). Overall, 74 of 285 (26.0%) Chinese subjects, 27 of 289 (9.3%) Senegalese subjects, and 12 of 102 (11.8%) United States Asian subjects were HBV DNA positive. Fig. 1 shows the prevalence of HBV DNA on Southern blot by age group. Some of the differences in HBV DNA seroprevalence between the populations could be accounted for by the different age distributions of our study samples, but the three populations exhibited marked differences in the age-related decline in the prevalence of viremia. Of particular importance is the lack of significant decline in the prevalence of viremia in the Chinese group between their 20s and 50s (trend = 0.15), a highly unusual feature that has not been described in other studies of HBV carrier populations. By contrast, in the Senegalese population, viremia declined sharply with age, such that only 3% remained HBV DNA positive in their 30s (trend = 0.03), compared with 30% in the Chinese group. In the Philadelphia Asian-American population, the decline with age was also significant, but the steepest decline occurred in middle age rather than in early adulthood (trend = 0.001). A substantial number of carriers remained HBV DNA positive by Southern blot throughout their reproductive years.

However, among those who remained HBV DNA positive by Southern blot, log mean viral titers did not differ among the three populations (test, controlling for age), although log mean viral titers did decline with age (r = -0.181; P = 0.05). Viral titers for carriers in their 20s, for example, were higher than titers for carriers in their 40s and 50s, but within the age groups there were no differences among the populations. This suggests that the longer maintenance of the viremic state in the Chinese and Asian-American carriers is not due to initially higher individual viral loads in this population. Productive viral infection is less frequently found in the Senegalese population, but when it is seen in Senegalese carriers, the viral load is comparable to that seen in the other populations (Fig. 2).

Resolution of active viral replication is usually accompanied by the disappearance of HBeAg from the blood and followed by the development of detectable anti-HBe. A total of 554 HBV DNA-negative samples were tested for anti-HBe. The proportion of positive samples differed significantly between populations (χ² = 12.25, 2 degrees of freedom; P = 0.002). In the Chinese group, 162 of 207 (78.3%) had detectable anti-HBe. In the Senegalese and United States Asian groups, 227 of 257 (88.3%) and 82 of 90 (91.1%), respectively, were anti-HBe positive.

Presence of HBV DNA below the limit of sensitivity of the Southern blot was assessed by PCR in the Senegalese and Chinese study populations. We selected a random subset of 66 Chinese and 73 Senegalese samples that were HBV DNA negative on Southern blot for PCR testing. Among the Chinese samples, 27 (40.9%) were positive, compared with 31 (42.5%) among the Senegalese samples (P = 0.87). The prevalence of PCR positivity among those samples that were negative by Southern blot was not significantly different by age group in either population. The prevalence of anti-HBe among Southern blot negatives was independent of PCR status and of population (data not shown). This finding implies that HBV replication may occur at very low levels in chronically infected individuals in both populations even after conversion to anti-HBe positive, although PCR positivity per se is not necessarily due to the presence of infectious viral particles in serum.
Geographic Variation in Viral Load among Hepatitis B Carriers

High-Titer Viremia Is Associated with Concomitant Liver Damage. We used serum GST as an indicator of liver damage, because this assay, unlike the more commonly used transaminase assays, can be performed on frozen serum samples stored for months or years (19, 22). In the Chinese and Senegalese groups, the presence of HBV DNA was significantly associated with the presence of serum GST. In the Chinese group, 60 (36.7%) HBV DNA-positive subjects had detectable serum GST, compared with 37 (13.5%) HBV DNA-negative subjects ($P = 0.02$). In the Senegalese group, the relationship to liver damage was even stronger: 27 (66.7%) HBV DNA-positive subjects were also positive for GST, compared with 17 (5.9%) HBV DNA-negative subjects ($P = 0.0001$). Among those positive for HBV DNA by Southern blot, however, median HBV DNA titers did not differ between GST-positive and -negative samples ($P = 0.62$; data not shown), suggesting that the quantity of virus in the serum is not an indicator of liver damage among viremic individuals.

Surface Antigenemia Is Increased in Senegalese Carriers. During viral replication and virion assembly, a large excess of HBsAg is produced in the infected hepatocyte and secreted into the blood such that the preponderance of HBsAg particles found in the serum contain no viral DNA. Production and secretion of HBsAg may continue in hepatocytes that contain integrated viral DNA and very low or undetectable levels of replicating virus. The significance of this continued production of HBsAg is unknown, but it is a frequent finding in healthy adult HBV carriers in whom no viral DNA is detectable in the blood even with the use of PCR amplification. We examined the titers of serum HBsAg in 382 samples from our 3 populations. Serum titers of HBsAg were significantly higher among those who were HBV DNA positive ($P = 0.0001$; Table I). Log mean HBsAg titers declined with age both in HBV DNA positives ($r = -0.233; P = 0.015$) and HBV DNA negatives ($r = -0.322; P = 0.0001$; Fig. 3) on Southern blot. Among HBV DNA-Southern blot positives, HBsAg titers were significantly and positively correlated with HBV DNA titers ($r = 0.775; P = 0.0001$).

Once age was accounted for, there was no difference in the log mean HBsAg titers among the populations ($P = 0.37$) for Southern blot HBV DNA positives, although titers were slightly higher among the Senegalese. Among Southern blot HBV DNA negatives, however, HBsAg titers were significantly higher in the Senegalese population compared with those of the Chinese and United States Asians ($P < 0.0001$). Median and range values for HBsAg titers by population and HBV DNA status are shown in Table I. HBsAg titers were not significantly higher among those positive for serum GST, regardless of HBV DNA status ($P = 0.93$).

Discussion

Comparison of these three populations revealed a much longer duration of productive viral infection in the Chinese population compared with the West African population. The productive
stage of infection, defined by high titers of virus in the serum detectable by Southern blot, generally terminates among Senegalese males by 30 years of age. By contrast, high-titer virus replication in Haimen City tends to persist longer, with approximately 25% of males remaining productively infected to age 65 years. Among the Asian-Americans studied, productive viral infection tended to persist longer than in the African population as well, but it decreased dramatically after the peak reproductive years. Prolonged high-titer virus production was positively correlated with serological evidence of concomitant liver damage. More prolonged high-titer virus production and liver damage in the Chinese population may explain their higher incidence of HCC compared to the West African population.

A notable strength of our study was our use of HBV carriers identified through routine screening rather than a clinic-based population. Nevertheless, the possibility of bias either in terms of generalizability of each population to its source or in the comparison of the populations to each other cannot be excluded. Although we have no evidence of significant birth cohort effects in these populations, if such effects existed, they may have affected our conclusions related to age effects. In the Senegalese population in particular, discharge or early retirement of viremic HBV carriers could have biased our results. Other studies of HBV DNA or HBeAg seroprevalence in African adults have not reported results by age group.

Geographical differences between Asia and Africa in the risk of perinatal transmission of HBV have been described in many studies but remain largely unexplained both in their origins and in their implications for different outcomes of infection (12, 14, 15). Perinatal transmission is more common in Asian carriers, due in part to the higher prevalences of HBeAg in women of reproductive age, but the origin of this difference in prevalence itself is unknown. Our data suggest that an earlier age of infection may also carry a higher risk of prolonged high-titer viremia, but age at infection alone does not seem to account for the observed differences in age-specific prevalence of HBV DNA among HBV carriers. Whereas Chinese HBV carriers maintained a high level of HBV DNA positivity through the fifth decade of life, Asian-American carriers experienced a dramatic reduction in viremia after the peak reproductive years. African HBV carriers experienced this reduction much earlier, such that HBV DNA positivity was rare in men in their 30s and older. Therefore, our data are consistent with the high level of perinatal transmission by Asian HBV carrier mothers observed by others but suggest that other factors must be responsible for the maintenance of viremia in the Chinese population in middle age and beyond, compared with the United States Asian population.

At the start of infection, HBsAg production results from the transcription of episomal viral DNAs found in the nucleus of the infected hepatocyte and involved in virus production (24–26). Viral DNA, however, randomly integrates into the host genome during prolonged infections. In many patients, viral production eventually ceases, but HBsAg production continues, with transcription apparently occurring from the integrated DNA. The cessation of virus replication in most or all hepatocytes is thought to signal a change in disease state, with active hepatitis reverting to a more quiescent state. In clinic-based studies, this transition is often preceded or accompanied by an exacerbation of liver inflammation, suggesting that an immune-mediated event clears the replicating virus (27). A similar mechanism of clearance seems to be involved in interferon treatment, when successful (28). In our study populations, viral clearance had already occurred in the majority of subjects by 20 years of age, despite the continued presence of significant HBV surface antigenemia, particularly in the Senegalese population. It is not known whether the same mechanisms of clearance apply to all HBV carriers, or whether observations from clinic-based studies can be applied to subjects who have remained asymptomatic throughout the course of their infections. Recent evidence from studies of transgenic mice have shown that suppression of HBV DNA replication may be ac-
complished by coinfection with another hepatotropic virus (29).

In humans, the reduction of HBV replication has been observed during the course of acute hepatitis A virus infection in chronic HBV carriers (30). The influence, therefore, of other viral, bacterial, and parasitic diseases that may cause inflammation of the liver should be explored as a potential explanation for the observed geographic differences.

The significance of the variable HBsAg titers observed in our three populations is unknown. HBsAg titers were highly variable in all of the populations studied, and the significance of these variations remains unclear. Although HBsAg titers did differ significantly between populations and declined with age, the titers themselves did not correlate with a surrogate indicator of ongoing liver damage, serum GST. Higher titers were, in fact, found in HBV DNA-negative subjects who were positive for anti-HBe, usually an indicator of less-severe liver damage in HBV carriers. Antigen titers, although significantly higher among HBV DNA-positive subjects, varied widely over a range from $10^{-4}$ to $10^{3} \mu g/ml$. Senegalese Southern blot HBV DNA-negative subjects had higher HBsAg titers than did HBV DNA-negative subjects in the other two populations, even after accounting for age. It is not known, however, whether differences in HBsAg titers reflect important differences in the natural history of the infection within the individual. Environmental and/or host genetic cofactors may play a role in producing the observed differences among populations. Genetic variants of the S (surface antigen) gene of the virus have been reported to have geographic specificity, with at least one strain unique to Africa (31). Whether African variants produce higher HBsAg titers is unknown. The higher antigen titers seen in Senegalese subjects compared with the other two populations may also reflect the effect of environmental hepatotoxins (e.g., aflatoxins), other hepatotropic infections, or as yet unidentified host factors. Whether it is due to host, viral, or environmental factors, this difference indicates that chronic HBV infection may have a substantially different natural history in Africa than it has in Asia.

In general, active viral replication is associated with ongoing liver damage in HBV carriers (32, 33), but the association of viral replication with HCC risk is still particularly uncertain. Moreover, the clinical significance of low-level viral replication detectable only by PCR is unclear. Population-based prospective studies of HCC risk in HBV carriers around the world have generally not examined HBV DNA and/or HBeAg as risk factors among carriers. In our study, viremia was significantly correlated with detectable GST in the serum, a marker of hepatocellular damage, and viremia was much more frequent and prolonged in the population at highest risk of developing HCC, the Haimen City group. United States Asian HBV carriers, however, also have high prevalences of viremia in early adulthood but seem not to have the extremely high risk of HCC seen in Haimen City and other HBV-endemic areas of Asia. Epidemiological studies of HCC risk in HBV carriers in the United States and other Western countries are needed to confirm this observation. Our observations are provocative, but confirmation must come from well-designed prospective studies of unselected groups of HBV carriers.

HBV carriers in Senegal are still at substantial risk for HCC, despite low levels of viral replication in adulthood (34). What is unknown, however, in the Senegalese population and in other HBV carrier populations, is whether HCC develops preferentially in those few who do remain HBV DNA positive. Ongoing studies in these three populations will be able to confirm whether HBV DNA positivity in adulthood is directly related to HCC risk. Our observations in this cross-sectional study, however, suggest that factors in addition to the duration of active viral replication per se, including ongoing liver damage by both viral and nonviral agents, must be essential in the ontogeny of HCC.

Acknowledgments

We thank Marline Hercog, Gail Duncan, Diane Faison, and Heidi Simmons for technical assistance and Dr. Eric Ross for review of the manuscript.

References


Geographic variation in viral load among hepatitis B carriers with differing risks of hepatocellular carcinoma.

A A Evans, A P O'Connell, J C Pugh, et al.


Updated version
Access the most recent version of this article at:
http://cebp.aacrjournals.org/content/7/7/559