Widespread Dissemination in England of a Stable and Persistent Hepatitis B Virus Variant

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(See the editorial commentary by Zöllner on pages 953–4)

Background. Outbreaks of acute hepatitis B among inmates of 6 prisons in 3 regions of northern England occurring from 1992 through 1994 were found to be associated with a single hepatitis B virus (HBV) variant, which was carried by 20 of the 24 case patients. We instigated a study of cases of acute hepatitis B to trace the spread and prevalence of this variant.

Methods. A denaturing gradient gel electrophoresis assay was optimized to detect the HBV variant, and cases of acute HBV infection in 3 regions in England occurring from 1990 through 1996 were screened for its presence. Samples from HBV-transmission incidents that were received for molecular investigation were also tested.

Results. The variant was identified in 117 (41%) of the 266 cases of acute hepatitis examined in representative regions in England. In North Humberside, but not in southeast England or the West Midlands, a trend toward an increase in the prevalence of the variant was observed. Furthermore, the same variant was identified in the case patients or the individuals implicated in transmission in 11 (22%) of 51 transmission incidents occurring in England from 1997 through 2002. The spread of the variant was primarily associated with injection drug use.

Conclusions. The finding of a single, genetically identical variant (over the 600 bp sequenced) occupying a large niche among the circulating viruses was unexpected. This finding has major implications for the use of DNA sequencing analysis in the investigation of chains of transmission. The study also highlights the need for better protection of at-risk groups through vaccination against HBV, a strategy that currently achieves poor coverage.

Britain has adopted a policy of selective, rather than universal, vaccination against hepatitis B virus (HBV) [1], and outbreaks of acute hepatitis B continue to occur. Vaccination coverage of at-risk groups, which include people with multiple sexual partners, prison inmates, and injection drug users (IDUs), is poor [2–4]. The molecular epidemiology of the HBV strains associated with these outbreaks has been studied, but results have been confined to reports of outbreaks in institutions [5–10] and local communities [11, 12]. Investigations have failed to link outbreaks occurring across wider regions. We describe here the widespread circulation of a particular HBV variant.

MATERIALS AND METHODS

Specimens. In 1994 and 1995, serum samples from inmates with serologically confirmed acute hepatitis B in 3 prisons in Hull (6 samples), a prison in Lancaster (4 samples), a prison in Durham (5 samples), and a prison in Liverpool (12 samples) were referred to the Central Public Health Laboratory (CPHL [Colindale, London]) of the Public Health Laboratory Service (now the Health Protection Agency) for molecular investigation into possible linkage. The onset of disease occurred in 1994 for all of the case patients from Hull, Lancaster, and Durham and occurred during the period 1992–1995 for the case patients from Liverpool. The HBV subgenomic nucleotide–sequencing data revealed that an overwhelming majority of the inmates were infected with a single HBV variant; that is, the DNA sequence was identical over a 400-bp surface (S) gene fragment and a 200-bp core (C) gene fragment. Here-
after, we refer to this variant as “HBVpv,” to indicate “prison variant.” We extended the investigation to determine the extent to which HBVpv had been circulating in the country.

Serum samples that were obtained from case patients with acute hepatitis B during the period 1990–1996 were collected from the following laboratories: the CPHL (119 samples), which receives samples from case patients across a large part of England and Wales—in particular, London, the Home Counties, southeast England, and Trent; the Hull Public Health Laboratory (108 samples), which receives specimen referrals originating in North Humberside; and the Birmingham Public Health Laboratory (70 samples), which receives specimen referrals from the West Midlands. The period 1990–1996 was chosen because it encompassed the period during which the outbreaks occurred in the prisons.

Samples are usually referred from general practitioners and other clinics and institutions to confirm a diagnosis of acute HBV infection. We selected samples solely on the basis of a positive result for IgM antibody to hepatitis B core antigen, which is the marker of acute disease. During the study, we did not investigate any consecutive samples from patients.

Study samples originating from the CPHL were randomly selected (30 samples per year) from the laboratory’s serum repository, and those from the Hull and Birmingham laboratories were derived from all the cases of acute hepatitis B diagnosed in the 2 laboratories. Samples obtained by the CPHL in 1991 and 1992 and those obtained by the Birmingham laboratory in 1996 were not available for study. Risk information relating to case patients whose serum samples were referred to the Hull and Birmingham laboratories was evaluated. The paucity of risk information provided with the serum samples referred to the CPHL precluded a similar analysis for the CPHL cases.

PCR amplification of DNA from the HBV S and C genes. Nucleic acid extraction from 100 μL of serum was done by using a guanidinium thiocyanate–silica method [13]. Extracts were subjected to first-round PCR amplification in 50-μL volumes containing 0.7 U of Expand High Fidelity polymerase mix (Roche Diagnostics), 50 μM of each deoxyribonucleoside triphosphate, 20 pmol of each PCR primer, and 10 μL DNA extract. Primers S5 and S6 were used for first-round amplification of a segment from the HBV S gene [5], and primers 1763 and 2032R were used for amplification of a segment from the C gene [14]. PCR was performed under the following conditions: denaturing at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, with a final extension at 72°C for 2 min. Nested PCR was done under the same conditions, except that 2 μL of the first-round PCR product, as template, and 20 pmol of each inner primer were used. For DNA amplification of the S gene, primers 109 and 585R were used, and primers 1778-E and 2017-B were used for DNA amplification of the C gene [14]. In all PCR experiments, the possibility of cross-contamination was ruled out by the use of appropriate measures, such as negative control samples, aerosol-resistant tips, and separate pre- and post-PCR rooms.

DNA sequencing. PCR products from the outbreak samples from the prisons and from the samples associated with transmission incidents, as detailed in table 1, were purified and submitted for DNA sequencing using the ABI Prism or the ABI BigDye DNA sequencing kits and the ABI 373 or 377 automated sequencer (PE Applied Biosystems). The MegAlign program of the Lasergene package (DNASTAR) was used to create multiple alignments and dendrograms, for comparison of the sequences.

Screening for sequence variation in HBV S and C gene amplicons, by denaturing gradient gel electrophoresis (DGGE). DGGE [15] was adapted to screen for sequence variation in DNA amplified from the HBV S and C genes. To generate amplicons suitable for DGGE, one of the inner primers in the nested PCR was replaced with GC-rich “clamping” primers, to prevent the products from undergoing complete denaturation during electrophoresis. To amplify S gene DNA for DGGE, primers 109-clamp (5′-CGCCCCCAGGGCGCAGGACATTGAGAATTTT-3′) and 585R were used. To amplify C gene DNA, primers 1778-E-clamp (5′-CGCCCCCAGGGCGCAGGACATTGAGAATTTT-3′) and 2017R-B were used. The final PCR products for the S and C genes were 517 bp and 298 bp, respectively.

Optimization experiments were done by using the first 17 samples received from the Hull laboratory for which the sequences of the HBV S and C genes were determined. In 3 samples, the sequences of both fragments were identical to those of HBVpv. In the other 14 samples, a 1–28-bp difference in the S gene and a 1–24-bp difference in the C gene were found. Varying the DGGE conditions showed that, for DGGE to achieve a 1-bp discrimination between PCR products, the gel needs to possess a gradient of 10%–60% of denaturants. A 10%-denaturant mix comprises 8% acrylamide (w/v; Protogel, National Diagnostics), 0.6× Tris-acetate EDTA buffer, 4% formamide (v/v), and 0.7 mol/L urea, whereas a 60%-denaturant mix comprises 8% acrylamide, 0.6× Tris-acetate–disodium EDTA buffer, 24% formamide, and 4.2 mol/L urea. Gradient gels were poured by using Ingeny Phor U2 electrophoresis apparatus (Genetic Research Instrumentation Ltd.), in accordance with the manufacturer’s instructions. PCR products (2–7 μL) obtained with the “clamping” primers were mixed with an equal volume of loading buffer and were loaded into wells (each gel can accommodate up to 48 samples). Separate gels were used to screen for variations in the sequences of the HBV S and C gene amplicons. Wells at the ends and the middle of the gels were loaded with PCR products from plasmids that had been constructed to carry HBVpv S or C gene inserts. Electrophoresis
Table 1. Summary of findings for 11 outbreaks and transmission incidents associated with the hepatitis B virus “prison variant” (HBVpv), 1997–2002.

<table>
<thead>
<tr>
<th>Setting, risk factor, incident cluster</th>
<th>Year(s)</th>
<th>Location(s)</th>
<th>No. of cases of acute infection with HBVpv(^a)</th>
<th>Transmitter infected with HBVpv</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hospital</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Blood transfusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1997</td>
<td>Liverpool</td>
<td>1/1</td>
<td>Yes(^b)</td>
</tr>
<tr>
<td>2</td>
<td>1998</td>
<td>Birmingham</td>
<td>1/1</td>
<td>Yes(^b)</td>
</tr>
<tr>
<td><strong>Surgery</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1997</td>
<td>Warrington</td>
<td>2/3</td>
<td>Not applicable(^c)</td>
</tr>
<tr>
<td>4</td>
<td>1998</td>
<td>London</td>
<td>1/2</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Community</strong></td>
<td></td>
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<tr>
<td><strong>Tattooing</strong></td>
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</tr>
<tr>
<td>5</td>
<td>1997</td>
<td>Hull</td>
<td>3/3</td>
<td>Not applicable(^c)</td>
</tr>
<tr>
<td><strong>Family</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2001</td>
<td>Chester</td>
<td>1/1</td>
<td>Yes(^d)</td>
</tr>
<tr>
<td><strong>Injection drug use</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1997</td>
<td>Hull</td>
<td>3/3</td>
<td>Not applicable(^c)</td>
</tr>
<tr>
<td>8</td>
<td>2000</td>
<td>Norwich</td>
<td>7/7</td>
<td>Not applicable(^c)(^e)</td>
</tr>
<tr>
<td>9</td>
<td>2002</td>
<td>Leicester</td>
<td>14/19</td>
<td>Not applicable(^c)(^e)</td>
</tr>
<tr>
<td><strong>Risk factor unknown</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>10</td>
<td>1997</td>
<td>Warrington</td>
<td>13/14</td>
<td>Not applicable(^c)(^e)</td>
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<tr>
<td></td>
<td></td>
<td>Whiston</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1998 and 1999</td>
<td>Ashford</td>
<td>2/14</td>
<td>Not applicable(^c)(^e)</td>
</tr>
</tbody>
</table>

\(^a\) Only HBV DNA-seropositive cases were considered.
\(^b\) Donor seronegative for hepatitis B surface antigen (HBsAg) at time of blood donation; recipient infected with HBVpv. Donor subsequently became positive for HBsAg.
\(^c\) Transmitter not identified.
\(^d\) Mother-child transmission that was presumed to be vertical.
\(^e\) Investigation done in response to upsurge observed in local incidence of acute hepatitis B.

was done in 0.6× Tris-acetate-disodium EDTA buffer at 60°C and at 100 V, for 18 h. DNA bands were visualized by UV transillumination after staining with SYBR Green I (Flowgen). Serum samples were considered to carry HBVpv if the PCR products derived from both the HBV S and C genes migrated to the same gel position as that for the referent HBVpv bands.

Search for HBVpv involvement in HBV-associated outbreaks and other transmission incidents. The CPHL had been conducting molecular investigations into hepatitis B outbreaks since 1993 [5]. Records of the investigations that involved molecular epidemiology were reviewed to determine the extent to which HBVpv could be identified in the outbreaks.

RESULTS

HBVpv in inmates of prisons in the north of England. All 15 serum samples from inmates in the prisons in Hull, Lancaster, and Durham and 9 of the 12 samples from inmates in the prison in Liverpool yielded amplifiable DNA from the HBV S and C genes. Direct sequencing of the PCR products showed that 5 of 6 samples from Hull, 3 of 4 samples from Lancaster, 3 of 5 samples from Durham, and all 9 samples from Liverpool (20 [83%] of 24 samples) carried HBV bearing the HBVpv S and C gene sequences. These sequences, which have been deposited in GenBank (accession nos. AY374226 and AY374227), permit HBVpv to be assigned to genotype A and the predicted hepatitis B surface antigen (HBsAg) subtype to adw2.

Prevalence of HBVpv in representative regions of England. Two hundred sixty-six HBV DNA–amplifiable samples assembled from the CPHL and from the Hull and Birmingham laboratories were screened by DGGE for the carriage of HBVpv S and C gene sequences. Figure 1 shows representative outcomes following DGGE of PCR products generated from the HBV S and C genes in a set of serum samples referred to the CPHL in 1990 and 1996. In the figure, DNA bands that migrated to the same position as the referent HBVpv bands are indicated with asterisks. Only serum samples yielding HBVpv–like bands for both the S and C genes were considered to carry HBVpv (in figure 1, these serum samples correspond to lanes 1, 5, 7, 12, 20–22, and 30 for the sample from 1990 and lanes 1, 3–5, 7, 15, 17–19, 23, 26, and 27 for the sample from 1996). Figure 2 illustrates the outcome of a DGGE study of PCR products...
Figure 1. Representative gels obtained by denaturing gradient gel electrophoresis of PCR products generated from the hepatitis B virus (HBV) surface (S) and core (C) genes in a set of serum samples referred to the Central Public Health Laboratory in 1990 and 1996. Lane P, referent HBV^prv^ ("prison variant") amplicons. Asterisks indicate DNA bands that migrated to the same position as the referent amplicons.
from the HBV C gene that were derived from the entire set of serum samples referred to the Hull laboratory from 1990 through 1996. The trend toward an increase in the number of cases of acute hepatitis B and in the proportion of amplicons bearing the HBV<sup>PV</sup> C gene sequence is clearly evident: by 1995 and 1996, an overwhelming majority of serum samples were carrying the HBV<sup>PV</sup> C gene sequence.

Changes in the proportion of serum samples carrying HBV<sup>PV</sup> for the 3 laboratories are summarized in figure 3. The increase in the proportion of samples carrying HBV<sup>PV</sup> was significant for samples from the Hull laboratory (P<.01, by the χ² test for linear trend) but not for samples from the CPHL and Birmingham laboratory. Although the differences among the 3 laboratories in the proportion of samples carrying HBV<sup>PV</sup> were not significant for 1990 and 1993, the differences were significant for 1994 and 1995 (P = .017 and P<.0001, respectively, by the χ² test).

Of the 58 case patients from Hull who were infected with HBV<sup>PV</sup>, 26 (45%) were IDUs, and 12 (21%) had a history of imprisonment; no risk information was available for the 20 other case patients (34%). Of the 26 case patients from Birmingham who were infected with HBV<sup>PV</sup>, risks associated with acute hepatitis B were as follows: male homosexual intercourse, 7 case patients (27%); overseas travel, 3 case patients (12%); injection drug use, 2 case patients (8%); heterosexual intercourse, 2 case patients (8%); medical treatment/injections, 2 case patients (8%); and body piercing, 1 case patient (4%). For 5 case patients (19%), no risk was identified, and no information was available for 4 case patients (15%).

**HBV<sup>PV</sup> in HBV-associated outbreaks and other transmission**
incidents. From 1995 through 2002, the CPHL conducted 51 investigations into hepatitis B outbreaks and transmission incidents in the United Kingdom, and the incidence of acute hepatitis B in each cluster ranged from 1–33 cases. Eleven (22%) of these investigations were of outbreaks and transmission incidents involving HBVPV in the case patients or the transmitters of infection and were located across geographically disparate regions. A summary of the characteristics of HBVPV-associated outbreaks and transmission incidents is shown in table 1. The transmitters of infection were grouped into the following 4 clusters: 2 associated with those who had received blood transfusions (clusters 1 and 2), 1 associated with surgeon-patient transmission (cluster 4), and 1 associated with mother-child transmission (cluster 6). For 7 other clusters (clusters 3, 5, and 7–11), no transmitter of infection was implicated, but HBVPV was identified among the case patients, in varying proportions.

Phylogenetic analysis. To further characterize the HBVPV sequence, we carried out a phylogenetic comparison of the longer gene fragment (the S gene) and 18 other genotype A sequences identified during investigations of transmission incidents and 4 genotype A sequences deposited in GenBank (accession nos. X75666, X51970, M57663, and X75669). The resulting dendrogram (figure 4) shows several very closely related sequences present in the CPHL samples. Sequences South East Health Protection Agency 5, 6, 8–10, and 14 and X75666 differed by only a single nucleotide.

Furthermore, a BLAST search of the GenBank database, using the PCR-product sequences from the HBVPV S and C genes, returned a large number of identical or very similar sequences from various studies, indicating that HBVPV is a common variant worldwide.

DISCUSSION
HBV has a low rate of endemicity in the United Kingdom [1]. Previous molecular studies of outbreaks [5–12] have supported the assumption that sporadic cases and outbreaks of acute hepatitis B are caused by HBV variants unlinked to one another. On the contrary, the studies described here show that a significant proportion of outbreaks of HBV infection and of transmission incidents are associated with a particular variant of HBV, HBVPV. To the best of our knowledge, defined HBV variants circulating in a population have not been described in the scientific literature. The closest example is a study of 30 epidemiologically unlinked patients with acute HBV infection in Samara, a Russian city [16]. In that study, a large majority of patients was found to carry HBV with identical subgenomic sequences, but the analysis was not extended to the determination of how the predominantly circulating variant was transmitted over time.

We first identified HBVPV in the clusters of cases of acute hepatitis B that occurred from 1992 through 1994 among inmates of prisons in northern England, and we found that >80% of the cases were associated with this particular HBV variant. We attribute these outbreaks to the sharing of needles and other equipment for the injection of narcotics. Prisons provide a unique environment for the transmission of bloodborne infections, owing to high-risk behaviors such as injection drug use and, to a lesser extent, men having sex with men [17–20]. Also, vaccination programs for the prevention of HBV infection have not been well implemented in prisons [19, 21–24]. None of the inmates included in this study had been vaccinated prior to infection.

Application of a high-throughput assay involving DGGE of
serum samples from case patients from 3 regions of England who had received a diagnosis of acute hepatitis B between 1990 and 1996 revealed that HBV<sup>PV</sup> was widespread in the country prior to, contemporaneous with, and following the outbreaks in the prisons. The high prevalence of HBV<sup>PV</sup> was indicated by the findings that 16%–37% of the set of study serum samples from the CPHL (mostly representing cases of acute hepatitis B in southeast England) and 25%–70% of the serum samples from the Birmingham laboratory (reflecting cases of acute hepatitis B in the West Midlands) contained HBV<sup>PV</sup> (figure 3).

Although no trend toward an increase in the proportion carrying HBV<sup>PV</sup> was observed for the samples from the CPHL and the Birmingham laboratory, a notable trend of an increase in the proportion of samples referred by the Hull laboratory was observed. This increase corresponded with the appearance of an outbreak of acute hepatitis B in the community that began in 1994 (figures 2 and 3), implicating HBV<sup>PV</sup> as the cause of the outbreak. Risk analysis showed that the increase in HBV<sup>PV</sup>-associated cases in North Humberside was associated with imprisonment and injection drug use. People released from prison tend to change sexual partners frequently and to continue to engage in injection drug use [3, 18, 19, 23]. Whether the community outbreak of acute hepatitis B was linked to the outbreaks in the Hull prisons in 1994 cannot formally be ascertained, although the chronology of events and the high proportion of former prison inmates among the case patients in the community are consistent with such a linkage.

That HBV<sup>PV</sup> may be found in subpopulations other than those consisting of IDUs and people with a history of imprisonment is suggested from the risk analysis for the HBV<sup>PV</sup>-infected case patients in the West Midlands. The analysis showed that sexual contact (both homosexual and heterosexual), rather than injection drug use or imprisonment, was the dominant risk factor associated with acute HBV<sup>PV</sup> infection. Analysis of the outbreaks of HBV infection and of other transmission incidents investigated by the CPHL from 1995 through 2002 also showed that HBV<sup>PV</sup> could be carried by a variety of people other than those who admitted to being IDUs; these people included a surgeon, blood donors, and customers of a tattoo parlor (table 1). Nonetheless, the prevalence of HBV<sup>PV</sup> among IDUs remained high, as exemplified by the clusters of cases in Norwich (cluster 8) and in Leicester (cluster 9).

The larger DNA fragment that we studied encompassed codons 40–174 of the HBV S gene, which brackets the major hydrophilic region of HBsAg. Mutations associated with vaccine escape and weak HBsAg signals in serological assays are located in this domain [25]. We sought such mutations and found HBV<sup>PV</sup> to be a wild-type variant. We did not look for mutations in the pre–C gene domain, some of which are known to abrogate the production of hepatitis B e antigen (HBeAg) [25], because serological testing of the original prison inmates infected with HBV<sup>PV</sup> had shown that the case patients carried HBeAg.

Despite the involvement of HBV<sup>PV</sup> in many chains of infection during ~10 years, no sequence changes were observed in the S and C genes. We found this to be surprising, particularly because these genes tend to carry more hypervariable loci than other HBV genomic domains [26]. Phylogenetic analysis that included other genotype A sequences from investigations of transmission incidents revealed several very closely related S gene sequences (figure 4). Analysis of more of the genome would indicate whether HBV<sup>PV</sup> and closely related sequences should be considered a distinct variant family or quasi species and to what extent they have penetrated the population of circulating HBV in the United Kingdom [27]. Whether such stability is specific to HBV<sup>PV</sup> or whether it could be conferred on any HBV variant that found a niche (such as among IDUs) favoring rapid and continuing transmission from hosts with acute infection is unknown.

One important consequence of a particular HBV sequence being maintained over a long period despite transmission through many cycles of infection is that epidemiological inferences about causation and transmission could be con-
founded. Nucleotide-sequencing assays increasingly are being used to establish that a particular HBV variant is carried by the source of a cluster of cases of HBV infection and to track chains of HBV transmission [5, 7–10, 12, 28–32]. When the same or very similar subgenomic sequences are identified, the conclusion reached is that recent transmission has occurred between infected individuals. In view of our findings, the use of PCR sequencing alone cannot be the basis for implicating an HBV variant in transmission events without a survey of HBV genomes concurrently in circulation. Reliance must continue to be placed on other, carefully collected epidemiological data. When the epidemiological and molecular data are correlated, insights into the source and spread of HBV infection may be revealed.

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