Outcome of lamivudine resistant hepatitis B virus infection in the liver transplant recipient

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Abstract

Background—In many transplant centres lamivudine is an important component of prophylaxis against, and treatment of, hepatitis B virus (HBV) graft infection. Drug resistant HBV species with specific polymerase mutations may emerge during lamivudine treatment.

Aims—To examine the clinical consequences of graft infection by lamivudine resistant virus.

Methods—The clinical course of four liver transplant patients who developed graft infection with lamivudine resistant virus was reviewed. The response of HBV infection to reduction of immunosuppression and to manipulation of antiviral therapy was assessed. For each patient, serum viral titre was measured and the viral polymerase gene was sequenced at multiple time points.

Results—High serum titres were observed following emergence of the lamivudine resistant species. Wild type HBV re-emerged as the dominant serum species after lamivudine withdrawal. All patients developed liver failure, and onset of liver dysfunction was observed when resistant virus was the dominant serum species. In three patients, liver recovery was observed when immunosuppression was stopped and when alternative antivirals were given. Wild type virus appeared to respond to ganciclovir, and to reintroduction of lamivudine. For one patient, introduction of famciclovir was associated with clinical, virological, and histological response.

Conclusions—Failure of lamivudine prophylaxis may identify patients at special risk for the development of severe graft infection. Treatment of graft reinfection should include reduction of immunosuppression, and systematic exposure to alternative antivirals. Viral quantitation and genetic sequencing are essential components of therapeutic monitoring.

Keywords: hepatitis B virus; lamivudine; prophylaxis; reinfection

Lamivudine is a nucleoside analogue that inhibits the replication of hepatitis B virus (HBV). It has been used for the treatment of HBV infection of immunocompetent and immunosuppressed patients. In the liver transplant setting, lamivudine has been used for prophylaxis, and for treatment of established graft infection. It was in the context of transplantation that the first reports of lamivudine resistant HBV emerged. The genetic changes associated with lamivudine resistance are, at least partially, understood. The resistant genotype requires a change at codon position 552 of the viral polymerase gene which causes a change from methionine to valine (M552V) or isoleucine (M552I). Reported cases of M552V are associated with L528M (methionine to leucine change at codon position 528). Other amino acid substitutions of uncertain significance have also been described.

The resistant species have also been observed in the immunocompetent during longer term treatment, and emergence in this setting has been associated with resumption of viral replication and with recurrent hepatitis.

The clinical course of infection with lamivudine resistant species (with and without continuation of lamivudine treatment) in immunocompetent and immunosuppressed patients needs to be studied. For instance, liver disease associated with resistant virus may be more or less aggressive than that associated with wild type HBV infection. If resistant virus is less aggressive, it may be preferable to continue lamivudine despite the emergence of drug resistance. Also, the response of lamivudine resistant virus to treatment with other antivirals needs to be studied. Resistance to lamivudine may be associated with resistance, reduced sensitivity, or enhanced sensitivity to other antivirals.

In this report we describe the clinical course of four patients who developed liver allograft infection with HBV as a result of the failure of lamivudine prophylaxis.

Methods

HBsAg/anti-HBe and IgM anti-HBc were measured in a semiquantitative assay (Amerlite, Ortho Clinical Diagnostics, Amersham, UK). HBsAg and anti-HBs titres were measured quantitatively against international standards using enhanced luminescent immunonassays (Amerlite, Ortho Clinical Diagnostics, Amersham, UK).

Two assays were used for measurement of serum HBV DNA. For prospective patient management, HBV DNA was measured with the Genostics assay (Abbott, Chicago, Illinois, USA). This is a relatively insensitive assay which measures DNA in picograms per ml of

Abbreviations used in this paper: HBV DNA, hyperimmune globulin; HBV, hepatitis B virus; LFT, liver function test.
serum. Values less than 3 pg/ml are considered negative, though 1 pg/ml corresponds to $10^5$–$10^6$ genomic copies/ml. Thus, when HBV DNA titre is low, an alternative, more sensitive assay is required. For this purpose, sera were stored at $-70^\circ$C, and HBV DNA titre was subsequently measured with the Roche Diagnostics Amplicor HBV Monitor assay according to the manufacturer’s package insert instructions. This polymerase chain reaction (PCR) based assay quantitates HBV DNA between the titres of 400 and 40 million genomic copies/ml of serum. Use of the two assays permits quantitation of serum HBV DNA over a 7 log$_{10}$ range.

For viral DNA sequence analysis, part of the polymerase gene was amplified by PCR using primers 5’-GCCCGTTTGTCCTATAAT-3’ (nt 446–463) and 5’-TAACCCCATCTTTTTGTTTG-3’ (nt 863–844). The PCR products were directly sequenced using the same primers and an Applied Biosystems automatic DNA sequencer.

Patients
Between 1/1/94 and 1/10/97, 26 patients with HBV associated liver disease underwent liver transplantation at the Queen Elizabeth Hospital. For the duration of this study period, patients presenting with fulminant HBV received hyperimmune globulin (HB Ig) as prophylaxis. During 1994, patients with DNA negative chronic HBV (negativity defined by the Genostics assay) received HB Ig as prophylaxis, and DNA positive patients received lamivudine as prophylaxis. Since 1995, all patients with chronic HBV associated liver failure requiring transplantation have received lamivudine as prophylaxis (irrespective of serum HBV DNA status).

During the study period, seven patients received HB Ig prophylaxis. Five continue HB Ig with no evidence of recurrent HBV infection. One patient suffered graft reinfection with an HB Ig surface antigen “escape” mutant. Another patient (patient 4) suffered an anaphylactic reaction to HB Ig, and was converted to lamivudine prophylaxis. Subsequently, lamivudine resistant HBV emerged.

During the study period, 19 patients received lamivudine as primary prophylaxis. During the early post-transplant period three died of causes unrelated to HBV infection. Following emergence of lamivudine resistant HBV, two patients died of recurrent HBV infection (patients 1 and 2). Fourteen survivors have been followed for 20–60 months post-transplant. Only 1/14 has clinical evidence of graft reinfection (patient 3).

Thus, we report the outcome of four patients who have developed graft reinfection with lamivudine resistant virus.

Patient 1
A 45 year old Asian woman with decompensated cirrhosis was referred to be considered for liver transplantation. At the time of initial assessment she had high levels of viral replication (HBeAg positive, HBV DNA 38 pg/ml (Abbott Genostics assay), HBV DNA >40 million genomic copies/ml (Roche Amplicor HBV Monitor assay)). She fulfilled the inclusion criteria for participation in a phase 2 study designed to assess the safety and preliminary efficacy of lamivudine monotherapy as prophylaxis. For the duration of this study period, patients presenting with fulminant HBV received hyperimmune globulin (HB Ig) as prophylaxis. During 1994, patients with DNA negative chronic HBV (negativity defined by the Genostics assay) received HB Ig as prophylaxis, and DNA positive patients received lamivudine as prophylaxis. Since 1995, all patients with chronic HBV associated liver failure requiring transplantation have received lamivudine as prophylaxis (irrespective of serum HBV DNA status).

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### Table 1 Sequencing data

<table>
<thead>
<tr>
<th>Patient</th>
<th>Specimen</th>
<th>Date</th>
<th>Codon 528</th>
<th>Codon 552</th>
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<tr>
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<td>Valine</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
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<td>Methionine</td>
<td>Valine</td>
<td></td>
</tr>
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<td>Methionine</td>
<td>Valine</td>
<td></td>
</tr>
<tr>
<td>#6</td>
<td>24/07/96</td>
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<td>Methionine</td>
<td>Valine</td>
<td></td>
</tr>
<tr>
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<td>18/03/97</td>
<td>Methionine</td>
<td>Valine</td>
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<td>19/05/97</td>
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<td>Methionine, valine, and isoleucine*</td>
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Part of the polymerase gene (nt 446–863) was amplified by PCR and directly sequenced using the same primers and an automatic DNA sequencer. Multiple serum samples were examined (#1 to #19) and are referred to in the text and figures. Multiple changes were observed, and the amino acids at polymerase codon positions 528 and 552 are shown.

*Direct sequencing of serum #11 suggested that a mixture of viral species was present—leucine or methionine at position 528, and methionine and valine or isoleucine at 552.*
Lamivudine resistant HBV infection in the liver transplant recipient

Lamivudine was increased to 300 mg/day, but serum HBV DNA did not decline during four months treatment at the higher dose. Lamivudine was stopped on 8/12/95 (specimen #3), and treatment with famciclovir (750 mg/day) was commenced at the next outpatient attendance one month later (5/1/96; fig 2). Famciclovir treatment was sustained at that dose for four months, but serum HBV DNA did not decline during famciclovir therapy.

The dominant HBV species in the patient’s serum during famciclovir treatment (18/3/96, specimen #4) was the lamivudine resistant genotype. Despite high levels of viral replication during lamivudine then famciclovir treatment, biochemical liver function tests (LFTs) remained almost normal. Subsequently, three months after famciclovir was withdrawn, progressive and severe disturbance of liver function was observed. At the time that serum aminotransferase began to rise (16/5/96, specimen #5 and 24/7/96, specimen #6), the dominant HBV species was the lamivudine resistant genotype. By 4/9/96 (specimen #7) genetic sequencing established that the dominant serum HBV species had reverted to a lamivudine sensitive genotype. Liver biopsy performed two months after the onset of liver dysfunction confirmed the presence of recurrent HBV infection with histological features of fibrosing cholestatic hepatitis. There was extensive expression of HBV antigens. The patient developed liver failure with jaundice and ascites.

Immunosuppression was gradually reduced during this period of progressive liver dysfunction. All immunosuppression was stopped on 19/9/96, and, in the absence of clinical improvement, treatment with ganciclovir was commenced two weeks later. At the time that ganciclovir was commenced, serum HBV DNA titre was >40 million genomic copies/ml and the dominant species had the lamivudine sensitive genotype. During the subsequent three week period, there was convincing improvement in the patient’s clinical condition and LFT’s. During the first nine days of ganciclovir treatment, serum titre declined more than 1 log_10. Unfortunately, she died of staphylococcal septicaemia on 22/10/96, nearly two years post-transplant. The source of the septicaemia was the Hickman intravenous catheter which was required for the prolonged administration of ganciclovir. Postmortem examination was refused.

PATIENT 2
A 61 year old white man with a small hepatoma complicating HBV cirrhosis was referred to be considered for liver transplantation. Investigations at that time confirmed the presence of a small (<3 cm size) single lesion with no evidence of vascular invasion and no overt extrahepatic spread. Serology confirmed high levels of viral replication (HBV DNA 10 pg/ml (Abbott Genostics assay), HBV DNA 10 018 160 genomic copies/ml (Roche Amplicor HBV Monitor assay)). The patient was suitable for inclusion in the previously

Figure 2  Response observed in patient 1 (HBV titre measured with Abbott Genostics assay) to administration of increased lamivudine (LAM) dose, famciclovir (FAM), ganciclovir (GAN), and cessation of immunosuppression. HBV was sequenced at multiple time points #2 to #7. CycA, cyclosporin.

Figure 3  Response of patient 2 to treatment with lamivudine (LAM).
Lamivudine treatment was commenced on 4/8/95, and it e\textsuperscript{2}V\textsubscript{ected a dramatic reduction in serum HBV DNA which was below the thresh-
old of the Amplicor assay (400 genomic copies/ml) after five weeks (12/9/95). HBV DNA was undetectable in serum for seven months post-transplant. Subsequently, serum HBV DNA titre increased exponentially (fig 3). At the time that severe biochemical liver dysfunction emerged (specimen #9), lamivudine treatment was ongoing, and the resistant genotype was the dominant serum species. Immunosuppression was reduced, and liver biopsy was performed on 24/10/96, then again on 18/11/96. Appearances were those of aggressive hepatitis, with extensive HBV surface antigen (HBsAg) expression and ductular proliferation. In these and subsequent biopsies, HBV core antigen (HbcAg) expression could not be shown, and there was no evidence of rejection.

Lamivudine treatment was stopped on 13/2/97, and five weeks later (18/3/97, specimen #10) the lamivudine resistant genotype was still the dominant serum species. Severe hepatitis persisted, and all immunosuppression was stopped on 29/3/97. Liver biopsy at that time confirmed hepatitis of moderate severity with extensive immature fibrosis progressing to early micronodular cirrhosis. On 19/5/97 further biochemical deterioration was observed. Sequencing identified a mixture of lamivudine resistant and lamivudine sensitive genotypes in serum at that time (specimen #11, 19/5/97).

On 22/5/97, ganciclovir treatment was commenced, but there was no apparent clinical or biochemical improvement. The mixture of HBV species persisted during ganciclovir treatment. One week later, lamivudine was added to ganciclovir, and both drugs were con-
tinued until the time of death (7/6/97). Application of the Amplicor assay confirmed that there was no significant decline of serum HBV DNA titre during ganciclovir or no decline during combination ganciclovir/ lamivudine treatment.

**PATIENT 3**

A 50 year old Chinese man with decompensated cirrhosis due to HBV infection was referred to be considered for liver transplantation. At the time of assessment, he had high levels of viral replication (HBV DNA 10 pg/ml (Abbott Genostics assay), HBV DNA 11 000 000 genomic copies per ml (Roche Amplicor HBV Monitor assay)). He was suitable for transplantation, and suitable for participation in the previously mentioned clinical study. Lamivudine e\textsuperscript{2}V\textsubscript{ected a reduction of serum HBV DNA, which was below the Amplicor detection limit two weeks after trans-
plantation. Eleven months after transplantation, recurrence of viraemia (detected by the Amplicor assay) was associated with the emergence of a lamivudine resistant species (speci-
men #13, 28/5/97). Liver biopsy confirmed the presence of graft reinfection.

Three months later, when serum HBV DNA had become detectable by the Abbott Genos-
tics assay, biochemical liver dysfunction developed, and liver histology at this time showed recurrent hepatitis B infection with widespread cytoplasmic staining for HBsAg and panacinar immunoreactivity for HbcAg (fig 4A). Subsequently, immunosuppression was stopped, treatment with lamivudine was maintained,
PATIENT 4

A 42 year old white man with HBV associated cirrhosis and diuretic resistant ascites was referred to be considered for liver transplantation. At the time of assessment, his serum was HBeAg positive but HBV DNA could not be detected with the Abbott Genostics assay (and therefore, he did not fulfil the criteria for inclusion in the previously mentioned study). Liver transplantation was undertaken on 4/1/95, and HBIg was administered according to protocol, such that 10 000 units were given during the anhepatic phase of the transplant operation, and 5000 units were administered on each of the first three postoperative days. Thereafter, further HBIg was given when serum anti-HBs titre declined below 100 IU/litre. On day 7 post-transplant the patient experienced an anaphylactic type reaction during HBIg administration. HBIg was discontinued and lamivudine prophylaxis (supplied for “compassionate” use by GlaxoWellcome) was commenced on day 15 post-transplant.

Lamivudine prophylaxis achieved profound inhibition of viral replication (serum HBV DNA below the Amplicor assay detection threshold until 22/8/95). Genetic sequencing of sera collected on 17/10/95 (specimen #15) confirmed that a lamivudine resistant HBV genotype had emerged (fig 7). Serum HBV DNA and HBsAg titres increased exponentially. Liver biopsy performed on 4/12/95 showed the presence of mild hepatitis associated with generalised intense HBcAg and HBsAg expression. Lamivudine treatment was stopped on 12/12/95 (specimen #16). By 26/6/96, the lamivudine sensitive genotype had become the dominant serum species (specimen #17). High levels of viral replication were sustained during transition from the lamivudine resistant to the lamivudine sensitive dominant serum species. Treatment with famciclovir (750 mg/day) was commenced on 24/4/96, but failed to effect a significant reduction of serum HBV DNA during a three month treatment period (treatment stopped on 4/8/96, specimen #18). Biochemical liver dysfunction developed during famciclovir treatment, and liver biopsy performed on 14/8/96 showed the presence of cholestatic hepatitis associated with extensive HBV antigen expression.

The patient developed liver failure with jaundice and ascites. All immunosuppression was stopped on 21/8/96. Treatment with ganciclovir commenced on 27/8/96. During the subsequent three month period, there was dramatic clinical improvement with resolution of jaundice and ascites. During this period, serum HBV DNA titre declined from 378 pg/ml to <3 pg/ml (the lower detection limit of the Abbott Genostics assay). According to the Amplicor assay (data not shown), a 2 to 3 log10 decline in serum HBV DNA titre was observed. Despite the observed clinical improvement, liver biopsy (4/11/96) showed more severe hepatitis associated with rapidly developing fibrosis, though diminished HBV antigen expression. Subsequently, during ganciclovir therapy, viral replication resumed, and liver dysfunction ensued. Liver biopsy showed severe hepatitis associated with areas of bridging necrosis, severe cholestasis, and extensive viral antigen expression. Serum viral titre was greater than 40 million copies per ml, and sequencing
confirmed the dominance of the lamivudine sensitive genotype (4/6/97, specimen #19). Treatment with lamivudine was commenced on 4/6/97 and effected a reduction of serum HBV titre of at least 2 to 3 log units. One week later, ganciclovir was added to the patient’s treatment, and further reduction of viral titre was observed. Two weeks after commencement of lamivudine, the dominant serum species was still the lamivudine sensitive genotype. At this time, the patient expressed a wish to be discharged from hospital. At home, he persisted with oral ganciclovir and lamivudine. He was admitted to his local hospital four weeks later. He had suffered a variceal haemorrhage, and underwent sclerotherapy and transfusion. He declined further treatment, and died at home soon after. At the last hospital attendance, the serum bilirubin had declined significantly. At that time, serum was not available for virological examination.

Discussion

These four patients developed severe graft dysfunction pursuant to the emergence of lamivudine resistant HBV infection following liver transplantation. Patients 1, 2, and 3 received lamivudine (without HBIG) as prophylaxis according to a protocol that required a pretransplant treatment duration of at least four weeks. The preliminary results of that clinical trial have been published, and confirm that this schedule of prophylaxis can provide sustained inhibition of HBV replication post-transplant for a majority of treated patients. In that study, failure of prophylaxis was invariably the result of the emergence of HBV species that were resistant to lamivudine. For patient 4 of the present report, lamivudine was provided on a compassionate basis.

The genotypic changes associated with phenotypic resistance have been described previously. Our initial description of lamivudine resistance included patients 1 and 4 of this report. At the time of that report, neither had developed graft dysfunction. Lamivudine resistance resulting in graft infection has also been reported by others. Published reports of lamivudine resistance in the context of liver transplantation are remarkable for the relatively constant timing of relapse post-transplant. Graft reinfection with lamivudine resistant species typically becomes apparent during the second half of the first post-transplant year. This observation, and the fact that pretreatment viral titre appears to predict post-transplant emergence of the lamivudine resistant species, suggest that the resistant virus may be present in the viral quasispecies prior to lamivudine exposure, persists in serum at the time of liver transplantation, then establishes graft reinfection.

The nucleoside analogues, famciclovir and ganciclovir, have been used to treat wild type HBV recurrence following transplantation. It is possible that the lamivudine resistant genotype may be more, or less, sensitive than the wild type to treatment with these agents. Published abstracts suggest that the lamivudine resistant species (both L528M/M552V and M552I) may be poorly responsive/insensitive to famciclovir treatment. Indeed, Aye et al observed polymerase changes (putatively associated with famciclovir resistance), including the L528M mutation, develop during famciclovir treatment of wild type HBV.

Examination of these four patients permits limited conclusions concerning the efficacy of famciclovir and ganciclovir. For patient 1, extremely high levels of viral replication (predominantly the lamivudine resistant phenotype) were sustained, and severe hepatitis developed during famciclovir treatment. Subsequently, when the wild type was dominant, a reduction of serum titre was observed during administration of ganciclovir after immunosuppression was stopped. The relative importance of these two measures (ganciclovir administration and cessation of immunosuppression) for the inhibition of viral replication cannot be deduced. However, the strategy was associated with a dramatic reduction of serum HBV titre and with an impressive clinical improvement. A similar response to the same combination of measures was also observed during treatment of wild type virus in patient 4.

For patient 2, hepatitis was aggressive from the time that viral titre achieved levels that were detectable with the virology assay. At that time during lamivudine therapy the dominant species was lamivudine resistant. This patient died from aggressive hepatitis seven months later. His clinical condition, LFTs, and serum viral titre were unresponsive to withdrawal of immunosuppression, and unresponsive to brief treatment with ganciclovir, then reintroduction of lamivudine. For this patient, repeated liver biopsies were remarkable for the absence of HBcAg. It is possible that the absence of HBcAg positive cells reflected vigorous immune mediated clearance of HBV infected hepatocytes.

For patient 3, an alternative antiviral strategy was chosen. To maintain the lamivudine resistant genotype, lamivudine therapy was continued. Then, the response of that genotype to treatment with famciclovir was assessed. The response observed when this genotype (L528/I552) was treated with famciclovir was remarkable. This response contrasts with the response observed to treatment with famciclovir of patient 1 (M528/V552), and is consistent with the observation of Aye et al that the presence of a leucine residue at polymerase codon position 528 may be an important determinant of famciclovir sensitivity and resistance.

Finally, after re-emergence of wild type HBV, patient 4 was eventually exposed to famciclovir, ganciclovir, and to further lamivudine treatment. There was no apparent virological response to treatment of wild type with famciclovir, and severe liver dysfunction developed during treatment. Subsequently, dramatic clinical, biochemical, and virological response was observed when immunosuppression was stopped and ganciclovir was given. Unfortunately, virological relapse was observed during ganciclovir treatment. Later, when liver failure again ensued, virological and biochemical improvement was effected by lamivudine.
treatment (with possible contribution by ganciclovir). This observation suggests that a patient who has previously developed lamivudine resistance may benefit from the later reintroduction of that drug for treatment of severe liver dysfunction. Unfortunately, severe graft damage resulting in portal hypertension had already developed, and the patient died.

It may appear that the administration of antivirals to these patients after the emergence of lamivudine resistance was somewhat haphazard and unstructured. Most of the virological data presented in this paper were available only in retrospect. The genotypic changes associated with lamivudine resistance are incompletely understood, and the genetic bases of resistance/sensitivity to famciclovir and ganciclovir have yet to be clarified. Though it may be predictable that wild type HBV will re-emerge after lamivudine withdrawal, the viral and host factors that determine the rate of re-emergence have yet to be studied. Our observations suggest considerable variation in this respect. For the management of future transplant patients who develop lamivudine resistant infection, we plan to continue treatment with lamivudine (as has been our strategy for patient 3). This will maintain the dominance of that species, and permit the systematic evaluation of its sensitivity to other antivirals. A similar approach might appropriately be adopted for the non-immunosuppressed with lamivudine resistant HBV infection.

Observations made for these four patients clearly show that the lamivudine resistant phenotype can cause severe graft damage. Severe liver damage developed during lamivudine resistant HBV dominance for patients 1, 2, and 3. We have no reason to believe that the lamivudine resistant virus is more virulent than the wild type, but we propose that emergence of the resistant species in patients who received lamivudine as primary prophylaxis in the transplant setting might identify patients who are at risk for the development of aggressive HBV recurrence.

For the future, accurate viral quantitation and genetic sequencing with rapid turnaround of results, will be prerequisites for the management of patients with graft reinfection after liver transplantation. When the genetic basis of drug resistance is better understood, prospective analysis of clinical specimens will be required for the appropriate selection of antiviral therapy.

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