Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial

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Abstract

***Background, aim, and methods*—Alpha interferon is the generally approved therapy for HBe antigen positive patients with chronic hepatitis B, but its efficacy is limited. Lamivudine is a new oral nucleoside analogue which potently inhibits hepatitis B virus (HBV) DNA replication. To investigate the possibility of an additive effect of interferon-lamivudine combination therapy compared with interferon or lamivudine monotherapy, we conducted a randomised controlled trial in 230 predominantly Caucasian patients with hepatitis B e antigen (HBeAg) and HBV DNA positive chronic hepatitis B. Previously untreated patients were randomised to receive: combination therapy of lamivudine 100 mg daily with alpha interferon 10 million units three times weekly for 16 weeks after pretreatment with lamivudine for eight weeks (n=75); alpha interferon 10 million units three times weekly for 16 weeks (n=69); or lamivudine 100 mg daily for 52 weeks (n=82). The primary efficacy end point was the HBeAg seroconversion rate at week 52 (loss of HBeAg, development of antibodies to HBeAg and undetectable HBV DNA).**

***Results*—The HBeAg seroconversion rate at week 52 was 29% for the combination therapy, 19% for interferon monotherapy, and 18% for lamivudine monotherapy (p=0.12 and p=0.10, respectively, for comparison of the combination therapy with interferon or lamivudine monotherapy). The HBeAg seroconversion rates at week 52 for the combination therapy and lamivudine monotherapy were significantly different in the per protocol analysis (36% (20/56) *v* 19% (13/70), respectively; p=0.02). The effect of combining lamivudine and interferon appeared to be most useful in patients with moderately elevated alanine aminotransferase levels at baseline. Adverse events with the combination therapy were similar to interferon monotherapy; patients receiving lamivudine monotherapy had significantly fewer adverse events.**

***Conclusions*—HBeAg seroconversion rates at one year were similar for lamivudine monotherapy (52 weeks) and standard alpha interferon therapy (16 weeks). The combination of lamivudine and interferon appeared to increase the HBeAg seroconversion rate, particularly in patients with moderately elevated baseline aminotransferase levels. The potential benefit of combining lamivudine and interferon should be investigated further in studies with different regimens of combination therapy.**

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Keywords: chronic hepatitis B; hepatitis B virus; nucleoside analogue; lamivudine; alpha interferon; combination therapy; HBeAg seroconversion

Until recently, the only generally approved treatment for chronic hepatitis B was alpha interferon, which is a natural antiviral agent but acts primarily by immunomodulation.[1] The efficacy of interferon is variable, with rates of hepatitis B e antigen (HBeAg) loss ranging from 15 to 37%.[2–4] Virological response rates are higher in patients with elevated serum aminotransferases which presumably reflects the immune activity to the virus.[5 6] Interferon treatment is associated with considerable but tolerable side effects in approximately 90% of patients.[7 8]

Lamivudine is an oral nucleoside analogue that inhibits hepatitis B virus (HBV) DNA synthesis by chain termination.[9] Previous clinical trials have shown that lamivudine treatment rapidly suppresses HBV replication, enhances aminotransferase normalisation, improves histological outcome, and has an excellent safety profile.[10–12] HBeAg seroconversion, the surrogate marker with predictive value for improved survival,[13 14] was observed in 16% of Asian patients after one year of lamivudine therapy.[12]

Since the two drugs have different mechanisms of action, we conducted a study to determine if combination treatment with both lamivudine and interferon had an additive effect against HBV and would lead to higher HBeAg seroconversion rates than either monotherapy.

Materials and methods

PATIENTS

Eligible patients included males and females, 16–70 years of age, with detectable hepatitis B surface antigen (HBsAg) and HBeAg in serum at the time of screening and for at least six and three months, respectively, before study entry; serum HBV DNA levels of at least 5 pg/ml at

Abbreviations used in this paper: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; ULN, upper limit of normal.

screening (solution hybridisation assay, Abbott, Chicago, USA); and evidence of inflammation by histology or by raised alanine aminotransferase (ALT) levels (1.3–10 × the upper limit of normal (ULN)) at screening and at least once three months before screening with no value falling within the normal reference range in the intervening period.

Patients were excluded at screening if they had been treated previously with interferon or had received antiviral medications within six months; were co-infected with hepatitis C, hepatitis D or HIV; had decompensated liver disease (serum bilirubin more than 2.5×ULN, prothrombin time prolonged more than three seconds, serum albumin less than the lower limit of normal, a history of ascites, variceal haemorrhage or hepatic encephalopathy); had evidence of liver disease of other aetiology (toxic, immune); or any contraindications specified for interferon.

The study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments. All patients gave written informed consent. The study was approved by the ethics committees at participating centres.

STUDY DESIGN

Patients eligible at screening returned for a baseline assessment within four weeks. At baseline (day 1), patients were randomised to receive one of the following three treatment regimens: (i) combination treatment: eight weeks of oral lamivudine 100 mg once daily followed by 16 weeks of lamivudine 100 mg once daily and alpha interferon (Intron A, Schering Plough, Kenilworth, USA) 10 million units three times weekly subcutaneously†; (ii) interferon monotherapy: eight weeks of oral placebo once daily followed by 16 weeks of placebo once daily and interferon 10 million units three times weekly; and (iii) lamivudine monotherapy: lamivudine 100 mg once daily for 52 weeks‡. Treatment was allocated in blocks of six per investigational centre (ratio of random assignment to the three treatment regimens: 2:2:2); the randomisation code was computer generated.

All treatment arms were blinded up to week 8. At week 8, investigators opened sealed envelopes corresponding to patients' treatment numbers which contained instructions on whether or not to dispense interferon. On opening the envelopes, the lamivudine monotherapy arm effectively became unblinded although the interferon and combination arms remained blinded. Results of HBV serology were kept blinded during treatment and follow up. Patients were followed up after treatment cessation to week 64 (end of the study): 12 weeks of follow up for lamivudine patients and 40 weeks of follow up for patients who received interferon or the combination treatment.

The primary outcome measure was HBeAg seroconversion at week 52 (loss of HBeAg, development of antibodies to HBeAg (anti-HBe), and undetectable HBV DNA). Secondary efficacy variables included histological response (reduction in Knodell score by at least 2 points in the biopsy at week 52), HBV DNA loss, and ALT normalisation at week 52.

MONITORING

Patients returned for assessments every four weeks after the baseline visit until the end of the treatment and thereafter every 4–8 weeks. Serum was analysed for HBV DNA, HBeAg, and anti-HBe at various times between baseline and the end of study, with key points at weeks 24, 52, and 64. Serum was analysed for the presence of YMDD variant HBV at week 52 and week 64. Biochemistry and haematology laboratory evaluations and adverse events were assessed at each clinic visit. Patients were requested to have had a liver biopsy within 12 months of the study baseline, and at week 52.

LABORATORY METHODOLOGY

Viral markers were assayed at a single reference laboratory (Covance, Harrogate, UK). Serum HBV DNA was quantified by a solution-hybridisation assay (Abbott, Chicago, USA) with a lower limit of quantitation of 3.0 pg/ml of serum (approximately 8×10^6 copies of HBV DNA, Eurohep standard). HBeAg, anti-HBe, and HBsAg were assessed by qualitative microparticle enzyme immunoassay (Abbott). The incidence of YMDD variant HBV DNA was assessed by a restriction fragment length polymorphism assay, as described by Lai and colleagues.[12] The assay performed at Glaxo Wellcome, Triangle Park, USA, had a lower limit of detection of approximately 500 copies of HBV DNA/ml of serum.

Liver biopsy specimens were randomly assigned a predetermined computer generated code at Glaxo Wellcome and sent for histological assessment to a single independent histopathologist who was blinded with respect to patient identity, treatment assignment, date, and sequence of biopsy specimen. The biopsy specimens were scored according to the Knodell histological activity index.[15]

STATISTICAL ANALYSES

Based on an estimated rate of HBeAg seroconversion at 52 weeks of 40% for interferon and lamivudine monotherapy and 65% for the combination therapy, a sample size of 210 patients was calculated to provide 80% power to detect a significant difference in HBeAg seroconversion rates between the combination treatment and interferon, and the combination and lamivudine monotherapy. The study was

†An initial eight week pretreatment period with lamivudine was included in the combination arm design to reduce HBV DNA load before the combination treatment as low HBV DNA levels have been significantly associated with improved response to interferon.[3 6]

‡The original study design had a lamivudine monotherapy arm with a six month treatment duration for comparison with the combination and interferon treatment arms. However, emerging results from phase II studies[11] indicated lamivudine therapy of longer than six months duration was required for significant HBeAg seroconversion. The protocol was consequently amended to extend the duration of the lamivudine monotherapy arm to 12 months.

not powered to establish whether the HBeAg seroconversion rates were equivalent between lamivudine and interferon monotherapies.

The primary population for the efficacy analyses was the intent to treat population (ITT). The ITT population was defined as patients with confirmed chronic hepatitis B (i.e. patients who were HBsAg positive for at least six months *at screening* and had evidence of ALT elevation (>ULN) and/or histological evidence of inflammation by a Knodell HAI score ⩾2 points) who were randomised to treatment.

A secondary analysis, for the primary end point only, was performed on a subpopulation of the ITT population referred to as the per protocol population. This population was redefined retrospectively due to the unexpected variability in hepatitis B virus markers between screening and baseline. The criteria for patient inclusion in the per protocol population were positivity for HBeAg and HBV DNA *at baseline*, use of trial medication according to the randomisation and protocol, and non-use of prohibited medications.

Safety data were analysed by treatment received for all patients who were given at least one dose of study medication ("as treated population").

Missing data for HBeAg, anti-HBe, and HBsAg parameters were assigned values based on the method of last observation carried forward. In the analysis of HBeAg seroconversion, a patient was considered to have seroconverted if it occurred prior to withdrawal or a missing HBV DNA value. In the analysis of ALT and HBV DNA, missing data were considered as failures (HBV DNA detectable or ALT above the normal reference range).

The Cochran-Mantel-Haenzsel test adjusted for centre, or Fisher's exact test was used to compare differences in proportions between treatment groups. A supplementary analysis of HBeAg seroconversion at week 52 for the ITT population was performed using generalised estimating equation analysis[16] to address the issue of small centres; the results were similar to those using Cochran-Mantel-Haenzsel. All p values are two sided.

Results

STUDY POPULATION

Fifty one centres from 15 countries participated in recruitment between July 1994 and June 1996. A total of 310 patients were screened, of which 230 patients were randomised to treatment. Patients who failed the screening were mainly those who did not demonstrate persistent HBV DNA positivity or those with ALT values <1.3×ULN.

Of the 230 patients randomised to treatment, 226 fulfilled the entry criteria of HBsAg positivity for longer than six months and evidence of disease activity at screening (ITT population). The numbers of patients in the ITT population randomised to the combination treatment, interferon monotherapy and lamivudine monotherapy were 75, 69, and 82, respectively (fig 1). The per protocol population comprised 180 patients: 15 patients had HBV serological ineligibility at baseline, 28 patients were non-compliant with the study medication, one patient received prohibited medications and eight patients were incorrectly dispensed medication (some patients appeared in more than one violation category).

The as treated population consisted of all 230 patients but was analysed by treatment received rather than by treatment allocated (fig 1).

All treatment arms were well matched with regard to baseline characteristics (table 1).

PRIMARY EFFICACY MEASURE (FIG 2)

HBeAg seroconversion

At week 52, the rate of HBeAg seroconversion was 29% (20/68) for the combination group, 19% (12/64) for interferon, and 18% (14/80) for lamivudine monotherapy (combination group *v* interferon: odds ratio 1.9 (confidence interval (95% CI) 0.8–4.4), p=0.12; combination

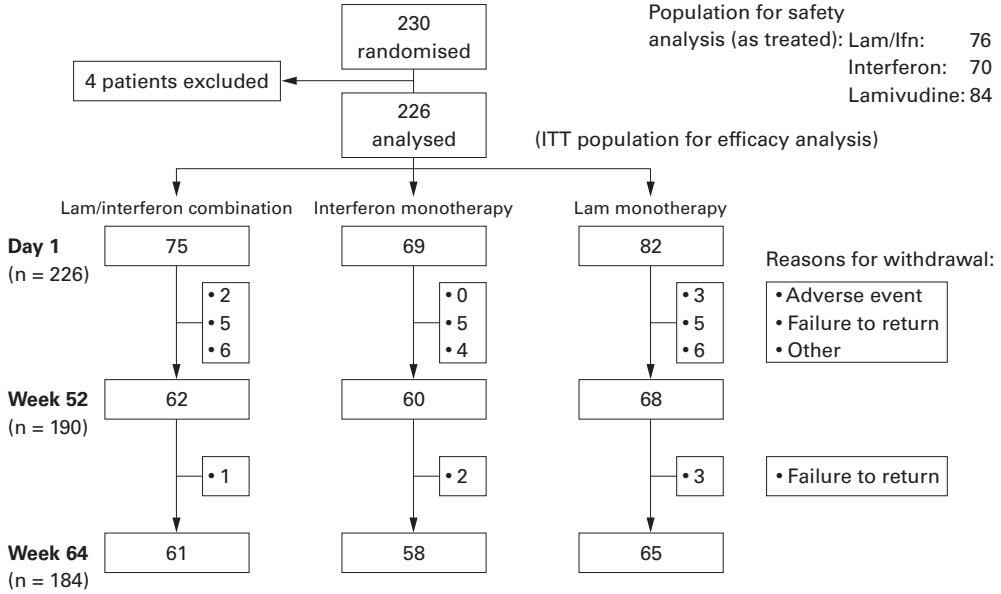

Figure 1 Progress of patients through the various stages of the trial.

Table 1 Patient characteristics at entry to the study

Characteristic	Treatment group: Lam/interferon (n=75)	Interferon (n=69)	Lamivudine (n=82)	Total (n=226)
Age (years) (median (range))	31 (15–60)	32 (16–70)	30 (16–69)	31 (15–70)
Sex (% male)	71	81	71	74
Weight (kg)	72.0 (42–115)	71.0 (45–115)	68.5 (45–118)	70.4 (42–118)
Ethnicity (%)				
Caucasian	59	65	65	63
Asian-Oriental	31	28	29	29
ALT (×ULN)				
Median (range)	2.2 (0.8–26.1)	2.4 (0.8–10.1)	2.6 (0.8–19.2)	2.4 (0.8–26.1)
Mean (SD)	3.2 (3.4)	3.1 (2.1)	3.3 (2.8)	3.2 (2.8)
<1×ULN (No (%))	4 (5)	3 (4)	4 (5)	11 (5)
HBV DNA (pg/ml)				
Median (range)	94.0 (1.5–786)	109.0 (1.5–1322)	136.0 (1.5–2264)	113.5 (1.5–2264)
Mean $\log_{10}$ (SD)	1.74 (0.75)	1.78 (0.77)	2.04 (0.66)	1.86 (0.73)
<3 pg/ml	4 (5)	9 (13)	2 (2)	11 (5)
Positive for HBeAg (No (%))	72 (96)	68 (99)	81 (99)	221 (98)
Positive for HBV DNA and HbeAg (No (%))	68 (91)	64 (93)	80 (98)	212 (94)
Knodell HAI score				
Median (range)	4 (0–14)	4 (0–13)	4 (0–12)	4 (0–14)
<2	8 (11)	6 (9)	11 (5)	18 (8)
Evidence of cirrhosis (No (%))	3 (4)	8 (12)	5 (6)	16 (7)

Evidence of cirrhosis is indicated by a score of 4 on the fibrosis component of Knodell histological activity index score. HBV DNA values below 3 pg/ml (lower limit of detection) have been set to 1.5 pg/ml in the calculation of summary statistics.

groups *v* lamivudine: odds ratio 2.0 (95% CI 0.9–4.7), p=0.10, ITT analysis). Of the 14 lamivudine treated patients who had seroconverted by week 52, 81% (9/11 of those followed up) were maintained off therapy through to week 64 (fig 2).

In the per protocol population analysis, the HBeAg seroconversion rate at week 52 was 36% (20/56) in the lamivudine-interferon combination group, 22% (12/54) in the interferon group and 19% (13/70) in the lamivudine group. The HBeAg seroconversion rate in the combination group was significantly higher than that for lamivudine monotherapy (odds ratio 3.3 (95% CI 1.2–8.8), p=0.02) but failed to reach significance compared with interferon monotherapy (odds ratio 2.3 (95% CI 0.9–5.5), p=0.07).

In a subgroup analysis, the HBeAg seroconversion rate was determined for three categories of baseline ALT. The HBeAg seroconversion rate in the total study population was different for the three categories, with the lowest rate for patients with serum ALT ⩽2×ULN (11%, 9/82) and the highest rate for patients with serum ALT levels ⩾5×ULN (38%, 13/34). The combination therapy appeared to increase the HBeAg seroconversion rate predominantly in those with baseline serum ALT >2× and <5×ULN (fig 2).

SECONDARY EFFICACY MEASURES

Histological findings

Paired liver biopsy slides, at pretreatment and at week 52, were available for 77% (174/226) of patients. The histological response (reduction in HAI score by ⩾2 points) was 37% (21/57) for patients receiving the combination treatment, 46% ((25/54) for interferon, and 49% (31/63) for lamivudine. Evidence of histological progression (worsening) of liver disease (increase in HAI score by ⩾2 points) was 30% (17/57) for the combination group, 31% (17/64) for interferon, and 11% (7/63) for lamivudine. No differences between the combination therapy and interferon monotherapy were observed.

ALT normalisation and loss of HBV DNA

Data for ALT normalisation and loss of HBV DNA are given in table 2. Data are presented as point prevalence at weeks 24, 52, and 64.

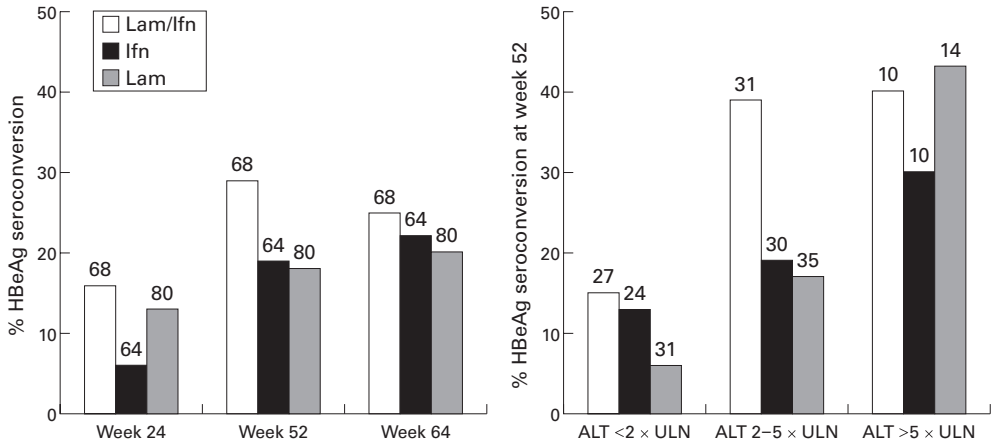

Figure 2 Percentage HBeAg seroconversion for the three treatment arms (lamivudine-interferon, interferon, and lamivudine) by various times (left) and by baseline ALT levels at week 52 (right). The number of patients in each category is given above the bar.

Table 2 Secondary efficacy measures: point prevalence in percentages (intention to treat population eligible for response at baseline)

Outcome measure	*Time (week)*	*Lam/interferon (n=68)*	*Interferon (n=64)*	*Lamivudine (n=80)*
HBeAg loss	24	19 (62)	11 (57)	14 (70)
	52	35 (55)	23 (56)	23 (60)
	64	33 (55)	29 (48)	21 (62)
HBV DNA loss	24	84 (62)	30 (57)	64 (70)
	52	36 (55)	29 (55)	60 (60)
	64	31 (55)	29 (49)	32 (63)
ALT normalisation	24	34 (62)	21 (58)	51 (72)
	52	38 (55)	29 (55)	57 (58)
	64	36 (50)	32 (50)	21 (63)

HBeAg loss is defined as undetectable (AS×YM, Abbott); HBV DNA loss is defined as HBV DNA values below the cut off (3 pg/ml, Abbott HBV DNA test); ALT normalisation is defined as ≤1.0×ULN. Number of patients is indicated in parentheses.

Incidence of YMDD variant HBV

The incidence of YMDD variant HBV was evaluated in all patients where serum samples were available at weeks 52 or 64, or both. YMDD variant HBV variants were not detected in any serum sample from interferon or combination treated patients.

At week 52, 21% (13/61) of patients who were treated with lamivudine were HBV DNA PCR negative, 31% (19/61) had a YMDD variant HBV, and 48% (29/61) had wild type HBV. At week 64, 21% (12/57) had YMDD variant HBV, indicating the re-emergence of the wild type virus after stopping lamivudine treatment.

SAFETY

The percentage of patients completing treatment was 92 (70/76) for the combination treatment, 91 (64/70) for interferon at 24 weeks and 83 (70/84) for lamivudine at 52 weeks. Withdrawals by week 52 are summarised in fig 1. Five patients withdrew before completing treatment because of adverse events; the reason for withdrawal in the lamivudine group was asymptomatic raised serum transaminases two weeks after the start of therapy (one patient) and asymptomatic elevated CPK levels (two patients); in the combination group, one patient withdrew because of fever, chills, and insomnia, and another patient because of fatigue, headache, and mental disturbance. There were no withdrawals caused by adverse events in patients treated with interferon only.

Other reasons for discontinuation included pregnancy, emigration, non-compliance or the patient's request to withdraw for non-specific reasons.

Table 3 Most common adverse events during treatment (number of patients)

	Lam/interferon (n=76)	*Interferon (n=70)*	*Lamivudine (n=84)*
Viral respiratory infections	32	37	25
Headache	71*	47	27†
Muscle pain	36	40	11†
Abdominal discomfort and pain	11	23	13
Diarrhoea	14	16	13
Malaise and fatigue	66	70	35†
Arthralgia	9	23	4
Anorexia	30	33	4†
Dizziness	9	19	8
Nausea and vomiting	33	34	19
Fever/chills	46	43	6†
Hair loss and alopecia	30	21	8†

Most common adverse events defined as those occurring during treatment in ≥15% of patients in any treatment group.
*p value: lam/interferon *v* interferon: 0.004 (borderline significant in view of multiple testing); †p value lam/interferon *v* lamivudine: <0.002 (significant after correction for multiple testing).

The most common adverse events are summarised in table 3. There was a high incidence of headache in the combination group (71%, 54/76) but other adverse events were similar in the combination and interferon groups. The incidence of adverse events for the interferon regimens was much higher than that observed for lamivudine therapy, despite the longer treatment duration; the difference was significant ($p<0.002$) for headache, muscle pain, anorexia, malaise and fatigue, fever/chills, and hair loss.

In approximately 20% of patients in the interferon and combination groups, the interferon dose was adjusted during the study.

Hepatitis flares (ALT levels ≥500 IU/l and >2×baseline) were observed during and after treatment. During treatment, flares occurred in 12% (10/82) of patients who received lamivudine, in 11% (8/70) of interferon recipients, and in 0% (0/75) of those given the combination therapy.

Post-treatment, the incidence of hepatitis flares was 13% (10/78) in the lamivudine group, 9% (6/68) in the interferon group, and 7% (5/74) in the combination group. Three of the hepatitis flares (two lamivudine, one interferon) were associated with elevation of serum bilirubin (>2×ULN); all events resolved spontaneously.

Discussion

In this study of HBeAg positive chronic hepatitis B patients of predominantly Caucasian origin, HBeAg seroconversion rates were similar after 52 weeks of lamivudine monotherapy (18%) and 16 weeks of interferon therapy (19%). However, a combination of 24 weeks of lamivudine and 16 weeks of interferon produced a higher HBeAg seroconversion rate (29%).

The rate of HBeAg seroconversion of 19% for the interferon monotherapy group appears low compared with the generally quoted rate of 33% for loss of HBeAg.[4] The low rate of HBeAg seroconversion in our study partly reflects the use of the most stringent definition of response (HBeAg seroconversion *v* loss of HBeAg). Also, our study population had lower mean baseline ALT values (3.2×ULN) compared with patients studied by Perillo and colleagues[3] (4.3×ULN) and a HBeAg loss rate of 37%. The rate of HBeAg loss for interferon in this study was 23% compared with 29% in a recently published European study[17]; in patients with a low mean baseline ALT, loss of HBeAg even fell to 15%.[2] These data reinforce the conclusion of a European meta-analysis based on individual patient data[5] that the effect of alpha interferon is relative to baseline ALT levels and that the absolute benefit seems greatest in patients with high serum ALT levels.

The HBeAg seroconversion rate after 52 weeks of lamivudine monotherapy (18%) observed in our trial was similar to the HBeAg seroconversion rate of 16% reported by Lai

and colleagues[12] after one year of lamivudine treatment in Asian patients.

Clearance of HBeAg after interferon treatment is reported to be sustained in approximately 90% of patients and associated with an improved clinical outcome in long term follow up studies.[14] All lamivudine treated patients who HBeAg seroconverted are currently being followed up long term; HBeAg seroconversion is reported to be approximately 90% at six months post-treatment.[18] Thus our data suggest that lamivudine for 52 weeks has the potential to induce prolonged HBeAg seroconversion in patients with chronic hepatitis B at a similar rate to that of a 16 week course of interferon treatment.

The combination of lamivudine with interferon appeared to be associated with a higher HBeAg seroconversion rate compared with either form of monotherapy. In this study, the efficacy of the combination therapy was most pronounced in patients with moderately elevated baseline ALT (2–5×ULN). In patients with high ALT levels (≥5×ULN), no additional effect of combining interferon and lamivudine on the HBeAg seroconversion rate was observed. Seroconversion based on baseline ALT categories (≤2×ULN, 2–5×ULN, ≥5×ULN) in the present study are consistent with the results of Liaw (data on file at Glaxo Wellcome) in Asian patients and underlines the importance of this baseline feature for the prediction of HBeAg seroconversion. These exploratory findings require confirmation in a prospective study.

Patients who received lamivudine monotherapy derived histological benefit, irrespective of their HBeAg seroconversion status,[12] whereas the histological response after interferon treatment is usually observed only in patients who have demonstrated a serological response.[19] Results from this study agree with a previous report[12] indicating improvement in histological inflammation in patients after one year's lamivudine treatment.

The incidence of YMDD variant HBV DNA after one year of lamivudine therapy was 31% in this study; this is higher than that previously reported in Asian patients (14%)[12] but similar to that in a recent series of Caucasian patients.[20] The reason for this discrepancy is uncertain but is probably related to differences in study populations. When lamivudine treatment was stopped at week 52 in patients with YMDD variant HBV, there was re-emergence of the wild type virus. The long term clinical outcome and significance of YMDD variant HBV are being evaluated in ongoing follow up clinical trials.

No serious side effects were observed in this study with the use of these two agents in combination, as previously suggested.[21] The high incidence of headache and hair loss needs to be evaluated further. The incidence of drug related adverse events was much lower with lamivudine monotherapy compared with interferon therapy, and similar to that observed in Asian patients receiving lamivudine or placebo.[12]

In conclusion, HBeAg seroconversion rates at one year were similar for lamivudine monotherapy and a standard course of interferon. Combination therapy may be more effective than either monotherapy. Studies with other regimens regarding duration of lamivudine and interferon therapy are needed to identify subgroups of patients in whom combination therapy may be the best treatment option.

Solko Schalm advised on the original protocol design, was a centre coordinator, performed data collection, advised on statistical analysis, and prepared the manuscript. Jenny Heathcote, Geoffrey Farrell, Janusz Cianciara, Morris Sherman, and Bernard Willems were centre coordinators, performed data collection, and advised on the manuscript. Amar Dhillon was the central study histopathologist. Judy Barber was the statistician and analysed the study data. Alison Moorat was the overall study coordinator, advised on data analysis, and contributed to the writing of the manuscript. Fraser Gray advised on data analysis and manuscript preparation.

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