Synergistic Inhibition of Hepadnaviral Replication by Lamivudine in Combination With Penciclovir *In Vitro*

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Lamivudine ([-]-β-L-2′,3′-dideoxy-3′-thiacytidine [3TC]) and penciclovir (9-[2-hydroxy-1-(hydroxymethyl)ethoxymethyl]guanine [PCV]) are potent inhibitors of hepatitis B virus (HBV) replication. Both drugs have entered phase III clinical trials for treatment of chronic HBV infection. 3TC and PCV are deoxycytidine and deoxyguanosine analogs, respectively, and their modes of action and how they interact are matters of both theoretical and practical interest. We compared the antiviral activities of 3TC and PCV alone and in combination in primary duck hepatocyte (PDH) cultures derived from ducklings congenitally infected with the duck hepatitis B virus (DHBV). 3TC and PCV inhibited DHBV replication to a comparable extent when used alone (50% inhibitory concentrations with 95% confidence intervals were 0.55 [0.50-0.59] μmol/L for 3TC and 0.35 [0.27-0.43] μmol/L for PCV), and in combination, the two nucleoside analogs acted synergistically over a wide range of clinically relevant concentrations. Synergy between PCV and 3TC was also observed in acutely infected cells and in "washout" experiments designed to assess the persistence of antiviral activity after drug removal. Furthermore, the combination was more effective in reducing the normally recalcitrant viral covalently closed circular (CCC) DNA form of DHBV than either drug alone. These results suggest that combinations of 3TC and PCV may act synergistically against HBV *in vivo*. (HEPATOLOGY 1997;26:216-225.)

Chronic hepatitis B virus (HBV) infection has been estimated to affect more than 350 million individuals worldwide, and currently available hepatitis B surface antigen–containing vaccines are of no benefit to those individuals who are already persistently infected with HBV and who constitute the major reservoir of potentially infectious virus.[1,2] Until very recently, the only therapeutic option for carriers of HBV was interferon alfa, but both short- and long-term responses to interferon are generally poor, even in the most successful studies.[2] Because the sequelae of uncontrolled HBV infection include chronic active hepatitis, cirrhosis, and primary hepatocellular carcinoma,[3] there is a clear need for the development of more reliable chemotherapy.[4] In the past, attempts to control chronic HBV infection by treatment with nucleoside analogs, which together constitute the largest class of antiviral agent, were unsuccessful or were compromised by associated toxicity.[5,6] Recent renewed interest in the potential of nucleoside analogs to control HBV has been stimulated by two main factors: 1) the discovery that HBV replication entails an obligatory reverse-transcription step[7] that is dependent on an unusual priming mechanism,[8,9] and 2) the development of many "new" and novel nucleoside analogs as potential inhibitors of the human immunodeficiency virus (HIV) reverse transcriptase (RT)[5,6] or herpes simplex virus DNA polymerase.[5] Nucleoside analogs already identified as specific inhibitors of HIV reverse transcription have been generally accepted as potential anti-HBV agents, but experience has shown that peculiarities of hepatocyte enzymology severely limit the number that are actually useful *in vivo*.[5]

Of the anti-HIV nucleoside analogs that also possess anti-HBV activity, those that have the unnatural "L" configuration are among the most active and selective;[6,10] they include (-)-β-L-2′,3′-dideoxy-3′-thiacytidine (lamivudine [3TC])[11-17] and its 5-fluoro derivative, FTC.[15,18] 3TC is a deoxycytidine analog that lacks the 3′ hydroxy group necessary for DNA chain extension and has a sulfur atom instead of the normal 3′ carbon in the pentose ring. After being identified as a potent and selective inhibitor of HIV replication,[19,20] 3TC was approved for compassionate use as an anti-HIV agent.[11,15] Subsequently, 3TC was found to inhibit HBV replication in human hepatoma cells *in vitro*,[15] in patients coinfected with HIV and HBV,[11] and in chimpanzees *in vivo*,[17] and is now undergoing phase III clinical trials against HBV.[14] Despite its demonstrable efficacy as an anti-HBV agent, little is known about its mechanism of action against hepadnaviruses. 3TC inhibits duck hepatitis B virus (DHBV) replication *in vitro*[16] and its triphosphate (3TC-TP) inhibits cell-free replication of DHBV DNA in viral core particles by terminating nascent DNA chains synthesized on endogenous viral DNA and RNA templates.[16] 3TC therefore appears to have the ability to inhibit both DNA- and RNA-dependent activities of the hepadnaviral DNA polymerase.

A second major group of potential anti-HBV agents distinct from those that were initially identified as inhibitors of HIV is comprised mainly of acyclic deoxyguanosine analogs originally developed to combat herpesvirus infections.[5,6] This group in-

Abbreviations: HBV, hepatitis B virus; HIV, human immunodeficiency virus; RT, reverse transcriptase; 3TC, lamivudine; DHBV, duck hepatitis B virus; ACV, acyclovir; GCV, ganciclovir; PCV, penciclovir; PDH, primary duck hepatocyte; CCC, covalently closed circular.

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cludes acyclovir (ACV),[21] ganciclovir (GCV),[21-27] and penciclovir (PCV),[9,27-31] which, despite their close structural similarity, have distinctly different activities against DNA-dependent DNA polymerases.[32,33] Although the triphosphates of acyclic guanine nucleoside analogs are comparably active against HBV-DNA polymerase-RT in cell-free assays, their antihepadnaviral activity in cell culture and *in vivo* differs substantially.[24,27,29-31] Relatively little is known about either the anabolism of the parent nucleoside analogs, or the precise mechanisms of action of their triphosphates. By contrast with 3TC-TP, PCV-TP and GCV-TP (but not ACV-TP) are not obligatory DNA chain terminators,[33] and members of this group have an additional mechanism of action against HBV that is not shared by 3TC-TP, that is, inhibition of the priming step for reverse transcription.[9]

Independent sites and mechanisms of action imply that triphosphates of 3TC and PCV may act additively against HBV-DNA polymerase-RT. However, prediction of *in vivo* interactions between the parent nucleoside analogs is difficult without detailed information about their metabolism. The collection of comprehensive data describing antiviral activity of the analogs alone and in combination is a useful initial step toward understanding their mechanisms of action. The aims of this study were, first, to confirm the efficacy of 3TC against chronic hepadnaviral infection by studying its effects on DHBV replication in primary duck hepatocytes (PDH); secondly, to determine how 3TC and PCV interact as inhibitors of DHBV replication in the PDH test system, particularly with DHBV covalently closed circular (CCC) DNA, the replicative species most resistant to antiviral therapy.[4,21]

MATERIALS AND METHODS

Drugs and Chemicals. 3TC, PCV, GCV, and ACV were gifts from SmithKline Beecham Pharmaceuticals (Harlow, Essex, England, or King of Prussia, PA), Syntex Australia (Sydney, New South Wales, Australia), and Wellcome Australia Ltd. (Sydney, New South Wales, Australia), respectively. All other chemicals and reagents were purchased from local suppliers and were analytical grade or the highest grade commercially obtainable. α-^{32}P-dCTP (specific activity, 3,000 Ci/mmol) used to prepare the DHBV probe was purchased from Amersham International, Amersham, England.

Animals. One-day-old Pekin-Aylesbury cross-bred ducklings congenitally infected with an Australian strain of DHBV were obtained from a commercial supplier. Viremia was monitored by dot-blot hybridization, and 7- to 14-day-old ducklings having stable viral titers of at least 1×10^9 viral genome equivalents per milliliter were selected for hepatocyte isolation.[21,22,27]

Cell Culture. Primary cultures of duck hepatocytes were prepared as described previously.[27] After isolation, hepatocytes were seeded into six-well plastic multiplates (Greiner, Frickenhausen, Germany) in Lebovitz-L15 medium (Gibco, Gaithersburg, MD) supplemented with 5% (vol:vol) fetal calf serum at a density of approximately 1.5 to 2.0×10^6 hepatocytes per well. PDH were allowed to attach overnight before the first medium change (on day 1 postplating) and were maintained with medium changes every second day. In addition, a well-characterized human hepatocyte–derived cell line, HuH-7,[34] was used for cytotoxicity testing. HuH-7 cells were grown in Dulbecco's modified Eagle medium supplemented with 5% (vol:vol) fetal calf serum.

Comparison of Anti-DHBV Activity of 3TC With That of Deoxyguanosine Analogs. The *in vitro* activity of 3TC as an inhibitor of DHBV replication was compared in parallel tests with activity of the acyclic guanine nucleoside analogs, ACV, GCV, and PCV. Replicate sets of congenitally DHBV-infected PDH cultures were exposed to different concentrations of 3TC, ACV, GCV, or PCV, each in fivefold dilution series starting at 100 μmol/L. In a second experiment, replicate sets of cultures were exposed continuously to 1.0 μmol/L 3TC, 1.0 μmol/L PCV, 2.5 μmol/L GCV, or 5.0 μmol/L ACV, concentrations of each drug found in preliminary experiments to cause $\geq$50% inhibition of replication after 9 days of continuous exposure. Control cultures were harvested every 2 days. Drug-treated PDH cultures were harvested on days 4, 8, and 10 postplating. DHBV replication was monitored by dot-blot hybridization.

Inhibition of DHBV Replication by 3TC and PCV Alone and in Combination. Replicate sets of congenitally DHBV-infected PDH cultures were exposed to 3TC and PCV (alone or in combination at fixed molar ratios of 1:4, 1:1, or 4:1) in the concentration range 0 to 20 μmol/L, starting on day 1 postplating. Viral replication was monitored by dot-blot hybridization after 9 days of continuous exposure to nucleoside analog(s).

Persistence of Antiviral Activity After Drug Removal: "Washout" Experiment. The persistence of antiviral activity after nucleoside analog removal may reflect the intracellular stability of its triphosphate.[27,31] In this experiment, the antiviral activity of different combinations of 3TC and PCV were compared after drug "washout." PDH cultures were exposed to 3TC and PCV (alone or in combination at a fixed molar ratio of 1:1) only for the first 5 days, then maintained for the next 5 days in drug-free medium. Drug concentrations were in the range 0 to 100 μmol/L. Cultures were harvested on day 10 postplating, and viral replication was assayed by dot-blot hybridization.

Inhibition of DHBV Replication in Acutely Infected PDH: "Rebound" Experiment. A study using acutely infected PDH was performed to investigate the effects of PCV and 3TC combinations on a much lower level of DHBV infection than typically found in chronically infected PDH preincubated with nucleoside analogs. This situation could be expected to mimic the *in vivo* end-of-treatment situation when residual nucleoside analog and virus are both present and "rebound" (i.e., resumption of viral replication to at least pretreatment levels) commonly occurs.[23] Uninfected PDH cultures were prepared as described above and allowed to attach overnight before exposure for 24 hours to halving dilutions of 3TC and PCV (alone or in combination at fixed molar ratios of 1:4, 1:1, and 4:1). The starting (highest) drug concentration in each case was 100 μmol/L. After 24 hours, PDH were infected with DHBV, using pooled high-titer sera from 4- to 5-week-old ducklings as the inoculum. Viral titer in the inoculum was estimated and expressed as viral genome equivalents by internal comparison with cloned DHBV DNA standards.[21] Infection was achieved by removing the culture medium, then incubating the cell monolayers for 2 hours with 300 μL per well of DHBV-positive serum diluted in culture medium to a final multiplicity of infection of approximately 5 to 10 viral genome equivalents per cell. Mock infection of control PDH was performed using DHBV-free duck serum. After 2 hours, the inocula were removed and replaced by fresh drug-free medium, in which cultures were maintained for a total of 7 days after infection. Cultures were harvested on day 10 postplating, and viral replication was monitored by dot-blot hybridization as described above.

Detection of DHBV DNA Replication and Analysis of Viral Replicative Species. Two different procedures were used to extract viral DNA from cell lysates. Total DNA extraction was performed as previously described[27] and assayed by dot-blot hybridization analysis after capillary transfer to positively charged nylon membranes (Boehringer Mannheim, Germany). Membranes were autoradiographed to visualize bound probe, then the amount of probe bound was quantitated by liquid scintillation counting using a Microplate Liquid Scintillation Counter (Top Count, Packard Instruments, Meriden, CT). Enrichment for viral CCC DNA was achieved by extraction in the presence of 500 mmol/L KCl as described elsewhere.[22] Individual DHBV DNA replicative species were analyzed by Southern blot hybridization after electrophoresis through 1.5% agarose gels and capillary transfer to positively charged nylon membranes according to standard procedures. Probe preparation, hybridization conditions, and autoradiography have been described previously.[22,27]

Detection of DHBV-Specific Protein Synthesis. Immunoblotting was performed as described elsewhere.[27] Polyclonal rabbit antibodies to

DHBV Pre-S antigen or to the carboxy-terminal part of the DHBV core protein were used to stain immunoblots. Bound antibody was detected using an enhanced chemiluminescence kit (Amersham Australia, North Ryde, New South Wales, Australia), according to the manufacturer's instructions. Detailed procedures have been reported earlier.[27]

Quantitation of Antiviral Effects. Image densities resulting from autoradiographs (DNA) and enhanced chemiluminescence (protein) exposures were measured with the aid of an imaging densitometer (model GS-67 with Molecular Analyst software; Bio-Rad Laboratories, Hercules, CA).[27]

Assays for Cytotoxic and Cytostatic Effects. On the day of harvesting, cell viability in PDH cultures was assessed by neutral red uptake.[35] Because primary hepatocytes normally remain quiescent during culture, this assay is an index of cytotoxic rather than cytostatic effects. To test for possible cytostatic effects, HuH-7 cells were used. For these assays, cells were seeded into 96-well flat-bottomed microtiter plates (Flow Laboratories, McLean, VA) at a density of $\approx 1 \times 10^4$ cells per well and allowed to attach for 4 hours, when the medium was replaced with fresh medium containing different concentrations of 3TC and PCV. Cells were exposed continuously to nucleoside analogs for 4 days, the time required for cell monolayers in drug-free control wells to reach confluence. Cell viability was then assayed by neutral red uptake.[35]

Data Analysis. Viral replication and protein synthesis in drug-treated PDH was expressed as a percentage relative to values for drug-free controls. Dose-response plots for individual drugs were generated and analyzed with the aid of TableCurve2D, a curve-fitting statistical software package from Jandel Scientic (San Rafael, CA). Three-dimensional dose-response surfaces[36] describing the combined activity of 3TC and PCV were generated and analyzed using TableCurve3D, also from Jandel Scientific. Further details are provided in the legends to Figs. 1 through 4 and Table 1. Subsets of data describing the effects of drug combinations were also analyzed by the median-effect method,[37] using the ComboStat software program (ComboStat Corp., Duluth, GA) and both mutually exclusive and mutually nonexclusive assumptions.

RESULTS

Cytotoxicity. Cell monolayers remained intact for the duration of all experiments and, when examined by phase-contrast microscopy, there were no observable differences in appearance between treated and untreated hepatocytes, nor was any measurable reduction in neutral red uptake seen in cultures exposed to total drug concentrations $\leq$100 μmol/L. When PDH were continuously exposed to 20-fold-higher concentrations of 3TC and PCV ($\geq$2,000 μmol/L) for 10 days, the concentrations required to cause a reduction in viability of 50% as measured by neutral red uptake were estimated to be $\approx$2,900 μmol/L (by extrapolation), 525 μmol/L, and 1,400 μmol/L for 3TC alone, PCV alone, and 3TC plus PCV at a ratio of 1:1, respectively. Cytostatic effects of drug combinations were investigated using HuH-7 cells grown in microtiter plates. Results, which are summarized in Table 1, suggest that the cytostatic effects of 3TC and PCV are no more than additive.

Comparison of Anti-DHBV Activity of 3TC With That of Deoxyguanosine Analogues. Replicate sets of congenitally DHBV-infected PDH were exposed continuously for 9 days to different concentrations of 3TC, PCV, GCV, or ACV. Cell lysates were assayed for DHBV DNA by dot-blot hybridization. The resulting data were analyzed using TableCurve2D, which generated logistic dose-response plots (not shown) by unweighted nonlinear regression analysis. All plots were consistent (correlation coefficients $\geq$ 0.96) with logistic-dose responses described by the equation $y = a / (1 + x/b^c)$, where a represents the control DHBV signal (y value defined as 100%); b is the transition center, defined as the midpoint between maximum and minimum y values (in these cases equivalent to the 50% inhibition endpoint); c is the transition width (which defines the slope of the curve); and x is the nucleoside analog concentration. (This equation is equivalent to the so-called "median effect" equation[37]; for further discussion, see Greco,[36] Berenbaum,[38] Martinez-Irujo,[39] and de Jong.[40]) Parameters for individual dose-response analyses, which are listed (with 95% confidence limits) in Table 1, show anti-HBV activity in the decreasing order PCV >3TC >> GCV > ACV, corresponding 50% inhibitory concentrations (in micromoles) being: 0.34, 0.55, 2.0, and 4.0, respectively.

In a second experiment, replicate sets of congenitally DHBV-infected PDH were exposed continuously to 1.0 μmol/L 3TC, 1.0 μmol/L PCV, 2.5 μmol/L GCV, or 5.0 μmol/L ACV, concentrations determined from previous experiments to consistently produce $\geq$50% inhibition of DHBV replication. Cultures were harvested at 2- or 4-day intervals, and DHBV replication was monitored by dot-blot hybridization and compared with untreated controls. Figure 1 shows the results graphically and confirms that, on a molar basis, the order of anti-DHBV activity is PCV > 3TC >> GCV > ACV. In this experiment, the intracellular DHBV signal decreased slightly over the first 4 days, then increased exponentially until day 10 postplating. The doubling time for intracellular DHBV in drug-free control cultures was estimated as 1.45 days, which increased in the presence of 3TC, GCV, or ACV to 12.0, 2.1, or 2.0 days, respectively. However, in the presence of 1 μmol/L PCV, the virus load decreased from day 4 with a half-life of approximately 3.3 days, close to the estimated half-life of DHBV CCC DNA under the same conditions.[41] Although all four nucleoside analogs inhibited DHBV replication, PCV was the only compound to cause a net decrease in intracellular DHBV over the 10-day incubation period, perhaps as a consequence of its ability to block the intracellular pathway that generates hepadnaviral CCC DNA[4,30] (see also Fig. 3).

Inhibition of DHBV Replication by 3TC and PCV Alone and in Combination in Congenitally Infected PDH. The 50% inhibitory concentrations for inhibition of DHBV DNA synthesis were estimated from preliminary experiments to be (with 95% confidence interval): 0.55 (0.5-0.6) μmol/L for 3TC and 0.35 (0.27-0.43) μmol/L for PCV. In further experiments, 3TC and PCV were used alone and at fixed molar ratios of 1:4, 1:1, and 4:1 at concentrations in the range of 0 to 20 μmol/L. The resulting data were plotted in three dimensions, and a dose-response surface was fitted to the data by nonlinear regression analysis with the aid of TableCurve3D using the parametric approach of Greco et al.[36] (Fig. 2A). The best-fit-overall dose-response surface generated by this method was consistent with the "Bliss independence" model, in which the combined inhibitory effect (Z) of 3TC and PCV combinations can be described by the equation $Z = X + Y(1 - X)$, where X and Y represent the fractional inhibitions of each analog alone.[36,40] All individual datum points lay within 2 SD (95% confidence limits) of the dose-response surface fitted using this equation. Analysis of subsets of data (each consisting of data sets in which 3TC and PCV were present at fixed ratios) was performed by using the ComboStat program.[37] Combination indices produced by these analyses

TABLE 1. Antihepadnaviral Efficacy of 3TC and PCV Alone and in Combination: Summary of Results

Cell and Infection Type	Nucleoside Analog or Combination	Correlation Coefficient r^2	Equation Parameters*			ComboStat Analysis†
			a	*b*	*c*	
Toxicity dose-response, proliferating cells						
HuH-7, uninfected	3TC	0.97	101 ± 5	530 ± 91	1.0 ± 0.2	—
	PCV	0.99	99 ± 2	782 ± 51	1.1 ± 0.1	—
	3TC + PCV 1:1	1.0	100 ± 1	652 ± 30	1.0 ± 0.1	ADD
Toxicity dose-response, quiescent cells						
PDH, chronic DHBV	3TC	0.99	101 ± 3.4	2347 ± 272	0.7 ± 0.1	—
	PCV	0.99	102 ± 4.1	524 ± 49	1.9 ± 0.3	—
	3TC + PCV 1:1	0.96	102 ± 5.7	1013 ± 234	0.8 ± 0.2	ADD
Antiviral dose-response: comparison of individual nucleoside analogues						
PDH, chronic DHBV	ACV	1.0	99 ± 1.2	4.0 ± 0.3	1.5 ± 0.1	—
	GCV	1.0	99 ± 1.0	2.8 ± 0.1	1.0 ± 0.03	—
	PCV	1.0	98 ± 1.1	0.35 ± 0.03	1.2 ± 0.1	—
	3TC	1.0	98 ± 1.4	0.55 ± 0.02	1.5 ± 0.1	—
Antiviral combinations: 9 days of continuous exposure						
PDH, chronic DHBV	PCV	1.0	98 ± 1.1	0.55 ± 0.02	1.5 ± 0.1	—
	3TC	1.0	101 ± 2.3	0.42 ± 0.05	0.8 ± 0.1	—
	3TC + PCV 4:1	0.99	98 ± 5.5	0.32 ± 0.07	0.8 ± 0.1	ADD/SYN
	3TC + PCV 1:1	0.99	95 ± 6.1	0.34 ± 0.08	0.9 ± 0.1	ADD/SYN
	3TC + PCV 1:4	0.99	98 ± 4.2	0.44 ± 0.08	0.8 ± 0.1	ADD/SYN
Antiviral combinations: 4 days of exposure, 5 days "washout"						
PDH, chronic DHBV	PCV	0.98	102 ± 3.3	0.5 ± 0.1	1.0 ± 0.2	—
	3TC	0.96	104 ± 5.6	1.1 ± 0.3	0.97 ± 0.2	—
	3TC + PCV 1:1	0.97	101 ± 5.0	0.6 ± 0.1	2.3 ± 1.1	SYN
Antiviral combinations: 2 days of exposure preinfection, 7 days "rebound"						
PDH, acute DHBV	PCV	0.97	100 ± 3.6	55.9 ± 11	0.63 ± 0.1	—
	3TC	0.98	97 ± 2.4	72.1 ± 8.2	1.51 ± 0.3	—
	3TC + PCV 4:1	0.93	97 ± 4.7	25.9 ± 5.4	0.95 ± 0.2	SYN
	3TC + PCV 1:1	0.99	101 ± 2.2	11.7 ± 1.1	0.72 ± 0.04	SYN
	3TC + PCV 1:4	0.97	99 ± 2.9	29.1 ± 4.0	0.76 ± 0.1	SYN

Abbreviations: ADD, additivity; SYN, synergy.

* The logistic dose-response equation $y = a/(1 + [x/b]^c)$ was fitted to the data by nonlinear regression analysis, and the correlation coefficients and equation parameters were determined using the TableCurve2D program (see text). In each case, the parameter *b* is equivalent to the 50% endpoint in micromoles. Data are means ± SE.

† Each data set was analyzed using ComboStat with Monte Carlo simulation and the mutually nonexclusive assumption (as defined in Belen'kii).[37] Results are for fractional effects >0.2, below which analyses were inconclusive.

were either less than, or not significantly different from, 1.0, confirming that effects of 3TC and PCV in combination were at least additive (data not shown).

Persistence of Antiviral Activity After Drug Removal: "Washout" Experiment. In this experiment, drug exposure was continued for only the first 5 days, followed by 5 days of drug-free "washout." The resulting data were subjected to dose-response surface analysis as described above. Results, which are shown in Fig. 2B, were again consistent with the "Bliss independence" model for drug interaction. ComboStat analysis of data subsets gave combination indices <1.0, consistent with synergy at all levels of inhibition, regardless of whether the mutually exclusive or mutually nonexclusive assumption was applied (data not shown).

Inhibition of DHBV Replication in Acutely Infected PDH: "Rebound" Experiment. 3TC and PCV combinations at fixed ratios of 4:1, 1:1, or 1:4 at concentrations in the range of 0 to 100 μmol/L were tested in one set of acutely infected PDH cultures under conditions designed to mimic drug withdrawal *in vivo*, i.e., low intracellular viral load and preexisting but progressively declining drug concentrations. The resulting data were subjected to dose-response surface analysis as described above. Results, which are shown in Fig. 2C, were consistent with the "Bliss independence" model. ComboStat analysis of data subsets were again consistent with synergy at all levels of inhibition, regardless of which mechanistic assumption was applied (not shown; but see Table 1).

Effects on DHBV DNA Replicative Species. Total DNA extracted from PDH after drug treatment was analyzed by Southern blot hybridization. Using this type of DNA extraction, only the relaxed circular, linear, and single-stranded replicative intermediates are detected. The results confirmed that both 3TC and PCV inhibited DHBV DNA replication in a dose-dependent fashion without having any selective effect on individual DHBV replicative species, either alone or when present in combination (data not shown). Effects of 3TC and PCV combinations on viral CCC DNA were investigated using an extraction procedure that enriches for this DNA species, followed by Southern blotting. The results, which are shown in Fig. 3, suggest that: 1) generation of DHBV CCC DNA is inhibited by 3TC and PCV in a dose-dependent fashion; 2) on a molar basis, 3TC is a less-effective inhibitor

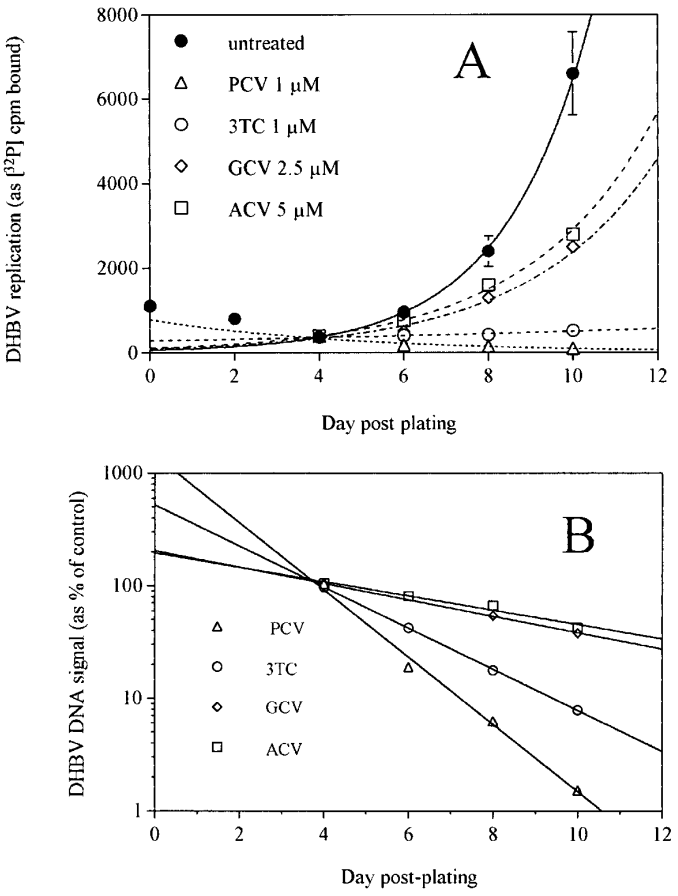

FIG. 1. Comparison of anti-DHBV activity of 3TC with activities of acyclic deoxyguanosine analogs. (A) Rate of inhibition of DHBV DNA replication in PDH cultures after 9 days of continuous exposure to 1 μmol/L 3TC, 1 μmol/L PCV, 2.5 μmol/L GCV, or 5 μmol/L ACV. Cells were plated on day 0 and maintained for 1 day in drug-free medium; they were harvested at 2- or 4-day intervals and lysates assayed for DHBV DNA. Exponential curves were fitted to data from days 4 to 10 and used to estimate the doubling time for DHBV replication in PDH in the absence and presence of nucleoside analogs. (B) The same data plotted with the DHBV DNA signal plotted as a percentage of the control (untreated signal). All analogs inhibited DHBV DNA replication, but only PCV caused a net decrease in the intracellular virus load. In the presence of 1 μmol/L PCV, intracellular DHBV DNA decreased with a half-life of aproximately 3.3 days, close to the estimated half-life of DHBV CCC DNA under the same conditions.[41]

than PCV; and 3) combinations of 3TC and PCV result in more effective inhibition than either drug alone, most clearly apparent at a 3TC:PCV molar ratio of 1:4.

Inhibition of DHBV-Specific Protein Synthesis: Link Between Active Viral DNA Replication and Antigen Expression. Immunoblot analyses using antibodies to DHBV-specific proteins showed inhibition that was dose-dependent but incomplete, even at drug concentrations as high as 4 μmol/L (results not shown). Different ratios of 3TC and PCV at a total concentration of 0.8 μmol/L, sufficient to inhibit DHBV DNA synthesis by ≈90%, under the same conditions (see Fig. 2A and Table 1) inhibited synthesis of Pre-S and core antigens to different extents. Sample results appear in Fig. 4 and show: 1) PCV substantially inhibited both core and Pre-S antigen synthesis, whereas 3TC strongly inhibited core antigen synthesis, but without affecting Pre-S antigen synthesis; 2) inhibiton of core antigen synthesis by PCV and 3TC was approximately additive; and 3) 3TC antagonized the inhibition by PCV of Pre-S antigen synthesis. Taken together with the results presented in Figs. 1 and 3, these results imply that core antigen expression is linked to active DNA replication and that Pre-S antigen synthesis is not linked to active DNA replication, but probably reflects transcriptional activity of functional viral CCC DNA.[4,24,25]

DISCUSSION

All anti-HBV agents investigated to date have been found to be virustatic rather than virucidal, necessitating long-term therapy.[4,42] Cessation of therapy with nucleoside analogs, in particular, has usually resulted in reversion of viral replication to at least pretreatment rates—the relapse phenomenon, which is most probably caused by intracellular persistence of HBV CCC DNA[21,23,41]—and persistence of virus in nonhepatocyte reservoirs.[25,30] Nowak et al.[41] recently calculated that, on the basis of estimates of rates of viral and hepatocyte turnover, 1 year of continuous treatment with an effective nucleoside analog such as 3TC could, at best, result in complete elimination of HBV, and even, at worst, could reduce the frequency of infected hepatocytes to about 8%. Apart from overlooking the refractoriness of HBV CCC DNA to nucleoside analogs, the simulations by these authors failed to take into account the tissue specificity of most nucleoside analogs or to consider the possibility that viral resistance or altered sensitivity may develop during long-term therapy. Indeed, the view that monotherapy will never be able to permanently check the progress of chronic HBV disease is becoming widely accepted, because both the significance of extrahepatic HBV replication[24,25,30] and the emergence of viral resistance[43-47] is increasingly being recognized. PCV and 3TC are among the most effective inhibitors of HBV replication developed to date; both have reached phase III clinical trials,[14,48] and how they interact is clearly of practical as well as theoretical interest. The major aims of this work were, accordingly, to confirm and quantify the antihepadnaviral activity of 3TC in PDH and to investigate interactions between 3TC and PCV using this *in vitro* model of hepadnaviral infection.

Results presented here confirm the potent anti-hepadnaviral activity of 3TC in primary duck hepatocytes *in vitro*. The efficacy of 3TC as an inhibitor of DHBV replication is similar to that of PCV (Table 1). Although the 50% inhibitory concentration (0.55 μmol/L) reported here for inhibition of DHBV replication by 3TC *in vitro* is about 10-fold greater than concentrations (0.05 μmol/L) reported to inhibit secretion of HBV from transfected human hepatoma cells by 50% to 90%[15,29] and 25-fold greater than 3TC-TP's estimated K_i (0.02 μmol/L) for HBV-DNA polymerase,[13] it is close to the K_i (0.8 μmol/L) estimated for 3TC-TP as an inhibitor of DHBV DNA polymerase.[16] Analysis of data from the latter report[16] permit an estimate of approximately 0.5 μmol/L for the EC_{50} for 3TC against DHBV RT activity in primary duck hepatocyte culture, which is also consistent with our results. To what extent species differences in activities, efficiencies, and allosteric characteristics of cellular and/or viral enzymes (for example, cellular deoxycytidine kinase, cellular deoxycytidine deaminase, viral RT-DNA polymerases) contribute to differences in estimates of activity derived from different test systems is presently unknown. Assuming PCV-TP shows similar kinetic characteristics as an inhibitor of DHBV and human HBV DNA polymerases, the greater potency of PCV on a molar basis (see Table 1 and Fig. 2) could be due to its

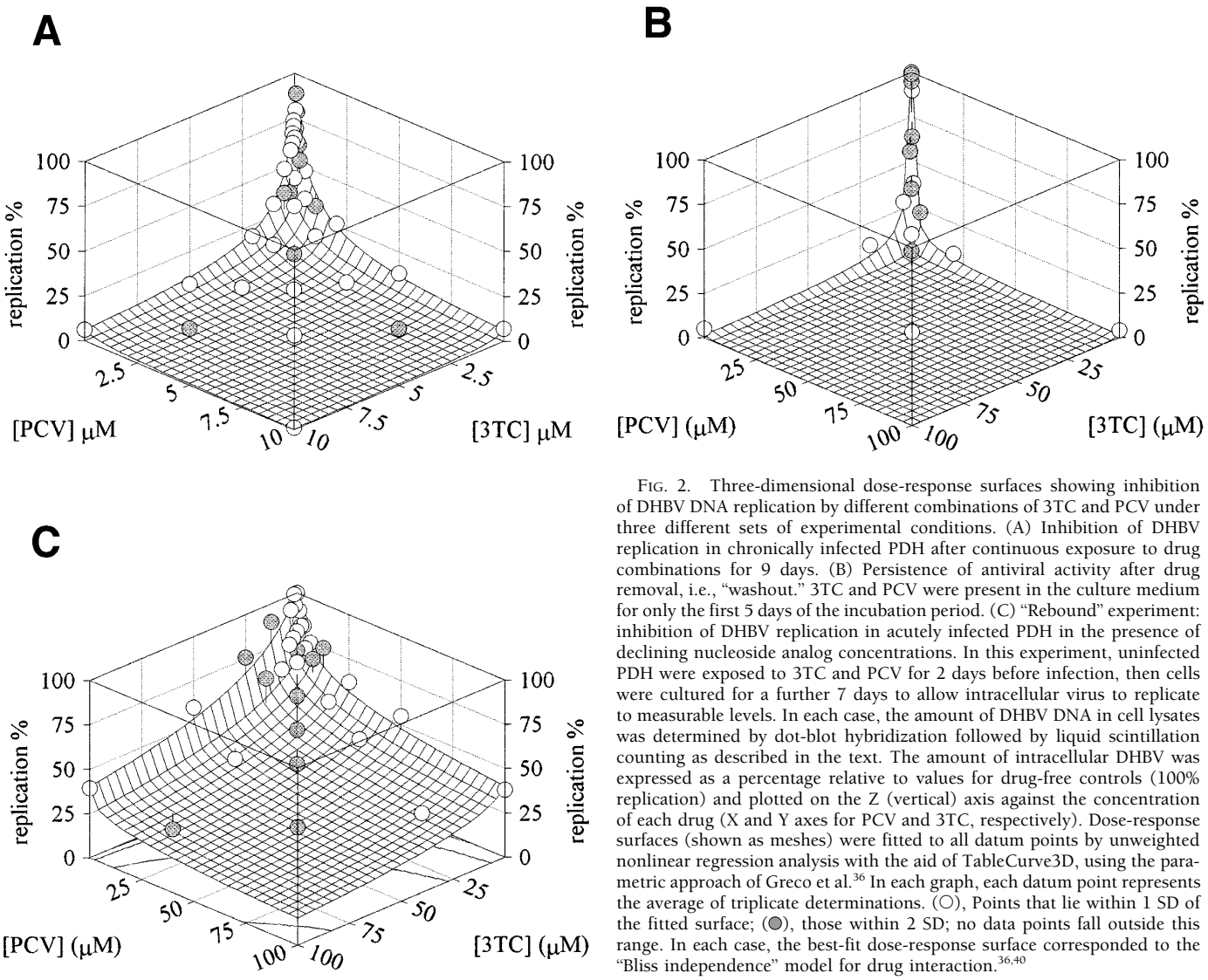

FIG. 2. Three-dimensional dose-response surfaces showing inhibition of DHBV DNA replication by different combinations of 3TC and PCV under three different sets of experimental conditions. (A) Inhibition of DHBV replication in chronically infected PDH after continuous exposure to drug combinations for 9 days. (B) Persistence of antiviral activity after drug removal, i.e., "washout." 3TC and PCV were present in the culture medium for only the first 5 days of the incubation period. (C) "Rebound" experiment: inhibition of DHBV replication in acutely infected PDH in the presence of declining nucleoside analog concentrations. In this experiment, uninfected PDH were exposed to 3TC and PCV for 2 days before infection, then cells were cultured for a further 7 days to allow intracellular virus to replicate to measurable levels. In each case, the amount of DHBV DNA in cell lysates was determined by dot-blot hybridization followed by liquid scintillation counting as described in the text. The amount of intracellular DHBV was expressed as a percentage relative to values for drug-free controls (100% replication) and plotted on the Z (vertical) axis against the concentration of each drug (X and Y axes for PCV and 3TC, respectively). Dose-response surfaces (shown as meshes) were fitted to all datum points by unweighted nonlinear regression analysis with the aid of TableCurve3D, using the parametric approach of Greco et al.[36] In each graph, each datum point represents the average of triplicate determinations. (○), Points that lie within 1 SD of the fitted surface; (●), those within 2 SD; no data points fall outside this range. In each case, the best-fit dose-response surface corresponded to the "Bliss independence" model for drug interaction.[36,40]

20-fold lower K_i (0.04 μmol/L for PCV-TP,[31] compared with 0.8 μmol/L for 3TC against DHBV DNA polymerase[16]); alternatively, it is possible that PCV metabolite(s) have additional, as-yet-unidentified mechanism(s) of action. For example, inhibition of hepadnaviral CCC DNA production and Pre-S antigen synthesis, which are unique to PCV[30] (also see Figs. 3 and 4) may perhaps be caused by interference by PCV anabolite(s) with guanosine triphosphate–dependent intracellular signal transduction or RNA processing. In any case, the 50% inhibitory concentrations and K_i for inhibition of cellular DNA polymerases by 3TC-TP in the presence of dCTP concentrations equivalent to its K_m are in the range of 15.8 to 175 μmol/L,[19] 20- to 200-fold higher than corresponding values for hepadnaviral RT-DNA polymerases, and large (>600-fold) differences in corresponding parameters for PCV-TP[29,31] are similarly indicative of the high selectivity of PCV-TP. Furthermore, experience with HIV-infected patients,[12] as well as results of initial phase III trials in HBV-infected patients,[14] indicates that 3TC is relatively safe and well tolerated. Results from limited clinical trials of famciclovir, the orally available form of PCV, are similarly encouraging and suggest that long-term PCV (famciclovir) therapy is not likely to be associated with significant adverse side-effects.[48] How 3TC and PCV will interact *in vivo* remains to be established.

Combination chemotherapy offers well-recognized advantages over monotherapy.[39,40] *In vitro* analyses constitute a useful preliminary stage in selection of combinations for clinical use, and a variety of different methods have been developed for analysis of data generated by combination experiments.[36-40] Lack of detailed information about the anabolism of either 3TC or PCV in primary hepatocytes makes it difficult to predict possible interactions; moreover, conclusions from drug combination tests may depend on assumptions implicit in the chosen method(s) of analysis.[36-40] Because synergy and antagonism are defined as greater or less than expected effects, respectively, the definition of the "expected" effect is crucial. Two alternative mathematical expressions termed "Bliss independence" and "Lowe additivity" have been widely used as the basis for predicting "expected" effects, but only the latter is free from implicit mechanistic assumptions about mechanisms of action (for a comprehensive review, see Greco et al.[36]). Even when test results are unequivocal, *in vitro* test systems are not ideal surrogates for the far more complex *in vivo* situation. However, our previous observations on the comparative *in vitro* and *in vivo* anti-DHBV activ-

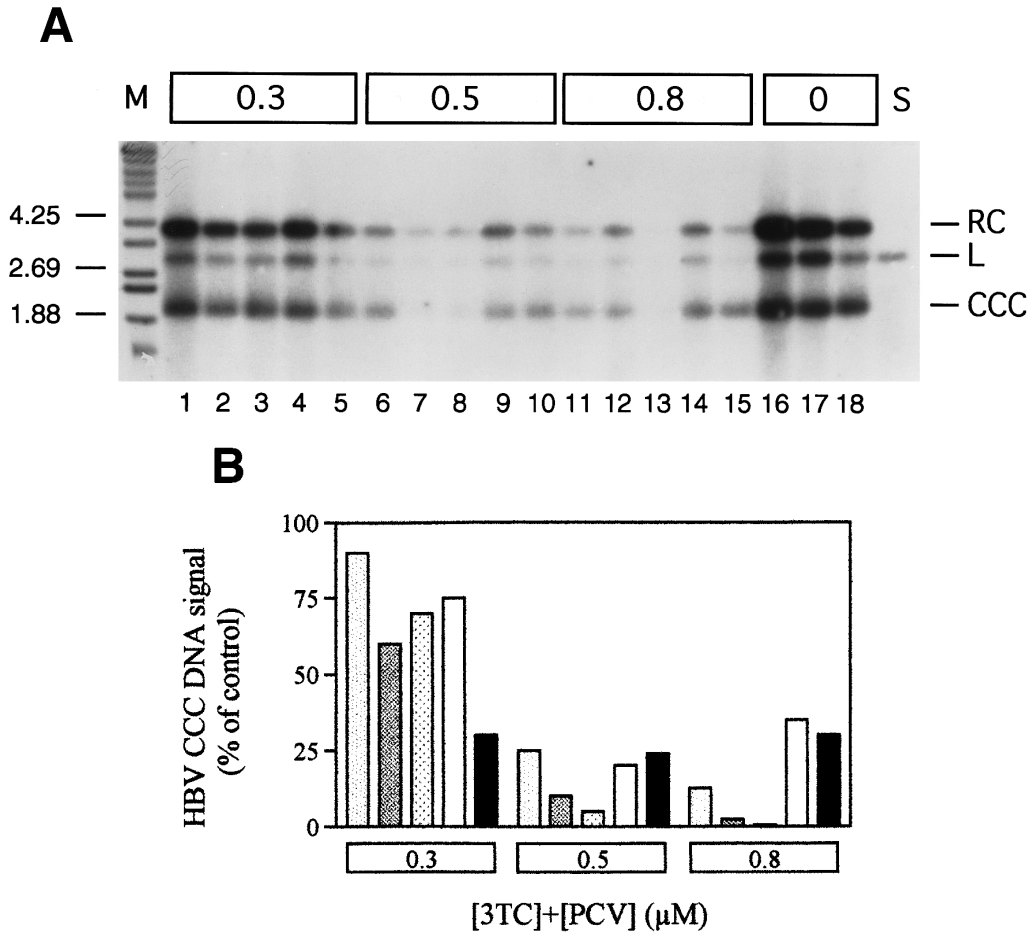

FIG. 3. Inhibition of DHBV CCC DNA generation by 3TC and PCV combinations. DNA extracted from PDH cultures by a CCC-DNA enrichment method was analysed by Southern blot hybridization, autoradiography, and densitometry. (A) An autoradiograph of a Southern blot. Each gel lane was loaded with 1 μg extracted DNA (estimated from UV absorbance at 260 nm). The leftmost lane (M) contains size markers, and the single band in the rightmost lane (S) is a linear DHBV DNA standard. The intervening were loaded with DNA extracted from cultures treated with total drug concentrations of 0.3 μmol/L (lanes 1-5), 0.5 μmol/L (lanes 6-10), or 0.8 μmol/L (lanes 11-15), respectively. Samples in lanes 16 to 18 were from untreated control cultures. Total drug concentrations are shown boxed above the autoradiograph; migration positions of relaxed circular (RC), Linear (L) and CCC DHBV DNA species are indicated on the right. The densitometer signal produced by the CCC DNA band in each lane of the autoradiograph was expressed as a percentage of the averaged signal given by the three untreated control lanes and plotted as a column graph (B). Graph columns and autoradiograph lanes run in the same order from left to right; numbers below each column correspond to lane numbers, and total drug concentrations are shown boxed below the graph. 3TC:PCV ratio: (□), 1:1; (■), 4:1; (▨), 1:4; (□), 1:0; and (■), 0:1.

ity of GCV[24,25,27] and PCV[27,30] are consistent with findings presented here.

The congenitally DHBV-infected duck would be an ideal model in which to test 3TC and PCV combinations, if, as expected, 3TC shows demonstrable anti-DHBV activity *in vivo*. At clinically achievable concentrations ($\leq$15 μmol/L for 3TC[12] and $\leq$20 μmol/L for PCV[49]), combinations of 3TC and PCV acted at least additively as inhibitors of DHBV DNA synthesis in congenitally infected PDH (Fig. 2A). Similar findings using the HBV-producing human hepatoblastoma cell line, 2.2.15,[28] support this conclusion. 3TC and PCV combinations were clearly synergistic as inhibitors of DHBV DNA replication in "washout" and "rebound" experiments (see Table 1 and Fig. 2B and 2C). Synergy between anti-HBV activities of 3TC and PCV may depend partly on allosteric interactions, because their triphosphates must ultimately compete for the same active site on the HBV-DNA polymerase. Independent action of PCV-TP in blocking the RT priming reaction[9] would effectively remove 3TC-TP binding sites; similarly, irreversible termination of nascent HBV-DNA synthesis as a result of 3TC incorporation[16] would reduce available PCV-TP targets. On the basis of these assumptions, it would be predicted that 3TC and PCV behave as mutually exclusive inhibitors of HBV replication. While this prediction may perhaps be demonstrably correct in cell-free enzyme assays, 3TC and PCV were found to behave as nonexclusive inhibitors of intracellular HBV-DNA synthesis in each of the situations studied here, because the overall data were best fitted by the "Bliss independence" model (see Table 1 and Fig. 2). An analogous situation pertains to combinations of 5′-triphosphates of various anti-HIV nucleoside analogs that behave mutually exclusively in cell-free assays but synergistically in cell culture.[50] Presently, we can only speculate on the mechanism(s) that result in synergy between 3TC and PCV, but it seems possible that, even when both nucleoside analogs are present, the total intracellular availability of analog triphosphates is insufficient (in absolute, rather than kinetic terms) to saturate all viral polymerase active sites. It is also possible that PCV phosphorylation, which is inefficient,[31] rather than PCV-TP affinity for the hepadnaviral

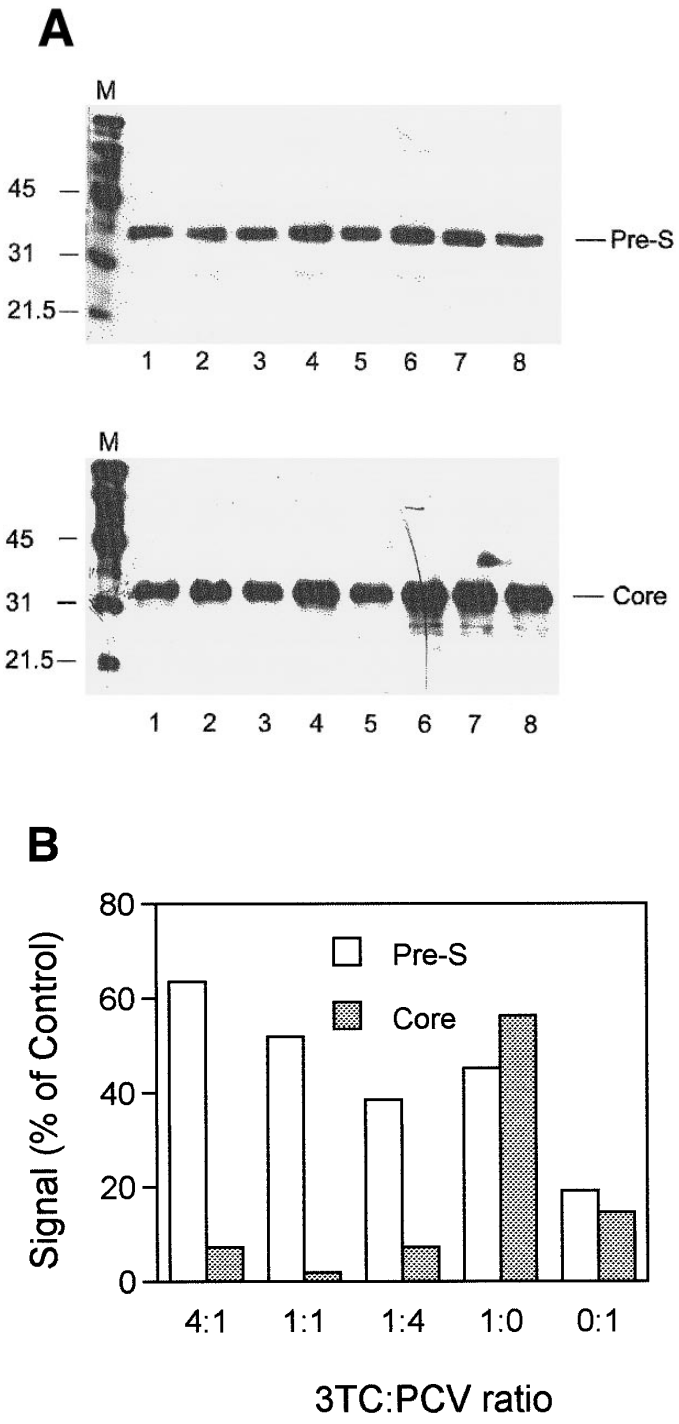

FIG. 4. Inhibition of DHBV-specific protein synthesis by 3TC and PCV in combination. Preparation and staining of immunoblots was as described previously.[27] (A) Each gel lane was loaded with 10 μg protein, and bound antibody was detected by enhanced chemiluminescence. The total drug concentration in each case was 0.8 μmol/L; lanes 1 to 5 correspond to 3TC:PCV ratios of 4:1, 1:1, 1:4, 1:0, and 0:1, respectively, and lanes 6 and 7 correspond to drug-free controls. Migration positions of molecular-weight markers (sizes in kilodaltons) and bands corresponding to DHBV core and Pre-S antigens are indicated. (B) Film exposures produced by enhanced chemiluminescence were analyzed by densitometry, and the data were used to plot the column graph, which compares the effects of various drug treatments (ordinate) on the synthesis of DHBV core and Pre-S proteins, expressed as a percentage (abscissa) of the averaged densitometer signal given by the corresponding drug-free controls.

polymerase (which is high),[31] is limiting for PCV's antiviral activity, whereas the opposite appears be true for 3TC, which is phosphorylated efficiently[13,51] but has relatively low affinity (as 3TC-TP) for the DHBV polymerase.[16] The crucial initial phosphorylation of 3TC is catalyzed by cellular deoxycytidine kinase,[52] an enzyme that, in vertebrate liver, is regulated by nutritional and hormonal factors.[53] Deoxycytidine kinases show broad substrate specificity and will phosphorylate not only deoxycytidine and its analogs, but also deoxyguanosine and deoxyadenosine and some of their analogs, albeit with low efficiency.[54] Because allosteric regulation of enzymes that metabolize nucleic acid precursors is common, it is possible that anabolism of 3TC, PCV, or both, may be increased in the presence of the other, and that one or more of their anabolites may deplete competing deoxynucleotide triphosphate pools by inhibiting ribonucleotide reductase.[55]

We did not find any evidence of synergistic toxicity of 3TC and PCV in any combination tested here, even in proliferating cells; moreover, the selectivity of 3TC and PCV alone and in combination gives a high safety margin. Although 3TC antagonized the inhibitory effect of PCV on Pre-S antigen synthesis (Fig. 4), it may be possible to minimize this effect by careful choice of drug-dose ratios or frequencies. We have previously observed that stimulation and/or intracellular accumulation of Pre-S antigen synthesis, believed to be due to nucleoside analog-induced imbalance in normal intracellular controls that regulate synthesis and assembly of hepadnaviral components, occurs during GCV therapy[25] and may be a common side effect of nucleoside analogs that, like 3TC and GCV, block HBV replication without significantly affecting HBV CCC DNA stability.

Whether the incidence of drug-resistance will be reduced by the use of 3TC and PCV combination is presently unknown, because cases of resistance have only recently been documented. 3TC-resistant strains of both HIV[45] and HBV[43,47] can develop *in vivo*, and 3TC-resistant mutants of DHBV have been engineered by *in vitro* site-directed mutagenesis of the polymerase gene.[46] Poor copying fidelity inherent to RNA polymerase and reverse-transcriptase activities,[56,57] both of which are involved in hepadnaviral replication, almost certainly accounts for the appearance of drug-resistant HBV mutants; on the other hand, the large amount of reading frame overlap in HBV genomes could be expected to reduce the fitness of double mutants. HBV mutations that apparently confer PCV resistance occur in one of the conserved polymerase protein domains in which mutation is also associated with 3TC resistance,[43,44,47] and can be induced by 3TC.[45,47] The potential for development of cross-resistance to PCV and 3TC therefore exists, especially as a result of prolonged 3TC monotherapy before PCV treatment.

For reasons outlined above, monotherapy with nucleoside analogs alone may never completely suppress hepadnaviral DNA synthesis. PCV is thus far unique in its ability to suppress regeneration of viral CCC DNA, which is normally resistant to antiviral therapy;[4,23,24,25,41] however, the antiviral effects of PCV are tissue and cell-specific,[30] and elimination of chronic HBV is probably not achievable by the use of PCV alone. Similarly, long-term use of 3TC alone is unlikely to completely eliminate chronic HBV infection despite claims to the contrary.[42] Data accumulated to date have identified 3TC and PCV as both safe and efficacious inhibitors of hepadnaviral replication, and evidence presented here and else-

where[28] suggest that use of PCV and 3TC in combination against chronic HBV infection would be clinically advantageous.

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