Inhibition of Duck Hepatitis B Virus Replication by 9-(2-Phosphonylmethoxyethyl)adenine, an Acyclic Phosphonate Nucleoside Analogue

A. J. NICOLL,1 D. L. COLLEDGE,1 J. J. TOOLE,2 P. W. ANGUS,3 R. A. SMALLWOOD,3 AND S. A. LOCARNINI1

Victorian Infectious Diseases Reference Laboratory, North Melbourne, Victoria 3051,1 and Austin and Repatriation Medical Centre, Heidelberg, Victoria 3084,3 Australia, and Gilead Sciences, Foster City, California2

Received 9 February 1998/Returned for modification 28 May 1998/Accepted 29 August 1998

The use of regimens that use nucleoside analogues for the treatment of chronic hepatitis B virus infection is often limited because of their high relapse rates. This is thought to be due to the persistence of virus in nonhepatocyte reservoirs and/or the viral covalently closed circular (CCC) DNA species in the nucleus of infected hepatocytes. We have evaluated the novel nucleoside analogue 9-(2-phosphonylmethoxyethyl)adenine (PMEA) in the duck model of hepatitis B. Eight Pekin-Aylesbury ducks congenitally infected with the duck hepatitis B virus (DHBV) were treated with PMEA at a dosage of 15 mg/kg of body weight/day via the intraperitoneal route for 4 weeks. At the end of the treatment period, four animals were killed and the remainder were monitored for a further 4-week drug-free period before analysis. The results were compared with those for eight age-matched, untreated controls. The levels of viremia, the total intrahepatic DHBV load, and CCC DNA, viral RNA, and protein levels were measured by Southern hybridization, Northern hybridization, and immunohistochemical and in situ hybridization (ISH) of sections of liver and pancreatic tissue. PMEA treatment reduced the viremia to undetectable levels, while the total viral DNA load in the liver was reduced by 95% compared to the control level. Viral RNA and protein levels decreased by approximately 30% in both liver and pancreas. ISH and IHC confirmed the PMEA-related intrahepatic changes and established that the amount of virus in bile duct epithelial cells (BDEC) was reduced by 70% during therapy. During the follow-up period all parameters of active virological replication returned to those for the age-matched controls. PMEA had no significant effect upon the number of virus-infected islet or acinar cells in the pancreas. PMEA at a dosage of 15 mg/kg/day has potent activity against DHBV found within hepatocytes and BDEC and inhibits DHBV replication in BDEC.

There are more than 350 million chronic carriers of the hepatitis B virus (HBV) worldwide, and these individuals are at significant risk of developing cirrhosis, hepatocellular carcinoma (2), or decompensated liver disease (17). In recent years investigators have developed a number of new nucleoside analogues which may be useful for the treatment of chronic hepatitis B infection. There has been major interest in the potential role of the nucleoside analogues famciclovir (5, 19, 25) and lamivudine (12, 20, 37), which have been shown to reduce viral replication in chronic carriers of HBV. However, long-term remissions after completion of treatment with these agents appear to be uncommon, with most patients experiencing a relapse or a rebound in viremia once use of the drug is ceased (12, 24). The two most likely sources for this recurrence are reservoirs of unaffected virus in cells against which the nucleoside analogue has no activity (23) and/or the intranuclear viral supercoiled or covalently closed circular (CCC) DNA species whose replicative intermediates are nonresponsive to conventional antiviral therapies (8, 21, 32).

Immunohistochemical and in situ hybridization (ISH) studies of livers and pancreases from ducks congenitally infected with duck HBV virus (DHBV) after ganciclovir (22, 23) and penciclovir (21) therapies have shown that hepadnaviral replication may continue in intrahepatic bile duct epithelial cells (BDEC) and pancreatic islet and acinar cells, despite significant suppression of viral replication within hepatocytes. Infection of BDEC and extraparenchymal tissues has been well documented in humans (4, 11, 30). However, the roles that these sites play in the pathogenesis and persistence of infection, as well as in relapse after the completion of nucleoside analogue therapy, have not been determined.

9-(2-Phosphonylmethoxyethyl)adenine (PMEA) is an acyclic phosphonate analogue of adenine which has been shown to be effective against cytomegalovirus (28), herpes simplex virus, and retroviruses (10, 28). Initial studies by Heijtink et al. (15) established its anti-HBV activity in stably transfected human hepatocellular carcinoma cell lines and primary duck hepatocytes infected with DHBV (14). PMEA does not require the initial phosphorylation step required by other nucleoside analogues (33) for intracellular activation before subsequent conversion to the active form. Furthermore, it has been shown to stimulate natural killer (NK) cell activity and interferon alpha production (6).

In the study described in this report we have performed a detailed in vivo evaluation of PMEA in the DHBV model of chronic hepatitis B. We have examined the effects of the compound upon the different viral replicative forms within the liver and determined its activity in hepatocytes, BDEC, and pancreatic cells.
were probed with a full-length DHBV DNA clone labelled with a field Hospital Ethics Committee guidelines and were overseen by the resident acid fixative (at a ratio of 3:1) for histological techniques (23, 29). Standard clinical laboratory methods (3). Each animal was weighed before the period. Full blood examination and liver function tests were performed weekly by killed. For autopsy, a lethal dose of sodium pentobarbitone (160 mg/kg; treatment arm were killed at the end of the therapy, while the remainder were maintained from a commercial supplier (3). The experimental strategy is shown not shown). Therefore, for the detailed virological studies, a dosage of 15 mg/kg/day for 28 days was selected.

Animals and treatment protocol. Sixteen 1-day-old female Pekin-Aylesbury ducklings congenitally infected with an Australian strain of DHBV were obtained from a commercial supplier (3). The experimental strategy is shown diagrammatically in Fig. 1. Eight ducks received PMEA at a dosage of 15 mg/kg once a day via the intraperitoneal route for 4 weeks. Eight control ducks received no treatment and were followed similarly. One-half of the animals from each treatment arm were killed at the end of the therapy, while the remainder were monitored for a further 4 weeks after the therapy finished before they were killed. For autopsy, a lethal dose of sodium pentobarbitone (160 mg/kg; Lethabarb, Virbac, Australia) was administered intravenously.

The level of viremia was determined by dot blot hybridization (3) with blood samples collected prior to therapy and then weekly throughout the 5-week study period. Full blood examination and liver function tests were performed weekly by standard clinical laboratory methods (3). Each animal was weighed before the time of each venesection. Liver and pancreatic tissues were collected postmortem and were either frozen for virological assessment or fixed in ethanol-acetic acid fixative (at a ratio of 3:1) for histological techniques (23, 29).

Treatments and all procedures were performed in accordance with the Fairfield Hospital Ethics Committee guidelines and were overseen by the resident veterinary surgeon.

Preparation of probes. Southern hybridization, RNA slot, and DNA dot blots were probed with a full-length DHBV DNA clone labelled with [32P]dCTP as described previously (31) by using the NEN Random Primer Plus extension kit (Dupont-NEN, Boston, Mass.). A second clone of DHBV DNA (350 bp segment) was labelled with digoxigenin (DIG) for in situ hybridization studies and was prepared by digoxigenin (DIG) for in situ hybridization studies and was prepared as described previously (18) with a randomly primed DIG labelling kit (Boehringer-Mannheim, Mannheim, Germany).

Detection of viral DNA from liver tissue. DNA was extracted from the liver as described previously (39). The results were expressed as the viral genome equivalents per cell by the method of Jilbert et al. (16). Ten-microgram aliquots of liver DNA were extracted by digestion with a commercially available detergent-compatible assay (DC protein assay kit; Bio-Rad) prepared from lysates in sodium dodecyl sulfate. After denaturation, the samples were subjected to nitrocellulose membranes for probing. The levels of DHBV core antigen (p28DHBcAg) and DHBV pre-S1 antigen (DHBpreS1Ag) expression were measured with the appropriate antibodies. The production of antibodies, immunoblotting, and the detection of bound antibody by enhanced chemiluminescence have been described elsewhere (23, 40), and quantitation was by densitometry as described above.

IHC and ISH. Specimens of liver and pancreas, fixed in ethanol-acetic acid, were processed for histology in the standard manner (23, 29). Immunohistochemistry (IHC) for DHBcAg and DHBpreS1Ag proteins was performed as described previously (23, 29). Each slide was examined under code, and the number of positive cells in four low-power microscopic fields was counted for each specimen (29). In the liver, the proportion of positively staining hepatocytes per low-power microscopic field was approximated and was expressed as a percentage. For BDFC, the number of positive cells per small portal tract was expressed as a percentage. In the pancreas, the number of acinar cells was counted as an absolute number per low-power field, and the islet cells were expressed as a percentage. The mean and standard deviation were then determined for each group. This method of quantitation of viral load has been published previously (29).

The procedure for IHC for the detection of DHBV DNA has been described in detail elsewhere (21, 29), with the proportion of positive cells scored in a fashion similar to that used for the IHC sections as described above.

Statistical analysis. Statistical analysis was performed with Minitab Statistical computer software (Philadelphia, Pa.). Statistical significance was determined by an unpaired Student’s t test. Significance was taken as a P value of <0.05.

RESULTS

All animals in the study demonstrated a steady increase in weight, and there was no sign of illness or toxicity in any of the treated or control animals. The results of all laboratory tests of hematological, renal, and hepatological function were within normal limits for both the control and the PMEA-treated groups (data not shown). In particular, no fluctuations in the alanine aminotransferase levels were seen during or after PMEA therapy, and they remained within the normal limits.

Serum DHBV DNA levels. The level of viremia was markedly lower in the PMEA-treated animals than in the control animals and remained undetectable throughout the treatment period. Within 1 week of the completion of therapy, serum viral DNA levels returned to the levels for the age-matched controls. The serum dot blots for the treatment and control ducks are shown in Fig. 2. During treatment with PMEA all measured markers of active viral replication decreased, and in particular, the levels of total viral DNA and CCC DNA fell by more than 90 and 47%, respectively. During the follow-up period, the levels of these markers returned to at least the pretreatment levels and, in the case of the viral RNA, exceeded the pretreatment levels by more than 30%.

Intrahepatic replicative markers. The results for the age-matched controls and for the treated ducks at the end of PMEA treatment and following the 4-week follow-up period are shown in graphical form in Fig. 3.

(i) Intrahepatic DHBV DNA levels. The intrahepatic viral DNA load in PMEA-treated birds was reduced by approximately 95% of the control level following PMEA treatment. The relaxed circular DNA, double-stranded linear DNA, and replicative intermediates such as single-stranded DNA were virtually undetectable by Southern blot analysis (Fig. 4A). In animals killed after the 4-week drug-free period, the intrahepatic DHBV DNA levels were not statistically different from those in the age-matched controls. Although the mean CCC DNA level was lower in the PMEA-treated birds than in the control birds (Fig. 4B), the difference was not statistically significant (Fig. 3).

(ii) Intrahepatic DHBV RNA levels. At the end of 4 weeks of PMEA treatment the level of DHBV RNA was not significantly different from that in the controls (Fig. 3).
The follow-up period a higher mean viral RNA level was seen in treated animals compared with the level seen in the age-matched untreated controls. The viremia levels indicate the marked suppression of DHBV DNA during PMEA therapy and relapse on treatment cessation compared to the DHBV DNA levels for control animals (138% ± 21%; P < 0.05).

(iii) Intrahepatic DHBV protein levels. The intrahepatic level of DHBcAg expression, as determined by immunoblotting, was reduced by 30% at the end of the PMEA treatment (data not shown), but this did not reach statistical significance (Fig. 3). PMEA also did not have a statistically significant effect upon intrahepatic DHBpreS1 Ag expression, but again, the level was reduced by almost 40% of that for the control animals (Fig. 3).

Histological assessment. There were no appreciable differences in the histology of the liver or pancreas in PMEA-treated, control, and followed-up animals. Mild steatosis and/or a low-grade inflammatory cell infiltrate in the portal tracts was seen in both PMEA-treated and control duck livers (data not shown).

(i) IHC for DHBV proteins. The results of immunohistochemical staining for DHBpreS1Ag and DHBcAg are summarized in Table 1. IHC for DHBpreS1Ag with the livers of control ducks demonstrated that 100% of the hepatocytes were stained in all cases and that 93% ± 8% of all BDEC were positive (Fig. 5A). No other cell types within the liver stained positive. As described for DHBpreS1Ag, no DHBcAg was detectable in hepatocytes of animals treated with PMEA for 4 weeks. The proportion of positive BDEC during PMEA therapy was 35% ± 20% (P < 0.05). The immunohistochemical pattern of staining for both DHBV antigens returned to the pretreatment pattern in the follow-up period.

When pancreatic tissue in control animals was stained for
DHBcAg and DHBsAg, islet cells and occasional acinar cells stained positive. There were no significant differences in the numbers of cells positive for DHBcAg or DHBsAg between the animals in the control group, the treatment group, and the follow-up group (Table 1).

(ii) ISH for DHBV DNA. The results of ISH for DHBV DNA with liver and pancreatic tissue are summarized in Table 2. In the control liver specimens, all hepatocytes and 89% of BDEC were positive for DHBV DNA (Table 2). For animals treated with PMEA, all hepatocytes became negative by the end of therapy, and the number of BDEC with DHBV DNA was significantly lower in the animals treated with PMEA ($P < 0.05$). During the follow-up period the levels for all parameters returned to pretreatment levels.

The ISH results for DHBV DNA in the pancreatic islets and acinar cells are presented in Table 2. No difference was seen between the PMEA-treated group and the control group in terms of the amount of DHBV DNA in either islet or acinar cells.

**DISCUSSION**

The results of this study demonstrate the potent activity of PMEA on serum and liver DHBV DNA levels, confirming the original in vitro findings of Heijtink et al. (15). PMEA produced a rapid antiviral response which was maintained during treatment. However, a relapse was seen on the cessation of therapy. These results indicate an antiviral efficacy equivalent to those of penciclovir and famciclovir (21) and greater than that of ganciclovir (23) when comparable dosages (10 mg/kg/day) were used.

PMEA may differ from other experimental nucleoside ana-

---

**TABLE 1.** Percentage of positively staining hepatocytes and BDEC in liver for detection of DHBV proteins in PMEA-treated animals, age-matched control animals, and followed-up animals by IHC.

<table>
<thead>
<tr>
<th>Group</th>
<th>% Positively staining cells in liver&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pancreas&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHBcAg HEP BDEC</td>
<td>DHBcAg HEP BDEC</td>
</tr>
<tr>
<td></td>
<td>% Islet cells No. of acinar cells</td>
<td>% Islet cells No. of acinar cells</td>
</tr>
<tr>
<td>Controls at end of treatment</td>
<td>100 88 ± 10 99 ± 8</td>
<td>89 ± 97 2.5 ± 1.3</td>
</tr>
<tr>
<td>Treated ducks at end of PMEA treatment</td>
<td>0 35 ± 20 76 ± 15&lt;sup&gt;d&lt;/sup&gt;</td>
<td>70 ± 52 0.8 ± 0.9</td>
</tr>
<tr>
<td>Controls at follow-up</td>
<td>100 82 ± 13 97 ± 3</td>
<td>31 ± 32 4.3 ± 1.5</td>
</tr>
<tr>
<td>PMEA-treated ducks at follow-up</td>
<td>100 75 ± 13 89 ± 3</td>
<td>49 ± 41 6.8 ± 2.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>The proportion of positively staining hepatocytes was approximated per low-power microscope field and was expressed as a percentage. For BDEC, the number of positive cells per small portal tract was expressed as a percentage.

<sup>b</sup>In the pancreas, the number of acinar cells was counted as the absolute number per low-power field, and the number of islet cells was expressed as a percentage.

<sup>c</sup>HEP, hepatocytes.

<sup>d</sup>Results statistically different ($P < 0.05$) from those for the age-matched controls.

---

**FIG. 5.** Immunohistochemical studies with untreated age-matched control and PMEA-treated duck livers. (A) Staining for DHBpreS1Ag with livers from untreated controls shows that all hepatocytes and the majority of BDEC (arrow) are positive. By the end of PMEA treatment (B), all of the hepatocytes and most of the BDEC are negative for DHBpreS1Ag, with relapses in both cell types during the follow-up period (C). Magnification, ×400.
logues evaluated for activity against HBV since it appears to reduce the viral protein and DNA loads within BDEC, a response which has not previously been documented with other nucleoside analogues (21). This effect was detected when individual cell types were compared by using immunohistochemical stains for the detection of DHBcAg and DHBpreS1Ag, even though there was only a modest antiviral effect on total liver DHBcAg and DHBpreS1Ag, as measured by immunoblot hybridization. The likely explanation for this discrepancy is that the immunoblotting technique is extremely sensitive and is unable to detect changes in the very high levels of intrahepatic viral protein expression that occur in this model. Interestingly, there was a suggestion that this agent may also affect the intrahepatic levels of viral CCC DNA and RNA; however, the differences between the groups were not statistically significant (Fig. 3).

The antiviral activity in BDEC is of importance because other studies have demonstrated that the levels of hepadnaviral proteins and nucleic acids in BDEC (4, 29) were unchanged during famciclovir (21) and ganciclovir (23) treatment. The reasons for this may include failure to deliver the antiviral agent to the site in adequate concentrations or the inability of this particular cell type to take up the agent or convert it to its active form. The demonstration of activity against hepadnaviruses within BDEC may indicate an important difference in the liver’s handling of this compound (21, 23). PMEA is an acyclic phosphonate, and so it requires only two phosphorylation steps within the cell to attain the active form. Phosphorylation by cellular enzymes to the triphosphate form is required by most nucleoside analogues to help provide further insight into the mechanism of action of such agents, including PMEA, are clearly indicated.

The development of new nucleoside analogues for the treatment of chronic hepatitis B is of considerable clinical importance. However, major issues must be resolved. Relapse following the cessation of therapy is almost universal, certainly as a result of the fact that persistent CCC DNA species remain functional within hepatocytes (8, 22, 32) but also probably as a result of persistent viral infection in nonhepatocytes, and more recently, antiviral resistance has been documented (1). In the current study, PMEA was shown to reduce viral replication in both hepatocytes and BDEC and thus may offer the important advantage of reducing the viral burden at sites that other nucleoside analogues do not affect. In addition, it may prove to be useful in the treatment of patients who have a relapse of HBV replication following the development of antiviral resistance since it does not appear to be affected by the same HIV and hepatitis C virus infections, with no adverse effects being reported to date. The bis(pivaloyloxy)methyl derivatives of PMEA have increased oral bioavailabilities and improved antiviral activity. Further studies of the immunomodulating effects of nucleoside and nucleotide analogues to help provide further insight into the mechanisms of action of such agents, including PMEA, are clearly indicated.

The development of new nucleoside analogues for the treatment of chronic hepatitis B is of considerable clinical importance. However, major issues must be resolved. Relapse following the cessation of therapy is almost universal, certainly as a result of persistent CCC DNA species remain functional within hepatocytes (8, 22, 32) but also probably as a result of persistent viral infection in nonhepatocytes, and more recently, antiviral resistance has been documented (1). In the current study, PMEA was shown to reduce viral replication in both hepatocytes and BDEC and thus may offer the important advantage of reducing the viral burden at sites that other nucleoside analogues do not affect. In addition, it may prove to be useful in the treatment of patients who have a relapse of HBV replication following the development of antiviral resistance since it does not appear to be affected by the same

<table>
<thead>
<tr>
<th>Group</th>
<th>DHBV DNA in liver (% positively staining cells)</th>
<th>DHBV DNA in pancreas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hepatocytes</td>
<td>BDEC</td>
</tr>
<tr>
<td>Controls at end of treatment</td>
<td>100</td>
<td>89 ± 5</td>
</tr>
<tr>
<td>Treated ducks at end of PMEA treatment</td>
<td>0</td>
<td>59 ± 26a</td>
</tr>
<tr>
<td>Controls at follow up</td>
<td>100</td>
<td>96 ± 4</td>
</tr>
<tr>
<td>PMEA-treated ducks at follow-up</td>
<td>100</td>
<td>82 ± 17</td>
</tr>
</tbody>
</table>

a The proportion of positively staining hepatocytes was approximated per low-power microscope field and is expressed as a percentage. For BDEC, the number of positive cells per small portal tract was expressed as a percentage. In the pancreas, the number of acinar cells was counted as an absolute number per low-power field, and the number of islet cells was expressed as a percentage.

b Results statistically different (P < 0.05) from those for the age-matched controls.
mutations as lamivudine (1, 41). However, cell-based assays will need to be performed in order to confirm this apparent lack of cross-resistance. Further clinical studies of PMEA against chronic hepatitis B certainly appear to be warranted.

ACKNOWLEDGMENTS

We thank the staff at the Biomedical Reference Laboratory for caring for the animals and Norbert Bischofberger of Gilead Sciences, San Francisco, Calif., for helpful discussions and for providing the PMEA. We are grateful to Tim Shaw and Scott Bowden for helpful discussions and to Y. Y. Wang for the preliminary molecular evaluation. A. J. Nicoll is supported by a postgraduate scholarship from the Gastroenterological Society of Australia, and this work was partly supported by a grant from the National Health and Medical Research Council of Australia.

REFERENCES


