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RESEARCH ARTICLE

Inhibition of hepadnaviral replication by polyethylenimine-based intravenous delivery of antisense phosphodiester oligodeoxynucleotides to the liver

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Antisense oligodeoxynucleotides (ODNs) appear as attractive anti-hepatitis B virus (HBV) agents. We investigated in vivo, in the duck HBV (DHBV) infection model, whether linear polyethylenimine (lPEI)-based intravenous delivery of the natural antisense phosphodiester ODNs (O-ODNs) can prevent their degradation and allow viral replication inhibition in the liver. DHBV-infected Pekin ducklings were injected with antisense O-ODNs covering the initiation codon of the DHBV large envelope protein, either in free form (O-ODN-AS2) or coupled to lPEI (lPEI/O-ODN-AS2). Following optimization of lPEI/O-ODN complex formulation, complete O-ODN condensation into a homogenous population of small (20–60 nm) spherical particles was achieved. Flow cytometry analysis showed that lPEI-mediated transfer allowed the intrahepatic delivery of lPEI/O-ODN-AS2 to increase three-fold as compared with the O-ODN-AS2. Following 9-day therapy the intrahepatic levels of both DHBV DNA and RNA were significantly decreased in the lPEI/O-ODN-AS2-treated group as compared with the O-ODN-AS2-treated, control lPEI/O-ODN-treated, and untreated controls. In addition, inhibition of intrahepatic viral replication by lPEI/O-ODN-AS2 was not associated with toxicity and was comparable with that induced by the phosphorothioate S-ODN-AS2 at a five-fold higher dose. Taken together, our results demonstrate that phosphodiester antisense lPEI/O-ODN complexes specifically inhibit hepadnaviral replication. Therefore we provide here the first in vivo evidence that intravenous treatment with antisense phosphodiester ODNs coupled to lPEI can selectively block a viral disease-causing gene in the liver. Gene Therapy (2001) **8,** 874–881.

Keywords: *hepatitis B virus; antisense oligonucleotides; polyethylenimine; antiviral therapy; cell uptake*

Introduction

Liver is an important target for delivery of therapeutic sequences that can knock out metabolic and viral diseases-causing genes. In this regard, chronic liver infection by hepatitis B virus (HBV) is one of the major health problems since it leads to the development of cirrhosis and hepatocellular carcinoma. Because actual anti-hepatitis B treatments such as interferon alpha and nucleoside analogs have only partial efficacy and considerable side-effects, there is need for the development of alternative therapeutic strategies.[1–3]

In the past decade, the interest in application of antisense technology to fight viral infections has been intense. Recent studies have demonstrated antiviral effects of antisense oligodeoxynucleotides (ODNs) for a number of different viruses such as influenza A virus,[4] herpes simplex virus,[5] human immunodeficiency virus,[6] human cytomegalovirus.[7] For HBV, several *in vitro* studies have reported efficient inhibition of viral replication and gene expression with antisense ODNs directed against different regions of the viral genome.[8–11] The antihepadnaviral efficacy of antisense ODNs *in vivo* has been investigated in HBx gene transgenic mice,[12] in woodchuck[13] and duck model.[14] Offensperger *et al*[14] showed that phosphorothioate ODNs (S-ODNs) directed against the 5′ region of duck HBV (DHBV) preS/S gene (S-ODN-AS2) strongly inhibited viral replication and gene expression in chronically infected ducks. However, in all these studies, chemically modified phosphorothiate ODNs were used in order to confer resistance to nuclease degradation. Their use for human therapy is hampered by high cost, toxicity, and the potential risk that chemically modified nucleosides will be incorporated into cellular DNA.[15,16]

Unmodified natural phosphodiester ODNs (O-ODNs) would present an interesting alternative to overcome these problems. However, they are rarely used as antisense agents because of their rapid degradation by serum and cellular nucleases.[17] Among various synthetic carriers, linear polyethylenimine (lPEI), a polymeric DNA-binding cation, appears of particular interest for O-ODNs

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protection from degradation and for increase of their circulation half-life time. The lPEI–DNA complexes enter cells via endocytosis and the endosome buffering capacity of lPEI prevents DNA from lysosomal degradation.[18] lPEI is easy to manipulate, has high stability, low cost and flexibility with respect to size of delivered polynucleotide.[19–21] Recently, lPEI has been shown to be an efficient vector for antisense ODN delivery into duck hepatocytes *in vitro* and *in vivo*.[22] In rodents, several *in vivo* studies have investigated lPEI-mediated reporter gene delivery for gene therapy of lung,[19,20] or neurological disorders,[23] as well as antitumoral therapy.[24] In all these studies successful transgene expression was obtained, but the therapeutic and antiviral aspects of this approach have not yet been investigated.

In this study we have addressed the question of whether lPEI-mediated intravenous delivery of antisense O-ODNs could inhibit hepatitis B virus replication in the liver. Due to the narrow host range of HBV that infects only humans and chimpanzees, the closely related duck hepatitis B virus (DHBV) infecting domestic Pekin ducks, provides a very useful model for testing antihepadnaviral strategies. We report here that intravenous injection of ducklings with lPEI-complexed antisense phosphodiester O-ODN-AS2, covering the initiation codon of the viral envelope protein, leads to a significant decrease in DHBV replication. We also demonstrate that lPEI-mediated transfer allows significant reduction of the O-ODNs dose required for efficient inhibition of intrahepatic viral replication. To our knowledge this is the first report demonstrating antiviral activity of lPEI-based intravenous delivery of phosphodiester antisense ODNs in the liver.

Results

Optimization of lPEI/ODN complex formulation for intravenous delivery

To determine the optimal conditions for intravenous lPEI/O-ODN administration, different ratios and concentrations of lPEI and O-ODN in 5% sucrose were tested. The structure and dimensions of the resulting complexes were analyzed by transmission electron microscopy of negatively stained complexes (data not shown). Optimal complex formulation for intravenous injection was found at a ratio of 6 equivalents of lPEI nitrogen per ODN phosphate (N/P = 6) at 0.6 μg/μl concentration, demonstrating complete ODN condensation into a homogenous population of small spherical particles, ie with a diameter of 20–60 nm (Figure 1). Zeta potential measurement of the complexes revealed a strong positive surface charge of +55 ± 5 mV (n = 3).

lPEI mediates efficient delivery of phosphodiester ODNs to the liver

To analyze the liver uptake of ODNs, lPEI/FITC-O-ODN complexes or FITC-ODN in free form were intravenously injected into ducklings and the hepatocyte-associated fluorescence was analyzed by flow cytometry 24 h later. As shown in Figure 2, the S-ODN-AS2 in free form was efficiently delivered to the liver following injection as assessed by high percentage (28.6%) of positive hepatocytes. In contrast, only weak cell-associated fluorescence (4.7% of positive hepatocytes) was detected after injection of O-ODN-AS2 in free form. The lPEI/O-ODN-AS2 complexes were found in 13.9% of hepatocytes, indicating that lPEI improves uptake of the O-ODN.

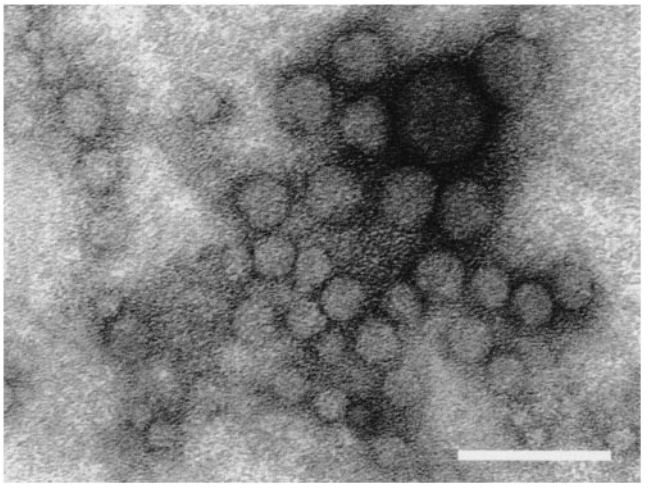

Figure 1 *Transmission electron microscopy of lPEI/O-ODN complexes. Preparation at N/P = 6 in 5% sucrose allows formation of an homogenous population of spherical complexes. Bar indicates 100 nm.*

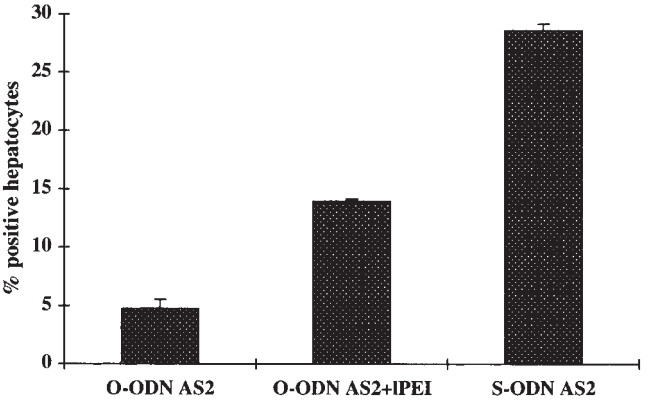

Figure 2 *Flow cytometry analysis of hepatocytes 24 h after ODN injection. Fluorescent phosphodiester O-ODN-AS2 in lPEI-complexed or in free form, or phosphorothioate S-ODN-AS2 were intravenously injected into 2-week-old Pekin ducklings, as detailed in Materials and methods. Animals were killed 24 h later and hepatocyte suspension was examined by flow cytometry. The mean percentage of FITC-positive cells from two animals injected with each ODN are represented. The autofluorescence of control cells was substracted.*

Next, confocal microscopy was used to visualize the differences in intrahepatic distribution of S-ODN and O-ODN. As illustrated in Figure 3, following injection of FITC-O-ODN-AS2 in free form the staining was almost negative (Figure 3c) and comparable to the hepatocyte autofluorescence of uninjected duck liver (Figure 3a). By contrast, following FITC-S-ODN-AS2 injection the majority of liver tissue showed a homogeneously diffused fluorescence (Figure 3b). Interestingly, injection of lPEI/FITC-O-ODN-AS2 complexes led to a different pattern, showing more intense staining but restricted to clusters of hepatocytes (Figure 3d). This distribution pattern was already observed 2 h after injection. Taken together, these data demonstrate that complexation with lPEI allows rapid and efficient delivery of O-ODN to the liver tissue.

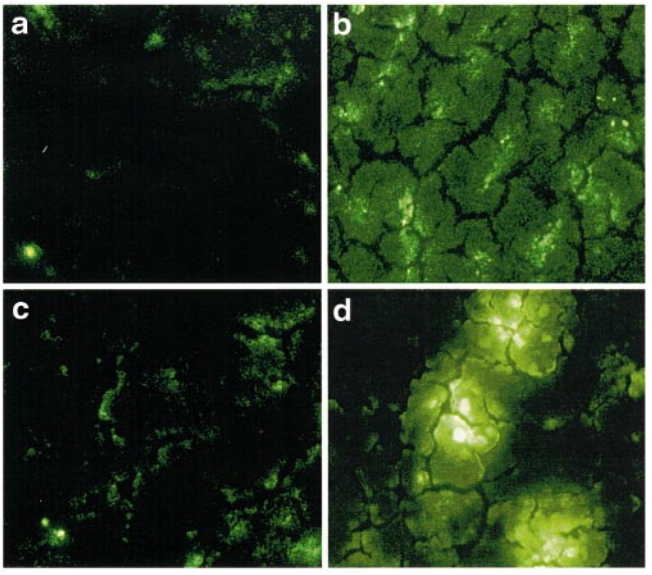

Figure 3 *Confocal microscopic images of liver sections 4 h after intravenous injection of FITC-labelled ODNs. Fluorescent staining of livers from (a) control uninjected duck (hepatocyte autofluorescence), ducks injected with (b) S-ODN-AS2 in free form, (c) O-ODN-AS2 in free form, and (d) lPEI-complexed O-ODN-AS2. Original magnification ×3000.*

Detection of ODNs in different organs

To assess whether other organs besides the liver retained the circulating S-ODN-AS2 and lPEI/O-ODN-AS2 complexes, sections of the kidneys, lungs and spleen were examined by fluorescence microscopy at 4 and 24 h after intravenous injection of FITC-ODN into ducklings. As summarized in Table 1, S-ODN-AS2 was found in all organs examined 24 h after injection from four out of four injected ducks, except for lungs where three out of four ducklings were positive, but fluorescent staining was very weak (data not shown). The fluorescence of lPEI/O-ODN-AS2 complexes was found in the spleen in three out of four, and in the kidneys in two out of four animals (Table 1), whereas the lungs remained negative in all four injected ducklings. Interestingly, distribution of lPEI/O-ODN-AS2 complexes in the spleen and kidney was similar to that found in the liver, ie limited to clusters of cells (data not shown).

Impact of antisense ODN treatment on viremia

DHBV-infected ducklings have been assigned into different treatment groups which received antisense therapy as summerized in Table 2. Viremia was followed daily in the ducks from all groups. It was not possible to investigate the effect of lPEI alone on viremia, since preliminary studies showed toxicity of the polycation in the soluble uncomplexed form (data not shown).

Table 1 Detection of ODNs in different tissues

Tissue	*S-ODN AS2*		*O-ODN AS2 + lPEI*	
	4 h	*24 h*	*4 h*	*24 h*
Kidney	4/4	4/4	0/4	2/4
Liver	4/4	4/4	4/4	4/4
Lung	0/4	3/4	0/4	0/4
Spleen	4/4	4/4	4/4	3/4

Ducklings were i.v. injected with 400 μg FITC-ODNs. At 4 and 24 h after injection, the distribution of ODNs over different organs was determined by fluorescence microscopy. The results are expressed as number of FITC-positive/number of injected ducklings.

Table 2 Therapeutic protocol

ODN administered	*lPEI*	*ODN dose*[a] *(μg/g bw/day)*	*No. ducks*
O-ODN AS2	+	1	5
O-ODN AS2	+	2	5
O-ODN AS2	+	3	3
S-ODN AS2	–	15	4
O-ODN AS2	–	3	4
O-ODN CTRL	+	2	3
Untreated controls	–	–	4

Antisense ODN treatment of DHBV-infected ducklings was started at day 4 after infection and followed for 9 days.
[a]Daily dose of administered ODNs, expressed in μg per g bw.

The follow-up of serum DHBV DNA titers in the untreated duck group showed the typical evolution of viremia in the DHBV-infected young ducklings, reaching high levels at day 6–7 after infection and followed by a decrease and fluctuation (Figure 4). Injection of a high dose (15 μg/g bw/day) of S-ODN-AS2 significantly reduced viremia ($P = 0.02$) in treated ducklings, as measured by the decrease in the total virus release during the treatment. Administration of O-ODN-AS2 in free form had no effect on serum DHBV DNA compared with the untreated controls, while a slight decrease was observed in animals injected with unrelated lPEI/O-ODN-CTRL complexes. The 1 μg/g bw/day dose of lPEI/O-ODN-AS2 complexes was ineffective in inhibition of virus release. By contrast, the treatment with 2 or 3 μg/g bw of lPEI/O-ODN-AS2 induced dose-dependent and significant decrease in viremia ($P = 0.05$ and 0.03, respectively). We have not observed loss of weight in lPEI/O-ODN-treated animals compared with the untreated controls (data not shown). In addition, no

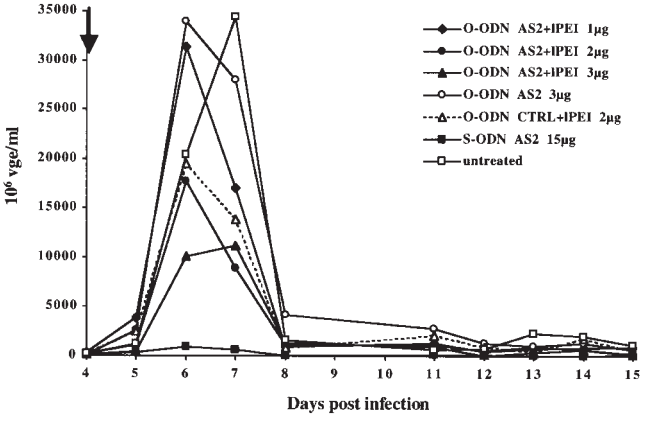

Figure 4 *Effect of ODNs administration on DHBV viremia. Viral DNA in duck serum was analyzed by dot blot hybridization during a 9-day course of ODN antisense treatment. The level of mean DHBV DNA titers in vge (virus genome equivalent)/ml for each group of ducks, based on individual viremia titers, is represented. Arrow indicates the beginning of treatment.*

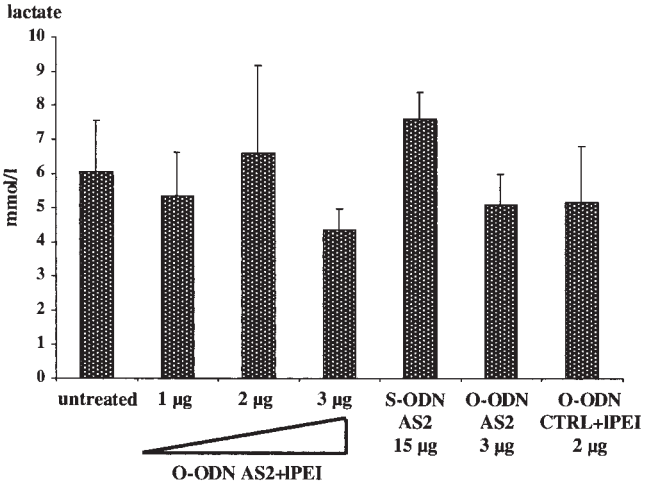

Figure 5 *Analysis of serum lactic acid levels at day 5 of ODN treatment of ducklings. Bars represent the mean of lactic acid concentration in mmol/l of serum for each duck group, based on individual titers. Standard deviations are indicated.*

increase in the lactic acid levels was detected in the sera of lPEI/O-ODN-AS2-treated duck group as compared with the S-ODN-AS2-treated and untreated controls (Figure 5). Moreover, the histological analysis of the autopsy liver samples revealed that DHBV infection induced only mild hepatitis characterized by portal infiltration that was similar for lPEI/O-ODN-treated and untreated ducks (data not shown). Taken together, these data suggest absence of toxicity following lPEI complex treatment.

lPEI-delivered O-ODN-AS2 decrease intrahepatic DHBV replication

To evaluate the effect of antisense ODNs on viral replication, intrahepatic DHBV DNA and RNA levels were analyzed at the end of the treatment. Southern blot analysis of viral DNA showed that treatment with either unrelated lPEI/O-ODN-CTRL complexes (Figure 6a) or O-ODN-AS2 in free form (not shown) had no effect on intrahepatic DHBV DNA, whose level was comparable

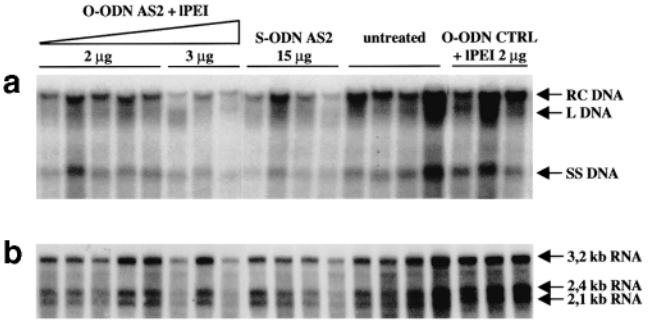

Figure 6 *Analysis of intrahepatic viral replication at the end of antisense ODNs treatment. (a) Southern blot of total liver DNA (5 µg/lane). The positions of relaxed circular (RC), linear (L), and single-stranded (SS) viral DNAs are indicated, as are the therapeutic protocols. (b) Total RNA analysed by Northern blot (3 µg of RNA/lane). Arrows show positions of the pregenomic RNA (3.2 kb) and RNAs (2.4 and 2.1 kb) encoding the large and small DHBV envelope proteins, respectively. Control for equal RNA loading was established by quantification of 28S RNA (data not shown).*

with that of untreated controls. As illustrated in Figure 6a, the treatment with a high dose (15 µg/g bw/day) of S-ODN-AS2 in free form decreased viral DNA synthesis as compared with the controls. No inhibition of intrahepatic viral DNA was observed in animals treated with 1 µg/g bw/day of lPEI/O-ODN-AS2 complexes (data not shown). By contrast, administration of 2 or 3 µg/g bw/day of lPEI/O-ODN-AS2 complexes led to a decrease, in a dose-dependent manner of DHBV DNA replicative forms (Figure 6a). In all treated animals, the viral covalently closed circular DNA (cccDNA) was still detected at the end of treatment (data not shown).

Northern blot analysis revealed that lPEI-/O-ODN-CTRL complexes (Figure 6b) and O-ODN-AS2 in free form (not shown) had no inhibitory effect on intrahepatic viral RNA levels. Following treatment with S-ODN-AS2 (Figure 6b) all forms of intrahepatic viral RNA were decreased. The pregenomic RNA (3.2 kb), that is the template of DHBV replication, was decreased to the same extent as the 2.4 and 2.1 kb RNAs encoding the large and small envelope proteins, respectively. Similarly, the treatment with lPEI/O-ODN-AS2 complexes resulted in a marked decrease in all viral RNAs, which was observed for 2 or 3 µg/g bw/day doses (Figure 6b).

Taken together, these results indicate that both the appropriate sequence of O-ODN and its complexation with lPEI were required to obtain the inhibition of intrahepatic DHBV replication. The densitometric analysis of Southern and Northern blots (Figure 7a and b) revealed that the decrease in DHBV DNA and RNA obtained with 2 or 3 µg of lPEI/O-ODN-AS2 complexes was significant as compared with untreated controls (P = 0.02 and 0.03, respectively). Interestingly, the decrease in viral DNA and RNA induced by lPEI/O-ODN-AS2 complexes at 3 µg dose was comparable with the decrease observed for S-ODN-AS2 in free form at a five-fold higher dose (Figure 7a and b).

Effect of antisense treatment on DHBV gene expression

Finally, we investigated whether reduction of DHBV RNA levels was associated with the decrease in viral envelope and capsid expression in the liver. Although both the 15 µg/g bw S-ODN-AS2 in free form and 3 µg/g bw lPEI/O-ODN-AS2 complexes inhibited DHBV RNA to the same extent, immunoblotting analysis revealed that the decrease in 32 kDa DHBV core and 36 kDa DHBV preS/S envelope protein expression were more pronounced in S-ODN-AS2-treated than in lPEI/O-ODN-AS2 complexes-treated animals (P = 0.02 and 0.07, respectively), as compared with untreated (Figure 8a and b), O-ODN-AS2 and lPEI/O-ODN-CTRL-treated controls (data not shown). Densitometric analysis of the immunoblotting forms showed that in animals treated with S-ODN-AS2 the DHBV core protein expression was inhibited to a larger extent than the DHBV envelope protein (Figure 8c).

Discussion

In the present study, we report that intravenously administrated natural phosphodiester antisense ODNs, when complexed to lPEI, can specifically inhibit hepadnaviral replication *in vivo*. To study the antiviral potential of antisense lPEI/O-ODN-AS2 complexes, we have used the DHBV-infected Pekin duck, which is a reference model

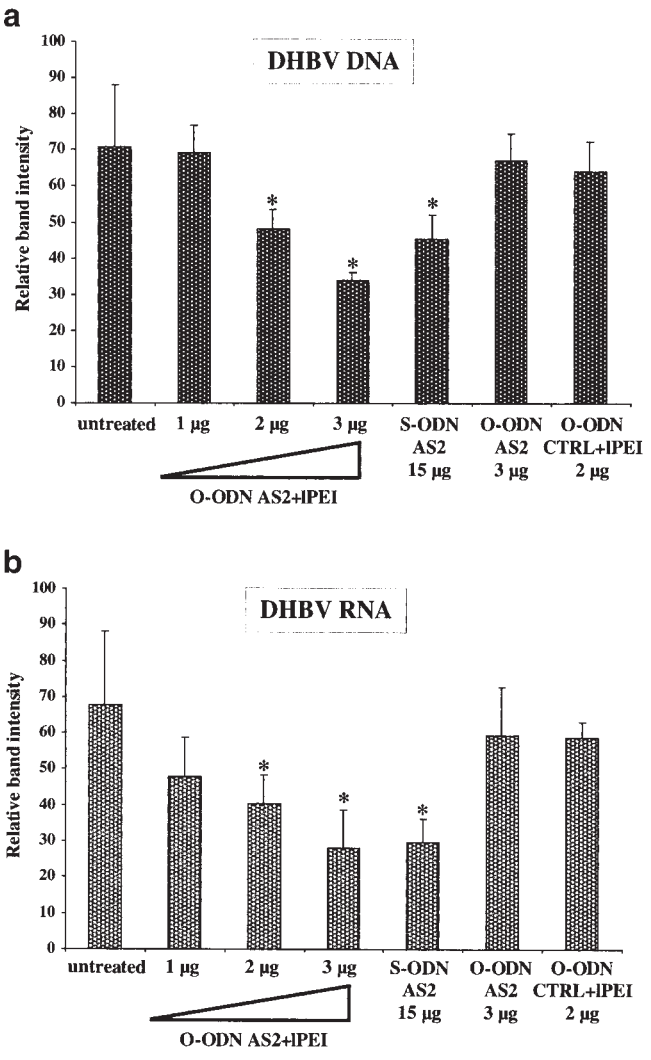

Figure 7 *Densitometric analysis of intrahepatic DHBV DNA and RNA levels at the end of treatment. Relative band intensity of all DHBV DNA replicative forms (a) from Southern blot (Figure 6a) and RNA forms (b) from Northern blot (Figure 6b) were quantified by densitometry. The bars represent the mean relative band intensity for each group of treated ducks. Standard deviations are indicated. *P < 0.05 was considered as significant compared with untreated controls.*

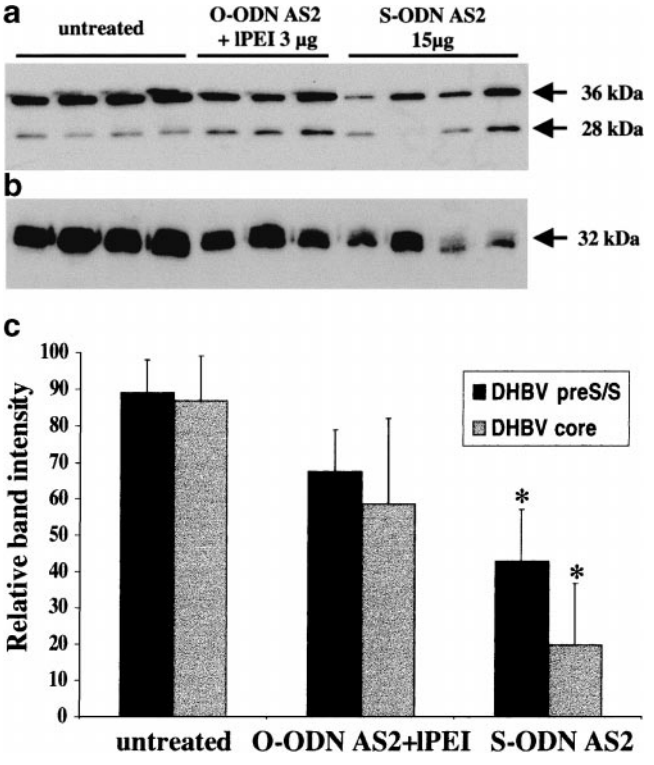

Figure 8 *Effect of antisense ODNs on DHBV protein expression in duck livers at the end of treatment. Liver proteins from untreated, S-ODN-AS2-treated and lPEI/O-ODN-AS2-treated ducks were revealed in immunoblotting assay with: (a) polyclonal anti-DHBV preS protein rabbit serum; arrows indicate the 36 kDa DHBV preS/S envelope protein and its 28 kDa cleavage product; (b) polyclonal anti-DHBV core protein rabbit serum; the position of the 32 kDa major DHBV core protein is indicated by arrow. (c) Densitometric analysis of immunoblottings presented above. The mean relative band intensity and standard deviations are shown. *P < 0.05 was considered as significant compared with untreated controls.*

validated by us and others for screening of anti-HBV approaches.[25–28] Offensperger *et al*[14] have previously reported in this model that antisense phosphorothioate S-ODN-AS2 covering the initiation codon of the DHBV envelope protein can efficiently inhibit DHBV replication. Since S-ODN are expensive and potentially toxic, we have tested whether the natural O-ODN-AS2 of the same sequence coupled to an efficient carrier could represent an alternative antiviral strategy. We have chosen lPEI because it is a particularly efficient polycationic vector with endosome buffering capacity that provides protection of complexed DNA from lysosomal degradation.[18] Following intravenous injection of FITC-O-ODN-AS2 in free form into ducklings, only weak hepatocyte-associated fluorescence was detected by flow cytometry, in agreement with their known rapid degradation by nucleases. Although the lPEI-mediated transport of O-ODNs was not liver-targeted, we observed a three-fold increase in their liver delivery following complex formation with lPEI. The small size (20–60 nm) of lPEI/O-ODNs complexes prepared in our study may have contributed to their filtration by liver sinusoids and successful delivery to hepatocytes. However, taking into account the strong positive surface charge of these complexes, we cannot exclude that after intravenous injection they interact with plasma proteins forming larger aggregates, a part of which could still be capable of passing through the liver sinusoids. After leaving the circulation the aggregated complexes may transfect several neighboring cells and this could explain the presence of the intensively stained lPEI/FITC-O-ODN clusters seen by confocal microscopy in the liver. A similar pattern of clusters of O-ODN-associated fluorescence was observed in the spleen and kidneys, but not in the lungs.

The main objective of this study was to investigate whether intravenous injection of low doses of unmodified antisense O-ODN-AS2 coupled to lPEI was able to inhibit hepadnaviral replication *in vivo*. The target region of ODN-AS2 is present on both the pregenomic (3.2 kb) and the subgenomic preS/S (2.4 kb) DHBV RNA. Since the pregenomic RNA is the template for reverse transcription, it is not surprising that all forms of viral RNA have been decreased following treatment with lPEI/O-ODN-AS2. In addition, a dose-dependent and significant decrease in intrahepatic DHBV DNA was observed in lPEI/O-ODN-AS2 complex-treated ducklings. We have

demonstrated that this effect was specific since both the O-ODN-AS2 in free form or the unrelated lPEI/O-ODN-CTRL complexes of the same size and linkage failed to have any effect on viral replication in the liver. The decrease in viremia in lPEI/O-ODN-AS2-treated animals was also dose-dependent and reflects the decrease in intrahepatic DHBV RNA and DNA. However, we cannot exclude that part of this decrease in circulating viral particles is due to their interaction with positively charged lPEI/O-ODN complexes or some free lPEI probably present in blood. The aggregates between lPEI, virions and serum proteins could have been depleted during the centrifugation step of serum samples preparation. Whether this contributed to the slight decrease in viremia observed in the control lPEI/O-ODN-CTRL-treated animals in the absence of a corresponding decrease in intrahepatic viral DNAs, RNAs and proteins, is unknown and would require further investigation.

We have observed a toxicity of lPEI in free form when intravenously injected into ducklings. lPEI is a positively charged macromolecule designed to interact electrostatically with the phosphates on DNA to form compact particles. In the absence of DNA this free macromolecule may interact with every negatively charged component such as cells and serum proteins, leading to toxicity. In addition, the 'naked' polycations were shown to be strong complement activators, but following complex formation with DNA the complement activation was significantly reduced.[29] We cannot exclude that after complex formation between lPEI and ODN there is still some free lPEI left. However, we have observed no toxicity in lPEI/O-ODN-treated animals as reflected by the absence of variations in duck weight, serum lactic acid levels and liver pathology as compared with the untreated controls.

Interestingly, the intrahepatic DHBV DNA and RNA levels were inhibited to comparable levels when ducklings were treated with 15 μg/g bw/day dose of S-ODN-AS2 in free form or a five-fold lower dose (3 μg/g bw/day) of lPEI/O-ODN-AS2 complexes. It may be possible that complexation with lPEI favors O-ODN-AS2 delivery to the hepatocyte nuclei, where the key steps of DHBV replication take place. This would be in agreement with the fact that PEI can promote transgene delivery to the nucleus of mammalian cells.[30] The comparison of intrahepatic DHBV proteins expression showed that S-ODN-AS2 induced a more marked decrease in viral core and preS/S proteins than the lPEI/O-ODN-AS2. This suggests that the intranuclear viral replication was inhibited in a comparable manner by both S-ODN-AS2 and lPEI/O-ODN-AS2 complexes, although a five-fold excess of S-ODN-AS2 more efficiently blocked the protein translation step within hepatocyte cytoplasm. The reduced intrahepatic production of DHBV core and DHBV preS/S proteins in the S-ODN-AS2-treated ducks may explain the more important decrease in their viremia as compared with the lPEI/O-ODN-AS2 complex-treated group. Whether the differences in the intrahepatic DHBV nucleic acids and proteins inhibition following treatment by lPEI/O-ODN and S-ODN are related to the mechanisms of their cellular uptake or intracellular distribution is actually not known and requires further investigation. Although the therapy with lPEI/O-ODN-AS2 at 2 and 3 μg/g bw/day led to a significant decrease in intrahepatic viral replication, we have not achieved complete viral elimination. To improve the efficacy of lPEI/O-ODN treatment one experimental approach would consist of using combination of antisense O-ODN directed to different parts of the viral genome. In addition, in this study the delivery of lPEI/O-ODN complexes was performed by the intravenous route and was not liver specific. The liver targeted delivery of lPEI/O-ODN via specific hepatocyte receptors may increase the efficiency of its transfer into cells and warrants further studies.

Taken together, we provide first experimental evidence that intravenous treatment with phosphodiester antisense ODN coupled to lPEI results in a specific and significant inhibition of hepadnaviral replication, suggesting its usefulness for therapy of hepatitis B. In addition, we demonstrate that the lPEI-mediated transfer allows the reduction of the amount of antisense O-ODNs required for efficient inhibition of hepadnaviral replication. The simple and inexpensive approach presented here can be of particular interest for different studies aiming to use the intravenous delivery of antisense O-ODN to block other viral or cellular disease-causing genes in the liver.

Materials and methods

Virus

A pool of viremic sera from ducklings infected with the DHBV strain sequenced by Mandart *et al*[31] was used as inoculum. This inoculum was quantified into virus genome equivalents (vge) by quantitative dot blot hybridization as previously described.[32]

Animals

Three-day-old Pekin ducklings (*Anas domesticus*) were intravenously inoculated with a DHBV-positive serum (5×10^7 vge per duck), as previously described.[24] Ducklings were maintained in accordance with the guidelines for animal care at the facilities of the Ecole Nationale Vétérinaire de Lyon, France.

Oligodeoxynucleotides

Unlabelled or 5′-fluorescein-labelled (FITC) phosphodiester (O-ODN) and phosphorothioate (S-ODN) 18-mers were synthesized by Birsner and Grob GmbH, Denzlingen, Germany. The antisense ODN-AS2[14] directed against the start of the DHBV preS region (nt 795–812: 5′-ATGTTGCCCCATCATAAA-3′) was used in S-ODN or O-ODN form. In addition, an unrelated O-ODN (O-ODN-CTRL) of the same size (5′-TGTTGTTGCCCATTATGT-3′) was used as a control for possible non-specific effects.

lPEI/O-ODN complex formation and zeta potential measurements

Linear lPEI (mean MW 22 kDa) was produced as previously described.[18] For intravenous delivery, complexes were prepared at a 6:1 lPEI nitrogen/ODN phosphate ratio (N/P = 6). Typically, 500 μg of O-ODN and the corresponding amount of a 1 M lPEI solution were each diluted into 400 μl of 5% sucrose and incubated for 10 min at room temperature (RT). Subsequently, the lPEI solution was added to the O-ODN solution, vortexed and incubated for 10 min at RT to allow complex formation. Immediately after incubation, the lPEI/O-ODN complexes were rapidly intravenously injected to ducklings avoiding their mixing with blood flow.

The zeta potential of the lPEI/O-ODN complexes was determined using a Zetamaster 3000 (Malvern Instruments, Orsay, France), as previously described.[33]

Electron microscopy of lPEI/O-ODN complexes

Carbon films were prepared by sublimation of freshly cleaved mica, recovered by flotation on Cu/Rh grids (300 mesh; Touzard and Matignon, Courtaboeuf, France), and dried overnight. Immediately before sample addition, grids were glow-discharged (110 mV, 25 s). A drop (5 μl) of sample solution was left on the grid for 1 min. Complexes were negatively stained with 30 μl of aqueous uranylacetate (1% wt/wt) for 20 s. Observations were performed at 80 kV with a Philips EM 410 transmission electron microscope.[30,33]

Therapeutic protocol

A total of 28 Pekin ducklings experimentally infected with DHBV as described above, was used in this study. Ducklings were randomly assigned into seven groups which were treated either with lPEI/O-ODN-AS2 complexes (1 to 3 μg/g bw/day), or with free S-ODN-AS2 (15 μg/g bw/day) as summarized in Table 2. Control groups included animals injected either with free O-ODN-AS2, or with unrelated lPEI/O-ODN-CTRL complexes, or untreated (Table 2). Ducklings received daily intravenous injection of ODN into the foot vein and in the retrooccipital sinus starting on day 4 after infection and over 9 days. Blood samples were collected daily for viremia follow-up. Cellular toxicity was evaluated by analyzing the serum lactic acid level 5 days after the beginning of treatment (Lactate PAP; BioMérieux, Marcy l'Etoile, France). In addition, animal weight was taken every day. All animals were killed 24 h after the end of therapy and specimens of liver tissue were frozen at –80°C for molecular analysis, and fixed for histological analysis.

Biodistribution of ODNs

To assess the organ distribution of antisense ODN, about 1 μg/g bw (400 μg per duckling weighing 350–400 g) of flurescein-labeled FITC-S-ODN-AS2 or FITC-O-ODN-AS2 (complexed to lPEI or in free form) was injected into the foot vein of 2-week-old DHBV-infected ducklings. Four groups of four animals were killed 4 or 24 h after ODN injection, and frozen liver, spleen, lungs and kidneys sections were examined by fluorescence microscopy (Leica, France; DMLB100). Intrahepatic localization of ODN was additionally analyzed by confocal microscopy (Zeiss, Germany; LSM). Excitation of green fluorescence of FITC-ODN at 488 nm was achieved with argon laser, with the resulting fluorescence wavelengths observed at 515–545 nm.

In adition, to quantify by flow cytometry the ODN delivery to the liver during the antisense therapy, the last treatment injection of ODN consisted of a mixture of unlabeled and 400 μg FITC-labeled ODN. Single-cell suspensions of liver cells were prepared by collagenase digestion of 0.5 g fresh liver tissue 24 h after ODN injection. The percentage of FITC-positive hepatocytes was assessed by flow cytometry (FACScan, Lysys II Software, Becton Dickinson) by analyzing fluorescence from 10000 individual hepatocytes.

Detection of viral replication

DHBV DNA was detected in 50 μl of duck serum by dot blot hybridization by using a full-length ^{32}P-labelled DHBV probe as described previously.[34] Total liver DNA was obtained from 0.2 g of frozen tissue homogenized in liquid nitrogen as described.[35] For intrahepatic DHBV DNA detection, 5 μg of the total DNA was subjected to electrophoresis on 1% agarose gel, followed by Southern blot analysis.[35]

Total RNA was isolated from frozen liver by liquid nitrogen homogenization and incubation in TRIZOL Reagent (Life Technologies). Three μg of purified RNA were glyoxalated, separated on 1% agarose gel and analyzed by Northern blot.[36]

Immunoblotting

Proteins were extracted from duck livers as previously described.[36] Immunoblotting of DHBV envelope and core proteins was performed on 200 μg of total proteins, using polyclonal rabbit antiserum directed against the DHBV preS region[34] or against DHBV core protein, anti-rabbit peroxidase conjugate (DAKO A/S, diluted 1:2000), and ECL chemiluminescence detection kit (Amersham), as previously described.[32]

Histopathology

Sections of fixed liver tissue were examined under code. For each sample, portal inflammation, ductular proliferation, lobular necrosis, and hepatocyte degeneration were semi-quantitatively scored according to their degree, as previously described.[36]

Quantification of viral markers and statistical analysis

The mean area under the curves of viremia, which reflects total virus production, was compared between duck groups. Comparative densitometry of autoradiographs were performed by densitometric analysis using a scanner apparatus (Canon, France; CLC 10). The comparison between antisense ODN-treated and untreated groups for all viral replication and gene expression markers was performed by the nonparametric Mann–Whitney test. Statistical significance was taken as $P < 0.05$.

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