ANTISENSE OLIGONUCLEOTIDES FOR THE INHIBITION OF HEPATITIS B VIRUS REPLICATION IN VIVO AND IN VITRO

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ABSTRACT

This study investigated the feasibility of using liposomes to increase the hepatic delivery and antiviral efficacy of phosphorothioate antisense oligodeoxynucleotides (PS-ODNs) for the treatment of hepatitis B virus infection. Ducks infected with duck hepatitis B virus were used as the *in vivo* model and the PLC/PRF/5 human hepatoma cell line containing the integrated hepatitis B virus surface antigen gene was used as the *in vitro* model. Inhibition of the hepatitis B surface gene expression was targeted, as this is essential for viral penetration and replication.

Phosphorothioate ODNs remained stable during the process of liposome entrapment, were stable in duck plasma for many hours, were rapidly cleared from the plasma when injected intravenously, intravenous injection of ODNs entrapped within liposomes enhanced delivery of the ODNs to the liver, and, inhibited duck hepatitis B virus replication. In addition, the intrahepatic distribution of a labelled free- and liposome-entrapped ODN was similar. Serum DHBV DNA levels fell rapidly, with a corresponding decrease in intrahepatic viral replicative intermediates and a fall in the target protein at the end of a 5 day treatment period. However, a marked inhibition of viral replication was also observed with high doses of a random sequence ODN.

*In vitro* experiments showed that antisense ODNs markedly inhibited HBsAg secretion into the cell culture medium, but the use of cationic liposomes for their transfection did not enhance the degree of inhibition. However, the cellular uptake of fluorescein-labelled ODNs was increased after one hour of incubation, and there was greater delivery to the nucleus of the cell. It is not certain that inhibition of viral replication or transcription was entirely through an antisense mechanism. Therefore, antisense ODNs inhibit HBV replication and liposomes may be effective vehicles to improve the delivery of antisense oligonucleotides to the liver for the therapy of hepatotropic viruses, but the non-specific effects of the antisense molecules remain a cause for concern.
STATEMENT OF ORIGINALITY

This thesis is based on original work carried out by the author from 1993 to 1995 in the University Department of Medicine at the Royal Free Hospital School of Medicine.

The experimental studies described in this thesis were designed by the author. The author was solely responsible for the collection of material, conduct of the experiments, and analysis of the data. The entrapment of oligodeoxynucleotides into liposomes was designed in liaison with the Centre for Drug Delivery Research, School of Pharmacy, University of London. Care of animals was performed by resident staff of the Comparative Biology Unit at the Royal Free Hospital. Where appropriate, assistance from colleagues has been acknowledged and the resulting publications have been listed. The author has prepared the manuscript and accompanying figures and tables.
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Finally, I wish to thank the South African Medical Research Council for funding my overseas research fellowship.
DEDICATION

This thesis is dedicated to

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LIST OF ABBREVIATIONS

3TC  3’-thiacytidine
AFP  Alpha fetoprotein
AIDS Acquired Immunodeficiency Syndrome
ALT  Alanine aminotransferase
AMP  Adenosine monophosphate
bp   base pair
BSA  Bovine serum albumin
C    core
cDNA complementary DNA
CF   Carboxyfluorescein
DAB  3,3’-diaminobenzidine tetrahydrochloride
ddC  Dideoxycytidine
ddG  Dideoxyguanosine
ddI  Dideoxyinosine
DHBsAg DHBV surface antigen
DHBV Duck hepatitis B virus
DMEM Dulbecco’s modified Eagles medium
DN   Dominant negative mutants
DOPE dioleoyl-phosphotidylethanolamine
DOTMA N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride
DR   Direct repeat
DRVs Dehydration-rehydration vesicles
DSPC distearoyl-phosphatidylcholine
EDTA ethylenediaminetetraacetate
FBS  Foetal Bovine Serum
FIAU Fialuridine
GSHV Ground squirrel hepatitis virus
HBcAg Hepatitis B core antigen
HBeAg Hepatitis B ‘e’ antigen
HBsAg Hepatitis B surface antigen
HBV  Hepatitis B virus
HCC  Hepatocellular carcinoma
HCV  Hepatitis C virus
HDV  Hepatitis delta virus
HPLC High performance liquid chromatography
IRES Internal ribosomal entry site
MHC  Major histocompatibility complex
mRNA messenger RNA
NADP Nicotinamide adenine dinucleotide phosphate
NCR  Noncoding region
NDS  normal duck serum
NS   Nonstructural
ODNs Oligodeoxynucleotides
ORF  Open reading frame
P    polymerase
PAGE Polyacrylamide gel electrophoresis
PC   Phosphatidylcholine
PCR  Polymerase chain reaction
PS   Phosphorothioate
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<td>PVP</td>
<td>Polyvinylpyrrolidine</td>
</tr>
<tr>
<td>RNase H</td>
<td>Ribonuclease H</td>
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<tr>
<td>S</td>
<td>surface</td>
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<tr>
<td>SA</td>
<td>stearylamine</td>
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<tr>
<td>SUVs</td>
<td>Small unilamellar vesicles</td>
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<tr>
<td>TdT</td>
<td>Terminal deoxytransferase</td>
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<td>TEMED</td>
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Chapter 1

INTRODUCTION

Statement of purpose
This study was conducted to investigate whether antisense oligodeoxynucleotides could be used to inhibit hepatitis B virus replication and gene expression, and if the use of liposomes as a delivery vehicle would enhance the antisense effect. Two models were used to investigate this: an in vitro model which used the PLC/PRF/5 human hepatoma cell line, and an in vivo model which used ducklings chronically infected with duck hepatitis B virus.

1.1 HEPATITIS B VIRUS
1.1.1 Natural history and epidemiology
Infection with hepatitis B virus (HBV) results in a broad spectrum of liver disease, ranging from subclinical infection through acute, self-limited hepatitis to fatal, fulminant hepatitis. Exposure to HBV, particularly when it occurs early in life, may result in an asymptomatic carrier state that can progress to chronic active hepatitis, cirrhosis of the liver, and eventually hepatocellular carcinoma. Complex viral and host factors determine this variable clinical outcome. Fundamental questions about the biologic aspects of HBV that have direct relevance to clinical practice remain unresolved. Immunocompromised patients, including those with human immunodeficiency virus infection, are at increased risk for chronic HBV infection.

It is estimated that there are 400 to 500 million HBV carriers in the world today (Moradpour & Wands 1995), and the World Health Organisation lists HBV as the ninth major cause of death world-wide (Hoofnagle 1990). In Africa and Asia, HBV is a disease of the general population, whereas in Europe and the USA it is found largely in certain high-risk groups: parenteral drug abusers, male homosexuals, promiscuous heterosexuals, health care workers, recipients of blood or blood products, and immigrants from areas of the world where HBV is common. In the United States, between 50 000 and 100 000 people acquire HBV infection each year, even though a highly effective vaccine is available. Approximately 5% to 10% of patients become long-term carriers of the virus; 90% of neonates infected become chronic carriers of the virus.
To date, no satisfactory medical treatment of chronic HBV infection is available. Alpha-interferon has been shown to be useful with a clinical, biochemical and serological remission in 30-40% of highly selected patients. However, it must be given by parenteral injection, is not without side-effects, and is ineffective in a high proportion of patients. New directions in treatment of HBV infection are therefore required.

1.1.2 Hepadnaviruses

The human HBV is a member of a family of viruses known as the hepadnaviruses. Other viruses in this family are the woodchuck hepatitis virus (WHV) of *Marmota monax*, the ground squirrel hepatitis virus (GSHV) of *Spermophilus beecheyi*, the duck hepatitis B virus (DHBV) of *Anas domesticus*, heron hepatitis virus, and Ross's Goose hepatitis virus. Among the features that define the hepadnavirus family are unique virion ultrastructure, characteristic polypeptide and antigenic composition, and common genome size, structure, and mechanism of replication (Robinson & Marion 1984). Common biological features include a striking tropism for hepatocytes and the common occurrence of persistent infection, with complete and incomplete viral forms found in high concentrations in the blood and lower concentrations in other body fluids continuously for years. These viruses replicate asymmetrically through a ribonucleic acid (RNA) template that requires reverse transcriptase activity. Infections with hepadnaviruses may be associated with acute and chronic hepatitis, immune complex-mediated disease, and hepatocellular carcinoma (HCC).

1.1.3 Molecular virology

Electron microscopy of partially purified preparations of HBV from human serum reveals 3 types of particles: (i) 43 nm double-shelled particles, termed "Dane particles" (after their discoverer) representing the intact virion; (ii) 22 nm spheres, usually present in $10^3$ - $10^6$-fold excess over virions; and (iii) filaments of 22 nm diameter and variable length (Robinson & Lutwick 1976; Dane *et al.*, 1970). The HBV virion consists of an outer shell composed of the virus-encoded envelope proteins and host-derived lipid components. The viral nucleocapsid displays a T3 symmetry and consists of 180 subunits of the viral core monomeric protein (Scaglioni *et al.*, 1996). The viral genome is located inside the nucleocapsid and consists of a partially double-stranded relaxed circular DNA
molecule of 3.2 kb in length that encodes, other than the envelope and core proteins already mentioned, the virus-specific polymerase necessary for replication in the hepatocyte. The level of circulating virions during the natural course of infection is variable and ranges from $10^5$ to $10^9$ viral particles per millilitre of serum. More abundant molecular species are the 22 nm subviral particles, with a concentration varying between $10^8$ to $10^{11}$ particles per millilitre. These spheres lack viral DNA or RNA and are therefore not infectious. They are highly immunogenic, and, for this reason, have been used in the generation of the first clinically effective HBV vaccines (Szmuness et al., 1980).

The viral genome contains four open reading frames (ORFs). The preS/S ORF of the HBV genome encodes for the proteins of the viral envelope and contains three in-frame initiation codons at the start of the preS1, the preS2 and S gene regions. There are therefore 3 envelope proteins produced depending on which of the 3 initiation codons is utilised: the LHBs (large) protein which consists of the preS1, preS2 and S gene products, which is converted by partial glycosylation in vivo to P39 and GP42; the MHBs (middle) protein which consists of the preS2 and the S gene products that is either glycosylated once or twice (GP33 or GP36); and the small protein (SHBs) encoded by the S gene and is present in glycosylated (GP27) or non-glycosylated (P24) form. SHBs is the most abundant polypeptide in all three HBV-associated particles, whereas MHBs is a minor component in all three. The preS1 sequence is essential for recognition of hepatocyte receptors. SHBs occur in stable subtypes that were originally defined by antibodies. Antigen reactivities that were present on all known HBs isolates were considered as determinant a. The best-known subtype determinants are d or y and w or r (Gerlich 1993). Determinant d has a lysine at position 122, and y an arginine. Likewise determinant w has a lysine at position 160 and r an arginine. These type-specific amino acid exchanges may, however, occur in quite divergent HBV genomes. The clinical relevance of these subtypes is not known.

The C gene codes for the core protein. It consists of 549 nucleotides (nt) coding for 183 amino acids (aa), and is preceded by the precore region made of 87 nt encoding 29 aa.

The core protein (p21°) is translated from pregenome-core-polymerase messenger RNA (mRNA), which does not include the entire precore region, while HBeAg is translated from longer mRNA with slightly different, diverse starting sites. The 21-kDa viral core
protein assembles into a 180-subunit nucleocapsid structure. This molecule is also involved in nucleic acid binding and promotes viral replication (Scaglioni et al., 1997). Translation of the precore gene results in a pre-core/core-related polypeptide designated p25. The 29-aa precore protein contains a signal peptide which directs the pre-core/core protein into the secretory pathway of the cell (Miyakawa et al., 1997). The first 19-aa of the protein are subsequently cleaved to generate a p22 intermediate protein product that is either translocated to the endoplasmic reticulum or released back into the cytoplasm. In the endoplasmic reticulum, p22 is cleaved at the carboxy terminus in an arginine-rich domain to create a 17-kDa soluble product, hepatitis B e antigen (HBeAg), that is secreted from the cell. The function of HBeAg in the biology of HBV infection is unknown, but is thought to induce immune tolerance. HBeAg is found in the serum of HBV-infected individuals, where it generally correlates with high levels of viraemia.

A functional precore gene appears not to be essential for viral replication, particularly in animals experimentally infected with the related duck hepatitis B virus and woodchuck hepatitis virus. With respect to HBV, genomes defective in HBeAg synthesis are frequently found in individuals with chronic infection. By far the most common mutation is the G to A at nt 1896 converting codon 28 in the precore region for tryptophan (TGG) to a stop codon (TAG) (Carman et al., 1989). This naturally occurring HBV mutant has been associated with fulminant hepatitis and high levels of viral replication, as well as with chronic infection (Carman et al., 1991). Thus, precore mutants with an HBeAg-minus phenotype cannot direct the synthesis and secretion of HBeAg because of the premature termination of the translation of the HBeAg precursor protein.

The genome is a circular partially double-stranded DNA with a single-stranded region of variable length (Figure 1). The long or L(-) strand is linear and of a fixed length of about 3200 nucleotides, the smallest of any animal DNA virus. The short or S(-) strand is of variable length, ranging from 50% to 100% of that of the L(-) strand. The L(-) strand of HBV contains 4 overlapping open reading frames (ORF) conserved in the sequences of different strains. In contrast, there are no conserved ORFs of any considerable length in the S(-) strand transcript. The L(-) strand thus carries the protein coding capacity of the virus and can therefore be considered as the minus strand. The 4 ORFs mentioned previously are considered in the HBV L(-) strand and the polymerase region overlaps with all the other three
Figure 1.1 Structure and genetic organization of the HBV genome. The broad arrows surrounding the genome represent the 4 large open reading frames of the L (-) strand transcript. The number of amino acids encoded by the coding sequences are indicated. The two thin arrows surrounding the broad arrows represent the two major HBV mRNAs. The partial restriction map and the numbering of the nucleotides indicated on the inner circle correspond to the ayw$_3$ genome. The positions of the initiation and stop codons of the coding sequences and the 5’ and 3’ ends of the two major transcripts are indicated. (Tiollais et al., 1985)
The positions of the 5' ends of the L(-) and S(+) strands are fixed, while the position of the 3' end of S(+) is variable. The maintenance of the circular structure of the genome is assured by base-pairing of the 5' ends of the two strands. At both sides of the cohesive ends there is an 11-base-pair (bp) direct repeat (DR) sequence, termed DR1 and DR2 respectively. They are approximately 225 bp apart in the mammalian viruses and appear to play a critical role in viral DNA replication. The P region codes for DNA polymerase, which also possesses a reverse transcriptase activity. It is essential for viral replication.

Mammalian hepadnaviruses carry in their genome a short ORF that has been designated 'X'. The HBx gene encodes for a 154-amino-acid long gene product called hepatitis B x protein (HBx). No definitive function has been assigned to HBx (Scaglioni et al., 1996). Since the X ORF is not present in the avian hepadnaviruses, it seems reasonable to propose that HBx may not be required for replication, but may play a regulatory role in the viral life cycle. Computer analysis of the X ORF reveals that HBx would be a hydrophobic non-secreted protein of 17 kDa. Many studies have suggested that HBx may act as a transcriptional regulator even though the protein does not bind to DNA directly. The HBx protein has also been reported to activate RNA polymerase II genes. HBx also appears capable of interacting with TATA binding proteins and may stimulate transcriptional activity (Henkler & Koshy 1996). Activation by HBx is mediated via the transcription factor, NFkB, which binds to a specific sequence in the enhancers of many inducible genes.

The ability of the HBx protein to transactivate a variety of promoters of cellular genes involved in cell growth has led to the hypothesis that HBx may contribute to the development of hepatocellular carcinoma (HCC) in chronic HBV infected individuals. This hypothesis is supported by the finding that HBx has the ability to transform mouse hepatocytes in vitro (Hohne et al., 1990). Functional X gene sequences are also frequently found in the integrated form of HBV DNA present in HCC tumour specimens (Wollersheim et al., 1988). It has been reported that transgenic mice expressing the X gene develop HCC (Kim et al., 1991). This observation implies a direct role for HBx in the pathogenesis of HCC.

The close correlation between X antigen staining and hepatitis in chronically infected woodchucks, and its persistence at high level in the livers of X transgenic mice that go
on to develop liver tumours, also suggest that the persistent expression of HBxAg is consistent with the development of HCC (Feitelson & Duan 1997). The inverse correlation between HBxAg staining in the liver and markers of virus replication in the serum suggests that HBxAg could be made independently from virus replication, probably from integrated templates of viral DNA. Additional evidence that HBxAg contributes to hepatocarcinogenesis is the appearance of altered foci, adenomas, and HCC in X-transgenic mice with persistently high levels of HBxAg expression, although these observations have not been reproducible (Feitelson & Duan 1997).

When p53 function is lacking, cells with damaged DNA continue to replicate and usually die or, rarely, transform. Point mutations, which inactivate this protein, have been found in many tumour types including HCC (Hollstein et al, 1991). HBxAg trans-activation of p53-responsive genes may be mediated by HBxAg-p53 complex formation, suggesting the presence of a p53 binding site within HBV DNA. Hence, HBxAg-p53 complex formation may alter genetic stability and cell cycle control, both of which are central to multistep carcinogenesis. HBxAg-p53 complexes have been found in the HCCs that develop in X-transgenic mice (Ueda et al, 1995). Co-staining of HBxAg and p53 in the same cells from altered foci, adenomas, and HCC nodules, further suggest a close relationship between these proteins (Feitelson & Duan 1997). In addition, HBx may influence the accumulation of specific aflatoxin-induced mutations, such as the activating mutation which causes a G to T transversion in the third position of codon 249 of the p53 gene (Aguilar et al, 1993). Thus, HBxAg-p53 complex formation seems to be an important step in viral hepatocarcinogenesis, by analogy to other DNA tumour viruses, but the significance of these complexes to transformation requires further characterisation.

Nucleotide homologies between HBV and WHV, GSHV, and DHBV are around 70%, 55% and 40% respectively (Tiollais et al, 1985). In general, the avian virus is the most divergent member of the hepadnaviruses, both at the level of nucleotide sequence and in biological properties (Ganem & Varmus 1987). The L (-) strand of DHBV contains only 3 ORFs - S, C, and P. The absence of the region X in DHBV may explain the absence of hepatocarcinogenesis in this model. The morphology of DHBV particles is somewhat atypical, and its encapsidated genomes contain a high proportion of molecules that lack a single-stranded gap. Although viral replicative intermediates are found in highest abundance in the liver, replication and gene expression also proceed to a lesser extent in
the pancreas, kidney and spleen (Tagawa et al, 1985). Another major distinction concerns routes of viral transmission. In nature, DHBV is transmitted almost exclusively by the vertical route. After hatching, ducks rapidly lose susceptibility to exogenous infection by three weeks of age even large parenteral doses of infectious virus rarely induce detectable viraemia (Mason et al, 1983).

1.1.4 Replication of hepadnaviruses

The replication mechanism of hepadnaviruses, discovered by Summers and Mason (1982) for DHBV, and confirmed later for HBV (Blum et al, 1984), differs strikingly from that of other DNA viruses. The central feature is the use of a RNA copy of the genome as an intermediate in replication, that is, a reverse transcriptase step similar to that of retroviral genome replication. The extremely small size of the hepadnaviral genome (3.0 to 3.3 kb), and the need to efficiently exploit this restricted genetic space results in a largely overlapping arrangement of both coding regions and regulatory elements. In contrast to retroviruses, extracellular hepadnavirions contain DNA rather than RNA; integration is not an obligatory step in replication: functional mRNAs are produced from several internal promoters on the circular DNA genome and RNA splicing does not appear to play a critical role in the basic replication cycle. The life cycle of HBV and its relatives in the animal kingdom can be divided into successive steps: attachment of the virus to the host cell, virus penetration into the cell, release of the viral genome, expression of viral gene products, replication of the viral genome, formation of virions, and release of the virus (Figure 1.2).

Viral entry into hepatocytes

It is generally believed that HBV interacts with specific cell-surface protein(s) followed by receptor-mediated endocytosis of virus into hepatocytes. Avian hepadnavirus envelope protein lacks the preS2 component. Some evidence has been provided for involvement of the preS1 protein in binding to liver membranes, as well as to HepG2 hepatocellular carcinoma cells (HCC) and other cell lines (Pontisso et al, 1989). Two groups of investigators have independently identified and cloned a hepatocyte-derived glycoprotein that binds with high affinity to DHBV particles (Klingmuller & Schaller 1993). This candidate DHBV receptor was identified as a novel member of the basic carboxypeptidase gene family and named gp170/180. The protein possesses some of the
characteristics of a viral receptor. The interaction of gp170/180 with the preS protein was inhibited by “wild type” DHBV virions in a dose-dependent manner (Scaglioni et al, 1996).

**Hepadnavirus replication**

The viral envelope is presumably removed by host factors and the nucleocapsid delivers the viral genome to the nucleus. DHBV nucleocapsids are found exclusively in the cytoplasm of infected hepatocytes (Pugh et al, 1989). Thus, according to present concepts of HBV replication, the encapsidation of viral nucleic acids probably occurs in the cytoplasm. In the nucleus the partially double-stranded viral genome is converted into covalently closed circular DNA (cccDNA). The formation of cccDNA requires a series of enzymatic reactions. These include: (1) completion of positive plus (+) strand DNA synthesis; (2) removal of the bond that links the polymerase to the 5' end of the negative (-) strand DNA; (3) removal of the RNA primer from the 5' end of (+) strand DNA; and (4) ligation of the DNA ends. These steps may be mediated by cellular enzymes. The cccDNA serves as the template for generation of viral transcripts, and therefore represents a key molecule in the hepadnaviral life cycle. It is organized as a viral minichromosome and is present in about 10-50 copies inside the nucleus of infected hepatocytes. Its half-life is estimated to be 3-5 days. The copy number of DHBV cccDNA has been shown to be regulated by preS proteins. Therefore, it appears that the envelope proteins not only play a role in virion uptake into the cell but may also be involved in the regulation of viral replication.

Hepadnaviruses replicate their DNA genomes by reverse transcription of an RNA intermediate designated pre-genomic RNA. The current model of HBV replication is based on data obtained with DHBV; however, it is believed that the same processes are probably conserved in the HBV replicative machinery as well. The pre-genomic RNA is generated by read-through transcription from the circular cccDNA. This species of RNA is more than a full genome in length and contains a terminal redundancy region as the 5' and the 3' end of the transcript. Present in the terminal redundancy region are two key cis-acting motifs: (1) a direct repeat (DRI), comprising a 12-nucleotide-long element. (2) ε, an RNA stem-loop structure required for RNA packaging into the nucleocapsids. Of the two copies of ε, the 5' end copy is the one used for encapsidation,
since deletion of the 5' end ε ablates RNA packaging and DNA replication, whereas deletion of the 3' end ε allows both reactions to occur (Seeger & Maragos 1990).

The pregenomic RNA is not only template for reverse transcription but also the messenger RNA of the polymerase gene (pol). Genetic studies have indicated that pol, or P protein, plays a key role both in RNA packaging and in priming DNA synthesis. Packaging of RNA and pol activity within the nucleocapsids are tightly coupled molecular events. The pregenome also serves as mRNA for the core protein, which has the intrinsic ability to self-assemble into icosahedrally symmetric particles. While the actual mechanism of P protein translation from the RNA pregenome of hepadnaviruses is still not clear, there is no doubt that P is synthesized separately from the core protein. P protein is incorporated into hepadnaviral capsids via an RNA-protein interaction with the encapsidation signal ε, rather than by a covalent bond to the core protein (Nassal & Schaller 1996). The highly basic C-terminal region of the protein acts as a nucleic acid binding domain, is required for RNA encapsidation, and is involved in proper reverse transcription. After binding of pol to pre-genomic mRNA, (-) strand DNA synthesis occurs. The 5' end of (-) strand DNA maps within the DRI region. Studies have shown that reverse transcription initiates in the 5' copy of ε in the pre-genomic RNA (Pollack & Ganem 1994). Pol binds to the 5' copy of ε present on its own RNA template, and this event is necessary to initiate both RNA packaging and DNA synthesis. During this step, pol acts as primer of reverse transcription, synthesizing a four-nucleotide-long DNA oligonucleotide that becomes covalently linked to the amino-terminal region of pol.

Following the synthesis of the first four nucleotides of (-) strand DNA, the polymerase-primer complex dissociates from the template and re-anneals with complementary sequences at the DRI located near the 3' end of the pre-genomic RNA where DNA synthesis continues. The (-) strand DNA is elongated within the nucleocapsids. As DNA synthesis proceeds, the pregenomic RNA is degraded by the pol derived RNAse H activity. When pol reaches the 5' end of the RNA template, it leaves an undegraded 18-nucleotide-long RNA molecule. This RNA oligomer is then transferred onto DR2, the second 12-nucleotide-long element identical to DRI, that is located downstream from the 5' end copy of DRI on (-) strand DNA-from where it primes (-) strand DNA synthesis. During (+) strand DNA elongation, the circularization of (-) strand DNA will occur. However, in mature virions, (+) strand DNA is not extended to full length.
Transcriptional control of HBV gene expression

Sequence analysis of the HBV genome reveals four open reading frames (ORF). These ORFs encode the core, envelope, polymerase (pol) and X genes as shown in Figure 1.1. The core ORF contains two in-frame translation initiation codons that allow for the synthesis of two distinct molecules, namely, the core and the hepatitis B e (HBe) proteins. The envelope ORF contains three in-frame translational initiation codons that direct the synthesis of the preS1, preS2 and S proteins. Thus, the compact HBV genome encodes, by partially overlapping ORFs containing transcriptional control elements, seven viral gene products. As a molecular consequence of this organization, the hepadnaviruses display a highly efficient transcriptional strategy that relies on the differential use of transcriptional initiation sites to synthesize both unspliced and spliced transcripts.

Thus, HBV infected hepatocytes express two classes of RNA transcript: (1) genomic transcripts of 3.5 kb. and (2) subgenomic transcripts of 2.4 and 2.1 kb. The 3.5 kb species serves as mRNA for the HBe, core and pol proteins as well as the template for reverse transcription into (-) strand DNA. The other two smaller species are templates for the envelope proteins. In addition to these species, a 0.7 kb transcript derived from the X gene region has been identified.

Viral assembly and secretion

Virion assembly is a series of complex events that takes place in the cytoplasm of HBV infected cells. Early steps involve the interaction of core proteins, DNA polymerase and pre-genomic RNA, and these components are assembled into nucleocapsids where viral DNA synthesis will take place. Interaction of the nucleocapsids with the viral envelope proteins occurs, but the domains on the preS/S proteins or on the nucleocapsids that mediate the interaction are not known. The first step in virion assembly overlaps with the beginning of DNA replication and consists of the priming of DNA synthesis at the 5' end of the pre-genomic RNA. This step does not require the presence of nucleocapsid proteins in vitro. Transfer of the viral pol to the 3' end-containing DR1 region and completion of viral DNA synthesis requires the presence of the viral nucleocapsids.
Core protein homodimers are formed when a threshold of core monomers is reached within the cytoplasm of the infected cell. These homodimers interact and form the spherical HBV capsid.

The overall replication strategy of the hepadnaviruses is therefore to use a built-in structured 5' proximal RNA signal as a specific binding site for P protein. On one hand, this complex triggers in a poorly understood fashion the addition of core protein dimers and nucleates capsid assembly. On the other hand the very same RNA-protein interaction initiates reverse transcription probably already inside the forming capsid. Owing to this central role in hepadnaviral replication, e has become an attractive target for interfering with the viral life cycle.

HBV DNA in the hepatocyte can exist in two states: free, or integrated into the host cellular chromosome. Free HBV DNA, which represents intermediate forms of replication, is detected during the acute stage and some chronic stages of HBV infection. Integrated sequences are mostly observed during chronic virus infection and especially in HCC. Free and integrated forms can coexist within the same sample but this does not necessarily mean that both states coexist in the same cell.
Figure 1.2: Replication cycle of HBV (Tiollais et al., 1985)

1. Entry into the cell of an extracellular virion containing the HBV genome with a short capped RNA (cp), the DNA polymerase (•) and the DNA-linked protein (*). DR1 and DR2 are indicated by 1 and 2.
2. Complete double-stranded open circular DNA.
3. Supercoiled covalently closed circular DNA.
4. Pregenome packaged into a nucleocapsid.
5. Core containing the complete (-) strand DNA.
7. Coated nucleocapsid giving rise to extracellular virion.
1.1.5 Antiviral therapy for HBV

Therapy of chronic hepatitis B is directed at eliminating viral replication. Typical endpoints of successful therapy are loss of HBV DNA and HBeAg from serum, decrease of ALT into the normal range, disappearance of symptoms (if initially present), and improvement of liver histology. Key steps in the life cycle of HBV that may be amenable to therapy include:

(i) viral attachment and penetration;
(ii) translocation to the nucleus and nucleocapsid removal;
(iii) viral transcription;
(iv) viral translation and post-translational modification;
(v) packaging of the viral genome;
(vi) viral DNA synthesis;
(vii) maturation of the viral nucleocapsid and formation of the envelope.

Development of therapies for chronic HBV has been facilitated by the active interest in antiviral drugs brought about by the search for treatments of AIDS and also by new in vitro and in vivo methods for assessing HBV replication. Animal models of HBV infection include several hepadnaviruses: duck hepatitis B virus, woodchuck hepatitis virus, and human HBV infection in chimpanzees. None of these models is ideal. The DHBV model is simple and convenient, but responses to antivirals do not always match those in humans. The woodchuck has been successfully used to screen a large number of antiviral compounds and is a good model for chronic infection, disease and carcinogenesis.

Tissue culture systems have also been used to assess antiviral agents. These include infection of primary duck, woodchuck and even human hepatocytes with the respective hepadnaviruses. More helpful in screening antiviral compounds, however, have been continuous hepatocyte cell lines that have been transfected with human HBV DNA. The most well characterised line is the 2.2.15 cells (Korba & Milman 1991) which were derived from a human hepatoblastoma (Hep G2) cell line transfected with HBV DNA which is stably incorporated into the cellular genome. A summary of drugs used in in vitro, in vivo and in clinical trials is shown in Table 1.1.
1.1.5.1 Immune modulators

Alpha interferon has been shown to be effective in inducing virologic and clinical
remissions in 25% to 40% of treated patients as compared to the spontaneous rate of 5%
to 15% in controls. Furthermore, the quality of remissions induced by interferon may be
superior to spontaneous remissions, in leading more commonly to complete
normalisation of enzymes and clearance of HBsAg in addition to clearance of HBeAg
and HBV DNA (Dusheiko 1995). However, using the sensitive method of polymerase
chain reaction (PCR), HBV DNA has been detected after loss of HBeAg and usually
persists until there is loss of HBsAg (Brechot 1993). Alpha-interferon induces HLA class
I expression on the hepatocyte membrane and increases the amount of lymphocytes and
natural killer cells HLA class II expressing in the liver. Binding of α-interferon to cellular
receptors also leads to inhibition of viral protein synthesis by induction of 2', 5'
oligoadenylate synthetase and double-stranded RNA-dependent protein kinases
(Dusheiko 1995).

There are several shortcomings in interferon therapy. Most importantly, the drug is
effective in only a proportion of patients. In non-responders, repeat courses of treatment
and higher doses are rarely beneficial and are not recommended. Alpha interferon also
has significant side-effects which can limit therapy, and it is expensive. Finally, the role of
interferon is still unsettled in special situations, such as in children, in patients with
unusual forms of the disease, in immunosuppressed patients, and in those with clinically
apparent cirrhosis or end-stage liver disease. The use of a short course of prednisone
followed by α-interferon therapy has also been tried in order to improve the response
rate; however, a large multicentre trial failed to demonstrate that this combination was
more effective than α-interferon alone (Perillo et al., 1990).

The effects of purified derivatives of thymic extract, thymosin fraction 5 and thymosin-α₁
have been studied in patients with chronic hepatitis B. These immune modifiers stimulate
the maturation and function of T cells and the production of interleukins and interferons.
In a pilot study, Mutchnick et al (1991) demonstrated that 6 months of thymosin
treatment resulted in a significantly higher HBV clearance rate (86%) than in placebo-
treated patients (20%). However, these results were not borne out in further large
studies. Other immunomodulatory agents which have been used include recombinant
preparations of interleukin-2 and granulocyte-macrophage colony-stimulating factor, which have been tested in small numbers of patients with chronic HBV (Martin et al., 1993). Unfortunately, low doses of these agents show little antiviral activity, and may be pro-inflammatory, while high doses show severe toxicity. Levamisole and transfer factor have also been used in an attempt to modulate the immune response (Dusheiko & Zuckerman 1991).

In a preliminary study, treatment with *Phyllanthus amarus*, a plant extract, resulted in a high proportion of carriers losing HBsAg (Thyagarajan et al., 1988). Extracts of this have been shown to inhibit the DNA polymerase of HBV *in vitro*. Another approach might be to provide the patient with a supply of activated cells. Examples of this include bone marrow transplantation and infusion of activated cells. Transplantation of bone marrow from donors immune to hepatitis B has occasionally been shown to result in clearance of HBV replication in recipients with chronic HBV infection (Lok et al., 1992). Studies are currently under way to infuse autologous ex-vivo activated lymphocytes into patients with chronic HBV. Another approach is to try to stimulate the immune system to respond specifically to components of the virus itself. Components of the HBV core protein have been shown to elicit the greatest HLA-restricted cellular immune response.

Interleukin-12 is a cytokine produced by phagocytic cells and other antigen presenting cells and several studies have demonstrated that IL-12 plays a critical role for efficient immune responses to intracellular pathogens in viral diseases (Naoumov & Rossol 1997). More recently, exogenous IL-12 has been shown to increase lymphoproliferative responses, increase cytokine production of peripheral blood mononuclear cells from chronic HBV carriers stimulated with HBeAg, and overcome the non-responsiveness to HBsAg (Vingerhoets et al., 1997). Others have shown that a substantial rise in IL-12 production, along with the induction of Th1 cytokines, is required for effective HBe seroconversion (Rossol et al., 1997). IL-12 has also been shown to inhibit HBV replication in transgenic mice (Cavanaugh et al., 1997).

Vaccine therapy, in preliminary, yet promising trials, has now been used for herpes simplex virus (HSV), leprosy, tuberculosis, leishmaniasis and HIV infection (Pol et al., 1997). A recent report of HBV clearance following the administration of hepatitis B vaccine (comprised of HBsAg) also suggests a role for specific immune stimulation (Pol
et al, 1997, Pol 1995; Pol et al, 1994). Forty-six consecutive chronic HBsAg carriers with biopsy-proven chronic hepatitis, including 11 with cirrhosis and active HBV replication were included in a pilot study. Patients received 3 standard doses of a 0.5 ml vaccine containing 20 μg of HBsAg and preS2 protein, with aluminium hydroxide as adjuvant, into the deltoid muscle at one month intervals. Six to 9 months after the first vaccine, a standard antiviral therapy (5 MU interferon α-2b thrice weekly subcutaneously for 4 months) was proposed to all patients and was accepted by 28 of the 46.

Over the 3-month period following the complete vaccination, serum HBV DNA became undetectable in 12 of the 46 patients (26.1%). Eight additional patients (17.4%) showed a significant decrease (more than 50%) in HBV DNA; these 8 finally lost HBV DNA, one 12 months after the first vaccine without other treatment and 7 after starting α-IFN within a mean time of 2.8 months. None of the patients cleared serum HBsAg. Eleven of the 42 HBeAg-positive patients (26%) developed anti-HBe antibodies 6 months after vaccination and 22 (52%) at the end of the mean follow-up period of 23 months. An exacerbation of hepatitis, as assessed by a marked increase in transaminase activities, preceded the disappearance of or the decrease in serum HBV DNA in 18 of the 28 (64%) patients who lost serum HBV DNA. Thus, anti-HBV vaccination stopped or markedly reduced HBV replication in 43.5% of patients with chronic HBV, whereas the rate of spontaneous HBV DNA clearance was reportedly about 7% per annum. It is possible that vaccine therapy might enhance the efficacy of α-interferon in patients with chronic HBV infection.

Akbar et al (1997) showed that 12 monthly intraperitoneal injections of HBV-transgenic mice with HBV vaccine containing 10.0 μg of HBsAg in complete Freund’s adjuvant resulted in complete clearance of HBsAg and HBeAg in sera in 25 and 30 of a total of 32 transgenic mice, respectively. In addition, 5 and 12 transgenic mice developed detectable levels of anti-HBs and anti-HBe in sera, respectively. Estimation of HBV DNA by PCR showed that vaccination resulted in a decrease of HBV DNA in sera. Placebo-recipient transgenic mice did not show any significant change in the titres of HBV markers after receiving 12 monthly injections of complete Freund’s adjuvant. Five monthly injections with vaccine were needed to demonstrate an initial change in HBV markers, and some transgenic mice became completely negative for HBsAg and HBeAg after 10 monthly injections.
DNA-based vaccination allows synthesis of a foreign protein/s \textit{in vivo} from injected plasmid DNA. An important feature of this method is that the viral protein enters the major histocompatibility complex (MHC) class I pathway of the cell, leading to the induction of cytotoxic T-lymphocyte responses. Theoretically, the presence of plasmid DNA within the transfected cells will allow sustained viral antigen expression \textit{in vivo}, with prolonged induction of both humoral and cell-mediated responses. A recent study tested the efficacy of DNA vaccines encoding the duck hepatitis B virus preS/S and S proteins in Pekin ducks (Triyatni \textit{et al.}, 1998). Plasmid pcDNA I/Amp containing the DHBV preS/S or S genes was injected intramuscularly three times at 3-week intervals. All preS/S and S-vaccinated ducks developed total anti-DHBs and specific anti-S antibodies with similar titres reaching 1/10 000 to 1/50 000 and 1/2 500 to 1/4 000, respectively, after the third vaccination. Vaccination of the ducks with either vaccine prevented the development of viraemia following virus challenge. The administration of DNA vaccination to chronic HBV-infected individuals may be a new form of antiviral therapy worth considering, as constant antigen presentation may enhance the immune response to HBV-infected hepatocytes.

\subsection{1.1.5.2 Nucleoside analogues}

Nucleoside analogues undergo phosphorylation and then compete with natural substrates for incorporation into the viral DNA sequence. They may inhibit viral DNA synthesis by causing chain termination, blocking binding sites on the DNA or RNA template, or by interfering with the metabolism and transport of the naturally occurring substrates. First generation nucleoside analogues include acyclovir, adenine arabinoside, ribavirin, azidothymidine, dideoxynosine (ddI) and dideoxycytidine (ddC). Adenine arabinoside (Ara A) and its more soluble analogue (Ara AMP), were the first nucleoside agents extensively studied for chronic HBV. These analogues were potent inhibitors of the HBV DNA polymerase, but responses were not sustained after discontinuing therapy (Hoofnagle \textit{et al.}, 1984). In addition to providing only temporary viral suppression, prolonged administration of Ara AMP led to disturbing neuromuscular toxicity (Guardia \textit{et al.}, 1986, DiBisceglie & Hoofnagle 1989), and further studies with this analogue were discontinued. Acyclovir is well tolerated; however, it was not a potent suppressor of HBV when evaluated in human trials (Guarascio \textit{et al.}, 1986). The dideoxynucleotides have also been demonstrated to have activity against HBV in the duck HBV model.
(Kassianides et al, 1989). Unfortunately, when one of the more promising agents, ddI, was administered to humans in a pilot study, the results were disappointing (Fried et al, 1992).

Currently, there is a second generation of nucleoside analogues being tested amidst great optimism. These agents include fialuridine (FIAU), famciclovir and 3'-thiacytidine (3TC or Lamivudine). Fialuridine, an agent with very potent antiviral effects against HBV, was unfortunately found to have severe and fatal side effects thought to be related to mitochondrial injury. The use of fialuridine was associated with lactic acidosis, fulminant hepatic failure, pancreatitis, myopathy and neuropathy resulting in the death of several patients treated in an experimental protocol (McKenzie et al, 1995). Fialuridine may have been singularly toxic because of its propensity to become incorporated within mitochondrial DNA.

Famciclovir is the oral form of penciclovir, an acyclic guanine derivative and a new nucleoside analogue, which has efficacy against the herpes simplex and herpes zoster viruses. In the Pekin duck animal model, both penciclovir and famciclovir reduced HBV DNA to undetectable levels within 2 days of start of treatment and maintained this suppression during the 21 days of treatment (Tsiquaye et al, 1994). In a double-blind placebo-controlled study with famciclovir in humans, a fall of more than 90% in HBV DNA levels was noted in six of 11 evaluable patients treated with a 10 day course of oral therapy. Experience in liver transplant patients with recurrent hepatitis B has also shown that famciclovir may be an effective antiviral agent (Schalm et al, 1995). This agent is now in phase III clinical trials.

The second generation nucleoside analogue with the most promising potential at present is Lamivudine. Lamivudine is a cytidine dideoxynucleoside analogue, the drug is a negative enantiomer of 3'-thiacytidine and is a potent inhibitor of HBV and HIV replication. It acts as a reverse transcriptase inhibitor to decrease HBV DNA synthesis through chain termination of the nascent proviral DNA (Dusheiko 1998). Lamivudine has an excellent safety profile when administered for prolonged periods in patients with HIV. It is phosphorylated within the cell to the triphosphate derivative, lamivudine 5'-triphosphate has an intracellular half-life of 17-19 h in HBV transfected HCC cell lines.
In a pilot study in which lamivudine was administered for 12 weeks in doses of 25 mg, 100 mg or 300 mg daily, serum levels of HBV DNA became undetectable in all patients treated with the two higher doses (Dienstag et al, 1995). In most patients, HBV DNA reappeared after therapy was completed and relatively few patients cleared HBeAg from serum. This suggests that lamivudine might be effective in suppressing but not eliminating HBV DNA replication. However, the emergence of HBV polymerase gene mutants resistant to lamivudine result in breakthrough infections in a substantial proportion of patients (Ling et al, 1996). A large extended treatment study is ongoing in Asia. To date, it has been reported that HBeAg has been lost in 13% and 16% of Chinese HBeAg positive patients after 1 year of treatment with 25 mg (n=142) and 100 mg (n=143) respectively, compared with 4% of 73 patients receiving placebo (Liaw et al, 1998). The combined effect of lamivudine and alpha-2b interferon (in previously untreated patients) has been assessed. Two hundred and twenty six HBeAg positive patients were randomised to receive lamivudine 100 mg for 52 weeks, lamivudine for 8 weeks and then concurrently with alpha 2b interferon, (to 24 weeks) or alpha 2b interferon alone (for 24 weeks) (Heathcote et al, 1998). Of the patients, 20%, 25% and 22% respectively seroconverted from HBeAg to anti-HBe at 64 weeks. In this study, histological improvement was noted in 38% of lamivudine-treated patients, 36% of the interferon treated patients and 28% of the lamivudine and alpha interferon patients. The available evidence suggests that the drug has an acceptable tolerance in patients with advanced cirrhosis who require liver transplantation and can be cautiously administered in these patients.

Resistance to lamivudine was associated with mutations which lead to amino acid site substitutions in the highly conserved YMDD motif, part of the active site of the polymerase (Ling et al. 1996). Substitutions of valine and isoleucine for methionine were found in some cases. Genotypic mutations were observed in 14% of Chinese patients treated with 25 mg or 100 mg after 1 year of treatment. YMDD mutations were observed in 38% of patients treated with lamivudine continuously for 2 years. The overwhelming majority of patients have a two log decline in HBV DNA from baseline which is accompanied by an improvement in serum aminotransferases and liver histology. In a one-year study, lamivudine was associated with substantial histologic improvement in many patients with chronic hepatitis B (Lai et al, 1998).
A new acyclic adenine nucleotide analogue with broad spectrum antiviral activity against retroviruses, hepadnaviruses and herpesviruses is adefovir dipivoxil (Bis-POM-PMEA). This drug acts as a chain terminator and has a high therapeutic index (>500). It has a high intracellular half-life of 16 to 18 hours (Renson et al., 1996). In a phase II study for chronic hepatitis B, adefovir dipivoxil as a once daily oral dose was well-tolerated and associated with significant and sustained reductions in serum HBV DNA levels for the duration of the dosing (Gilson et al., 1996). Furthermore, preliminary in vitro data has shown that there is lack of cross resistance to this agent for the human hepatitis B DNA polymerases which express lamivudine resistance codons (Xiong et al., 1997). Lobucavir is a new guanosine nucleoside analogue with broad spectrum antiviral activity. In a double-blind, placebo-controlled, randomised study involving 22 subjects, lobucavir was well-tolerated and associated with a 2- to 4-log reduction in serum HBV DNA levels during 28 days of dosing (Bloomer et al., 1997).

Intravenous ganciclovir has been used to treat HBV recurrence after liver transplantation in nine patients (Gish et al., 1996). Serum HBV DNA levels decreased by a mean of 90%, and four of nine patients had undetectable levels of HBV DNA. HBV DNA levels increased after treatment was stopped in most patients. There were no major side-effects of ganciclovir therapy. BMS-200475 is a potent and selective deoxyguanosine analog with the potential for treating chronic HBV infection. Direct comparison of BMS-200475 with other nucleoside analogs in the 2.2.15 cell culture assay demonstrated that BMS-200475 was the most potent inhibitor of HBV replication, with superior in vitro potency to that of lamivudine (Innaimo et al., 1997). It also had a high selectivity index.

The antiviral activity of 2',3'-dideoxy-fluoroguanosine (FdG) was evaluated for DHBV: it resulted in a dose-dependent inhibition of viral replication with a nearly complete inhibition at a concentration of 1 μM in vitro, and a 90% reduction in DHBV DNA replication in infected ducklings (Hafkemeyer et al., 1996). Another study using this agent showed inhibition of HBV production in cell lines, and inhibition of both DHBV and HBV DNA-polymerases at low concentrations in vitro (Schroder et al., 1998). In a recent study, the in vivo administration of L-FMAU (2'-Fluoro-5-Methyl-β-L-Arabinofuranosyl-Uracil), a new class of thymidine analog, by the oral route to experimentally infected ducklings showed a potent inhibition of viral replication which
was not associated with any significant toxicity (Aguesse-Germon et al., 1998). It also produced a dose-dependent inhibition of viral DNA replication in 2.2.15 cells with a 50% inhibitory concentration of 0.1 µM (Balakrishna et al., 1996).

1.1.5.3 Molecular biologic therapy

Molecular biologic approaches to therapy take advantage of the knowledge of the molecular biology of HBV. Antisense oligonucleotides have been shown to inhibit gene expression in vitro and in vivo (Wu et al., 1992; Offensperger et al., 1993). Ribozymes are small RNA molecules that are able to cleave RNA. They may be a more effective tool than antisense oligodeoxynucleotides (ODNs) since they possess both antisense and RNA cleavage activities. The enzymatic nature of ribozymes may facilitate effectiveness even at low levels of expression. After binding, they should irreversibly destroy their target sequence and, moreover, be capable of multiple turnover. Ribozyme genes can be delivered via various viral vector delivery systems and are expressed constitutively within the target cells, thereby overcoming the toxicity and degradation problems associated with the direct injection of antisense DNA. Stable expression of the anti-HBV ribozymes in liver cells could theoretically lead to lifelong intracellular immunity against HBV (Wands et al., 1997). An anti-HBV ribozyme can potentially be multifunctional, targeting both the pregenomic RNA as well as the viral mRNAs (Hoofnagle & Lau, 1997). In addition, by targeting the ribozymes to small highly conserved regions of the viral genomes, the possibility of generating escape mutants can be greatly reduced.

Two hairpin ribozymes were generated and shown to be capable of cleaving HBV RNA in vitro (Welch et al., 1997). The cDNAs for each of the anti-HBV ribozymes were cloned into retroviral vectors. Expression of these ribozymes, but not their disabled counterparts, in Huh 7 cells resulted in inhibition of HBV particle production and their tetraloop variants demonstrated even higher antiviral activity, reducing HBV production by up to 83%. Some workers are attempting to target the highly conserved RNA encapsidation signal ε with specific ribozymes; due to its multifunctionality this RNA region should not easily tolerate mutations (Nassal, 1997). Ribozymes have also been shown in vitro to cleave HBV RNA molecules and inhibit transcription of HBV proteins (Weizsaecher et al., 1992).
Hepatitis delta virus (HDV) is a small single-stranded RNA satellite of HBV. Although it is a human pathogen, it shares a number of features with a subset of the small plant satellite RNA viruses, including self-cleaving sequences in the genomic and antigenomic sequences of the viral RNA. The self-cleaving sequence is critical to viral replication and is thought to function as a ribozyme \textit{in vivo} to process the products of rolling circle replication to unit-length molecules (Been & Wickham 1997). A minimal natural ribozyme sequence with proficient \textit{in vitro} self-cleavage activity is about 85 nucleotides long and adopts a secondary structure with four paired regions (P1 to P4).

The functional inactivation of viral proteins by modified viral gene products, also termed dominant negative (DN) mutants, has gained substantial interest (von Weizsacker \textit{et al}, 1996). These are polypeptides that are able to interact and/or disrupt the function of their native counterparts. Core proteins of the woodchuck hepatitis virus and HBV, when fused at their truncated C-terminus to the viral small surface antigen, efficiently inhibited viral replication. The hepadnaviral core protein represents a particularly promising target for DN mutants because it is central to several functions critical for viral replication: it is the building block of the viral nucleocapsid and plays an essential role in both packaging of the RNA pregenome into core particles and the subsequent maturation of viral DNA. von Weizsacker \textit{et al} (1996) tested various core protein mutants for their potential antiviral activity by cotransfection with a replication-competent DHBV construct into the avian hepatoma cell line LMH. Carboxy-terminal, but not amino-terminal, DHBV core mutants inhibited DHBV replication by up to 90% at an effector-to-target ratio of 1:10. Antiviral activity was species-specific and caused by post-translational interference with viral replication.

Scaglioni \textit{et al} (1996) studied the DHBV and placed the DN mutant constructs in recombinant retroviral and adeno-viral expression vectors. Transient expression of the DHBV molecular equivalent of the WHV and HBV DN constructs inhibited wild-type DHBV replication by 98%. Recombinant retroviral and adeno-viral vectors containing the HBV and DHBV DN complementary DNAs (cDNAs) were used to transiently and stably transduce hepatoma-derived cell lines constitutively expressing replicating wild-type virus. These studies showed that the DN core mutants were powerful inhibitors of HBV and DHBV replication when delivered intracellularly and appear as promising antiviral agents for gene therapy of persistent viral infection of the liver.
Antisense RNAs recognise and specifically bind to target RNA sequences, usually mRNA, both in prokaryote and eukaryote cells. They may be delivered to target cells using viral delivery systems, such as retroviruses or adenoviruses. In addition, antisense RNA may be expressed from transcription units stably integrated into the host genome. Putlitz et al. (1998) studied subgenomic fragments of the HBV genome with respect to the property of inhibiting HBV replication when intracellularly expressed in the antisense orientation. Antisense RNAs derived from the HBV genome specifically inhibited HBV replication and antigen expression in human hepatocellular carcinoma (Huh 7) cells by 60-75%. DNA sequences corresponding to the identified RNAs had no effect on HBV replication, indicating that inhibitory effects were mediated by RNA. Transcripts corresponding to the inhibitory subgenomic fragments were present at high levels. One antisense RNA was found to reduce the amount of pregenomic RNA encapsidated into core particles as a molecular mechanism of antiviral effects.

In another study, two retroviral vectors carrying an antisense gene from the HBV preS/S or preC/C were constructed and used to transfect the human hepatoblastoma cell line 2.2.15 (Ji & Si 1997). The inhibitory effect of antisense gene transfer, mediated by retroviral vectors on the expression of HBV antigens, appeared as early as day 3 after transfection, reached a maximum on day 5 and persisted for 11 days. On day 5 after introduction, antisense preS/S inhibited HBsAg and HBeAg expression by 71% and 23%, and the antisense preC/C inhibited HBsAg and HBeAg by 23% and 59%, respectively. HBV DNA production in the supernatant of cells transfected with either sequence was also reduced on day 5, but the viability of the cells was not affected.

Double-stranded RNA molecules have shown some efficacy against chronic HBV in a pilot study (Hahm et al. 1994). Triple-helix forming oligonucleotides directed against the HBV core promoter region have also been shown to inhibit HBV-specific gene expression in vitro (Ito et al. 1997).

Despite promising in vitro data, the lack of reports on in vivo effectiveness of antisense oligonucleotides is related to their: (i) instability in serum, which, however, can be improved by backbone modification; (ii) inability to reach their target site because of nonspecific disposition; (iii) poor cell penetration; and (iv) adverse pharmacokinetics.
including a short half-life (5 to 60 minutes) and high renal excretion (50%) in mice, rats, monkeys and humans (Agrawal 1992; Agrawal et al, 1995).

Table 1.1 lists antiviral agents that have been evaluated in cell culture, animal models and in humans with chronic HBV. Results of testing in vitro reflect results on HBV DNA in cell culture systems; results in vivo reflect the effects on levels of viral DNA in animal models of disease; results in humans reflect analysis of whether the agents result in clearance of HBV DNA and HBeAg and long-term remissions of disease.
<table>
<thead>
<tr>
<th>AGENT</th>
<th>Effect in Vitro</th>
<th>Effect in Vivo</th>
<th>Effect in Humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine arabinoside (and monophosphate)</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Adefovir dipivoxil</td>
<td>NT</td>
<td>NT</td>
<td>++</td>
</tr>
<tr>
<td>Lobucavir</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
</tr>
<tr>
<td>Ganciclovir</td>
<td>+</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Suramin</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Forscanet</td>
<td>++</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>+</td>
<td>?</td>
<td>NT</td>
</tr>
<tr>
<td>Dideoxynucleotides: ddi</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>ddC, ddA, and ddG</td>
<td>++</td>
<td>++</td>
<td>NT</td>
</tr>
<tr>
<td>Carbocyclic deoxyguanosine (2’ CDG)</td>
<td>++</td>
<td>++</td>
<td>NT</td>
</tr>
<tr>
<td>Fluoro-iodo-arabinofuranyl uracil (FIAU)</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Famciclovir</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-Thiacytidine (3TC, Lamivudine)</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2',3'-dideoxy-3'-fluoroguanosine</td>
<td>++</td>
<td>++</td>
<td>NT</td>
</tr>
<tr>
<td>BMS-200475</td>
<td>++</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>2'-Fluoro-5-Methyl-β-L-Arabinofuranosyl</td>
<td>++</td>
<td>++</td>
<td>NT</td>
</tr>
<tr>
<td>Antisense oligonucleotides</td>
<td>++</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Ribozymes</td>
<td>++</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Antisense RNA</td>
<td>++</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Dominant negative mutants</td>
<td>++</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Levamisole</td>
<td>-</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>Phyllanthus amarus</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Alpha interferon</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Beta interferon</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gamma interferon</td>
<td>+</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>Interleukin 2</td>
<td>-</td>
<td>NT</td>
<td>?</td>
</tr>
<tr>
<td>Interleukin-12</td>
<td>+</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Thymosin</td>
<td>-</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Granulocyte-Macrophage CSF</td>
<td>-</td>
<td>NT</td>
<td>?</td>
</tr>
<tr>
<td>Short course of corticosteroids</td>
<td>-</td>
<td>NT</td>
<td>-</td>
</tr>
</tbody>
</table>

NT = not tested; ? = tested in pilot studies with promising results. - indicates mild inhibition. ++ indicates moderate inhibition, - indicates no inhibition.
1.1.6 Potential targets for future antiviral therapy

Any future antiviral therapy will have to be based on the potential targets within the HBV replication cycle. Some of the potential targets are as follows:

**Early events in infection:** A successful soluble receptor analogue for HBV could block viral adsorption and infection. This would be useful in post-exposure prophylaxis (eg. needlestick injuries, neonatal exposures) as an adjunct to passive and active immunisation.

**Viral nuclear transport, uncoating and transcription:** It is likely that most of these steps are mediated by host cell enzymes. Nothing is known of the machinery for nuclear translocation and uncoating, therefore at this time rational drug design to inhibit these steps is not yet possible.

**Reverse transcription:** The major proteins that carry out this reaction are of viral origin. Now that sequences required for encapsidation are being identified, antisense oligonucleotides could be designed to inhibit their recognition, thereby preventing encapsidation of genomic RNA. Since this step is obligatory for reverse transcription, it might be a useful target. Nucleoside analogues that either inhibit reverse transcriptase or chain elongation are also attractive therapy means, as in the treatment of HIV infection.

**Translation and post-translational processing:** Antisense oligonucleotides that anneal to viral coding regions may be of value in inhibiting viral gene expression. Such a strategy would reduce the load of circulating virions and might hinder the horizontal recruitment of new infected hepatocytes.

**Viral assembly and secretion:** Interaction of the nucleocapsids with the viral envelope proteins occurs before secretion from the cell, but the domains on the preS/S proteins or on the nucleocapsids that mediate the interaction are not known (Scaglioni et al, 1996). As these steps are carried out by cellular enzymes, it is likely that inhibition of these enzymes will result in considerable cellular toxicity. Antisense therapy that inhibits production of envelope or core proteins, or dominant negative core mutants may be useful in inhibiting this step.

1.2 Duck hepatitis B virus

In 1980 a group of investigators from the Fox Chase Cancer Centre in Philadelphia found a virus in the sera of Pekin ducks which appeared to be a member of the human
hepatitis B-like family of viruses (Mason et al, 1980). This virus had a diameter of 40 nm and an appearance in the electron microscope similar to that of human HBV. The DNA genome of the virus was circular and partially single-stranded, and an endogenous DNA polymerase associated with the virus was capable of converting the genome to a double-stranded circle with a size of about 3000 bp. An analysis for viral DNA in the organs of infected birds indicated preferential localisation in the liver, implicating the organ as the site of virus replication. In all these aspects, the virus had a striking resemblance to human HBV and appeared to be a new member of the family, which also includes ground squirrel hepatitis virus and woodchuck hepatitis virus.

Among the features that associated DHBV with the hepadnavirus family are unique virion ultrastructure; characteristic polypeptide and antigenic composition; and common genome size, structure, and mechanism of replication. Common biological features of hepadnaviruses include a striking tropism for hepatocytes and the occurrence of persistent infection, with complete and incomplete viral forms in high concentrations in the blood and lower concentrations in other body fluids continuously for years. However, the genome of DHBV has only 3 ORFs, lacking the X gene which codes for the X protein, suggesting that this protein is not essential for viral replication (Fig 1.3).

DHBV has been found in domestic ducks in parts of China and in up to 10% of Pekin ducks in many commercial flocks in the USA. Vertical transmission is the major, or perhaps the only, natural route of transmission of DHBV among Pekin ducks. Histologic studies of livers of ducks from Chi-tung county in China revealed some degree of hepatitis in most, and no correlation between severity of hepatitis and the presence of virus in serum or viral DNA in the liver, suggesting that non-viral factors are involved in at least some hepatitis in this duck population (Omata et al, 1983). Cirrhosis, a significant sequel of chronic infection in man, has been observed in ducks, but it has not yet been correlated with virus infection (Omata et al, 1983). No cirrhosis has been found in ground squirrels or woodchucks. The incidence of hepatocellular carcinoma is rare in ducks infected with DHBV, unlike that in woodchucks or humans infected with HBV. The differences in hepatitis and tumour formation associated with the different hepadnavirus infections of different hosts are striking. Whether these differences are due to differences in the pathogenicity of these closely related viruses, genetic differences in the host, or to environmental factors remains to be determined.
Figure 1.3: Genetic organisation of the duck hepatitis B virus genome
(Offensperger et al. 1993)
Replication of DHBV has been shown in extrahepatic sites (Mason et al, 1980). Replicating forms of DHVB DNA are found in the pancreas as well as the liver of infected ducks, but not of woodchucks or ground squirrels (Mason et al, 1981). Active replication of DHBV starts in the liver after infection, and is followed by replication in the pancreas, the kidney, and the spleen (Tagawa et al, 1985). The incubation period is shortened when larger amounts of virus are inoculated, but the sequential occurrence of viral replication in these organs remains the same. More recently, natural and experimental infection of wild mallard ducks with DHBV has also been shown (Lambert et al, 1991).

The duck experimental system has been used in several studies to screen antiviral agents for HBV (Zuckerman 1987; Kassianides et al, 1989; Niu et al, 1990; Hung L-F et al, 1991). A number of features make the duck system attractive for investigation of an HBV-like virus. First, ducks breed well in captivity, allowing an investigation of the route of vertical transmission as well as the role of genetically controlled factors in the development of chronic viraemia. Second, egg inoculation of DHBV appears to produce viraemia in the duckling, allowing the generation of a reliable supply of infected animals and permitting, in principle, the passage of the virus in the duck vector with each generation. Eggs and young ducklings are readily obtained from commercial suppliers, making the system easily accessible to investigators. Finally, levels of DHBV DNA can easily be measured in serum and the different states of viral DNA in liver can be demonstrated by standard hybridization techniques. However, it is not an ideal system to assess antiviral therapy; some nucleoside analogues have been shown to be potent inhibitors of viral replication in vivo, but their neurotoxicity precluded their use in humans (Guardia et al, 1986; DiBisceglie & Hoofnagle 1989).

Although the chimpanzee has long served as a surrogate host for humans in modelling HBV infection, the chronic disease in this model is less severe and hepatocellular carcinoma has not been observed (Roggendorf & Tolle 1995). The woodchuck is currently used in many laboratories to study pathogenesis of hepadnavirus infection, molecular mechanisms of HCC development, and cell tropism of hepadnaviruses. Significant hepatitis has been observed in wild caught animals that are persistently infected, and the disease may be severe, progressive, and lead to death (Robinson et al, 1984). Woodchucks are also used to study different approaches for new vaccines to
hepadnaviruses and evaluation of antiviral drugs in chronic WHV infection. Woodchucks are also a highly sensitive model to test viral inactivation procedures. The natural route of WHV infection in the woodchuck, is thought to be the same as for HBV, by blood and secretions, in utero or at birth (Roggendorf & Tolle 1995). Experimental inoculation of colony-bred neonatal woodchucks with standardized virus challenge pools results in uniform kinetics of WHV infection and predictably high rates of chronicity (60-70%).

Woodchucks chronically infected with WHV at birth almost inevitably develop HCC. HCC develops in approximately one-third of persistently infected woodchucks per year (Robinson et al, 1984). This incidence is much higher than that seen in humans infected with HBV, in which approximately 0.1% of middle-aged male HBsAg carriers in Taiwan were shown to develop HCC per year (Beasley et al, 1981). Southern blot analysis of genomic DNA from a large number of woodchuck HCCs provide evidence of integrated WHV sequences in about 90% of tumours, both in chronic carriers and tumours of woodchucks which seroconverted. Cirrhosis is present in 80% of humans with HCC, but this has not been observed in woodchucks. HCC develops during active acute and chronic inflammation in woodchucks, with high levels of viral DNA in serum, whereas in HBV-associated tumours evidence of viral replication is low or absent, and active inflammation is rare (Robinson et al, 1984). No HCC has been observed in DHBV infected or uninfected ducks followed as long as two years, and none has been reported in the commercial flocks in which DHBV has been studied in the USA (Omata et al, 1983; Marion et al, 1984). Clearly HCCs do occur in domestic ducks in China, but the limited data do not suggest that active DHBV infection is as common in ducks with tumours as is hepadnavirus infection in humans or woodchucks with HCC, and DHBV DNA sequences have not been detected in a number of tumours.

The first animal model of HBV infection, chimpanzees, were rarely used in antiviral trials because of animal protection and high costs. WHV infection of woodchucks is a model in which the virus and associated disease have been relatively well characterized. The woodchuck model has the advantage of employing the virus most closely related to HBV, but the disadvantage of being relatively inaccessible or expensive for most laboratories. In contrast, the equally well-characterized DHBV duck model is available around the world and has consequently been used more often to test potential antiviral substances. Drawbacks to the DHBV-duck system are that DHBV is much less similar
to HBV than WHV and GSHV, and that avian metabolism and pharmacokinetics of the tested drugs may differ significantly more from those of mammals.

Some differences between the DHBV model and the WHV model are outlined in Table 1.2.

**Table 1.2: Comparison of the duck and woodchuck models of hepatitis B virus.**

<table>
<thead>
<tr>
<th>Duck hepatitis B virus</th>
<th>Woodchuck hepatitis B virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avian virus</td>
<td>Mammalian virus</td>
</tr>
<tr>
<td>40% nucleotide homology with HBV</td>
<td>70% nucleotide homology with HBV</td>
</tr>
<tr>
<td>3 ORFs</td>
<td>4 ORFs</td>
</tr>
<tr>
<td>Absence of X gene</td>
<td>Presence of X gene</td>
</tr>
<tr>
<td>Poor disease model</td>
<td>Good disease model</td>
</tr>
<tr>
<td>Vertical transmission only</td>
<td>Horizontal and vertical transmission</td>
</tr>
<tr>
<td>Replication in extrahepatic sites</td>
<td>Replication in liver only</td>
</tr>
<tr>
<td>Low propensity for HCC</td>
<td>High propensity for HCC</td>
</tr>
<tr>
<td>Ducks available world-wide</td>
<td>Woodchucks only found in certain regions</td>
</tr>
</tbody>
</table>

**1.3 Antisense oligodeoxynucleotides**

Antisense oligodeoxynucleotides (ODNs) are synthetic DNA molecules which offer the potential to block the expression of specific genes within cells. Inhibition of gene expression by antisense ODNs relies on the ability of an ODN to bind a complementary messenger RNA (mRNA) sequence and prevent translation of the mRNA (Wagner 1994). Formation of the mRNA-DNA duplex would also, in theory, then suppress or prevent the process of translation of the targeted message into protein (Figure 4). If the cell, or virus, required that protein for growth and/or viability, these cells should be markedly diminished in the presence of the antisense compound. Clinical trials are now in progress to evaluate the therapeutic potential of antisense ODNs in several human diseases, including acute myelogenous leukaemia and infection by HIV-1, cytomegalovirus and human papillomavirus.
Figure 1.4 Blockade of translation by antisense oligodeoxynucleotides. Normal gene transcription of DNA into mRNA is followed by translation of mRNA into protein. Antisense oligodeoxynucleotides complementary to a portion of mRNA bind mRNA, preventing translation, either by the steric effect of the binding process itself or, possibly, by inducing degradation of the mRNA by RNase (Askari & McDonnell 1996).

The idea of utilising antisense ODNs as sequence-specific inhibitors of gene expression was proposed in 1967 by Belikova et al. (1967). However, at the time, organic synthetic methods did not exist to produce a sufficient yield of oligomers longer than tri- or tetramers. By 1978, Zamecnick and Stephenson, taking advantage of improved synthetic techniques, were able to demonstrate the sequence-specific inhibition of Rous sarcoma virus genetic expression in an in vitro culture system (Zamecnick & Stephenson 1978; Stephenson & Zamecnick 1978). By 1981, the development of phosphoramidite chemistry and the ease of automation of the synthetic process revolutionised the field because of the high yield of each coupling reaction. The last few years have witnessed an explosion of interest in antisense research.

The use of antisense ODNs as therapeutic agents presupposes that six criteria can be satisfied:
(i) the ODNs can be synthesised easily and in bulk;
(ii) the ODNs must be stable in vivo;
(iii) the ODNs must be able to enter the target cell;
(iv) the ODNs must be retained by the target cell;
(v) the ODNs must be able to interact with their cellular targets;
(vi) the ODNs should not interact in a non-sequence-specific manner with other macromolecules.

1.3.1 Oligonucleotide modifications

The vulnerability of unmodified or naturally-occurring phosphodiester ODNs to nuclease present in various extracellular fluids and in different intracellular compartments limits their therapeutic potential. These molecules are rapidly hydrolysed by 3'-5'-exonuclease activity both in plasma and intracellularly. Oligonucleotides have been modified to enhance stability, most frequently at the phosphodiester internucleotide linkage by substitution of a non-bridging oxygen with, for example, a sulphur atom (phosphorothioate), or a methyl group (methylphosphonate). The importance of stability is confirmed by a study that compared phosphorothioate ODNs with phosphodiester ODNs of identical sequence; in this case, the stabilizing modification was essential for high activity (Wagner et al., 1993).

Figure 4 shows the structure of an ODN with a natural phosphodiester linkage and various substituents at a nonbridging oxygen atom. These backbone-modified oligomers are significantly more resistant to nucleases, having half-lives in serum in excess of 24 hours as compared to about 2 minutes for unmodified oligonucleotides (Cantin & Woolf, 1993). The issue of oligonucleotide stability is controversial because differences in the experimental approach for measuring stability have resulted in widely disparate half lives being reported for ODNs in various environments.

It has been suggested that RNase H may play a role in the antisense effect by cleaving the mRNA strand of the duplex formed between mRNA and the antisense ODN (Stein, 1992). Phosphorothioate (PS) ODNs, at least when at a concentration lower than that of the complementary mRNA, will act as a substrate for RNase H activity (Stein & Cheng...
Although methylphosphonates show enhanced stability to nucleases, heteroduplexes formed with them are not substrates for RNase H. They need to be used at relatively high concentrations (50-200mM), perhaps because they cannot act via the RNase H pathway. The substitution of phosphorus by sulphur for one of the nonbridging oxygen atoms in PS ODNs produces a compound that retains its net charge and aqueous solubility.

Hybridization of an ODN to its target RNA depends on several factors, including length, base composition, secondary structure, concentration and any chemical modifications to the ODN. Components of the intracellular environment can profoundly influence hybridization of the ODN to its target in ways that cannot yet be predicted (Cantin & Woolf 1993). The intracellular location of both the oligomer and the target mRNA is likely to be an important determinant of oligomer hybridization efficiency and hence efficacy (Neckers & Whitesell 1993), but the magnitude of this effect is presently unknown.

Figure 1.5. Structure of an oligonucleotide with a natural phosphodiester linkage and various substituents at a non-bridging oxygen atom (Cantin & Woolf 1993).
Length is an important parameter to consider when designing antisense ODNs. 15-mers are a good choice on both practical and theoretical grounds. They are relatively easy to synthesize, and recognition at the level of 12-15 bp provides uniqueness in the range of 8 million to 500 million bp, that is, a 15-bp sequence is likely to be found only once in 500 million bp of DNA. Much larger oligomers, eg. 21-mers, may be less effective, perhaps because of reduced solubility or permeability.

Although most successful antisense experiments target the oligomer to the cap and/or initiation codon region of the mRNA (Wagner 1994), no general conclusions can be drawn with regard to the optimal target site. One criterion to consider when selecting viral target genes is that they should specify essential functions and be expressed early in the viral replicative cycle, prior to genome replication. Genetic variability must also be considered when choosing a target sequence, especially in highly mutable viruses. Target regions in which mutations would result in loss of function are ideal. Examples of such functionally constrained targets are splice signals, poly-adenylation signals, translational initiation codons, and structured binding sites for RNA regulatory factors, such as the HIV tat and rev gene proteins. In principle, multiple genes should be targeted where feasible, to maximise inhibition and minimise the probability of resistant strains developing.

Evidence suggests that charged ODNs (eg. PS-ODNs) are taken up by receptor-mediated endocytosis (Leonetti et al., 1993). Uptake depends on temperature, energy, and concentration and is also saturable; these attributes are consistent with the postulated existence of a cellular receptor. Uptake of charged oligomers is a relatively inefficient process, only a small fraction of the input oligomer becomes cell associated, and a significant amount of that remains bound to the cell membrane (Neckers et al., 1992).

Various schemes have been devised to improve ODN uptake, including conjugation to polylysine or cholesterol. ODNs conjugated with polylysine at either the 5' or 3' end are more resistant to serum nucleases and show enhanced antisense activity as compared to unmodified oligomers (Cantin & Woolf, 1993). The mechanism by which polylysine potentiates the antisense effect is unclear.
1.3.2 Non-specific effects of antisense oligodeoxynucleotides

In several instances, non-sequence-specific inhibitory effects have been observed with phosphorothioates. PS-ODNs are polyanions, and as such are capable of binding the same proteins that other polyanions, especially heparin, also bind. This binding is primarily based on a charge interaction, but more recent evidence indicates that the binding may depend in part on base sequence as well (Stein 1995). Non-specific effects have been observed when antisense PS-ODNs were used to inhibit HIV replication (Stein 1992). These effects are very likely caused by interference with reverse transcriptase. PS-ODNs, in a length-dependent but relatively sequence-independent manner, bind to recombinant soluble CD4 at or near the HIV-1 binding site (Stein & Cheng 1993).

Even so-called sequence-specific phosphorothioates, if sufficiently long and if used at sufficiently high concentration, can exhibit these non-sequence-specific but potentially therapeutic effects. It may therefore be quite difficult to determine precisely which observed effects are antisense in nature, and which are caused by a complex mix of sequence-specific plus non-sequence-specific phenomena. Batch variations in oligomer purity are another source of non-specific effects, and contamination with cytotoxic triethyl ammonium ions during HPLC purification of oligomers is known (Cantin & Woolf 1993). Another complicating factor is the observation that the affinity of PS-ODNs to proteins is increased if the oligomer contains four contiguous guanosine residues. It is not clear why the G-quartet motif contributes to this increased affinity.

1.3.3 Limitations of antisense oligodeoxynucleotides

The main problems encountered with *in vitro* utilisation of synthetic oligomers deal with metabolic stability, cell penetration, intracellular distribution, availability of the nucleic acid or protein target, and processing of target-oligomer complexes. *In vivo* applications must cope with large-scale production and manufacturing costs, mutagenicity, immunogenicity, and body distribution.

The metabolic stability of an ODN is related to its susceptibility to endo- and exonucleases in plasma and within cells. Phosphodiester ODNs are rapidly degraded by
these enzymes, whilst PS ODNs are more resistant to degradation while retaining its antisense activity. PS ODNs are polyanions, and as such are capable of binding the same proteins that other polyanions also bind. If PS ODNs localise to the cytoplasm of the cell, the target mRNA may not be accessible to RNase H, a nuclear enzyme required for its biological activity.

One of the main problems encountered with the antisense approach is the choice of the targeted sequence. Factors that govern the identification of a target site are likely to be related to the length of an ODN, binding affinity and accessibility of the target RNA (Wagner 1995). The knowledge of the mode of action of antisense ODNs is still rather fragmentary and may be different from one gene to another. Moreover, mRNA secondary structures and their interaction with proteins are important points to consider. For example, the structure of important targets like the encapsidation signal ε of the pregenomic RNA of HBV and the secondary structure of the 5' noncoding region of HCV must be accessible to the antisense molecule.

To be effective antisense agents, ODNs must possess suitable pharmacokinetics to allow them to traffic to diseased tissues by using non-toxic and cost-effective doses, slide through cellular membranes (which present a formidable obstacle to most highly charged macromolecules such as nucleic acids), clamp to the targeted region of RNA with high affinity and specificity, and maximally inhibit translation of the disease-causing protein (Wagner 1995).

1.3.4 Studies with antisense oligodeoxynucleotides for viral infections

This concept has been successfully applied in vitro to inhibit influenza virus, Rous sarcoma virus, herpes simplex virus, human T cell leukaemia virus type I, human immunodeficiency virus, vesicular stomatitis virus, human papillomavirus and hepatitis B virus (HBV) (Cantin & Woolf 1993). In vivo studies have also been performed for duck hepatitis B virus (Offensperger et al., 1993). Neoplastic diseases treated with antisense ODNs include acute and chronic myelogenous leukaemias. A PS-ODN targeted to c-myb mRNA was recently reported to inhibit restenosis in a rat model of balloon angioplasty (Simons et al., 1992).
There has been a strong interest in identifying a potent oligonucleotide inhibitor of HIV replication. The PS ODN (GEM 91) which binds to the gag region of HIV RNA was selected because of its potent antiviral effects \textit{in vitro} (Agrawal & Tang 1992). Phase I and II trials with this antisense agent have commenced (Kilkusie & Field 1997). To date, safety has been demonstrated for doses up to 4.4 mg/kg by continuous infusion and 3 mg/kg by repeated intermittent, 2-hour infusion. Matsakura \textit{et al} (1989) also evaluated the inhibition of HIV replication by PS ODN targeted to the HIV \textit{rev} RNA. They reported sequence specificity of the antiviral effect and the expected effects on the HIV mRNA profile. They also demonstrated that the PS 28-mer homopolymer dC is a potent inhibitor of HIV infection. Anazondo \textit{et al} (1995) demonstrated that a partially phosphorothioated 20-mer targeted to a well conserved coding region of the gag gene inhibited both expression of mRNA for the viral precursor p55 protein, the p55 protein and its cleavage product p24 in COS cells stably transfected with plasmids containing the \textit{gag-pol} region. Anti-retroviral effects of ODNs by nonantisense mechanisms have been amply demonstrated (Kilkusie & Field 1997).

Studies in herpes simplex virus infection have investigated antisense ODNs targeted to the splice donor/acceptor sites of the immediate early pre-mRNAs of IE 4, a transactivating protein (Field 1998). A sequence-specific inhibition of viral replication was reported; a methyl phosphonate 12-mer targeted to the IE mRNA splice donor site was effective in reducing virus growth by 80% at 100 \text{\mu M}, whereas the same ODN in which the central two residues were inverted was inactive. The same active ODN applied locally reduced ear infection in mice. Peyman \textit{et al} (1995) targeted the translation start site of IE 110 mRNA. To minimize non-specific effects they evaluated an array of 20-mers that were phosphodiester ODNs except for two PS nucleotide residues at both the 3' and 5' ends. The most potent compound inhibited virus cytopathology at 9 \text{\mu M}, and a 2-nucleotide shift in sequence reduced efficacy by about nine-fold. Mismatched ODNs were inactive at 80 \text{\mu M}.

Two key genes of the Epstein Barr virus, the EBNA-1 which is required for maintenance of cell transformation, and BZLF 1 which is required for reactivation of latently infected cells, have been the subjects for design and evaluation of antisense ODNs. Pagano \textit{et al} (1992) evaluated unmodified phosphodiester ODNs complementary to the coding region just 3' of the AUG on the EBNA-1 mRNA, and observed that prolonged treatment of
Raji cells using relatively high ODN concentrations (40 μM) resulted in a progressive reduction of EBNA-1 proteins and in EBV DNA copy number. Similar treatments with the sense control ODN were ineffective. When PS ODNs were used in similar studies, antisense specificity was also observed at lower doses (5 μM), but scrambled and control sequences were partially effective.

In studies to inhibit cytomegalovirus, a series of ODNs complementary to the translation start sites, coding regions, intron/exon region and 5’ caps in RNAs including the DNA polymerase, and immediate early genes IE1 and IE2 were evaluated (Azad et al, 1993). The most potent of these was a 21-mer (ISIS 2922) against the coding region of IE2, with an IC₅₀ of about 0.1 μM. Whereas unrelated ODNs were less active in both the reduction of IE2 and virus replication, mismatches in ISIS 2922 which substantially reduced hybridization did not alter antiviral effects. A potent 20-mer PS ODN complementary to the splice donor site of the immediate early gene, UL 36, has been identified (Pari & Anders 1993). This gene is essential for human CMV origin of replication-dependent DNA synthesis, and based on antisense studies it was proven essential for virus replication. Sequence specificity for inhibition of viral replication was established by comparison of the efficacy of UL36ANTI with the sense, reverse and unrelated sequences. The nucleotide sequence of UL36ANTI has now been the basis for the chemically modified hybrid GEM 132 which is presently in clinical evaluation for intravitreal and non-ocular treatment of CMV diseases (Field 1998).

Based on DNA sequence diversity, there are over 65 types of human papillomavirus (HPV), with each type having preferred anatomical sites of replication. A PS ODN (ISIS 2105) targeted to the translation initiation site of the E2 mRNA for HPV types 6 and 11 inhibited transactivation of chloramphenicol acetyltransferase (Cowsert et al, 1993). Other control ODNs were ineffective at the same concentration (5 μM). Recent studies have identified a PS ODN targeted to HPV E1 helicase transcript, which was active in cell culture studies and in a mouse xenograft model of HPV replication (Lewis et al, 1997). An active 20-mer PS ODN targeted to the E1 translation start site had an EC₅₀ of 30 nM in a cell based assay and was sequence specific.
1.3.5 Antisense oligodeoxynucleotides for hepatitis C virus (HCV)

Hepatitis C virus, a member of the flaviviridae family, has been shown to be the major cause of parenterally acquired non-A, non-B hepatitis. The viral genome of HCV is a positive-sense, single stranded RNA molecule of approximately 9.5 kb that encodes a single polyprotein of approximately 3011 amino acid residues. The polyprotein is processed by cellular and viral proteases to generate structural proteins (the nucleocapsid and the envelope E1 and E2) and nonstructural (NS) proteins (NS2 to NS5). The coding sequence of the RNA genome is preceded by a 5' noncoding region (NCR) of 324 to 341 nucleotides, which is highly conserved among all strains of HCV. This NCR forms an extensive and stable secondary structure and serves as an internal ribosomal entry site (IRES), essential for efficient cap-independent viral translation and probably also necessary for HCV replication.

Alt et al (1995) analyzed the inhibitory effect of antisense PS-ODN on HCV gene expression in an in vitro test system and in cell culture. Three antisense PS-ODN were directed against different stem loop structures in the 5' NCR of the HCV RNA and another was targeted to a region including the start AUG of the polyprotein precursor. At the highest concentration tested (4.14 μmol/l), the ODN complementary to nucleotides 264 to 282 of the HCV RNA showed the most profound inhibitory effect (76 ± 3%) of the ODNs targeted to the NCR. The antisense ODN directed against the region comprising the start codon of the polyprotein precursor was very efficient in inhibiting viral translation under all experimental conditions tested. This ODN showed a dose-dependent inhibition with a maximum of 96 ± 1% suppression of translation at a concentration of 4.14 μmol/l.

Vidalin et al (1996) explored three potential genomic targets for inhibition of HCV translation by antisense ODN located in the HCV IRES and the initiator AUG flanking sequences. namely (i) the pyrimidine-rich region (nt 103 to 138), (ii) the initiator AUG region (nt 338 to 377), and (iii) the third stem-loop region (nt 134 to 161). The efficiencies and specificities of three chemically modified ODNs, β-PO, α-PO and β-PS, were analysed with two in vitro translation systems, rabbit reticulocyte lysate and wheat germ extract. Inhibition of gene expression was tested with a vector encompassing the full-length 5'-NCR and the viral nucleocapsid sequence. Six ODNs displaying sequence-
specific inhibition ranging from 62 to 96% mapped in the pyrimidine-rich tract and in the
initiator AUG codon were identified. Both α- and β-PO ODNs were found to be equally
active.

Another study investigated the effects of antisense ODNs on expression of HCV RNA
and core protein (Hanecak et al., 1996). The investigators utilized immortalized human
hepatocytes constitutively expressing the HCV 5’ NCR and core protein coding region
to evaluate inhibitory effects of antisense ODNs. Two PS ODNs, one complementary to
sequences within the 5’ NCR and the other complementary to HCV sequences spanning
the translation start codon, caused sequence-dependent reductions in HCV RNA and
core protein levels. Characterization of a truncated HCV RNA product produced in cells
treated with one of these PS ODNs suggested that activation of RNase H resulted in
cleavage of the HCV RNA strand within the ODN-RNA complex. The two ODNs
diminished viral translation and replication in a cell-free protein synthesis system and cell
culture system respectively.

More recently, Wu and Wu (1998) administered antisense ODNs in the form of
asialoglycoprotein-polylysine complexes to Huh 7 cells in transient transfections of a
plasmid containing a luciferase gene immediately downstream from an HCV NCR insert.
 Luciferase activity expressed under control of a cytomegalovirus (CMV) promoter
HCVluc was measured. Complexed antisense ODN directed against a sequence in the
NCR of the HCV genome inhibited luciferase activity in Huh 7 cells by 20% at 10 μmol/l
and 85% at 60 μmol/l, and was comparable by an excess of asialoglycoprotein.
1.4. Liposomes

The purpose of a drug delivery system is to maximise the amount of an active drug, or its duration of action, at a target site within the body where desirable therapeutic effects will occur, and to minimise the amount of drug or duration of exposure at other sites where toxic effects or drug loss may occur. One widely used approach for controlled drug delivery is the use of phospholipid vesicles or liposomes. Liposomes are composed of one or more closed, concentric phospholipid membranes surrounding an internal ("encapsulated") aqueous compartment (Gregoriadis 1991). Highly polar, water-soluble drugs can be entrapped in the internal aqueous space of the liposome, while lipophilic agents can partition into and become part of the lipid bilayer. Liposomes of various composition can bind to cell surfaces and are taken up by various endocytic processes.

A large proportion of parenterally administered liposomes were taken up by the liver and spleen by endocytosis, and localized predominantly in the lysosomes of the liver (Juliano & Aktar 1992). After digestion in the lysosomes, liposomal contents are released intracellularly. Being particles rather than molecules, liposomes can only leave the circulation at sites where there are relatively large gaps or "fenestrations" between the endothelial cells lining the blood vessels. Such a fenestrated endothelium is characteristic of the hepatic and splenic sinusoids and, to a lesser extent, the capillaries of lymphoid organs and bone marrow. Virtually all material delivered to cells by means of liposomes must initially pass through endosomes or phagosomes (Juliano & Aktar 1992).

Advantages of liposomes as drug carrier systems include low or no toxic effects, biodegradability, low immunogenicity, and a high degree of versatility allowing easy manipulation of size and surface characteristics including covalent coupling of ligands for specific cell-surface receptor recognition (Scherphof et al. 1989). The scope of liposome technology can be broadened and the function of liposomes optimised by a variety of structural manipulations, including the use of cell-specific ligands anchored on their surface. Efficient entrapment of drugs in liposomes using minimal amounts of lipids is an important requirement for their use as a drug carrier. The small size of the vesicles is important to prevent them from being removed from the circulation rapidly, to end up in the reticuloendothelial system. Techniques have now been developed in which high entrapment values of up to 80% of the starting material can be obtained for large
multilamellar dehydration-rehydration vesicles (DRVs) (Gregoriadis & Florence 1993). Microfluidization of solute-containing DRVs produces smaller liposomes (down to 100 nm in diameter) which retain up to 100% of the originally entrapped solute (Gregoriadis & Florence 1993).

Several studies have demonstrated the value of liposomes in delivering antisense therapy in vitro. Stability studies have shown that ODN entrapped in the internal aqueous compartment of liposomes (lip-ODN) were effectively protected from environmental degradation in vitro (Thierry et al. 1992). Identical concentrations of free \(^{32}\)P-ODN and lip-\(^{32}\)P-ODN were incubated in 10% serum-containing culture medium. Analysis on a denaturing 20% polyacrylamide gel after incubation for different times showed that free-ODN was mostly degraded after 30 minutes, whereas lip-ODN showed no detectable degradation up to 1 week after incubation. The same investigators also showed that cellular accumulation of unencapsulated PS-ODNs was 18-fold and 13-fold lower than those using lip-PS-ODNs in leukaemia MOLT 3 cells and lung carcinoma A549 cells respectively (Thierry & Dritschilo 1992). They also showed, using a human squamous carcinoma cell line, that there was specific cellular uptake of lip-ODN, and that empty liposomes could saturate ODN penetration by occupying receptor-mediated endocytosis sites.

The inhibition of P-glycoprotein synthesis, coded for by the multidrug resistant gene mdr-1, was shown to be markedly enhanced in vitro when an antisense ODN was delivered entrapped into liposomes, than when given in the free form (Thierry et al. 1993). Leonetti et al (1990) described the in vitro use of antibody-targeted liposomes containing a 15-mer complementary to the 5' end region of the mRNA encoding the N protein of the vesicular stomatitis virus. More than 95% reduction of viral multiplication was achieved in mouse L929 cells. The amount of non-encapsulated ODNs required for antiviral activity was 100 times higher than for the liposome-encapsulated antisense ODN.

Cationic liposomes have also been used to deliver antisense ODNs to cells in vitro. The liposomal vesicles are made from a positively-charged lipid DOTMA (N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride), and DOPE (dioleoyl-phosphotidylethanolamine) in a 1:1 (wt/wt) ratio. The nucleotide material is not
entrapped into the liposomes, but is complexed by ionic interaction between the negative charges of the phosphate groups on the nucleotide chain and the positive charges present at the surface of the cationic liposomes (Felgner et al, 1987). DOTMA facilitates fusion of the complex with the plasma membrane of tissue culture cells, resulting in both uptake and expression of the DNA. Using a human umbilical vein endothelial cell line, it was shown that DOTMA increased by at least 1000-fold the potency of an antisense ODN that hybridized to the AUG translation initiation codon of human ICAM-1 (Bennett et al, 1992). In addition, cellular uptake was markedly increased and the ODN localised to the nucleus as well as discrete structures in the cytoplasm.

Preliminary work using liposomally-entrapped antiviral agents in DHBV infection has been completed by Professors Dusheiko and Gregoriadis at the Royal Free Hospital (unpublished data). A suppression of DHBV DNA by liposomally-encapsulated antiviral treatment with acyclovir and dideoxycytidine has been observed in these experiments. The stability of liposomes in the duck plasma and serum has been determined and the fate of injected liposomes has been ascertained. In these experiments, dehydration-rehydration vesicles containing distearoyl-phosphatidylcholine (DSPC) plus cholesterol or small unilamellar vesicles with DSPC have proven to be encouraging vehicles. The disposition of liposomes and liposomally-entrapped drug has been measured using radiolabelled liposomes.

Thus, delivery systems which may enhance cellular uptake and/or enhance efflux from endosomal compartments will be useful in the delivery of antisense agents. Liposomes, as carriers for the delivery of antisense compounds, may provide a system which allows both goals to be achieved. They offer a natural delivery system which is not only biocompatible and biodegradable, but can potentially enhance cellular uptake of ODNs. In addition to protecting ODNs from enzymatic degradation, liposomes also offer the potential of producing controlled and sustained release formulations. Targeting liposomes to cells using surface antibodies may also be possible.

1.5 The PLC/PRF/5 cell line

Many aspects of the biology of HBV infection are not amenable to experimental investigation because of the failure of the virus to replicate in tissue culture systems. In
addition, work is impeded by the lack of available susceptible animals other than chimpanzees. However, model systems for the study of hepatitis B surface antigen (HBsAg) have become available in the form of human hepatoma cell lines which produce this antigen.

The first of these cell lines, the PLC/PRF/5 cell line described by Alexander et al. (1976), has been shown to produce HBsAg similar in size, morphology, and polypeptide composition to the form that occurs in the serum of infected individuals. It is derived from the tumour of a 24 year old Shangaan male from Mozambique with hepatocellular carcinoma. Efforts to detect other markers of HBV such as the core antigen, e antigen, and DNA polymerase in cells and media of these cultures have proved unsuccessful. The HBsAg retains its immunoreactivity as judged by radioimmunoassay and reverse passive haemagglutination.

The genome of the PLC/PRF/5 cells contain several copies of integrated HBV DNA (Rivkina et al., 1988). The cells have a doubling time of 35-40 hours and a plating efficiency of 40-50%. No virus particles have been found in the cells by ultrastructural examination (Alexander et al., 1976). Serial medium samples removed from PLC/PRF/5 cultures show a progressive accumulation of HBsAg during growth and maintenance (Copeland et al., 1980). In addition, the HBV transcripts from this cell line have been identified using subgenomic single- and double-stranded HBV DNA probes (Hathiramani et al., 1988). The major viral mRNA of 2.1 kb associated with the synthesis of HBsAg in these cells is identical in size with the main transcript in the liver of non-replicating HBsAg-positive chronic carriers. It appears to include part of the preS region and the S and X regions and to exclude core and about two-thirds of preS.

1.6 Studies using antisense therapy for HBV

1.6.1 In vitro inhibition of HBV

Goodarzi et al. (1990) studied the effect of a series of antisense ODNs on the expression of the HBsAg gene of human HBV using the PLC/PRF/5 cell line. Of a number of antisense ODNs tested, synthetic 15-mers directed at the cap site of mRNA and regions of the translational initiation site of the HBsAg gene were found to be highly effective.
and inhibited viral gene expression by as much as 96%. The inhibition was specific to the HBsAg gene and appeared to be at the level of translation.

Blum et al (1991) used human hepatoma cells (HuH-7) to analyse the effect of antisense ODNs on HBV gene expression and replication. They showed that cotransfection of HBV DNA with an ODN of antisense polarity (ATC-40) against the polymerase gene completely blocked HBsAg and HBeAg synthesis as well as HBV replication. The same ODN of sense polarity (GAT-40) had no effect on viral antigen production or replication. This study proved that HBV-specific antisense ODNs could block viral gene expression and replication.

Wu and Wu (1992) used HepG2 cells (2.2.15) transfected with HBV and demonstrated specific inhibition of HBV gene expression in vitro by targeted antisense PS-ODNs. A 21-mer ODN complementary to the polyadenylation signal for human HBV was complexed to a soluble DNA-carrier system that was targeted to hepatocytes via asialoglycoprotein receptors present on those cells. In the presence of complexed antisense DNA, the concentration of HBsAg in the medium, and HBV DNA in the medium and cell layers was 80% lower than in controls after 24 hours of exposure. There was no significant increase in HBsAg concentration in the presence of complexed antisense DNA during the next six days. Total protein synthesis remained unchanged by exposure to complexed antisense sequences under identical conditions.

The same group of investigators subsequently also showed that pretreatment of HuH7 cells with the same targeted antisense-complexed DNA prevented subsequent infection with an HBV-plasmid at a level of 6.5 x 10^6 copies of plasmid per cell (Nakazono et al, 1996). This inhibited the amount of newly synthesized, core-associated viral DNA to undetectable levels as assessed by quantitative PCR. At concentrations less than 1.0 μmol/l, there was no significant effect on HBsAg concentration. However, at 5.0 μmol/l complexed antisense, HBsAg concentration was decreased by 60%. At 25 μmol/l, inhibition reached 97%. The inhibition lasted 6 days and was dose dependent. Controls consisting of antisense alone and a random ODN complex showed no significant effect on any of the parameters under identical conditions.
Wands et al. (1993) showed that a specific region of the HBV polymerase mRNA was highly susceptible to attack by antisense constructs, and that these ODNs blocked both the pregenomic 3.5 kb and the subgenomic 2.4/2.1 kb mRNA species in HCC cells, resulting in complete inhibition of HBV replication and gene inhibition \textit{in vitro}. By contrast, 30-mer antisense ODNs derived from the core or X-gene did not inhibit viral protein (HBsAg and HBeAg) synthesis and HBV replication.

Recently, a group of investigators reported the inhibition of DHBV by antisense PS-ODNs in primary duck hepatocyte cultures \textit{in vitro} as well as in DHBV-infected Pekin ducks \textit{in vivo} (Offensperger et al. 1993). Nine different antisense ODNs were evaluated \textit{in vitro}: 4 targeted against the preS/S region, one at the start of the polymerase region, and 4 at the preC/C region. Incubation with the 9 antisense ODNs for a period of 10 days led to a decrease of viral replicative intermediates for all ODNs tested. Two ODNs, however, showed a particularly high efficacy: one directed against the start of the preS region, and another against the direct repeat II (DR II) region, resulting in a strong inhibition of viral replication with only residual single-stranded, relaxed circular and ccc DNA species left. Viral replication was inhibited by more than 90\% by these ODNs.

\subsection{1.6.2 \textit{In vivo} inhibition of HBV}

The effect of the most potent antisense ODN in the study by Offensperger et al. (1993), against the start of the preS region, was also evaluated \textit{in vivo}. Chronically infected ducklings were treated with daily intravenous injections for 10 days. The \textit{in vivo} administration of this ODN resulted in a dose-dependent inhibition of viral replication with a nearly complete elimination of viral DNA from liver cells at a daily dose of 20 \(\mu\)g per gram of body weight. A total number of 14 ducklings were treated with this ODN in 5 consecutive series, and, without exception all ducklings showed a nearly complete inhibition of viral replication after treatment with this ODN, indicating that the antisense effect observed was highly reproducible. Western blot analysis showed a block of DHBV gene expression with disappearance of viral preS and S antigens from serum and viral preC and C antigens from the liver. No direct hepatotoxic effects were observed after 14 days of therapy.

The administration \textit{in vivo} of a sense PS-ODN complementary to the above ODN, and a random sequence ODN, to DHBV-infected ducklings did not cause a reduction in viral
replication, demonstrating the specificity of action of the antisense ODN \textit{in vivo}. This was the first study demonstrating the feasibility of antisense ODN therapy of a viral infection \textit{in vivo}, and that intravenous application was very effective.

1.7 Preliminary studies with liposomes in ducks

Initial studies performed by Varagona and Dusheiko (unpublished data) in collaboration with Gregory Gregoriadis investigated the feasibility of using liposomes as drug carriers to deliver antiviral agents to the liver of DHBV-infected ducklings. They studied, \textit{in vitro} and \textit{in vivo}, the stability of different liposome preparations used to deliver antiviral therapy for the inhibition of DHBV replication, and the uptake of solutes entrapped within these liposomes. Stability was monitored by measuring changes in membrane permeability to 6-carboxyfluorescein (CF) entrapped at a concentration that, because of self-quenching, prohibits its fluorescence. When, for any reason, liposomes leak the dye, its escape and ensuing dilution in the surrounding medium enable it to fluoresce, thus providing an immediate and easily measurable index of membrane permeability.

Small unilamellar vesicles (SUVs) of neutral charge with entrapped CF were prepared from phospholipid and cholesterol (1:1 molar ratio). These were prepared by the dehydration-rehydration method (Kirby \\& Gregoriadis 1984). Liposomes with entrapped CF were separated at room temperature from the unentrapped dye by passing the supernatant through a Sepharose 6B-CL (Pharmacia) column equilibrated with PBS. Liposomes, eluted at the end of the void volume in about 1-2 ml, were subsequently dialysed at 4°C against PBS in order to eliminate any subsequently diffusing CF until use. Duck fresh blood, serum or plasma was mixed with CF-containing liposomes in a volume ratio of 5:1 in order to simulate the dilution in blood or plasma upon injection. In control experiments 0.5 ml of PBS was mixed with 0.1 ml liposomes as above. All blood, serum, plasma and PBS samples were incubated at 42°C and at time intervals, duplicate 10 µl samples were pipetted into 4 ml of cold PBS. CF was measured in the absence and presence of Triton X-100 (1% final concentration) and latency estimated from 

\[ 100(Dye_t - Dye_f)/Dye_t \] 

where \( t \) and \( f \) denote total dye (in the presence of Triton X-100) and free dye respectively.
*In vivo* stability, plasma clearance and organ distribution was assessed using a single dose of intravenously injected $^{125}$I-labelled polyvinylpyrrolidone ($^{125}$I-PVP) in the free and liposome-entrapped DRV's. Ducks were injected with identical amounts (414,105 cpm/5 ml) of free or entrapped $^{125}$I-PVP and blood samples were taken at time points to measure the radioactivity on a gamma counter. At the end of a 24 hour period, organs were harvested, weighed and radioactivity measured as for plasma samples.

The efficacy of antiviral therapy for free versus entrapped acyclovir in Pekin-Aylesbury ducks chronically infected with duck hepatitis B virus (DHBV) was compared. Ducks with stable chronic DHBV infection were treated with daily injections of identical doses (20 mg bid) of acyclovir for 5 days, either free or entrapped into DRV-DSPC liposomes. Eight 5-month old ducks infected with DHBV at one day post-hatch were divided into 3 groups. The first group of 3 ducks were treated with 20 mg bid of free acyclovir injected intravenously for 5 days, the second group of 3 ducks were treated with 20 mg bid of liposome-entrapped acyclovir injected intravenously for 5 days, and the third group consisted of 2 untreated controls ducks. Blood samples for measurement of serum DHBV DNA levels were taken before treatment, on each treatment day, and on days 2 and 4 following the end of the treatment phase. DHBV nucleic acids were analysed by molecular hybridization using a full-length DHBV clone; DNA was extracted from duck livers and analyzed by Southern hybridization.

The results indicated that DSPC SUVs were much more stable, especially in serum and whole blood than SUV PC. The latency of SUV PC in whole blood was only about 40% after 24 hours, and fell further thereafter. Similarly, DRV DSPC were much more stable than DRV PC, particularly in whole blood. Overall, the SUVs were more stable than the DRV's. The plasma clearance of free and liposomal $^{125}$I-PVP entrapped into DRV DSPC over a 24 hour period, showed that, for both forms, initial plasma clearance of $^{125}$I-PVP was rapid, with a subsequent gradual decline and virtually no radioactivity detectable after 24 hours. Uptake to the liver and spleen was much greater for the liposome-entrapped than the free $^{125}$I-PVP, with minimal uptake in the heart, lungs and kidneys. Hepatic uptake was approximately 50% for the liposome $^{125}$I-PVP compared to 20% for the free form. Splenic uptake per gram of tissue was higher than liver uptake in both forms, but much more for the liposome $^{125}$I-PVP (27,507 vs 2,249 cpm/gram) than the free form (2,705 vs 1,272 cpm/gram).
The effect of 5 days treatment with free and liposome-entrapped acyclovir showed that, in both treated groups, DHBV DNA became undetectable by dot-blot hybridization after the first day of treatment, remained undetectable during the treatment phase, and reappeared within 2 days after cessation of therapy. The untreated control group showed no loss of DHBV DNA throughout the study period. Southern blot hybridization of liver samples at the end of treatment showed a marked reduction of DHBV replicative intermediates. However, there was a greater reduction in episomal forms with persistence of supercoiled and partially double-stranded forms.

These initial studies investigated the feasibility of developing the DHBV infected duck model for *in vivo* studies of liposomally entrapped antiviral drugs or nucleic acids. They confirmed (i) the stability of liposomes containing cholesterol in duck circulating fluids *in vitro* and *in vivo*, (ii) the predominant uptake of solutes entrapped within liposomes by the liver and spleen, and (iii) suggested that the suppression of replication of DHBV by specific antiviral agents given in the liposome-entrapped form enable this model to be developed further for antiviral targeting. In this study, DSPC SUVs were found to be the most stable in duck fluids. The uptake of liposome-\(^{125}\)I PVP injected *in vivo* was mainly to the liver; this uptake was much greater than free \(^{125}\)I PVP. These data indicate that in this model, a large proportion of liposomes are delivered with sufficient precision to the liver to be effective drug carriers in the treatment of hepatotropic viruses. Although the uptake per unit mass was greater to the spleen than the liver, the mass of the liver is far greater than that of the spleen in ducks.

This study did not determine whether specific targeting of liposomes to hepatocytes occurred *in vivo*. Liposome-acyclovir was at least as effective as free acyclovir in the inhibition of DHBV replication, although viral DNA re-appeared in the serum soon after cessation of therapy. It remains to be seen whether smaller doses of entrapped acyclovir can produce the same effect as higher doses of free acyclovir, and, whether a longer duration of treatment with entrapped drug is able to eradicate the more resistant forms of viral DNA, such as supercoiled DNA. These initial feasibility studies indicated that liposomes may be an attractive delivery vehicle for the treatment of HBV infection with antisense oligodeoxynucleotides, with an improvement in hepatic delivery.
Chapter 2

MATERIALS AND METHODS

2.1 Aim and strategy

This aim of this study was to determine whether antisense oligodeoxynucleotides could be used to inhibit hepatitis B virus replication and gene expression, and if their delivery to the liver and efficacy could be enhanced with the use of liposomes. The primary model for testing the efficacy of therapy was to use ducklings chronically infected with duck hepatitis B virus. This model would be created by acquiring neonatal ducklings from a breeder and inoculating them within 24 hours with serum which was known to contain high levels of infectious virus particles. The second model was an \textit{in vitro} model; this would use the PLC/PRF/5 human hepatoma cell line which contains integrated HBV DNA and secretes large quantities of HBsAg into the culture medium.

The antisense ODN chosen for the \textit{in vivo} studies was the 18-mer against the initiation of the pre-S region, which caused marked inhibition of DHBV replication in the study by Offensperger \textit{et al} (1993). That study tested 9 antisense sequences and this sequence was found to be the most potent inhibitor of viral replication and gene expression. Therefore, this antisense ODN was used for all \textit{in vivo} studies. The strategy was to perform a pilot \textit{in vivo} study first to determine whether a small dose of antisense ODN could inhibit DHBV replication in the liposome-entrapped form. No published literature was available suggesting a dose in the entrapped form, but the study by Offensperger \textit{et al} (1993) had shown that a high dose (20 \textmu g/gm bw) of antisense ODN was necessary to produce a therapeutic effect. If this dosage was not successful, it was planned to conduct experiments to determine whether the lack of success was due to a lack of sequence specificity, a lack of delivery of the ODN to the liver, or the use of a subtherapeutic dosage. Sequence specificity would be tested by amplification of the pre-S region of the DHBV genome by polymerase chain reaction and sequencing this region to determine if the target sequence was present in the viral isolates used in this study.

Biodistribution and plasma clearance studies in uninfected ducklings were planned to compare the \textit{in vivo} behaviour of liposome-entrapped ODNs to free ODNs. This would
provide information as to whether the liposome form of delivery increased hepatic uptake compared to the free form. For this purpose, ODNs would be labelled with a radioisotope which could be easily detected in tissues.

In parallel with this, in vitro studies would be conducted using the PLC/PRF/5 human hepatoma cell line. The aim of these experiments would be to inhibit expression of the HBsAg gene into the culture medium using antisense ODNs. The antisense sequences to be used would be the ones with the greatest potency demonstrated in the study by Goodarzi et al. (1990). The efficacy of liposome transfected ODNs would be compared with transfection of naked ODNs. In all experiments, random sequence ODNs would be used to determine specificity. Cellular uptake and intracellular distribution would be studied using ODNs labelled with fluorescein and viewed under fluorescence microscopy.

For the in vivo studies, ducklings were injected with an infectious viral inoculum one day after birth and considered to be chronically infected by the end of the first week. Transmission of DHBV to newly hatched ducklings invariably results in the development of persistent infection, while infection of adult ducks is usually transient (Jilbert et al., 1988; Jilbert et al., 1992). Jilbert et al. (1988) inoculated thirty-five 1-day-old Pekin-Aylesbury ducks intravenously or intraperitoneally with high doses of DHBV (7.5 X 10^7 DNA genome equivalents; sufficient to deliver virus to ~ 10% of liver cells) and the time-course of infection was examined by Southern blot, dot blot, and in-situ hybridization and by immunohistochemistry. Infection of the liver was first detected by the presence of a virus antigen and virus DNA in randomly scattered hepatocytes on Day 1-2 postinoculation and in the serum (DHBV DNA) on Day 3 postinoculation. From day 4 onward, a remarkably steady state of balance ensued between virus release and virus clearance from the circulation. Almost all hepatocytes contained DHBV DNA and DHBsAg from day 4 onward. Initially, levels of DNA and DHBsAg varied from cell to cell, possibly as a reflection of asynchrony in virus replication. From day 4 onward, virus DNA levels per cell become more uniform. In general, serum levels of DHBV DNA paralleled the extent of DHBV replication within the liver.

In further studies, Jilbert et al. (1996) examined the effect of DHBV dose on the incubation period of infection in neonatal ducklings using as their inoculum, pooled
serum from congenitally DHBV-infected ducks which contained $9.5 \times 10^5$ DHBV DNA genomes per millilitre. As in humans, the onset of viraemia was inversely related to the dose of inoculated virus. However, in contrast to results in humans, even inoculation with the equivalent of one virus DNA genome produced relatively short incubation periods with DHBV DNA and surface antigen detectable in the blood by Day 14 or 29 postinoculation. To further explore the kinetics of infection after low dose inocula, they inoculated newly hatched ducks intravenously with sufficient DHBV to infect only ~0.0001% of total liver cells and then examined autopsy tissues harvested daily from Days 3 to 16 postinoculation. Infection was first detected on Day 4 postinoculation in approximately 0.035% of hepatocytes and spread finally to involve the entire hepatocyte population. The exponential increase in the percentage of infected cells from Day 4 to Day 14 postinoculation suggested that there were no major delays in virus replication within the liver. By Day 14 postinoculation replication was seen in >95% of hepatocytes, with a mean doubling time of 16 hours.

In this study, the incubation period to onset of viraemia was prolonged with very low dose inocula, but never beyond 29 days, in contrast to HBV in adult humans in which the appearance of circulating HBsAg after low dose inocula ($10^7$) was delayed to greater than 90 days (Jilbert et al., 1996). This finding reinforces the view that neonatal duck hepatocytes are highly permissive to infection, that immune responses do not play a major role in suppressing infection at this age, and that once infection is initiated even by one virion, it rapidly progresses to involve all susceptible cells. Increases in the percentage of infected hepatocytes from 0.0001 to 0.035% could therefore be realistically achieved by Days 4-5 postinoculation.

In addition, Fukuda et al. (1987) examined the effect of age and outcome of DHBV infection and found that 1- and 3-day old ducklings inoculated with a constant dose of DHBV ($\sim 1 \times 10^8$ DHBV genomes) developed persistent viraemia, whereas ducks inoculated at 5 days of age and older showed persistent or transient viraemia. Jilbert et al. (1998) further confirmed that the ability of ducks to mount immune responses to resolve DHBV infection is linked to the age of the duck at the time of inoculation. Omata et al. (1984) also reported that 100% of 1-day-old ducklings become chronic carriers of DHBV by the inoculation of DHBV-positive serum.
Therefore, there is sufficient evidence to indicate that intravenous inoculation of neonatal ducklings with a high dose of infectious virions results in persistent infection within one week. This would enable lower doses of antisense ODNs to be used, resulting in considerable savings in cost and time, as ducks grow rapidly in size within the first few weeks of life.

This chapter details materials and methods commonly used during the course of this project. Methods used once only are described only in the relevant chapters. All reagents used were of molecular biology grade.

2.2. Dot blot hybridisation

2.2.1 Sample preparation and spotting
This was performed by a modification of the method initially described by Scotto et al (1983). DNA was extracted from 50μl serum with 20μl 10% NP-40 and 20μl 3% β-mercaptoethanol. The mixture was incubated for 5 minutes at room temperature and then placed on ice. The DNA was then denatured with 180μl 1M NaOH and 90μl 2M NaCl, and kept on ice until the mixture was used for spotting onto a membrane. Serum samples with known quantities of DHBV DNA, or cloned DHBV DNA containing known amounts of DNA, were also spotted onto the same membrane in graded dilutions as standards. Each hybridization included negative controls consisting of uninfected duck serum (without virus).

The nylon membrane (Hybond™-N, Amersham UK) was cut to fit the dot blot manifold (Hybridot, Bethesda Research Laboratories). The membrane and 2 layers of Whatman 3MM paper were soaked in 6x SSC before mounting, with the membrane above the paper. After fastening the screws of the manifold, a gentle vacuum was applied. Up to 180μl of the reaction mix was spotted; each sample was deposited in a well and allowed to filter under vacuum. Each well was washed twice with 0.5M Tris-HCl (pH 7.5)/2.5 M NaCl. The membrane was gently removed under continuous vacuum, blotted between 2 sheets of absorbent paper, air dried and the DNA cross-linked to the membrane using UV irradiation for 4 minutes.
2.2.2 Pre-hybridization, hybridization and washing

Blots were pre-hybridised in a heat-resistant plastic bag for 4 hours at 65°C in 6x SSC/10mM EDTA, 5x Denhardt's solution (50x stock solution contains 1% bovine serum albumin, 1% Ficoll, 1% polyvinylpyrrolidone), 0.1% SDS and 100μg/ml denatured fragmented salmon sperm DNA. Hybridisation with a heat-denatured 32P-labelled cloned DHBV DNA probe (vide infra) was performed in the same solution at 65°C overnight. The blots were then removed from the hybridisation bag and washed in 2x SSC, 0.1% SDS twice at room temperature, then in 0.2x SSC, 0.1% SDS once at room temperature and once at 65°C, then in 0.1x SSC, 0.1% SDS thrice at 65°C.

Autoradiography was performed at -70°C using Kodak XAR film and intensifying screens. More than one autoradiographic exposure time was used for the same membrane to maximise low signals by long exposure, and shorter exposures were used to avoid scanning bands for which the film was completely blackened. The signal was quantified using scanning densitometry (BioRad GS-670 imaging densitometer). In each hybridization experiment, a standard curve was generated from known quantities of DHBV DNA from cloned DNA or known quantities in serum; levels in the test samples were read off the standard curve. The lower limit of detection was approximately 4 pg/ml.

2.2.3 Preparation of DHBV DNA standards

Standards for quantification of DHBV DNA levels in duck samples were prepared from cloned DNA. The insert was cloned in plasmid DNA (pBR322) and was donated by Dr. T. Harrison. The reaction was set up as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>160 μl</td>
</tr>
<tr>
<td>10 x TE (pH 8.0)</td>
<td>20</td>
</tr>
<tr>
<td>salmon sperm denatured DNA (400 ng/ml)</td>
<td>10</td>
</tr>
<tr>
<td>Plasmid DNA containing DHBV DNA insert (276.4 pg/μl)</td>
<td>10</td>
</tr>
<tr>
<td>3M NaOH</td>
<td>20</td>
</tr>
</tbody>
</table>

This reaction was incubated for 10 min at 37°C, then placed on ice before the addition of:
2M Tris pH 7.1/1.5M NaCl 220 μl
3M HCl 20
5M NaCl 40
Total 500 μl

The standards were loaded onto the membrane in amounts ranging from 200 to 0.1 μl.

2.2.4 Preparation of DHBV DNA probe

A full-length purified DHBV DNA clone was labelled by random priming (Multiprime, Amersham International, UK) for all hybridisation experiments for the detection and quantification of DHBV DNA in serum and liver. Approximately 25 ng of the DNA was heat denatured for 5 minutes, and then chilled on ice. The reaction was set up as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA solution (denatured)</td>
<td>20 μl</td>
</tr>
<tr>
<td>Unlabelled dNTPs (dATP, dGTP, dTTP)</td>
<td>12</td>
</tr>
<tr>
<td>Buffer (Multiprime, Amersham UK)</td>
<td>5</td>
</tr>
<tr>
<td>Primer (Multiprime, Amersham UK)</td>
<td>5</td>
</tr>
<tr>
<td>[α-32P]dCTP (~ 3000 Ci/mmol) (50μCi)</td>
<td>5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1</td>
</tr>
<tr>
<td>Klenow fragment, DNA polymerase</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

The mixture was mixed gently by pipetting up and down, then incubated at 37°C for 1 to 2 hours. The efficiency of labelling was measured by precipitation by trichloroacetic acid (TCA). One μl of the reaction volume was diluted to 10 μl in distilled water for measurement of the specific activity of the probe. Two μl of this was spotted onto a glass fibre disc for measurement of total counts. Another 2 μl was spotted onto another glass fibre disc and washed 3 times for 5 minutes each with 10% cold TCA. Each disc was inserted into a separate scintillation vial, filled with 10 ml scintillation fluid, and counted in a Kontron Betamatic V counter for 1 min each. Percentage incorporation of the label was calculated as counts in precipitated DNA/total counts x 100. Typical incorporation ranged from 40-70% and typical probe specific activities ranged from 2.5 x 10^7 to 1.4 x 10^8 cpm/μg.
2.3 Oligonucleotide labelling

2.3.1 Oligonucleotide probe labelling

An oligonucleotide probe was used to detect the presence of injected PS-ODNs in duck tissues, and to quantify ODNs by comparison with standard amounts of ODN. End-labelling at the 5' end was performed using \([\gamma-^{32}P]ATP\) and T4 polynucleotide kinase. The reaction was set up as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligonucleotide (27 pmole)</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>10 x kinase buffer</td>
<td>2</td>
</tr>
<tr>
<td>([\gamma-^{32}P]ATP) (50 μCi)</td>
<td>5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>9.5</td>
</tr>
<tr>
<td>T4 polynucleotide kinase (20 units)</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>20 μl</td>
</tr>
</tbody>
</table>

The reaction was incubated at 37°C for 1-2 hours. The efficiency of labelling was measured by absorption to DE81 paper (Whatman, UK). One μl of the mixture was removed and diluted to 10 μl in distilled water. Two μl of the dilute probe was spotted onto DE81-paper and air dried for total radioactivity counts. Another 2 μl was spotted and washed 3 times for 5 minutes each with 0.5 M Na₂HPO₄. Percentage incorporation was calculated as above. Typical incorporations were 50-60% and typical probe specific activities were 1.9 to 7.4 × 10⁸ cpm/μmol.

2.3.2 Labelling for liposome entrapment

PS-ODNs were end-labelled at the 3' end with \(^{35}S\) using Terminal deoxytransferase (TdT). This was necessary to track the ODN during the procedure, and to measure the amount of PS-ODN which was finally entrapped into the liposomes prior to therapy. The reaction was set up as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODN (10 pmol)</td>
<td>6 μl</td>
</tr>
<tr>
<td>5 x TdT buffer</td>
<td>4</td>
</tr>
<tr>
<td>([\alpha-^{35}S]dATP)</td>
<td>5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>4</td>
</tr>
<tr>
<td>TdT (20U)</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>20 μl</td>
</tr>
</tbody>
</table>
The reaction was incubated at 37°C for 2 hours. For the purposes of large scale entrapment for *in vivo* antiviral studies, several reactions were performed in parallel and the labelled ODNs from each reaction were pooled to increase the specific activity. Percentage incorporation was calculated after washing off free label from the ODN with 0.5 M Na₂HPO₄, as described above. Incorporation values ranged from 43% to 75%.

Labelled ODN was separated from unincorporated nucleotides by Sephadex G50 column chromatography. The bottom of a disposable syringe was plugged with a small amount of sterile glass wool. In the syringe, a column of Sephadex G50 equilibrated in TE buffer was prepared. The column was washed with TE buffer (10mM Tris-Cl/1 mM EDTA, pH 8.0). The sample was then applied to the top of the column in a 50 to 200 µl volume. When all of the sample had entered the column, several hundred µl of TE buffer was added carefully to the top. Additional TE was added as needed during the fractionation. Fractions of 4 drops each (100 µl) were collected in fresh tubes. Radioactivity was measured in a liquid scintillation counter; the leading peak contained the larger fragments, while the trailing peak contained unincorporated dNTP. The fractions from the leading peak were pooled and counted to determine the total activity. Labelled ODN and unlabelled ODN were mixed, and then entrapped into small unilamellar vesicles. Total starting counts for radioactivity was obtained from the labelled ODNs only.

### 2.4 Southern blotting for intrahepatic DHBV DNA

At the end of the *in vivo* antiviral studies, DNA was extracted from post-mortem liver samples, precipitated with absolute ethanol, electrophoresed on an agarose gel, transferred to a nylon membrane and hybridized with a full-length DHBV DNA probe. The procedure is outlined below.

The liver specimens were stored at -70°C until DNA was extracted. A pestle and mortar was cooled with liquid nitrogen. About 500 mg of liver tissue was ground to a fine powder under liquid nitrogen with the pestle. The powder was added to 10 ml of lysis buffer (10mM Tris pH 8.0, 10mM NaCl, 10mM EDTA, 0.5% SDS, 20 µg/ml Bovine pancreatic RNase), mixed gently and allowed to stand for 10 min before pouring into a 50 ml universal tube. Proteinase K was added to a final concentration of 20 µg/ml. The
mixture was then incubated at 50°C for 2-3 hours. 5M NaCl was added to provide a final concentration of 0.1M.

An equal volume of salt saturated (SS) phenol/chloroform (1:1) was added, mixed gently, and the mixture centrifuged at 3000 rpm for 20 min. The upper aqueous phase containing the DNA was carefully removed and transferred to a fresh tube. The organic lower phase and the interface were discarded. Extraction of DNA with SS-phenol/chloroform was performed once more and transferred to a fresh tube. An equal volume of chloroform was then added and centrifuged at 2000 rpm for 20 min. The upper aqueous phase was retained.

Two volumes of ice cold absolute ethanol was added to the upper aqueous phase and the mixture incubated at -20°C overnight. This was then centrifuged at 4°C at 15,000 rpm for 30 min in a Europa 4M ultracentrifuge. The ethanol was discarded, leaving a DNA pellet at the bottom of the tube. The pellet was air dried before dissolving in TE pH 8.0. The concentration of DNA in each sample was measured by UV spectroscopy at 260 nm. Typical concentrations of total DNA ranged from 0.300 to 1.035 mg/ml. Ten µg of total DNA was loaded into each well of a 1% agarose gel, together with 2 µl of 6 x blue-orange loading dye (0.25% Bromophenol blue, 0.25% Xylene cyanol, 0.4% Orange G). A dilute 1 kb size marker was also loaded onto one of the lanes.

DNA was resolved by agarose gel electrophoresis at 120V for 2 hours in 1 x TBE buffer (20X stock solution contains 121gm Tris base, 61.7 gm boric acid, 7.44 gm Na₂EDTA, 2H₂O, and water to a final volume of 1L). The gel was then stained with ethidium bromide (0.5 µg/ml) in water for 15 min, then rinsed in water. DNA bands were then viewed under UV light and the gel photographed. The gel was then soaked in a denaturing solution of 5M NaCl/10M NaOH at room temperature for 45 min. It was then rinsed and soaked in a neutralising solution of 1M Tris (pH 8.0)/1.5M NaCl for 45 min. DNA from the gel was then transferred to a nylon membrane by capillary transfer, by the method described by Southern (1975). The buffer used for the transfer was 10 x SSC, and the transfer of nucleic acids from the gel to the membrane was allowed to proceed overnight.

After overnight transfer, the membrane was air dried. The DNA was then crosslinked to the membrane under UV light for 4 min. The membrane was then stored between sheets
of Whatman 3 MM paper in a dry place at room temperature until used for hybridization. Prehybridization, probe preparation, hybridization and washing of the membrane was performed as per the dot blot protocol. However, during the hybridization procedure, a second probe was also used for hybridization with the 1 kb DNA size marker. This was the same DNA molecular weight marker labelled with $\alpha^{32}\text{P}d\text{CTP}$ by random priming. After washing, the membrane was exposed for autoradiography at -70°C for varying lengths of time.

2.5 Entrapment of oligonucleotides into liposomes

Entrapment was performed in Professor Gregoriadis’s laboratory in the Centre for Drug Delivery Research at the School of Pharmacy, University of London. Entrapment into liposomes was effected by the dehydration-rehydration method, described in detail by Kirby and Gregoriadis (1984) and Gregoriadis et al (1996). ODNs were entrapped into positively-charged small unilamellar vesicles (SUVs) composed of phosphatidylcholine (PC), dioleoylphosphatidylethanolamine (DOPE) and stearylamine (SA) in a molar ratio of 2:2:1 by the preparation of dehydration-rehydration vesicles (DRVs). For the preparation of neutral liposomes, cholesterol was substituted for SA. Briefly, PC (16 mmol), DOPE (16 mmol) and SA (8 mmol) were dissolved in chloroform and placed in a round-bottomed flask. The chloroform was evaporated using a rotary evaporator, leaving a lipid film on the sides of the flask.

Following addition of 2 ml distilled water, the mixture was left at room temperature for 45 minutes to form multilamellar vesicles (MLVs). In order to restrict hydrated lipid to vesicles of the smallest size possible, it is necessary to use a method which imparts energy at a high level to the lipid suspension. This was performed by high power sonication (probe sonicator) in an ice bath to produce SUVs. The mixture was centrifuged at 2000 x g to remove any multilamellar vesicles or titanium debris in the sample. Aliquots of the SUV-containing supernatant were removed and put into tubes for lyophilization. To each aliquot (1 ml), 1 ml of the material to be entrapped was added. The mixture was frozen at -20°C for 1 hour and then freeze-dried at a vacuum of at least 0.1 torr for about 18 hours.
The resulting powder was rehydrated by adding a small volume (0.1 ml) of distilled water and then left at room temperature for 30 min. DRV's began to form (Gregoriadis et al 1993) while the mixture stood for 30 minutes at room temperature (Figure 2.1). Then, each sample was brought to a volume of 5 ml with a solvent that was isotonic (PBS, pH 7.4) to the substance in the liposome interior. DRV formation was complete after another 30 minutes at room temperature. Separation of unincorporated material was achieved by centrifugation, making use of differences in the buoyant densities of liposomes and unentrapped solute. The liposomes were collected by diluting the mixture to about 8 ml with the isotonic diluent and centrifuging at 35,000 rpm for 35 minutes at 4°C. The pellet contained the DRVs, which were then suspended in 1 ml of 0.1 M sodium phosphate buffer supplemented with 0.9% NaCl, pH 7.4 (PBS), while the unentrapped material was in the supernatant.

**Figure 2.1** Preparation of liposomes with high-yield drug entrapment. A solution containing the drug is mixed with a suspension of 'empty' water-containing liposomes and the mixture freeze-dried. The inset shows intimate contact of flattened liposomal membrane structures and of drug molecules in a dry environment. Liposomes (DRVs) formed upon controlled rehydration contain much of the original drug in the entrapped form inside the vesicle (Gregoriadis 1990).

The size of the liposomes was then reduced by the help of a Microfluidizer M110S (Microfluidics, Newton, MA, USA). The Microfluidizer has been characterized as, and shown capable of, producing large quantities of SUVs reproducibly (Mayhew 1984). It has the advantage that the size distribution is uniform, without the need for terminal
filtration, and the liposomes are smaller than the smallest easily prepared multilamellar vesicles. The machine was set for a 4500 psi driving pressure and the SUVs were collected after 2 to 4 passes through the device. The SUVs were then centrifuged at 50 000 rpm for 40 minutes. The resulting pellet was then resuspended in 1 ml of PBS (pH 7.4). The size of the liposomes was determined by photon correlation spectroscopy, using a Malvern Model 4700 apparatus (Malvern Instruments, Malvern, UK) equipped with a 25-mWhelium/neon laser (Gregoriadis et al 1993), and the average diameter of the final vesicles was approximately 200 - 300 nm. The percentage of the starting material entrapped into small vesicles was calculated by measurement of the radioactivity in the ODN added to the lipids and in the final preparation (counts in pellet/total counts added x 100).

2.6 Maintenance of cell culture

For the in vivo studies, a human hepatoma cell line, the PLC/PRF/5 cell line, was used. The cells contain integrated hepatitis B viral DNA, and secrete large amounts of HBsAg into the medium. The cells were a gift from Dr. Jim Owen of the Royal Free Hospital.

2.6.1 Materials used
Dulbecco’s Modified Eagles Medium (DMEM), containing Na pyruvate, 4.50 g/l glucose, 3.70 g/l Na bicarbonate, without L-glutamine (Imperial Laboratories, UK).
Foetal Bovine Serum (Imperial Laboratories, UK).
L-glutamine 200 mM (Imperial Laboratories, UK).
Penicillin 10 000 IU/ml and Streptomycin 10 000 µg/ml (Imperial Laboratories, UK).
Trypsin 0.25% and EDTA 0.02% (Imperial Laboratories, UK).
Dulbecco’s Phosphate buffered saline (PBS) without CaCl₂ and MgCl₂ (Sigma Cell Culture, St. Louis).
Vented tissue culture flasks 75 cm² (Falcon, Becton Dickinson, UK).
24-well multiwell tissue culture plate (Falcon, Becton Dickinson, UK).
96-well plate (Falcon Primaria, Becton Dickinson, UK).

2.6.2 Maintenance
Cells stored in liquid nitrogen were thawed rapidly at 37°C and plated onto 75 cm² tissue culture flasks. All work with cell cultures was performed using an aseptic technique in a
laminar flow hood. The cells were maintained in DMEM containing 10% foetal bovine serum, 2mM glutamine and 100 μg/ml penicillin/streptomycin. The flasks and multiwell plates were stored in an incubator at 37°C in 5% CO₂. The medium was changed every 2 days.

2.6.3 Subculture of cells
The cells were subcultured when they reached confluence. The medium was withdrawn and discarded. PBS (10 ml/75 cm²) was added to the flask, the cells rinsed and the rinse discarded. Trypsin 0.25%/EDTA 0.02% (2 ml/75 cm²) was added to the flask, the monolayer covered completely and left for 30 to 60 seconds. The trypsin was withdrawn and discarded, and the cells were incubated with the residue until they detached when the flask was tilted. 10 ml fresh medium was added and the cells were dispersed by repeatedly pipetting over the surface. Dispersed cells were counted in a haemocytometer. One ml of the cell suspension was seeded into a new flask containing 9 ml of fresh medium (1:10 split ratio). The flask was capped and returned to the incubator. For seeding onto multiwell plates in studies with antisense ODNs, a cell density of 1-2 x 10⁵ cells/ml was used.

2.6.4 Preservation of cells
At the time of subculture, or when cells were no longer required in the near future for experimentation, aliquots of cells were frozen to be thawed out at intervals as required. This minimised genetic drift and guarded against accidental loss by contamination. Cells were grown up to the late growth phase and trypsinized. They were resuspended at approximately 5 x 10⁶ - 2 x 10⁷ cells/ml in culture medium containing 20% serum and 10% dimethyl sulphoxide (DMSO). The cell suspensions were dispensed into 2 ml cryovials and sealed. The vials were then placed in the neck plug of a liquid nitrogen cylinder to cool at 1°C/min, before being transferred to a cane in the cylinder for storage. One vial was thawed to assess viability of the cells by re-plating in a culture flask.

2.7 Measurement of HBsAg in medium
The level of HBsAg in the cell culture medium was measured using the Wellcozyme HBsAg (Murex Diagnostics, UK) sandwich enzyme immunoasssay, as per the manufacturer's instructions. All samples were diluted to 1:10 before testing. Briefly, 50
μl of the conjugate was added to the microwell, the conjugate containing mouse monoclonal antibody to HBsAg labelled with alkaline phosphatase in a serum base. To this, 150 μl of sample or control (positive or negative) was added. The wells were then covered with a lid and incubated at 37°C for 60 min in a humid incubation box. After incubation, the wells were washed 5 times for about 30 seconds each with a multichannel pipette, the wash fluid containing piperazine and soluble magnesium and zinc salts.

Fifty μl of a substrate containing NADP was added to each well, and the plate was again incubated at 37°C for 20 min. Thereafter, 100 μl of the amplifier containing alcohol dehydrogenase with INT violet dye was added to each well and incubated for exactly 10 min at room temperature while colour developed. Fifty μl of stop solution containing 2M sulphuric acid was then added to each well. The absorbance of each well at 492 nm (A\text{492}) was read after 10 min using a microwell plate reader.

In each assay 2 positive and 2 negative control samples were included; these were supplied with the kit. The mean A\text{492} of the positive control had to be more than 0.5 above the mean A\text{492} of the negative control for the assay to be valid. In each assay, known quantities of HBsAg were also used to produce a standard curve. This international standard for HBsAg, containing a total of 100 units of the protein, was supplied by Dr. Morag Ferguson of the National Institute of Biological Standards and Control, Hertfordshire, UK. The standard and the collaborative study leading to its preparation has been previously described (Seagroatt \textit{et al} 1982). It was used in dilutions ranging from 0.1 IU/ml to 10 IU/ml. The A\text{492} of the standards was used to generate a standard curve, and HBsAg concentrations of the unknown samples were read from the curve in IU/ml.

2.8. Measurement of alpha-fetoprotein in cell culture medium

Alpha-fetoprotein (AFP) was measured in the medium at the end of the antisense experiments with the PLC/PRF/5 cell line, to determine the effect of the therapy on a controlled non-targeted protein. This was measured by a competitive radioimmunoassay technique (AMERLEX-M, Amersham International plc, UK). This technique depends on competition between AFP present in the sample and 125I-labelled AFP (tracer) for a limited number of binding sites on a rabbit anti-AFP antibody. Separation of the
antibody-bound fraction is effected by addition of a second antibody bound to magnetizable polymer particles, followed by magnetic separation and decanting of the supernatant. The amount of tracer bound is inversely proportional to the concentration of AFP present.

All standards were diluted with 1 ml sterile water. One hundred µl of standards, controls or samples were pipetted into appropriate tubes. One hundred µl of tracer was then added to each tube. The tube with the total counts (containing no AFP) was set aside. Rabbit anti-AFP polyclonal antiserum (100 µl) was dispensed into all tubes. The tubes were vortexed, covered and incubated at 37°C for 4 hours. One ml of donkey anti-rabbit IgG antibody suspension was then dispensed into each tube, the tubes vortexed and incubated at room temperature for 15 min. The rack containing the tubes was left on the separator base for 15 min, while the tubes containing the blank (to correct for background activity) and total counts were kept separate.

The supernatant was decanted with the tubes still on the rack, leaving the antigen-antibody complex at the bottom of the tube, attracted to the magnetic separator base. Activity from all tubes, including the blank, total, standards and samples, were then counted on a gamma counter for 1 min. A standard curve was generated from the standard samples and values for the test samples were read off the curve. To determine the background level of AFP in foetal bovine serum, AFP was also measured in fresh DMEM containing 10% FBS which had not been incubated with any cells. The levels of AFP ranged from 10 to 12 ng/ml, which was much lower than levels in medium incubated with PLC/PRF/5 cells.
2.9 Measurement of total protein concentration of cells from in vitro studies

Total cellular protein was measured in the wells from the 96-well plate at the end of the in vitro antisense studies. After all the supernatant was removed and stored, the cells were washed twice with PBS. Twenty μl trypsin/EDTA was added to each well and withdrawn after 30 to 60 seconds; the residue was incubated with the cells until they detached. Two hundred μl of PBS was added to the cells and the cells were dispersed. The cells and the PBS were decanted into a microcentrifuge tube and centrifuged at 8000 rpm for 10 min. The PBS was decanted, leaving a protein pellet. This was snap-frozen in liquid nitrogen and stored at -70°C until total protein concentration was measured.

Total protein concentration was measured with the Bio-Rad Protein Assay (Bio-Rad Laboratories, UK). This is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein. The microassay procedure was used for the 96-well plate. Bovine serum albumin (BSA) was used to prepare a range of standards from 1 to 25 μg/ml; this was used to perform a standard curve each time the assay was performed. All standards and samples were made up to 0.8 ml with PBS in a clean dry test tube; 0.8 ml of PBS was used as a reagent blank in a separate tube. Dye reagent concentrate was added in a volume of 0.2 ml. The test tubes were mixed several times by gentle inversion, to avoid excess foaming. After a period ranging from 5 min to 1 hour, the OD_{595} versus the blank reagent was measured by spectroscopy in disposable cuvettes. Total protein values in μg/ml was determined for each sample by reading it off the standard curve.

2.10 Polyacrylamide gel electrophoresis for oligonucleotides

This was used to determine whether the PS-ODNs were degraded into smaller ODNs during various procedures or exposure to biological fluids. The ODNs were usually labelled with a radioisotope, electrophoresed on a 20% polyacrylamide/7M urea gel, the gel dried under vacuum, and exposed for autoradiography. The procedure was as outlined below.
Preparation of 20% polyacrylamide/7 M urea gel:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>42 gm</td>
</tr>
<tr>
<td>40% Acrylamide (Acrylamide: Bisacrylamide = 19:1)</td>
<td>50 ml</td>
</tr>
<tr>
<td>10 x TBE</td>
<td>10 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>to total 100 ml</td>
</tr>
</tbody>
</table>

The above reagents were mixed in a beaker with a magnetic stirrer, while the mould for the vertical gel apparatus was assembled. When the reagents had dissolved, 0.45 ml of 10% Ammonium persulphate (APS) and 50 μl of TEMED were added to polymerise the gel immediately prior to casting it.

The apparatus was then filled with buffer (1 x TBE) and the samples loaded into the wells. A small amount of bromophenol blue was used as a tracking dye to follow the progress of the electrophoresis; the dye migrates at the same rate as a 12 base ODN. Cool water was passed through the electrophoresis tank to prevent overheating of the glass plates. The gel was electrophoresed for about 2 hours at 500 V, while the progress of the tracking dye was monitored. Thereafter, the gel was removed and fixed in 50% methanol/10% acetic acid/10% glycerol for 30 min, again in fresh fixative for another 30 min, and overnight in fresh fixative. It was then dried under vacuum at 80°C for approximately 4 hours before exposure for autoradiography at -70°C.

A $^{32}$P-labelled oligonucleotide size marker (GibCo BRL Paisley, UK.) was also electrophoresed on the same gel. It consists of a set of 19 single-stranded oligonucleotides increasing in length in 1 base increments from 4 to 22 bases. The labelling reaction for the marker was as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligonucleotide marker (50 ng)</td>
<td>1 μl</td>
</tr>
<tr>
<td>10 x kinase buffer</td>
<td>2</td>
</tr>
<tr>
<td>$[^{32}P]ATP$ (3000 Ci/mmol)</td>
<td>5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10</td>
</tr>
<tr>
<td>T4 polynucleotide kinase (20 units)</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>20 μl</td>
</tr>
</tbody>
</table>

The reaction was incubated at 37°C for 2 hours before loading onto the gel.
2.11 Design of *in vivo* antiviral studies

Animal use and care were in accordance with the Animals (Scientific Procedures) Act 1986 as stated by the Home Office. United Kingdom. A license was acquired from the Home Office for all the procedures performed during the course of this study. All ducks were housed, and *in vivo* experiments performed, in the Comparative Biology Unit at the Royal Free Hospital and School of Medicine. Aylesbury ducks were used for the first antiviral study. However, as these birds grow to a large body mass, they require a large dose of ODN for therapy, thus increasing the cost of the experiments. In all subsequent experiments, Mallard ducklings were used as they grow to a smaller size. It has also been shown that Mallard ducks are also a natural host for DHBV infection (Lambert *et al* 1991).

One-day old hatchlings were acquired from a breeder. On the same day, all ducklings were inoculated intravenously via the external jugular vein with duck serum containing \( \sim 10^9 \) to \( 10^{10} \) DHBV virions per ml, diluted in PBS. Fourteen days later the ducks were bled and infection was confirmed by dot blot hybridization. With this method of neonatal infection, all ducks were infected in most experiments. Only ducks that were definitely infected were used for subsequent antiviral study with antisense ODNs.

Infected ducks with the lowest weights were selected for the antiviral studies. The dose of antisense ODN was titrated with the weight during the treatment course. All ducks were treated for 5 days with daily intravenous injections of appropriate therapy. A blood sample was taken just prior to the first dose of treatment, thereafter daily before each dose, and 24 hours after the last dose. All injections and bleeds were performed at 24 hour intervals. Blood samples were cooled at 4°C after phlebotomy, and serum was separated by centrifugation at 3 000 rpm for 10 min before storage at -20°C until further analysis. Twenty four hours after the final dose (day 5 post-treatment), the ducks were killed with an overdose of Pentobarbital. Postmortem liver samples were washed in PBS and stored at -70°C until DNA was extracted.

Thus, 2 pretreatment and 5 post-treatment serum samples were available from each duck used in the experiment, in addition to liver tissue at the end of the treatment phase. In the final experiment, the antiviral therapy was commenced one week earlier, after confirming
the development of persistent infection in all ducks 7 days after inoculation with infectious serum.

2.12 Supply of PS-ODNs

All ODNs were acquired from commercial companies. The ODNs for the pharmacokinetic study, the initial in vivo antiviral study, the in vitro antisense studies and the primers for PCR were purchased from the Oswel DNA Service (Edinburgh). ODNs used in high dose for later in vivo studies were purchased from Genosys (Cambridge, UK) and Cruachem (Glasgow, UK).

Briefly, ODNs were synthesised on an automated synthesiser (ABI 394) on a 10 μmol scale. They were then purified by HPLC on a reverse-phase column. The product was deprotected in concentrated ammonia (55°C, 6 hours), evaporated and desalted on a NAP-25 column. The purified ODNs were then analysed by capillary zone electrophoresis.
Chapter 3

PILOT STUDY AND CONFIRMATION OF TARGET SEQUENCE

3.1 Aim and strategy

The aim of this section of the work was to perform an in vivo pilot study to compare the efficacy of free and liposome-entrapped ODNs and to assess the viral load before, during and after treatment. This would provide some information on whether any effect could be seen with a low dose of liposome-entrapped antisense ODN, and whether any obvious toxicity was observed with intravenous injections of liposomes or ODNs. There are no previous studies for comparison using liposomes as a delivery agent for antisense therapy in vivo, thus doses required for an effect with liposome-ODN could not be estimated. If no response was seen, then the presence of the target sequence in the viral isolates would be checked.

3.2 In vivo pilot study

3.2.1 Study design

Twelve neonatal Pekin-Aylesbury ducklings were inoculated with serum containing a high titre of infectious virions (10^9 to 10^10 virions/ml) from a duck used previously in the Comparative Biology Unit. The ducks were bled 14 days later to confirm that they were infected. The presence of chronic infection was confirmed by dot blot hybridization for DHBV DNA. All 12 ducks were chronically infected.

The antisense ODN chosen for the study was an 18-mer phosphorothioate previously shown to inhibit DHBV replication in vivo (Offensperger et al, 1993). It is complementary to the initiation region of the pre-S gene of the genome, corresponding to nucleotides 795-812 (numbering of nucleotides begins at the initiation codon of the core gene). This ODN was shown to cause marked inhibition of viral replication in the livers of ducks treated intravenously with a high dose (20 μg/gm body weight) for 14 days (Offensperger et al, 1993). It was the most potent of nine ODNs tested in vitro in a primary duck hepatocyte culture in that study. The sequence of this ODN is 5'-AAA-TAC-TAC-CCC-GTT-GTA-3'.
Ten mg of this ODN was synthesised by Oswel DNA service and a trace amount (10 pmole) labelled with \( \alpha^{-3^5}\text{S} \)dATP by terminal deoxytransferase for monitoring during liposome entrapment. The efficiency of incorporation of the label was 75%, with a final activity of \( 1.3 \times 10^7 \text{ dpm/mg of ODN} \). A total of 7 mg was used for encapsulation into liposomes, and the remaining 3 mg was retained for use as free ODN.

The ODNs were entrapped into liposomes composed of DSPC/cholesterol in a molar ratio of 1:1 by the dehydration-rehydration procedure. Unentrapped ODNs were separated and used again to increase the amount entrapped. This was performed 4 times and the final amount entrapped was 2.154 mg, an entrapment efficiency of 30% \( (2.154/7.0 \times 100\%) \). The mean diameter of the SUVs was 124 nm after microfluidization.

Nine chronically infected ducks were used in the antiviral experiment. The total amount of ODN entrapped was divided between 4 ducks, and each of these was allocated to receive the treatment in 5 divided doses given at 24 hour intervals. Thus, each of these ducks was given a fixed dose of 107.7 \( \mu \text{g} \) of liposome-ODN daily for 5 days. Another 4 ducks were given the same treatment regimen of free-ODN. The remaining ODN was used to treat one duck with one mg of free-ODN daily for 3 days. Blood was taken for dot blot analysis prior to the first treatment, on the 4th treatment day, on the 7th and 14th day after the start of treatment. A percutaneous non-surgical liver biopsy was performed with a Menghini needle under sodium pentothal anaesthesia on day 5 of treatment, by the method described by Varagona et al (1991). All ducks were killed 14 days after starting treatment, by an overdose of the anaesthetic agent, and samples of liver tissue were stored at -70°C.

By the time the first dose of treatment was given the ducks were 31 days old. The mean weight of the ducks was 1877 gm at the beginning of the treatment phase (range 1752 to 1960 gm). At the time of the percutaneous liver biopsy, one duck in the liposome-treated group died from an accidental overdose of the anaesthetic agent. All other ducks survived the treatment with free or entrapped antisense ODN without any discernible adverse effects.
3.2.2 Serum DHBV DNA response to treatment

Two serum samples were taken prior to treatment, 1 during treatment, and 2 post-treatment. Dot blot hybridization was performed on all these samples, with known quantities of cloned DHBV DNA as standards. The autoradiograph from the procedure is shown in Figure 3.1. Cloned DNA standards are shown, ranging from 5528 pg to 27.64 pg. A lower standard containing 2.764 pg of DNA showed no signal. The signals on the autoradiograph are from the equivalent of 25 µl of serum.

The values of DHBV DNA were converted and expressed as pg/ml of serum. The levels of DHBV DNA before, during and after treatment are shown in the figure 3.1.

The results of this experiment showed that there were wide variations in the serum levels of DHBV DNA over the 5 weeks during which levels were measured. None of the ducks lost serum viral DNA and there was no significant fall in viral load with the dose of antisense ODN used in this experiment. However, no overt toxicity was observed with free- or liposome-ODN, and all the ducks survived the therapy.

There were several possibilities for the lack of inhibition of viral replication in this pilot study. It was possible that the target sequence in the pre-S region of the genome was not identical to that present in the viral genome used in the previous study by Offensperger et al (1993). The same antisense sequence was used as in that study, but the target sequence had not been confirmed. Another possibility was that the fate and biodistribution of the ODNs, particularly in the liposome form, was not as predicted. This could not be assessed in this experiment because the ODNs were only trace-labelled with $^{35}$S and the activity could not be measured in tissues. Viral replication takes place mainly in the liver: liposomes were used as a delivery agent because they are known to reach the liver in large quantities. However, whether a greater amount of ODN gets to the liver rather than other organs had not been proven previously.
Figure 3.1: (a) Autoradiograph from dot blot hybridization of serum samples from 9 ducks. The first 2 rows are pre-treatment samples, the third row is from the fourth day of treatment, and the next 2 rows are post-treatment samples. One duck in the free-ODN group died from an accidental overdose of the anaesthetic at the time of the percutaneous liver biopsy. The last row is a set of cloned DNA standards. (b) Changes in mean (±SEM) serum DHBV DNA levels in ducks chronically infected with DHBV that received a 5-day course of free-ODN, liposome-ODN, or one duck that received a high dose of free ODN (1 mg/day x 3 days) are shown on a log scale. The treatment phase is shaded.
It was also possible that the ODNs were degraded by plasma exo- and endonucleases before reaching their target in the liver. Lastly, the dose of ODN injected may have been too small to have a therapeutic effect. An antisense effect with free-ODN was shown in Offensperger's study (1993) with 20 μg/gm body weight. The weights of the ducks were quite high when treatment was commenced, and the dose used was very small, even in the liposome-ODN group.

It was decided to investigate these possibilities, before performing further in vivo antiviral studies with antisense therapy. The first question to answer was whether the target sequence was present in our viral isolates. To achieve this, the pre-S region of the viral genome was sequenced. The gene was amplified by polymerase chain reaction (PCR), cloned in plasmid DNA, and the clones sequenced by the dideoxy chain termination method.

3.3 Amplification of the pre-S gene by PCR

3.3.1 Primers
Primers were designed from a complete nucleotide sequence of a German duck hepatitis B virus (Mattes et al, 1990). The primers flank a 512 bp fragment of the pre-S region of the gene. The forward primer was a 16 base oligonucleotide with the sequence 5'-AAT-ATT-TAA-CCA-GGC-T-3' and the reverse primer was an 18 base oligonucleotide with the sequence 5'-GTA-TTC-CCC-CGA-AGG-TAC-3'.

3.3.2 Polymerase chain reaction (PCR)
Fifty μl of known DHBV DNA-positive serum by dot blot hybridization from a duck used in the initial antiviral study was heated at 95°C for 5 min. Sterile distilled water was added (10 μl) to the serum and the mixture was centrifuged for 10 min at 8000 rpm. The supernatant was used for the template DNA.

A master mix was prepared to a volume of 95 μl per sample as outlined below. Five μl of template DNA was then added. Appropriate precautions were taken to prevent contamination of the reaction.
Master mix per sample:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X Taq buffer</td>
<td>10 μl</td>
</tr>
<tr>
<td>dNTPs (5 mM)</td>
<td>4</td>
</tr>
<tr>
<td>Distilled water</td>
<td>73.5</td>
</tr>
<tr>
<td>DHBV1 primer (50 pmol)</td>
<td>0.5</td>
</tr>
<tr>
<td>DHBV2 primer (50 pmol)</td>
<td>0.5</td>
</tr>
<tr>
<td>Taq DNA polymerase (2.5U)</td>
<td>0.5</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>95 μl</td>
</tr>
</tbody>
</table>

The 10 x reaction buffer contained 200 mM (NH₄)₂SO₄, 750 mM Tris-HCl pH 9.0, 0.1% (w/v) Tween. A plasmid vector pBR322 containing the full length DHBV genome (plasmid A49) was also used as a positive control. Sterile distilled water was used instead of template DNA for the negative control.

The DNA was amplified for 35 cycles in a Perkin Elmer 9600 automated thermal cycler according to the following protocol:

- Denaturation at 94°C for 1 min
- Annealing at 50°C for 1 min
- Polymerization at 72°C for 2 min.

After the final cycle, polymerization continued for 10 min at 72°C.

The PCR products were then electrophoresed on a 2% agarose gel. Ten μl of PCR product was loaded into each well, together with 2 μl of 6 x loading dye. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml) and viewed under UV light. The results are shown in Figure 3.2. Thus the amplified product was now available to be cloned and sequenced. The PCR products were stored at -20°C until needed for cloning.

3.4 Cloning of the PCR product

All pipette tips, glassware, eppendorfs and reagents to be used were autoclaved. Strict aseptic technique was used in all procedures involving the handling of bacteria.
**Figure 3.2:** 2% agarose gel electrophoresis of PCR products. Lanes 1 and 5: DHBV-positive serum; lanes 2, 4 and 7: plasmid vector pBR322 containing the full length DHBV genome; lane 3 and 6: reagent mix without template DNA; lane 9: φX174 *Hinf I* markers.

### 3.4.1 Preparation of agar plates

LB (Luria-Bertani) agar was prepared as follows:

- Bacto-tryptone 2 gm (DifCo Laboratories, Michigan)
- Bacto-yeast extract 1 gm (DifCo Laboratories, Michigan)
- NaCl 2 gm

This was made up to 175 ml with sterile water and the pH was adjusted to 7.5 with NaOH. Bacto-agar 3 gm (GibCo Laboratories, UK) was then added and the volume
made up to 200 ml. The agar was autoclaved for 40 min, then allowed to cool to 45°C in a water bath. As soon as the agar was cooled, ampicillin was added to provide a final concentration of 50 μg/ml and the agar swirled gently. Approximately 10 ml was then poured into sterile petri dishes on a level surface.

3.4.2 Ligation of PCR product to vector

PCR products were cloned using the pCR™II cloning vector (Invitrogen, San Diego, CA). It contains the lacZ gene for blue-white colour selection, ampicillin and kanamycin resistance genes, and has 3932 nucleotides (Figure 3.3). The non-template dependent activity of thermostable polymerases used in the PCR adds single deoxyadenosines to the 3’-end of all duplex molecules. The vector is supplied as a linear molecule with the 3’ dT overhangs ready for insertion of the PCR product. It is modified at the unique EcoRI site during preparation so that the inserted PCR product is flanked on each side by EcoRI sites.

![Figure 3.3](image-url)  
Figure 3.3: Schematic representation of the pCR II plasmid cloning vector showing the different genes. The cloning site is within the lacZ gene.

The ligation reaction was set up at a 1:1 molar ratio of vector:PCR product. The formula to determine the amount of PCR product to be ligated with 50 ng of vector was as follows:
X ng PCR product = \((Y \text{ bp } \text{PCR product}) \times (50 \text{ ng vector})\) = \(\frac{512 \times 50}{3932} = 6.5 \text{ ng}\)

The concentration of the PCR product was estimated from the agarose gel to be 20 ng/µl. The product was diluted to provide ~ 6.5 ng in 1 µl for the ligation reaction. The ligation was performed on ice as follows:

- Sterile water: 5 µl
- 10 x Ligation buffer: 1 µl
- Resuspended plasmid vector: 2 µl
- PCR product: 1 µl
- T4 DNA ligase: 1 µl
- Total: 10 µl

The reaction was incubated at 14°C overnight.

### 3.4.3 Transformation

A water bath was equilibrated to 42°C. One vial of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄, 20mM glucose) was warmed to room temperature. Previously prepared LB agar plates with ampicillin were incubated in a 37°C incubator to remove excess moisture. The microfuge containing the ligation reaction was centrifuged briefly and then placed on ice. β-mercaptoethanol (0.5 M) and one 50 µl vial of frozen competent *E. coli* cells (Invitrogen, San Diego, CA) were thawed on ice. The *E. coli* strain used was INVαF¹, which is a recombination-negative strain used for stable replication of high copy number plasmids. Two µl of β-mercaptoethanol (0.5 M) was pipetted into the vial of competent cells and mixed by stirring with a pipette tip, taking care not to cause any mechanical lysis.

One µl of the ligation reaction was pipetted directly into the competent cells and tapped gently. The vial was incubated on ice for 30 min, then transferred to a 42°C waterbath and incubated for exactly 30 seconds before placing on ice for 2 min. Prewarmed SOC medium (450 µl) was added to the vial. The vial was then placed in a microcentrifuge rack and secured horizontally with tape. It was then shaken at 37°C for 1 hour at 225 rpm in a rotary shaking incubator.
The LB agar plates containing ampicillin (50 μg/ml) were prepared by spreading 25 μl of X-gal (40 mg/ml stock in dimethylformamide) on top of the agar with a sterile glass spreader. The X-gal was allowed to diffuse into the agar for about 1 hour. The vial with the transformed cells was placed on ice. One hundred μl of the transformation vial was spread onto separate LB agar plates prepared with antibiotic and X-gal. The plates were inverted and placed in a 37°C incubator overnight. About 90% of the colonies grown overnight were white, indicating that the insert was present and had interrupted the lacZ gene, which is essential for the production of β-galactosidase; X-gal turns blue in the presence of this enzyme.

3.4.4 Small scale plasmid preparation

One litre of LB medium was prepared as follows:

- Bacto-tryptone 10 gm
- Bacto-yeast 5 gm
- NaCl 10 gm
- Sterile water to 1 litre

The pH was adjusted to 7.5 with NaOH and the medium autoclaved. After cooling, ampicillin was added to provide a final concentration of 50 μg/ml. Five ml aliquots of medium were pipetted into 9 universal bottles. Eight white colonies from 2 agar plates were picked with previously autoclaved toothpicks and added to the broth in each of the 8 universals. The final universal was used as a negative control, containing no colony. The universals were incubated at 37°C at 120 rpm in a rotary shaking incubator. Growth of bacteria, as evidenced by a cloudiness of the liquid medium, occurred overnight in all 8 universals cultured with colonies, but not in the negative control. The universals were stored at 4°C until plasmid DNA purification.

3.4.5 Plasmid DNA purification

Plasmid DNA was purified from the host cells using the QIAprep-spin plasmid purification procedure (QIAGEN, Chatsworth, CA). This is based on a modified alkaline lysis method and the adsorption of DNA on to silica in the presence of high salt. DNA is eluted in a small volume of low salt buffer.

Eight separate 1 ml cultures of E. coli grown in LB medium were centrifuged at 5 000g for 5 min. The supernatant was discarded and the pellet containing the bacterial cells
 retained. The pellet of cells was resuspended completely in 250 μl of resuspension buffer containing 50mM Tris-Cl, pH 7.5/10mM EDTA/100 μg/ml RNase A. Two hundred and fifty μl of lysis buffer (0.2M NaOH/1% SDS) was added, the solution inverted to mix a few times and incubated for 5 min at room temperature. Chilled neutralisation buffer (2.55M Potassium acetate) was added in a volume of 350 μl, mixed immediately by gentle inversion several times, and incubated on ice for 5 min. The solution became cloudy and viscous. The mixture was centrifuged for 10 min at maximum speed in a 1.5 ml microfuge tube. A compact white pellet was formed, separate from the supernatant.

A QIAprep-spin column was placed in a 2 ml collecting tube and the supernatant was applied to the column by pipetting. The column and collecting tube were centrifuged for 60 seconds at maximum speed, and the flowthrough fraction drained from the tube. The column was washed with 0.75 ml of a wash buffer containing 200mM NaCl/20mM Tris-HCl, pH 7.5/5 mM EDTA pre-diluted in 95% ethanol, and centrifuged at maximum speed for 60 seconds. The wash buffer was drained from the existing tube and the column recentrifuged for an additional 60 seconds to remove residual wash buffer. The column was then placed in a clean 1.5 ml microcentrifuge tube and the DNA eluted by adding 100 μl TE, pH 8.5 (10mM Tris-HCl/1mM EDTA) and centrifuged for 30 seconds.

3.4.6 Restriction digest of plasmid DNA

The presence of the inserted PCR product within the plasmid vector was confirmed by performing a restriction enzyme digest of the plasmid DNA with EcoR1. This was performed on each of the 8 colonies cloned and purified, as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td>10 μl</td>
</tr>
<tr>
<td>Digestion buffer (reaction 3, Promega)</td>
<td>2</td>
</tr>
<tr>
<td>Distilled water</td>
<td>6</td>
</tr>
<tr>
<td>EcoR1</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>20 μl</td>
</tr>
</tbody>
</table>

The reaction was incubated at 37°C overnight in a waterbath. Ten μl of each reaction was then electrophoresed on a 1% agarose minigel, together with linear vector DNA and size markers. The gel was stained with ethidium bromide and viewed under UV light. All eight lanes containing the digest showed 2 distinct DNA bands, one corresponding to ~4
000 bp and another to ~500 bp (data not shown). The lane with the linear vector DNA showed a single band in the region of ~4 000 bp. Thus the insert consisting of the pre-S gene was present in all 8 of the colonies cloned.

3.4.7 Alkali denaturation of double-stranded plasmid DNA.
The plasmid DNA to be sequenced was denatured with 2 M NaOH. The denaturation reaction was set up as follows in 8 microfuge tubes:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td>10</td>
</tr>
<tr>
<td>2M NaOH</td>
<td>2</td>
</tr>
<tr>
<td>2mM EDTA</td>
<td>2</td>
</tr>
<tr>
<td>Distilled water</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
</tr>
</tbody>
</table>

The reaction was incubated at 37°C for 30 min. Thereafter 3M Na acetate was added, followed by 70 μl of absolute alcohol. The DNA was precipitated at -70°C for 15 min, then centrifuged at maximum speed for 5 min and the alcohol decanted. The pelleted DNA was then washed with 70 μl of 70% alcohol and centrifuged at high speed for 5 min. All the alcohol was then discarded, the pellet air dried completely, and then dissolved in 7 μl of distilled water. The ssDNA was stored at -20°C for the sequencing reactions.

3.5 Sequencing

Sequencing of the pre-S gene of DHBV was performed by the dideoxy chain termination method on the ssDNA. The main steps involved before gel electrophoresis are annealing of the template and primer, the labelling reaction and the termination reaction. The reactions were performed with the aid of a commercial kit (Sequenase 2.0, USB, Cleveland, USA).

3.5.1 Annealing template and primer

For each set of 4 sequencing lanes, a single annealing reaction was used, as follows:
Primer (-40) 0.5 pmol

(5'-GTTTTCCAGTCACGAC-3') 1 µl

Reaction buffer

(200mM Tris-HCl, pH 7.5/100mM MgCl2/250mM NaCl) 2

DNA 7

Total 10 µl

The capped tube was warmed to 65°C for 2 min, then cooled slowly to room temperature over 30 min. Once the temperature was below 35°C, annealing was considered to be complete and the tube was placed on ice.

3.5.2 Labelling reaction

Labelling mix (5 x concentrate containing 7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP) was diluted 5-fold with distilled water. T7 DNA polymerase (Sequenase version 2.0 13 units/µl) was diluted 1:8 with enzyme diluting buffer (10mM Tris-HCl, pH 7.5/5 mM DTT/0.5 mg/ml BSA) to the desired amount for the number of reactions and kept on ice until used. The labelling reaction was performed on ice as follows:

Template-primer (above) 10 µl

DTT 0.1M 1.0

Diluted labelling mix 2.0

[α-35S]dATP (1000 Ci/mmol) 0.5

Diluted T7 DNA polymerase 2.0

Total 15.5 µl

The reaction was mixed thoroughly and incubated for 2-5 min at room temperature.

3.5.3 Termination reactions

Four microfuge tubes per reaction were labelled G, A, T and C. Into each of the 4 tubes 2.5 µl of ddGTP, ddATP, ddTTP or ddCTP termination mixes was added respectively. The tubes were prewarmed at 37°C for at least 1 min. When the labelling incubation was complete, 3.5 µl was removed and transferred into each of the tubes marked G, A, T and C. The contents of each tube were mixed, centrifuged briefly to collect the solution at the bottom of the tube, and returned to the 37°C waterbath.
The incubation was continued for a total of 5 min. Four μl of stop solution (95% formamide/20mM EDTA/0.05% Bromophenol Blue/0.05% Xylene Cyanol FF) was added to each of the termination reactions and mixed thoroughly. The samples were then ready to run on the sequencing gel.

### 3.5.4 Denaturing polyacrylamide gel electrophoresis

An 8% denaturing polyacrylamide gel was assembled for sequencing. All reagents were of electrophoresis grade. The gel was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>37.4 gm</td>
</tr>
<tr>
<td>10 x TBE</td>
<td>8 ml</td>
</tr>
<tr>
<td>40% Acrylamide/Bisacrylamide (29:1)</td>
<td>16 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>to 80 ml total volume</td>
</tr>
</tbody>
</table>

The mixture was stirred with a magnetic stirrer until clear. Just before pouring the gel, 650 μl of 10% ammonium persulphate and 100 μl of TEMED were added. Care was taken to prevent any air bubbles from being trapped within the gel. The gel was allowed to set at room temperature for 15 min. When ready for electrophoresis, the gel was pre-run for 30 min. Thereafter, the samples were heated to 75°C for 2 min and 2.5 μl was loaded immediately into each lane of the gel. The gel was run at 1.5 kV with TBE as the buffer. After 5 hours, the buffer in the lower well was changed and 3M acetic acid was added to it. The gel was run for a further 2 hours. The apparatus was then dismantled and the gel fixed in 10% acetic acid/10% methanol for 15 min, dried on 3 MM Whatman paper for 2 hours at 80°C under vacuum. The dried gel was then exposed for autoradiography at room temperature for 36 hours.

### 3.6 Results

Six of the 8 clones were successfully sequenced. Approximately 200 nucleotides of the insert could be read from the autoradiograph. All 6 clones showed the presence of the target sequence complementary to the antisense PS-ODN sequence which was used as the therapeutic agent in the pilot study (Figure 3.4). This sequence was TTT-ATG-ATG-GGG-CAA-CAT.
3.7 Discussion

Thus far it has been shown that a very low dose of antisense ODN does not inhibit DHBV replication in vivo, either in the free or the liposome-entrapped form. There were no overt toxic effects either from the PS-ODN or from the liposomes at the doses used. The in vivo model that was chosen seemed to be practical, because experimental chronic infection could easily be induced, and viral load could be quantified in serum to assess the effects of antiviral therapy. One of the major drawbacks noted was that the weight of the Pekin-Aylesbury ducks increased quite markedly within the first few weeks of life. This would entail large doses of antisense therapy, and concomitant high costs, in order to achieve appropriate therapeutic levels. In order to diminish this, therapy would need to be started as soon as possible once chronic infection was confirmed.

The antisense sequence used was correct, as the target complementary sequence was present in the region of the genome which was targeted. Therefore, this was not the reason for the lack of an antiviral effect in the pilot study. Other reasons for the lack of efficacy therefore needed to be examined. The distribution and hepatic delivery of liposome-entrapped ODNs compared to free-ODNs would thus need to be studied to determine the feasibility of using this form of delivery to improve the efficacy of antisense therapy for chronic DHBV infection.
Figure 3.4: Sequences from 6 clones of the pre-S gene showing the presence of the target sequence complementary to the antisense ODN used in the pilot study.
Chapter 4

BIODISTRIBUTION AND STABILITY STUDIES OF FREE- AND LIPOSOme-ENTRAPPED PHOSPHOROTHIOATE ANTISENSE OLIGODEOXYNUCLEOTIDES IN DUCKS

4.1 Introduction

Prior to using liposomes as a delivery vehicle to increase hepatic delivery of antisense ODNs for the treatment of chronic hepatitis B, it was necessary to confirm that the entrapped ODN was stable during the entrapment process and in duck plasma. The plasma and urine clearance kinetics, and the fate of the entrapped ODN also needed to be determined. The results of these studies would be necessary to determine the use of liposomes to augment antisense therapy in vivo.

To achieve these objectives, the ODN was labelled so that it could be detected in duck tissues, such as plasma, urine, liver and other organs. In initial in vivo studies, $^{35}$S was used to label the ODN. However, this proved to be a weak label as the specific activity was not high enough to allow reliable counts in most of the organs. Experiments were performed twice with this label, but the results were not reliable and the counts in organs were all very low. Micro-autoradiography was attempted to study the distribution of the ODN in the liver using this label; however very few silver grains were noted, making interpretation impossible. The in vivo studies were thus repeated and $^{125}$I was used to label the ODN.

4.2 Materials and Methods

4.2.1 Oligonucleotide synthesis and labelling.

An 18-mer PS ODN previously shown to inhibit DHBV replication in vitro and in vivo was used for all the studies (Offensperger et al. 1993). The nucleotide sequence was 5'-AAA-TAC-TAC-CCC-GTT-GTA-3', which is complementary to the pre-S region of DHBV (nucleotides 795-812). The ODNs were synthesized by the solid phase phosphoramidite method (Oswel DNA Service, Edinburgh, UK). After deprotection and cleavage the ODN was purified by reverse phase HPLC.
ODNs were trace-labelled with $^{125}$I-dCTP using terminal deoxytransferase (TdT) as described previously. Un-incorporated label was removed by Sephadex G-50 gel filtration and labelled ODN was mixed with unlabelled ODN to give a specific activity of $2.4 \times 10^6$ cpm/mmol. To assess the in vitro stability of ODNs in normal duck plasma and during liposome entrapment, PS-ODN was labelled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase as described previously.

### 4.2.2 Liposome entrapment

ODNs were entrapped into positively-charged liposomes composed of phosphatidylcholine (PC), dioleoyl phosphatidylethanolamine (DOPE) and stearylamine (SA) in a molar ratio of 2:2:1 by the dehydration-rehydration method (Kirby & Gregoriadis 1984; Gregoriadis et al., 1996), as outlined in Chapter 2. The size of the liposomes was determined by photon correlation spectroscopy, using a Malvern Model 4700 apparatus (Malvern Instruments, Malvern, UK) (Gregoriadis et al., 1993), and the average diameter of the final vesicles was found to be approximately 200 nm. The amount entrapped was 50.3% of the starting material.

### 4.2.3 Analysis of integrity of oligonucleotides during liposome entrapment

To assess whether the ODNs were degraded during the process of liposome entrapment, the ODN was labelled with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase as described. Free label was separated from the labelled ODN by Sephadex G-50 gel filtration. Labelled ODN was then entrapped into positively-charged SUVs as described. Before liposome entrapment, at each step during entrapment, and after entrapment, samples were stored for analysis of ODN size to determine any degradation.

ODNs were extracted from liposomes by incubation with 1% Triton X-100 at 37°C for 1 hour and electrophoresed on a 20% PAGE/7M urea gel. The size of the ODN was compared with a labelled oligonucleotide size marker (4 to 22 bases) (GibCo BRL Paisley, UK). The gel was fixed in 10% acetic acid/50% methanol/10% glycerol solution, and then dried before autoradiography at -70°C, as described in Chapter 2.
4.2.4 *In vitro* plasma stability of the PS-ODN

Twenty-five pmol of PS-ODN was labelled with \([\gamma^{32}\text{P}]\text{ATP}\) and T4 polynucleotide kinase to a specific activity \(4.4 \times 10^4\) cpm/pmol ODN. Twenty \(\mu l\) of \(^{32}\text{P}\)-labelled PS-ODNs were incubated in 250 \(\mu l\) of normal duck plasma at 37°C for 24 hours with gentle shaking. Two \(\mu l\) of labelled ODN was stored as a control before addition to plasma, and 10 \(\mu l\) samples were taken at 2, 15, 30, 60 min and 4, 6 and 24 hours after exposure to the plasma and diluted to 50 \(\mu l\) with PBS for analysis. Samples were analysed by electrophoresis on a 20% PAGE/7M urea gel, and compared with a \(^{32}\text{P}\)-labelled oligonucleotide size marker (GibCo BRL), before the gel was fixed, dried and exposed for autoradiography as described above.

4.2.5 Kinetic and tissue distribution study

Animal use and care was in accordance with the Animals (Scientific Procedures) Act 1986 as stated by the Home Office, London, UK. Ten 12-day old uninfected Mallard ducklings (204 ± 17 grams) were used in the study. Animals were injected intravenously via a neck vein with a single bolus intravenous injection of approximately 1 mg of ODN made up to 1 ml with phosphate-buffered saline (PBS). Four ducklings were injected with liposome-ODN, four control ducklings with free-ODN, and two with 1 ml of PBS. After intravenous injection, each animal was placed in a cage and fed with a commercial diet and water *ad libitum*. Plasma samples were taken at 2, 15, 30 and 60 min, and 4, 6 and 24 hours into EDTA-containing tubes. Total excreted urine and faeces was collected between 0-1, 1-4 hours, 4-6 hours and 6-24 hours. This was collected in a tray placed below each cage housing the duck.

One duckling from each of the ODN-treated groups was killed at 1 hour, one from each at 4 hours, and the remaining 6 ducklings (2 per treatment group and 2 PBS-treated) at 24 hours after treatment. Ducklings were killed with an overdose of sodium pentobarbital anaesthesia and the organs were collected from each bird. Organs were washed in PBS, weighed and stored at -70°C until further analyses. Plasma was separated by centrifugation and 100 \(\mu l\) was used for counting. Radioactivity was measured in plasma samples, tissue samples, urine/faeces and aliquots of the injected ODN using a gamma counter (Riagamma 1271; LKB Wallac, Finland). Total
radioactivity in the ducks' blood was calculated assuming the blood volume to be 10% of the body weight (Campbell 1995).

4.2.6 Intrahepatic distribution of ODN

To assess the intrahepatic distribution of injected ODNs, autoradiography of frozen sections of liver was performed. After the ducklings were killed, liver samples were immediately fixed in 4% paraformaldehyde for 4 hours, washed in PBS and dehydrated overnight in 15% sucrose in PBS. Thereafter, samples were embedded in OCT compound (Tissue-Tek. Miles Diagnostic Division, Inc, USA), frozen rapidly in isopentane/liquid nitrogen, and stored at -70°C. Five μm cryostat sections were cut and placed on glass slides pre-coated with poly-L-lysine that were then air-dried, wrapped in cling film, and stored at -20°C until use.

When they were needed, cryostat sections were brought to room temperature. Slides were dipped vertically into photographic emulsion (Hypercoat emulsions, Amersham, UK) prewarmed to 42°C in a dark room. Each slide was dipped into the emulsion for approximately 3 seconds to avoid different emulsion thickness across the slides. The slides were dried vertically at room temperature, allowing the emulsion to set and dry. After drying, the slides were stored in a rack in a light-tight box at 4°C for varying periods of exposure. Slides were developed for 3 min (Kodak Microphen), washed and fixed in 1:4 Hypam fixative for 5 min (Ilford, UK), counterstained with haematoxylin and blueing solution, differentiated in acid alcohol, before dehydrating with 100% alcohol and mounting with DPX. Sections were then examined by light microscopy for the presence and distribution of silver grains. Sections from ducklings injected with PBS were used as negative controls.

4.2.7 Detection of ODN in plasma by hybridization

Plasma samples from ducks killed at 1 hour and at 4 hours were also used to detect the presence of ODNs in the circulation by a hybridization method, modified from Temsamani et al (1993). Briefly, 250 μl of plasma was incubated with 250 μl of DNA extraction buffer (0.5% SDS, 10 mM NaCl, 20 mM Tris HCl, pH 7.6/10 mM EDTA) and proteinase K (1mg/ml final concentration) for 90 min at 60°C. After incubation, 200 μl of distilled water was added and the samples were extracted twice with 500 μl of
phenol/chloroform/isoamyl alcohol (25:24:1), and once with 500 µl of chloroform. Before blotting, the samples were heated at 90°C for 5 min and mixed with 40 µl of 20 x SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.0). A nylon membrane (pore size 0.45 mm) and Whatmann 3 MM paper were soaked in 10 X SSC buffer. The membrane was placed in a slot blot apparatus (Minifold II, Shleicher and Schuell) and a vacuum applied. The wells were first rinsed with 100 µl of 20 x SSC buffer, the samples loaded and the wells were then rinsed again with 100 µl of 20 X SSC. Known quantities of injected ODN were also loaded to create a standard curve. The membrane was then removed and cross-linked under UV light for 10 min.

An 18-mer oligonucleotide complementary to the injected ODN was 5'-end labelled with [γ-³²P]ATP and T4 polynucleotide kinase. The specific activity of the probe was 87.9 mCi/mmol. The membrane was prehybridized for 1 hour at 45°C in hybridization buffer (6 X SSC, 10 X Denhardts solution, 50 mg/ml salmon sperm DNA, 0.1% SDS) and hybridization was performed overnight at 45°C. The membrane was washed 3 times in 6 x SSC/0.1% SDS for 5 min each time at the hybridization temperature, and exposed for autoradiography at -70°C.

4.3 Results

4.3.1 Analysis of integrity of ODN during liposome entrapment
ODNs were not degraded during any of the stages of liposome entrapment, as shown by a single 18-base band on the 20% PAGE/7M urea gel (Figure 4.1).

4.3.2 Plasma stability of ODN
Electrophoresis on a 20% PAGE/7M urea gel of the PS-ODN from plasma showed a single band at all time points sampled, indicating that the ODN remained intact over 24 hours with no evidence of degradation (Fig. 4.2). The reason for the tail of radioactivity above each band is not clear.
Figure 4.1: Assessment of the integrity of $^{32}$P-ODN on a 20% PAGE/7M urea gel during liposome entrapment. Lanes (1) before addition to SUVs; (2) before freeze-drying; (3) after rehydration; (4) after centrifugation; (5) after microfluidization; (6) after separation of unentrapped ODN; (7) entrapped ODN. Lane M: Oligo(dT)$_{4-22}$ ladder (GibCo BRL, Paisley, UK).

Figure 4.2: Assessment of in vitro stability of the ODN on exposure to duck plasma from 2 min to 24 hours. Lane 0: Control labelled ODN before addition to plasma.
4.3.3 *In vivo* plasma clearance:

Plasma levels of PS-ODN were rapidly depleted after intravenous administration in both treatment groups, with a half-life of approximately 1-2 min. Figure 4.3 shows the rapid clearance of the ODN from plasma, as a percentage of the ODN injected, with a little more liposome- than free-ODN remaining in the circulation at the end of the 24 hour period.

![Graph showing plasma clearance](image)

**Figure 4.3:** Mean (±SEM) *in vivo* plasma clearance of free- and liposome-ODN in 4 ducks per group, expressed as a percentage of the injected amount (100%) detectable in plasma.

4.3.4 Biodistribution

The percentage of the injected dose detected in each of the organs sampled is shown in Table 4.1. The greatest proportion of the injected ODN was present in the liver in both treatment forms. However, the percentage of injected liposome-ODN retained in the liver at 1, 4, and 24 hours was 82.8%, 67.3%, and 25.8% (mean of 2 ducks at 24 hours) compared to 13.9%, 10.8%, and 2.7% for free ODN, respectively (Figure 4.4). Thus, the hepatic uptake and retention of liposome-ODN was far greater than for free-ODN at all time points measured. The ratio of liposome-ODN to free-ODN uptake in the liver for paired ducks measured at corresponding times ranged from 6.0 to 9.7 (Table 4.1). The amounts present in other organs were far less, and similar for both treatment groups, with the exception of the airsacs which retained a greater proportion of liposome-ODN than free-ODN at 1 and 4 hours. The amount of ODN present in the brain was the lowest in both groups. Uptake in the spleen was much lower than hepatic uptake, even for the
liposome-ODN. However the mean weight of the liver in all ducklings was 18.8 ± 1.5 grams compared to 0.3 ± 0.1 grams for the spleen.

Table 4.1: Percentage of injected oligodeoxynucleotide retained in each organ at different times. One and 4 hour values are for individual ducks, and 24 hour values are for a mean of 2 ducks.

<table>
<thead>
<tr>
<th>Time &amp; treatment</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidneys</th>
<th>Airsacs</th>
<th>Heart</th>
<th>Brain</th>
<th>Liposome/Free uptake in liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr: Liposome</td>
<td>82.8</td>
<td>1.13</td>
<td>2.9</td>
<td>8.8</td>
<td>1.22</td>
<td>0.16</td>
<td>6.0</td>
</tr>
<tr>
<td>Free</td>
<td>13.9</td>
<td>0.58</td>
<td>2.5</td>
<td>1.9</td>
<td>0.6</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>4 hrs: Liposome</td>
<td>67.3</td>
<td>0.77</td>
<td>0.8</td>
<td>10</td>
<td>0.29</td>
<td>0.1</td>
<td>6.2</td>
</tr>
<tr>
<td>Free</td>
<td>10.8</td>
<td>0.6</td>
<td>0.74</td>
<td>0.52</td>
<td>0.28</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>24 hrs: Liposome</td>
<td>25.8</td>
<td>0.38</td>
<td>0.3</td>
<td>0.37</td>
<td>0.14</td>
<td>0.08</td>
<td>9.6</td>
</tr>
<tr>
<td>Free</td>
<td>2.7</td>
<td>0.39</td>
<td>0.17</td>
<td>0.18</td>
<td>0.03</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

% retention of ODN

![Bar graph showing the percentage of injected ODN present in the liver of each duckling at the times when they were sacrificed. The 24 hr bars each represent a value for a duck, as 2 ducks per group were killed at 24 hr.](image)

Figure 4.4: Bar graph showing the percentage of injected ODN present in the liver of each duckling at the times when they were sacrificed. The 24 hr bars each represent a value for a duck, as 2 ducks per group were killed at 24 hr.
4.3.5 Excretion of ODN

Ducks excrete urine and faeces through the cloacae, therefore it was not possible to differentiate excretion via urine from that via faeces. The cumulative excretion of the free-ODN after 1, 4, 6, and 24 hours was 8.7 (± 1.5)%, 16.5 (± 0.8)%, 17.2 (± 0.9)% and 18.6 (± 2.1)%, respectively. For the liposome-ODN, the excretion was 10.2 (± 2.4)%, 19.8 (± 2.1)%, 25.9 (± 1.4)% and 28.9 (± 1.6)%, respectively. Thus, a large proportion of the injected material was excreted within 24 hours in both treatment groups, as is shown in Figure 4.5.

![Cumulative excretion of [125]PS-ODN in urine](image)

**Figure 4.5:** Cumulative excretion of free- and liposome-ODN in duck urine over 24 hours. Each data point is a mean (± SEM) percentage of the initial injected dose.

4.3.6 Intrahepatic distribution of ODN

Hepatic sections exposed to photographic emulsion were developed after 2, 5, 12 and 28 days for estimation of the distribution of silver grains. The strongest signal was seen after 28 days exposure, as shown in Figure 4.6. This shows a section from a duckling injected with PBS and killed after 24 hours, and sections from the ducklings killed at one hour after injection with either free- or liposome-ODN. The number of grains was sparse in the ducklings injected with either PBS or free-ODN, but much greater in the duckling injected with liposome-ODN.
Figure 4.6: Intrahepatic distribution of labelled ODN on autoradiography after 28 days exposure. A, Duck injected with PBS killed 24 hours after injection; B, Duck injected with free-ODN killed 60 min after injection; C, Duck injected with liposome-ODN killed 60 min after injection.
This observation was consistent with the much greater proportion of injected ODN present in the liver of liposome-ODN injected ducklings by gamma counting, as shown in Figure 4.4. The distribution of the silver grains was uniform and even, rather than confined to specific cells. However, cell morphology was not adequate to differentiate Kupffer cells from other intrahepatic cell types. Double staining with an antibody to duck Kupffer cells would have been useful to identify these cells and assess the density of silver grains around them compared to hepatocytes. However, this antibody was not available at the time of this experiment.

4.3.7 Detection by hybridization

Known quantities of injected ODN ranging from 0 to 1000 ng were spotted onto the nylon membrane and subjected to hybridization as before to determine the sensitivity and specificity of the method. The sensitivity was found to be between 0 and 3.1 ng of ODN (Figure 4.7). ODN was detectable in plasma up to 30 min after injection, and in 3 of the 4 ducks at 15 min. The amount of ODN spotted onto the membrane was the total amount extracted from 250 µl of plasma. Therefore, if necessary the total quantity in the circulation can be calculated by correcting for the total blood volume, and producing a standard curve after densitometry.

![Standards](image)

**Figure 4.7** Quantification of plasma ODN by hybridization on a slot blot manifold. Standards are (from top to bottom signals) 1000, 100, 50, 25, 12.5, 6.25, 3.1 and 0 ng of ODN. F and L indicate plasma from ducklings injected with free- or liposome-ODN, respectively. Ducks F1 and L1 were killed at 240 min after injection, ducks F2 and L2 were killed at 60 min after injection.
4.4 Discussion

Thus, these data suggest that (i) phosphorothioate oligodeoxynucleotides are stable in duck plasma for many hours; (ii) they remain stable during the process of liposome entrapment; (iii) they are rapidly cleared from the plasma when injected intravenously; and that (iv) there is greater uptake of liposome-entrapped oligonucleotides by the liver after intravenous injection. Thus liposomes may be effective vehicles to improve the delivery of oligonucleotides to the liver for the therapy of hepatotropic viruses. The duck model was chosen because it is a natural host for duck hepatitis B virus infection. Uninfected ducklings were used in this study because the fate of the injected ODN might have been altered if viral sequences complementary to the ODN were present in the duck body fluids such as plasma.

There was no difference in the plasma clearance kinetics between the 2 treatment groups, although a little more of the liposome- than the free form was present in the circulation at the end of the 24 hour period. Excretion of the ODN was probably via the urine, in keeping with its water-soluble properties (Stein & Cheng 1993). The liposome-ODN was excreted to a greater extent than the free-ODN, especially after the first 4 hours. However, the number of ducks in the study was small, and reduced further after 4 hours, as ducks were killed at different time points. A similar study in mice also showed that about 30% of PS-ODN was excreted in urine after 24 hours (Agrawal et al, 1991), and another study showed that the ODN were completely eliminated in the urine over 3 days, predominantly as parent compound (Iversen 1991).

If the ODNs were degraded to smaller molecules during the process of liposome entrapment, then liposomes would be unsuitable as a delivery vehicle for them. In vitro, there was no degradation of the ODN by plasma exo- or endonucleases, and the molecule was intact after 24 hours at 37°C. This is one of the main properties of phosphorothioate-modified ODN, as opposed to the naturally occurring phosphodiester-bonded molecule which is degraded in vivo within 5 minutes in some animal species et al, Zhang 1995). The most notable difference between the 2 treatment groups was the markedly increased hepatic uptake of the liposome-ODN. It suggests that liposomes are an attractive non-viral vector to increase the delivery of drugs to the liver. This is the first study to show this using ODNs. It is known that liposomes preferentially target the
reticulo-endothelial system. Thus they would be adsorbed largely by the spleen and Kupffer cells of the liver. However, the uptake to the spleen was much less, partly due to the greater mass of the liver (mean hepatic mass 63 times the mean splenic mass). The spleen, compared to the liver, is a very small organ in ducks.

All the injected radiolabel was not accounted for in the biodistribution studies. The total radioactivity in the organs and excreta for the 1 and 4 hour liposome-ODN ducklings approximates 100%, and the 24 hour liposome-ODN approximates 57%. However, the total for the free-ODN injected ducks only approximates 30%, 34% and 33% for the 1 hour, 4 hour and the mean of the two 24 hour ducklings, respectively. It is possible that some of the ODN, particularly free-ODN, was retained in organs that were not examined, such as muscle, bone and cartilage, or the stool. Only the supernatant from the excreta (urine), and ODN present within this, was measured. It may also be possible that some of the excreta was not collected accurately, hence underestimating the excretion of free-ODN via this route. Once again, the small numbers of birds per group makes estimation difficult.

Being particles rather than molecules, liposomes can only leave the circulation at sites where there are relatively large gaps or "fenestrations" between the endothelial cells lining the blood vessels. Such a fenestrated endothelium is characteristic of the hepatic and splenic sinusoids and, to a lesser extent, the capillaries of lymphoid organs and bone marrow. Most liposome formulations are rapidly cleared (within minutes) from the bloodstream and accumulate within the liver and spleen (Juliano & Akhtar 1992).

In this study, autoradiography of thin sections of the liver was performed to demonstrate whether the liposome-ODN was preferentially targeted to any specific cell type, particularly Kupffer cells. Kupffer cells could not be identified by light microscopy; however, the distribution of entrapped ODNs within the liver was uniform and not concentrated around any particular cell type. In future studies, this important question can best be resolved by specifically identifying relative uptake within duck Kupffer cells and hepatocytes, for example by using a monoclonal antibody to duck reticuloendothelial cells, with or without electron microscopy.
It was not possible to assess whether the ODN detected in the tissues and urine remained intact, or was partially degraded. An enzymatic method was used for labelling the ODN, allowing only small quantities to be labelled. ODN was undetectable by analysis of urine, liver and plasma samples on a 20% PAGE/7M urea gel soon after the birds were sacrificed to determine the size of the ODN (data not shown). However, other similar studies using free-ODN in mice, in which each ODN molecule was labelled, have shown that the molecule is degraded after several hours in the liver and kidney, although it remains intact \textit{in vivo} in plasma for many hours (Agrawal \textit{et al}, 1991; Zhang \textit{et al}, 1995). Another limitation of this study was the small number of ducks used, which did not permit detailed pharmacokinetic analysis of mean distribution and elimination half-life for the 2 forms of delivery.

4.5 Conclusion

In summary, this study demonstrated that the 18-mer PS-ODN complementary to the pre-S gene of DHBV is stable in duck plasma, and that intravenous injection of this ODN entrapped within liposomes enhances delivery of the ODN to the liver. There are two potential advantages to this increased hepatic delivery in clinical practice: firstly, lower doses can be used thus reducing the high production costs of these synthetic agents, and, secondly, there may be a reduced potential for unwanted side-effects because of dose reduction and smaller quantities reaching non-target sites. Thus, there seems to be a reasonable possibility for the application of liposome technology for the delivery of antisense oligonucleotides. An obvious application may be for delivery of oligonucleotides useful in therapy of intracellular infections, such as HBV and HIV. The next step would be to use this agent in chronically-infected ducklings to assess whether a lower dose of liposome-ODN can achieve the same or better efficacy on suppression of DHBV replication. In the following chapters, these possibilities will be explored in more detail.
5.1 Introduction

The PLC/PRF/5 cell line is derived from the tumour of a 24 year old Shangaan male from Mozambique with hepatocellular carcinoma (Alexander et al., 1976). The genome of the PLC/PRF/5 cells contains several copies of integrated HBV DNA (Rivkina et al., 1988), and the cells produce HBsAg similar in size, morphology, and polypeptide composition to the form that occurs in the serum of infected individuals. The HBsAg retains its immunoreactivity as judged by radioimmunoassay and reverse passive haemagglutination.

The constituent expression of HBsAg from this cell line and the simple biochemical composition of this immunoreactive antigen produced by these cells make them an attractive model to study the ability of antisense ODNs to inhibit the expression of the gene for the HBsAg. Serial medium samples removed from the cultures show a progressive accumulation of HBsAg during growth and maintenance (Copeland et al., 1980), and the cells are not known to harbour any transmissible endogenous or adventitious agents (Tabor et al., 1981).

The PLC/PRF/5 cell line has been used to study antisense ODNs inhibition of the expression of the HBsAg gene. Goodarzi et al. (1990) investigated the effect of a series of antisense ODNs on the expression of the HBsAg gene in this cell line. Initially, they chose five target sites for antisense ODNs in the region of the pre-S and S genes of the HBV genome and synthesised 12- to 15-mers of ODNs complementary to their mRNA. These sites included the cap site of the mRNA transcribed from the SPII promoter (cap site/SPII), a region inside the pre-S2 gene (inside/pre-S2), the translation initiation site of the S gene (initiator/S gene), a region about 10 bases downstream of the initiation site of
the S gene (inside I/S gene) and a region around the middle of the S gene (inside II/S gene). As a control, two ODN with random sequences were also used; one a 15-mer and the other a 12-mer. The degree of inhibition of this series of ODNs varied depending on the target site. In particular, antisense ODNs directed against the cap site/SPII, inside/pre-S2, initiator/S gene and inside I/S gene showed a similar and high degree of inhibition of as much as 96%. In sharp contrast, the antisense ODN directed against the inside II/S gene showed essentially no inhibitory effect. Both control ODNs had little effect on the expression of HBsAg.

This group of investigators also showed that increasing amounts of ODN directed against the initiator/S gene resulted in increased inhibition of the expression of HBsAg. Furthermore, at similar concentrations, phosphorothioate ODNs had a greater inhibitory efficiency than phosphodiester ODNs of the same length and sequence. Based on these findings, it was planned to study the effect of some of the same antisense ODNs on the inhibition of HBsAg expression in the PLC/PRF/5 cell line, using PS-ODNs instead of phosphodiester ODNs, and using liposomes in an attempt to improve cellular delivery and enhance the inhibitory effect.

5.2 Materials and Methods

Three ODNs used in the study by Goodarzi et al. (1990) were used in all the in vitro studies. The two antisense ODNs were directed against the cap site from the SPII promoter (anti-pre-S2) and against the translation initiation site of the S gene (anti-S). The target regions of the genome, sequences and complementary sites were as follows:

<table>
<thead>
<tr>
<th>Function</th>
<th>Sequence (5' to 3')</th>
<th>Complementary site*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cap site/SPII</td>
<td>GATGACTGTCTCTTTA</td>
<td>3188 to 3202</td>
</tr>
<tr>
<td>Initiator/S gene</td>
<td>GTTCTCCATGTTGG</td>
<td>149 to 163</td>
</tr>
<tr>
<td>Non-complementary</td>
<td>TTGCCGAGCGGGGTA</td>
<td>--</td>
</tr>
</tbody>
</table>

*Corresponds to numbering published by Ono et al (1983)

All ODNs for the in vitro studies were synthesised by Oswel DNA Service, University of Edinburgh, U.K. Several experiments were performed using different liposome preparations, different ODN preparations and different study designs. However, all the
experiments used the same 3 ODN sequences shown above. The methods and results of each experiment are presented together, with a discussion at the end of each section.

5.3 Free versus liposome-entrapped ODNs

In this experiment, the 3 ODNs were used in a 24-well microtitre plate in a free form, or entrapped into positively-charged liposomes, or into neutral liposomes. The mean diameter of the liposomes was 165 nm. Cells were grown to confluence in a 24-well plate; all the medium was aspirated and stored for pre-treatment HBsAg levels. Pre-treatment levels of HBsAg were standardised by plating the cells at the same density in all wells; the levels all fell within a narrow range of 13-15 IU/ml (Fig. 5.1). The cells were then washed twice with PBS to remove any residual HBsAg. Fresh medium containing antisense or non-complementary ODN in the free or entrapped form was then added to the cells in a final concentration of 4.4 μM, based on the available amount of entrapped ODN. Untreated control wells were incubated with DMEM containing 10% FBS only.

Some medium (=1 ml) was removed daily for 5 days thereafter, and replaced with fresh medium after each aspiration. The removed medium was stored at -20°C. At the end of the treatment phase, all the medium and cells were stored at -20°C. Each observation was carried out in duplicate. HBsAg was measured at a 1:10 dilution from samples taken on day 0 (pre-treatment), day 3 and day 5 post-treatment, together with known standards, on the same ELISA plate. As this was a preliminary experiment, measurements on other days were not performed. Figure 5.1 shows the response to treatment with the ODNs in a graphic form, showing the 3 treatment groups separately. Each data point is a mean of 2 values; SEM is not shown because there was much overlap between the error bars, which obscured the data points. With the free-ODN treated group and the control untreated wells, there was a decline in levels with all the ODNs used. A similar decline was seen with positively-charged and neutral liposomes, although the effect was not as marked with the neutral liposomes. With the neutral liposomes there was some increase in HBsAg levels at the end of the treatment period. It would have been more accurate to express the antigen level in IU per number of cells, but the cell density and viability was not measured in any of the in vitro experiments performed.
Figure 5.1: Inhibitory effects of antisense ODNs on HBsAg before treatment (day 0), and
days 3 and 5 after treatment using free-ODNs, ODNs entrapped within positively-charged
liposomes (+ Liposomes) and within neutral liposomes (N-liposomes). #8165 is against the
Cap site/SP2, #8166 against the Initiator/Gene S, and #8167 is non-complementary to any
region of the HBV genome. Controls are untreated wells.
The decrease in levels with the non-complementary ODN is suggestive of non-specific activity with PS-ODN. However, the decrease in the controls may be due to contact inhibition of growth after the cells reached confluence. As shown later in figure 5.3, the cumulative levels of HBsAg in all untreated wells reached a plateau after 3 days, suggesting no new secretion of HBsAg. Therefore, no firm conclusions can be reached from the results of this preliminary experiment.

5.4 LIPOFECTIN to enhance cellular uptake of antisense oligodeoxyynucleotides

Lipofectin® reagent (Life Technologies, Paisley, Scotland) is a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), and dioleoyl phosphotidylethanolamine (DOPE) in membrane filtered water, which forms 250 nm liposomes. It is suitable for the transfection of DNA into cells. Lipofectin reagent reacts spontaneously with DNA to form a lipid-DNA complex. The fusion of the complex with cells results in the efficient uptake and expression of the DNA. DNA is thought to bind initially to the surface of the liposome by ionic interactions. The DOTMA-containing vesicles interact with the cell membrane, possibly by ionic interactions. In the case of conventional liposomes (DRVs), the ODNs need to be encapsulated before addition to cells and may be restricted to only a few cell types, such as phagocytic cells. With Lipofectin the nucleotide material is not entrapped into the liposomes, but is complexed by ionic interaction between the negative charges of the phosphate groups on the nucleotide chain and the positive charges present at the surface of the cationic liposomes (Felgner et al, 1987). No pre-formulation of the liposome with the ODN is required with Lipofectin.

DOTMA-containing vesicles have the advantage that ODNs can be introduced into large numbers of cells. In one study (Bennett et al, 1992), DOTMA-containing lipid vesicles not only enhanced the rate of ODN uptake into cells but also markedly changed the subcellular distribution of the ODN, localising the ODN in the cell nucleus. The main disadvantages of DOTMA are toxicity in high doses and markedly decreased activity of the liposomes in the presence of serum. The toxicity varies with the type of cell, the duration of exposure to DOTMA, and the density of the cell culture. DOTMA increased by at least 1000-fold the potency of an antisense ODN that hybridised to the AUG translation initiation codon of human ICAM-1 in a human umbilical vein endothelial cell
Successful transfection with Lipofectin is dependent on the optimisation of transfection conditions for each cell type. This depends on the optimal amount of Lipofectin reagent, DNA or ODN concentration, incubation time of Lipofectin reagent-DNA complexes with cells, and cell density at the time of transfection. The manufacturers recommend starting with 1-2 μg DNA for 35 mm culture dishes, and a 6 hour incubation time (range 2-24 hours). The optimal amount of Lipofectin suggested is 2-25 μl (equivalent to 2-25 μg), and the cells should be about 40-60% confluent at the time of transfection.

**5.4.1 Procedure for transfection of cells with Lipofectin**

Cells were seeded at a density of ~1-2 x 10^5 cells/ml in DMEM supplemented with 10% FBS onto a Falcon 24-well flat bottom tissue culture plate (Becton Dickinson, New Jersey); growth area 1.88 cm², diameter 15.5 mm. In later experiments a 96-well plate was used (growth area 0.32 cm², volume 0.36 ml). The cells were grown to 40-60% confluence, usually after 18-24 hours at 37°C in 5% CO₂. For each transfection, the amount of ODN to be used was diluted into 100 μl in serum-free medium (Opti-MEM I Reduced serum medium, GibCo BRL). For each transfection, the amount of Lipofectin (1 mg/ml stock solution) to be used was diluted separately into 100 μl serum-free medium. The 2 solutions were combined, mixed gently, and incubated at room temperature for 10-15 min. The solution became cloudy after mixing.

Serum-containing medium was aspirated from the cells, and each well was washed twice with serum-free medium. For each transfection, 0.8 ml serum-free medium (without antibacterial agents) was added to each tube containing the Lipofectin reagent-ODN complexes. This was mixed gently and the complexes were overlaid onto the cells. The plates were then incubated for 5-24 hours at 37°C in a CO₂ incubator. Thereafter, the serum-free medium containing ODNs was removed and replaced with normal growth medium containing serum and antibacterial agents. The plates were then incubated at 37°C. The medium was changed daily; the old medium was stored at -20°C for analysis. At the end of the treatment period, cells were scraped off the plates, frozen in liquid nitrogen and stored at -70°C. The amount of ODN that was transfected into the cells and
the amount remaining in the medium could not be assessed, as the ODNs were not labelled with a radioactive label.

5.4.2 Initial experiments with Lipofectin

Initial experiments were performed to obtain an estimate of the amount of Lipofectin, the amount of ODN, and the transfection time required to observe an inhibition of HBsAg production. In the preliminary experiments, free ODNs were not used as a control; wells treated with Lipofectin-ODN were compared to untreated control wells, which were first incubated with serum-free medium for the same duration as the other wells before changing the medium.

Firstly, 2 pg of ODN (manufacturers' recommendation) (~0.39 μM) was transfected with 6 μg of Lipofectin. The cells were incubated with the ODN and Lipofectin for 18 hours, after which the serum-free medium was aspirated and replaced with fresh serum-containing medium with antibiotics. The medium was changed daily until the end of 5 days from the time of transfection. Thereafter, 2 μg of ODN was transfected with 10 μg of Lipofectin for 16 hours before changing the medium. The duration of the experiment and the methodology was the same as the first. All the above transfections were performed in duplicate. Both these experiments showed no significant inhibition of HBsAg production when the levels were measured in the medium on days 3 and 5 after treatment.

In the next experiment, it was decided to use higher doses of both ODNs and Lipofectin. A dose of 10 μM ODN with 20 μg Lipofectin was tried in triplicate. Transfection of ODNs was allowed to proceed for 16 hours before changing the medium. At the end of 16 hours, the cells in all the wells treated with Lipofectin were non-adherent and dead. Cells in the untreated control wells were adherent and alive. Thus, it was likely that this dose of Lipofectin was toxic for the cells. Although the dose of ODN was much higher than that used in the 2 initial experiments, it was unlikely that this dose was too high, resulting in toxicity to the cells. In the study by Goodarzi et al (1990), higher concentrations of ODN were used to achieve inhibition of HBsAg without toxicity.
In the next experiment, 10 μM ODN was used with 10 μg Lipofectin and transfected for 6 hours. Untreated control wells were treated in the same way as the treated wells, with serum-free medium for 6 hours before replacement with serum-containing medium. In addition, to control for any toxic effect of the Lipofectin, one set of wells was treated with Lipofectin only without any ODN. All experiments were performed in triplicate. The medium was changed daily until the end of the 5th day. No cellular toxicity was observed on light microscopy. HBsAg levels in the medium were measured on day 0 (before addition of ODN), 3 and 5. The results are shown graphically in Figure 5.2.

![Graph showing HBsAg levels](image)

**Figure 5.2** Effect on HBsAg levels in medium from 2 antisense and a non-complementary ODN complexed with Lipofectin before treatment (day 0), days 3 and 5 after treatment. Control wells were untreated and Lipofectin only wells were not treated with any ODN. Each data point represents the mean (±SEM) of 3 wells. Anti-S gene is against the Initiator/S gene and anti-pre-S2 is against the Cap site/SPII.

HBsAg levels in the medium continued to rise in the control untreated wells, in the Lipofectin-treated wells, and those treated with the non-complementary ODN. By contrast, levels fell in wells treated with each of the antisense ODNs. With the ODN against the cap site/SPII (anti-pre-S2), the levels fell by day 3 and continued to fall until day 5. In the wells treated with the ODN against the Initiator/S gene, the levels fell by day 3 but had risen again by day 5.
The results from this experiment, although preliminary, were encouraging, as an inhibition of the target protein was observed with the 2 antisense ODNs but not with the non-complementary ODN. Also, information was obtained about the dose of Lipofectin and ODN required to achieve a therapeutic effect, and an estimate of the transfection time.

5.5 Comparison of free-ODN, Liposome-ODN and Lipofectin-ODN

An experiment was performed to compare the effect of liposome versus Lipofectin delivery of antisense ODNs to PLC/PRF/5 cells to inhibit HBsAg production. Free ODNs were also used. Control wells were treated with either medium only, empty liposomes, Lipofectin only, or serum-free medium.

5.5.1 Materials and Methods

Cationic liposomes were prepared as dehydration-rehydration vesicles (DRVs) from PC: DOPE: SA in a molar ratio of 16: 16: 4. The concentration of ODN used was 10 μM in all ODN-treated wells, since the previous experiment showed that this dose could achieve a desirable biological effect. Cells were seeded at a concentration of \(-1 \times 10^5/\text{ml}\) and grown to 80% confluence in two 24-well plates, the medium was then aspirated and the cells washed twice with PBS. All experiments were performed in triplicate and the medium was made up to a total volume of 1 ml with the treatment. For the LPF-treated wells, 10 μg of Lipofectin was used, and the transfection time was 6 hours, after which serum-free medium was replaced with serum-containing medium.

Cells were treated for 5 days without a change of medium. After every 24 hours, 20 μl of medium was aspirated and diluted to 200 μl in PBS. This was stored at -20°C until analysis of HBsAg by EIA. At the end of the 5th post-treatment day all the medium was aspirated and stored. To assess cell viability and cytotoxicity, the incorporation of \(^{35}\text{S}\)-methionine was measured at the end of the experiment. The method is described briefly below (Freshney 1992).

After all the supernatant was aspirated on the final day, the cells were washed twice with PBS. They were incubated for 4 hours at 37°C in DMEM without glutamine or L-methionine. After 4 hours, the medium was removed and discarded, and the cells were
washed twice with PBS. 10 μCi [\( ^{35} \text{S} \)]-methionine (specific activity 40-500 mCi/mmol) (Amersham, U.K.) was added to each well in a total of 1 ml of methionine-free DMEM. After overnight incubation (~16 hours) at 37°C, the medium was removed and discarded.

The cells were fixed with methanol for 10 min, the methanol discarded and the cells air-dried at room temperature. After the cells had been washed gently with cold PBS the plates were placed on ice. The monolayers were then washed for 10 min in ice-cold 10% trichloroacetic acid (TCA) to remove the unincorporated \( ^{35} \text{S} \), followed by 2 further 5 min washes. The TCA was washed off with methanol and the monolayer air-dried. The fixed cells were treated with 0.5 ml 0.3N NaOH/1% SDS and left at room temperature for 30 min to solubilize the protein. The contents of each well was then mixed well and transferred to a scintillation vial, 10 ml liquid scintillation fluid (Cocktail T) added, and counted on a β-counter (Kontron Betamatic V) for 2 min.

5.5.2 Results and Discussion

The medium in the wells treated with liposome-entrapped ODNs (DRVs) and empty DRVs appeared cloudy and turbid during the experimental period. Many of the cells from these wells detached with time and appeared to die. HBsAg levels were measured on day 0 (pre-treatment), days 1, 3 and 5 post-treatment. The HBsAg response to the different treatments is shown in Figure 5.3. The incorporation of \( ^{35} \text{S} \)-methionine is shown in Figure 5.4 as a mean ± SEM.

The results show that, in the untreated control wells, HBsAg levels continued to rise over the 5 day period, with a peak at 72 hours, before reaching a plateau. The liposome-entrapped ODNs appeared to have a dramatic effect on HBsAg secretion, reaching undetectable levels from the first post-treatment day. However, incubation of the cells with empty liposomes produced an identical effect. This observation, coupled with the fact that the cells in these wells were detached, indicates a toxic effect from the DRVs; thus the reduction in the level of HBsAg was at least partly due to cell death. A trypan blue exclusion test would have confirmed this suspicion. The possible sources of toxicity could include the positive charge conferred by the stearylamine, the size of the vesicles, or impurities in the preparation. The cloudiness of the medium could indicate aggregation of lipids, which might have been in a very high concentration. A similar toxic effect was not observed in the wells treated with Lipofectin reagent, which contains
DOTMA to confer the positive charge, and is prepared commercially. However, the cells treated with liposome-entrapped ODN did not all die, because there was \textsuperscript{35}S methionine incorporation by cells in these wells.

The non-complementary ODN showed the same or greater degree of HBsAg inhibition in the medium as the antisense ODNs did. This similarity suggests a non-specific effect of the PS-ODNs, possibly due to the highly negative charge of these molecules, indicating that the inhibition observed with the antisense ODNs was not sequence-specific. Radiolabelled amino acids were shown to be incorporated into cells from all the wells. Paradoxically, cells incubated with liposome-ODN incorporated the \textsuperscript{35}S-methionine most avidly, although these appeared to be the least viable on light microscopy. The untreated control wells showed the lowest incorporation of the radiolabelled amino acid. These results indicate that certain liposome preparations may be toxic to cells \textit{in vitro}. No definite advantage of Lipofectin-ODN over free-ODN was observed. The inhibition of HBsAg secretion into the medium was not sequence-specific, as it was also observed with the non-complementary ODN. This could, however, be related to the purity of the oligonucleotide preparations used, or the highly negative charge of the phosphorothioate moiety. The fact that charged ODNs are polyanions is frequently overlooked (Stein & Cheng 1993).
Figure 5.3: HBsAg levels in medium after cells were incubated with either free-ODNs, liposome-entrapped ODNs, or ODNs complexed with Lipofectin. Controls were untreated wells, Lipofectin alone wells were not treated with any ODN, empty liposomes contained no ODN, and serum-free medium was treated with Opti-MEM for 6 hours. #1521 is against the initiator/S gene, #1522 is against the cap site/SPII, #1865 is a non-complementary ODN. All data points are a mean of 3 experiments ± SEM.
Incorporation of $[^{35}\text{S}]$-Methionine Into Cells after 5 Days Incubation

![Bar graph showing counts/min from $[^{35}\text{S}]$-methionine incorporated into cells for the synthesis of new protein at the end of 5 days incubation with ODNs or various controls. Each bar is the mean ± SEM of 3 replicates. F = free, L = liposome-entrapped, LPF = Lipofectin. #1521 is against the initiator/S gene, #1522 is against the cap site/SPII, #1865 is a non-complementary ODN.]

5.5.3 Control experiment for $[^{35}\text{S}]$-methionine incorporation

The reasons for the high level of incorporation of $[^{35}\text{S}]$-methionine could be that the cells were starved of methionine for too long (4 hours), coupled with the long incubation time (16 hours) with the labelled amino acid. To determine whether this was the case, a control experiment was designed with control untreated cells to compare cellular incorporation of radiolabelled amino acid to the amount of residual radioactivity in the medium. Cells were plated again onto a 24-well plate in the same density as previously. After incubation for 5 days in DMEM with 10% FBS (with no ODNs), the medium was discarded. The monolayer was washed in an identical way with PBS, and incubated with methionine-free medium for 4 hours. After discarding this medium and washing the cells, 4 wells were incubated with 10 μCi $[^{35}\text{S}]$-methionine in methionine-free medium for ~16 hours. The supernatant was aspirated and stored for measurement of radioactivity, and the cells were treated in the same way as previously (fixed with methanol, cold-precipitated with 10% TCA and lysed with 0.3N NaOH/1% SDS). The radioactivity in each of the cell monolayers was compared with its respective supernatant. The results are shown in Table 5.1.
Table 5.1: Radioactivity in cells (precipitate) and supernatant after incubation with 10 μCi \([^{35}\text{S}]\)-methionine in methionine-free medium.

<table>
<thead>
<tr>
<th>Well No.</th>
<th>Precipitate (cpm)</th>
<th>Supernatant (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 070 480</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1 063 323</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>967 167</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>962 473</td>
<td>0</td>
</tr>
</tbody>
</table>

\([^{35}\text{S}]\)-methionine could not be detected in the medium after incubation with the cell monolayer for 16 hours. Thus, all the available methionine was incorporated into the cells for protein synthesis. This confirmed that the cells were starved for too long and/or exposed to the radioactive mixture for too long, resulting in an avid uptake of the amino acid. The measurement of protein synthesis by this method was not a reliable indicator of cell viability in this study. Therefore, other methods were used in subsequent experiments.

5.6 Dose-response experiments with HPLC-purified free- and Lipofectin-oligodeoxynucleotides

5.6.1 Introduction

One of the reasons for the non-sequence-specific effects of antisense PS-ODNs, especially with \textit{in vitro} studies, relates to the purity of the synthetic ODNs (Cantin & Woolf 1993; personal communication. Prof. Tom Brown). The ODNs used in the previous experiments were not purified by high performance liquid chromatography (HPLC). Purification of ODNs synthesised on solid phase supports is necessary for most purposes since these ODNs are contaminated by shorter ODNs, due to incomplete reaction within each cycle by ODNs still carrying protecting groups owing to incomplete deprotection at the end of the synthesis (Pingoud \textit{et al.}, 1989). Contamination can also occur by ODNs that have undergone side reactions, in particular depurination due to the acid treatment for the removal of the 5'-protecting group at the end of each cycle. Since in solid phase synthesis, intermediates are not purified during chain assembly, erroneous products accumulate and must be removed after synthesis. This is best achieved conveniently and effectively by HPLC. Batch variations in oligomer purity are another
source of non-specific effects, and contamination with cytotoxic trityl ammonium ions is known (Cantin & Woolf 1993).

In order to determine the effect of a range of doses necessary to achieve inhibition of HBsAg production, a dose-response experiment with free ODNs was performed. This would provide information about the minimum dose required to achieve inhibition of the target protein, and how the inhibition varied with variation in the dose. In the same experiment, Lipofectin was also used to transfect cells, using the same doses as the free-ODNs.

A control protein secreted into the medium by the PLC/PRF/5 cells was used for comparison: this would provide information about the specificity of the inhibition of HBsAg. PLC/PRF/5 cells secrete several human plasma proteins into the medium; these include transferrin, caeruloplasmin, \( \alpha_1 \)-antitrypsin, \( \alpha_2 \)-macroglobulin, plasminogen, complement C3 and \( \alpha_1 \)-acid glycoprotein and \( \alpha \)-fetoprotein (Knowles et al, 1980).

Initially, transferrin was measured in the medium from control wells at the end of some of the previous experiments to determine whether levels in the medium were high enough to be detected by a routine assay. Transferrin was the control protein measured in the original study by Goodarzi et al (1990); in that study it was measured by ELISA using polyclonal antibody raised against purified human transferrin. A radial immunodiffusion (RID) assay for human transferrin was tried (NOR-Partigen-Transferrin, Behring, Germany), as an ELISA was not available. The RID plate consists of 5 \( \mu \)l wells embedded in agarose containing monospecific antiserum to human transferrin in the agarose gel layer. Ten samples of medium from different control wells were tried, and compared to human serum from 2 patients (positive controls). After incubation for more than 48 hours at room temperature, no precipitin rings were seen from any of the 10 test samples of medium incubated with cells. Both samples of human serum showed distinct precipitin rings. Thus, this assay was not sensitive enough to detect transferrin from the supernatant of PLC/PRF/5 cells.

Attempts to measure caeruloplasmin levels in the medium by a radial immunodiffusion kit (Bindarid, Birmingham, UK) were also unsuccessful because of undetectable levels. \( \alpha \)-antitrypsin detection by turbulometry also showed no detectable levels in the
medium. Although α-fetoprotein (AFP) is not secreted in large quantities into the cell culture medium (Knowles et al., 1980), detection of this was achieved in samples using the AMERLEX-M AFP RIA kit (Johnson and Johnson, Amersham, UK). This assay showed easily detectable quantities of AFP, with levels increasing after a few days incubation. Therefore, AFP was used as the control protein secreted into the cell culture supernatant.

5.6.2 Materials and Methods

Experiments were performed in a flat-bottom 96-well plate (growth area 0.32 cm², volume 0.36 ml) (Falcon Primaria, Becton Dickinson, UK). Cells were seeded at a density of ~1 x 10⁷/ml. Each of the 3 ODNs was used in doses of 5, 10, 15 and 20 μM, in the free form and complexed with Lipofectin, and each treatment was in triplicate. Four wells were untreated controls incubated with DMEM only and 4 with Lipofectin only. All treatments were mixed in a total volume of 200 μl.

After seeding overnight, cells were ~70% confluent. The medium was removed and discarded. Cells were washed once with PBS, and a second time with PBS for the free-ODN-treated wells, and serum-free medium for the Lipofectin-ODN-treated wells. The amount of Lipofectin used for transfection was 2 μg in 200 μl (equivalent to 10 μg in 1 ml). The Lipofectin was allowed to complex with the ODN for 15 min at room temperature. The cells were incubated for 6 hours at 37°C in 5% CO₂. After this period the medium was removed from all the wells and replaced with fresh DMEM with 10% FBS. Therefore, the transfection time was the same for both free- and Lipofectin-ODNs. Twenty μl of medium was removed 24, 48, 72 and 96 hours after addition of the ODNs and diluted to 200 μl in PBS. This was stored at -20°C until HBsAg was assayed.

After 96 hours all the medium was aspirated and stored. The cells were washed twice with PBS and trypsinised with 20 μl 0.25% Trypsin/0.02% EDTA for a few minutes. The trypsin was discarded and the cells mixed well with PBS, decanted into a microfuge, and centrifuged at 8 000 rpm for 10 min. The PBS was decanted, the cell pellet snap-frozen in liquid nitrogen, and stored at -70°C. Total cellular protein was measured in the cell pellet by resuspending in PBS and using the BioRad microassay procedure (Bio-Rad Laboratories, UK), expressed as μg protein/ml. Alpha-fetoprotein was measured as a control protein from the medium aspirated after 96 hours with the AMERLEX-M AFP
RIA kit. HBsAg was assayed from all the stored samples in a dilution of 1:10 (20 µl medium into 200 µl PBS).

5.6.3 Results

Figure 5.5 shows the levels of HBsAg in culture medium in response to the free- and Lipofectin- non-complementary ODN. Figure 5.6 shows the response to the ODN against the cap site/SP II, and Figure 5.7 to the Initiator/S gene ODN. Each data point represents the mean (± SEM) for 3 values. As this experiment was designed to show the cumulative increase in HBsAg over time, or its inhibition, the first measurement of HBsAg was taken 24 hours after addition of ODNs to the cells.
Figure 5.5: Effect of 5, 10, 15 and 20 μM non-complementary free- and Lipofectin-ODN on HBsAg concentration in culture medium. LPF = Lipofectin.

Figure 5.6: Effect of 5, 10, 15 and 20 μM free- and Lipofectin-ODN against the cap site/SPII of the HBV genome on HBsAg concentration in culture medium. LPF = Lipofectin.
Control wells treated with DMEM only or Lipofectin followed by DMEM showed the expected daily rise in HBsAg in the culture medium. The non-complementary ODN, either in the free form or complexed with Lipofectin, caused no inhibition of HBsAg, although the levels were lower than the respective controls (Fig. 5.5). No dose effect was seen with the non-complementary ODN; all four doses produced the same effect on HBsAg levels.

The greatest inhibition of HBsAg was seen with the antisense ODN against the translation initiation site of the S gene (initiator/gene S) (Fig. 5.7). However, the free antisense ODN caused greater inhibition than the Lipofectin-ODN, with the 20 μM dose causing significantly greater inhibition than the 5 μM dose. By contrast, the dose-response curve of the Lipofectin-ODN was similar to the non-complementary ODN. The antisense ODN directed against the cap site from the SPH promoter showed some inhibition of HBsAg secretion, which did not show a dose-related response, and was also not as profound as the anti-gene S ODN. With Lipofectin transfection, all the doses had an almost identical effect. To control for non-specific effects from the PS-ODNs, α-fetoprotein levels in the medium per microgram of total cellular protein were measured for each well and compared to untreated control wells. The results are shown in Table 35.
5.2. None of the treated wells showed levels of α-fetoprotein which were lower than the control wells. However, most wells had much higher levels than the controls.

Table 5.2: Alpha-fetoprotein values in culture medium (ng) per microgram of total cellular protein. Results are expressed as the mean ± SEM for 3 experiments; untreated and Lipofectin treated values are the mean of 4 experiments.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Free (ng/µg cell protein)</th>
<th>Lipofectin (ng/µg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td>27 ± 2</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>Non-complementary ODN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µM</td>
<td>164 ± 81</td>
<td>314 ± 132</td>
</tr>
<tr>
<td>10 µM</td>
<td>50 ± 10</td>
<td>184 ± 88</td>
</tr>
<tr>
<td>15 µM</td>
<td>51 ± 21</td>
<td>225 ± 84</td>
</tr>
<tr>
<td>20 µM</td>
<td>32 ± 3</td>
<td>234 ± 137</td>
</tr>
<tr>
<td>Anti-preS2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µM</td>
<td>70 ± 32</td>
<td>221 ± 96</td>
</tr>
<tr>
<td>10 µM</td>
<td>101 ± 49</td>
<td>201 ± 102</td>
</tr>
<tr>
<td>15 µM</td>
<td>75 ± 43</td>
<td>182 ± 54</td>
</tr>
<tr>
<td>20 µM</td>
<td>40 ± 12</td>
<td>177 ± 78</td>
</tr>
<tr>
<td>Anti-S gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µM</td>
<td>64 ± 18</td>
<td>180 ± 143</td>
</tr>
<tr>
<td>10 µM</td>
<td>313 ± 223</td>
<td>99 ± 78</td>
</tr>
<tr>
<td>15 µM</td>
<td>201 ± 82</td>
<td>48 ± 4</td>
</tr>
<tr>
<td>20 µM</td>
<td>225 ± 121</td>
<td>156 ± 67</td>
</tr>
</tbody>
</table>

5.6.4 Discussion
These experiments used HPLC-purified ODNs to inhibit HBsAg production and secretion into the culture medium. The results showed a marked difference between the more potent of the 2 antisense ODNs, and the non-complementary sequence, in inhibition of HBsAg production. However, the HBsAg production with the non-complementary ODN did not rise to the same extent as the untreated controls. The inhibitory effect of the anti-S gene ODN was greater as the dose was progressively increased. This differed from the results of earlier experiments performed. The progressive increase in HBsAg levels with the non-complementary ODN, together with the absence of a fall in the control protein secreted into the medium, indicates that nonspecificity was not a major confounding variable with the HPLC-purified antisense ODNs. Hence, the inhibition was probably through an antisense mechanism. In the study by Goodarzi et al (1990), the most potent of all the ODNs tested was also the one
directed against the region of the initiation site of the S gene, with a dose of 17.4 μM causing a 96% inhibition of HBsAg in the medium.

The effect of the antisense ODN was maintained for at least 96 hours after a single dose, indicating the potency of these molecules in blocking gene expression. The effective dose range of free antisense ODN was between 10 and 20 μM, with 20 μM blocking production of the target protein almost completely. The need for pure ODNs for \textit{in vitro} experiments has been recognised previously, as the cells are prone to direct cytotoxic damage from impurities in the preparation (Cantin & Woolf 1993). Batch variations in oligomer purity may also be a problem, and contamination with cytotoxic triethyl ammonium ions is known. However, a high level of purity implies a greater production cost and a lower yield. This is not practical for \textit{in vivo} or clinical therapy.

The most surprising finding of this experiment was the lack of enhanced efficacy in the inhibition of HBsAg production with the antisense Lipofectin-ODN. One would have expected cellular delivery and biological efficacy to improve markedly, partly because of the ionic interaction between the cationic DOTMA and the anionic cell membrane. It may be that the amount of Lipofectin used for the transfection was too small (2 μg). Even then, the effect should have been at least the same as the free-ODN. However, Lipofectin has not been universally successful in effecting cellular delivery. It has been found previously with HL60 cells, that DOTMA was ineffective at increasing uptake of antisense ODNs (Stein 1992). Furthermore, Lipofectin may also be toxic to cells and have non-sequence specific effects on protein translation.

Attempts to detect and measure intracellular HBsAg and mRNA for HBsAg were not made, as this was beyond the scope of this study. However, levels of mRNA, measured by hybridization and densitometry, were unchanged in the study by Goodarzi \textit{et al.} (1990). A fall in intracellular HBsAg levels with antisense therapy would be specific for a mechanism causing interference in intracellular synthesis of the target protein. Lack of significant change in the mRNA levels in untreated control cells, with a concomitant fall in intracellular HBsAg levels, would indicate that the inhibition of HBsAg expression was at the level of translation and not transcription. It would also prove that synthesis of new HBsAg, rather than secretion, was inhibited.
Another unusual finding was the marked variability in the levels of α-fetoprotein per μg of cellular protein, which was in many wells much higher than in the medium from the untreated control cells. This could be due either to a high level of AFP in the medium or a low value for the total cellular protein. In most cases, this was due to a low value for the total cellular protein. It is possible that, since newly synthesised HBsAg by PLC/PRF/5 cells forms a large part of the intracellular protein prior to secretion into the medium, inhibition of its production would cause a concomitant decrease in the total amount of cellular protein. Alternately, the variation in AFP secretion could be a natural variation from cell to cell. Cell viability, using trypan blue exclusion, was not measured in this study; the levels of α-fetoprotein per μg of cellular protein per 10⁴ viable cells would have been a more accurate representation of the effects of the ODNs on control protein production and secretion.

The fact that a biological effect was observed with an antisense ODN implies that the molecules were reaching their intracellular target, either nuclear or cytoplasmic. However, whether the Lipofectin-ODN reached their intracellular targets remains questionable, because a biological effect was not observed. The next experiment was designed to answer the question of cellular uptake with the 2 different modes of cellular delivery.

5.7 Cellular uptake and localisation of free- and Lipofectin-ODNs

Since the biological efficacy with the Lipofectin-ODN was less than the free-ODN, it was decided to investigate the cellular uptake of these 2 forms of delivery by using fluorescein-labelled ODNs and comparing the cellular uptake and localisation with fluorescent microscopy.

5.7.1 Materials and methods
The method used to test for cellular uptake and fluorescence microscopy was adapted from Bennett et al. (1992). PS-ODNs were obtained from Oswel DNA Service using the same 3 sequences that were used in all the previous in vitro experiments. The ODNs were fluorescein-labelled at the 5’ end of each molecule, and purified by HPLC. PLC/PRF/5 cells were seeded onto tissue culture chambers on a Permanox glass
microscope slide (Lab-Tek®, Nunc Inc., Naperville, Ill) at a density of \( \sim 1 \times 10^5 \) cells/ml. After overnight incubation the cells were 50-60% confluent.

ODNs were prepared at a final concentration of 10 µM in 750 µl total medium, either as free- or complexed with Lipofectin, using the same procedure as the previous experiment. A higher volume of total medium was used because the volume of the cell culture chamber was more than the volume of a 96-well plate; however, the molar concentration was the same as the previous experiment. Free-ODNs were mixed with DMEM and Lipofectin-ODNs were mixed with serum-free medium, respectively. The amount of Lipofectin used was 7.5 µg per chamber, equivalent to the final concentration used in the previous experiment. Cell monolayers were first washed 3 times with either D-PBS or serum-free medium prior to addition of the ODNs. To control for background fluorescence, control chambers without ODN treatment were treated in an identical fashion. After ODNs were added to the chambers, the cells were incubated at 37°C in a 5% CO\(_2\) incubator for 1 hour. The chambers were then removed from the incubator, the medium aspirated, and the cells washed 4 times with D-PBS. The monolayer was fixed with 10% buffered formalin for 20 min at 25°C, after which the cells were washed a further 3 times in D-PBS and mounted in aqueous mounting medium (10% glycerol in PBS). The subcellular localisation of the fluorescein-labelled ODN was determined by fluorescent microscopy, using a fluorescent microscope (Carl Zeiss, Oberkochen, Germany). Photography was performed using Fujichrome 1600 RSP 135 film.

5.7.2 Results

Figure 5.8 shows the photomicrographs of the fluorescent microscopy.
Figure 5.8: PLC/PRF/5 cells incubated with free- or Lipofectin-ODN for 1 hour at 37°C in 5% CO₂ after fixing in 10% buffered formalin and washing four times with D-PBS. A, untreated control cells; B, cells incubated with free-ODNs; C, cells treated with Lipofectin-ODN. (x 40).
The control well with no fluorescein-labelled ODN showed weak diffuse background fluorescence, which could only be photographed with an increase of the exposure time to 8 sec. There was a marked difference between the appearance of the cells treated with Lipofectin-ODN and the free-ODN. The cells treated with Lipofectin-ODN had a marked increase in uptake of the label compared to the free-ODN, exhibiting bright nuclear fluorescence with some cytoplasmic staining, primarily as bright punctate structures. In the absence of Lipofectin, the ODN appeared to have localised within the cytoplasm and the periphery of the cell membrane. In the free-ODN treated cells none of the ODN appeared to reach the nucleus, producing a negative outline on microscopy (Fig. 5.8).

The nuclear localisation of both the antisense and non-complementary ODNs was increased with Lipofectin, implying that this effect was not related to the ODN sequence, or the presence of a complementary or sense sequence within the cell. The intracellular distribution of the labelled ODN was not an artefact of the fixing conditions, as the endogenous fluorescence from untreated cells was very different.

5.7.3 Discussion

Lipofectin, or DOTMA-containing lipid vesicles, not only enhanced the uptake of the antisense ODN into cells, but also markedly changed the subcellular distribution of the ODN. In the absence of Lipofectin, the ODN appeared to be associated with cytoplasmic structures consistent with endosomal or lysosomal vesicles. These findings are in agreement with other reports examining the cellular distribution of ODNs (Loke et al., 1989; Yakubov et al., 1989; Bennett et al., 1992). The major difference in the distribution of the ODN in the presence of Lipofectin was the localisation of the ODN in the cell nucleus. Using light microscopy, it was not possible to identify conclusively whether the ODNs were contained within the cytoplasmic vesicles or associated with the surface of the vesicles.

Previous studies have demonstrated nuclear localisation of phosphorothioate and phosphodiester ODNs microinjected into the cytoplasm of the cell (Chin et al., 1990; Leonetti et al., 1991; Clarenc et al., 1993). Kinetic experiments were not performed in this study, however, data from another study showed that, in the presence of Lipofectin, the ODN first enters the cell through the cytoplasm and then accumulates in the nucleus.
(Bennett et al, 1992). Using microinjected FITC-tagged free ODNs it has been shown that translocation to the nucleus is nearly complete 1 min after microinjection (Leonetti et al, 1991). In that study, fluorescence remained at a maximum level for a relatively short period of time and decreased thereafter, becoming undetectable 6-10 hours later. This could reflect ODN degradation. However, PS-ODNs were retained for a much longer time in the nucleus than the phosphodiester ODN (Leonetti et al, 1991).

Although ODNs were shown to enter the cells in this experiment, the mechanism of cellular uptake was not examined. It has been shown that free ODNs are actively transported across the plasma membrane in a temperature-dependent, saturable, and structurally specific manner via an endocytic process (Loke et al, 1989). This transport may involve binding of ODN to an 80-kDa plasma membrane protein. It is not known whether the lipid from the Lipofectin remains associated with the ODNs within the nucleus. Another advantage of Lipofectin vesicles is that ODNs can be introduced into large numbers of cells.

This study did not determine whether the ODN in the cell remained intact, or whether the fluorescence was due to free label. In a study using an $^{35}$S-labelled 28-mer PS-ODN, Gao et al (1993) investigated the uptake and distribution of the ODN by different human cells. They found that accumulation of the ODN was higher in carcinoma cells grown in monolayers than in leukaemia cells grown in suspension culture. The intracellular concentration was significantly higher (60-fold) than in the medium, and the amount accumulated was dependent on the extracellular concentration. The integrity of the $^{35}$S-labelled 28-mer PS-ODN was examined on a 15% polyacrylamide-urea gel. More than 90% of the compound in the cells and in the culture medium was found to be intact after a 72-hour incubation at 37°C. One of the cell lines used in that study was the PLC/PRF/5 hepatoma line as was used here: the ratio of nucleus/cytoplasm $^{35}$S activity in these cells after 48 hrs at 37°C was 0.3. The results from Gao et al (1993) would seem to confirm that the fluorescence observed in this study was unlikely to be from free fluorescein label degraded from the rest of the ODN, but rather from the intact molecule.

The fact that PS-ODNs appear to concentrate within the nucleus has important advantages for the mechanism of action of these molecules. PS-ODNs are known to act by stimulating RNase H activity. RNase H is a nuclear enzyme; thus the ODNs are
localised in the same cellular compartment. However, translation takes place in the
cytoplasm of the cell, rather than the nucleus; this may explain the lack of HBsAg
inhibition with Lipofectin in this experiment. Furthermore, many of the essential steps in
the replication cycle of HBV, such as nucleocapsid assembly and DNA synthesis, take
place in the cytoplasm. Therefore, the Lipofectin-ODNs may be delivered rapidly to the
wrong cellular compartment.

It is not known whether cationic lipids, in addition to enhancing and modifying
distribution of the ODNs, enhance antisense activity by increasing the rate at which the
ODN hybridises to its target mRNA. The advantage that Lipofectin has over other cell
culture delivery systems is that no pre-formulation of the liposome with the ODN is
required. However, Lipofectin cannot be used for \textit{in vivo} or clinical studies because of its
toxicity and markedly decreased activity in serum.

The reason for a lack of efficacy with Lipofectin seen in the previous experiment is not
clear, as the Lipofectin-complexed fluorescein-labelled ODNs not only entered the cells
rapidly, but markedly enhanced cellular uptake and nuclear localisation after one hour at
37°C, compared to the effects of free-ODN. It is possible that the amount of Lipofectin
used was insufficient (2 μg). The dose was calculated from the experiment which showed
an effect in the 24-well plate using 10 μg in 1 ml of medium. Batch to batch variation is
not a possible explanation, as the same supply of Lipofectin was used in all the
experiments.

\textbf{5.8 Conclusion}

The cellular uptake studies have shown encouraging results that liposomes may be useful
in increasing cellular delivery of antisense oligonucleotides. Potent inhibition of gene
expression with antisense agents was also confirmed in these \textit{in vitro} experiments, with a
dose response. This now needs to be applied to the \textit{in vivo} model, which has significantly
different properties from a cell monolayer, including the presence of phagocytic cells. In
addition, a human carcinoma cell line has rapidly dividing cells, which are easier to
transfect with DNA or ODNs than a liver with chronic hepatitis B virus in an intact
animal or a human subject.
Chapter 6

IN VIVO ANTIVIRAL STUDIES WITH LIPOSTONE-ENTRAPPED AND FREE ANTISENSE OLIGODEOXYNUCLEOTIDES

6.1 Introduction

The experimental work performed thus far showed that PS-ODNs were stable in duck plasma, were stable during liposome entrapment, and, that intravenous injection of PS-ODNs entrapped within liposomes enhanced delivery of the ODNs to the liver. It was also shown that the target sequence to the chosen ODN sequence was present in the pre-S region of the DHBV genome of the viral isolate used in ducks in this study.

In vitro, ODNs delivered complexed with cationic liposomes increased cellular delivery, although increased biological efficacy was not demonstrated. There seems to be a reasonable possibility for the application of liposome technology for the delivery of antisense oligonucleotides to the liver for chronic hepatitis B virus infection. The next step was to use this agent in chronically-infected ducks to investigate whether a lower dose of liposome-ODN could achieve the same or better efficacy on suppression of DHBV replication than free-ODNs.

6.2 High dose free versus liposome-entrapped antisense oligodeoxynucleotide inhibition of DHBV replication in vivo

The pilot study with a small dose of antisense ODN showed no reduction in serum DHBV DNA after a 5-day course of therapy. Therefore, it was decided to commence antiviral testing with a high enough dose that had proven antiviral efficacy. In the study by Offensperger et al (1993), it was found that a dosage of 20 μg/gram body weight (bw) of the same ODN was necessary to inhibit viral replication. Therefore, it was decided to try this dose to determine whether a similar effect could be achieved with free- and liposome-entrapped antisense ODNs.
6.2.1 Material and Methods

The same 18-mer PS-ODN that was used in the \textit{in vivo} pilot study, and the biodistribution and stability studies, was used; it was directed at the initiation codon of the pre-S region of DHBV (nt 795 - 812). The ODN was obtained commercially (Genosys, Cambridge, UK). ODNs were trace-labelled enzymatically with \(\alpha^{35}\text{S}\text{dATP}\) using Terminal deoxytransferase (TdT) for liposome entrapment. To increase the specific activity of the labelled ODNs, 4 reactions were performed in parallel and the reactions were pooled. The incorporation for the 4 reactions was between 43 and 58%. Labelled ODN was separated from un-incorporated label by Sepadex G-50 column chromatography. The specific activity of the labelled ODN was \(8.5 \times 10^7\) cpm.

Entrapment was into positively-charged small unilamellar vesicles by the dehydration-rehydration method. Liposomes were prepared from PC: DOPE: SA in a ratio of 2: 2: 1 (PC = Phosphatidylcholine; DOPE = Dioleoyl phosphatidylethanolamine, SA = Stearylamine). The DRVs were microfluidized for 2 cycles and the vesicles had a mean diameter of \(\sim 300\) nm.

Neonatal Mallard ducklings were inoculated with DHBV serum containing infectious virions. Chronic infection was confirmed 14 days later by dot-blot hybridization (day -3). Chronically-infected ducks were then treated intravenously with single daily doses (20 \(\mu\text{g/g bw/day}\) of either free or liposome-entrapped antisense ODNs for 5 days. All the treatment regimens were prepared to a total volume of 1 ml with sterile PBS, and daily dosages were adjusted to account for the increase in weight. Nine ducks were used in total, 3 ducks each per treatment group, in addition to 3 untreated controls. Ducks were bled daily (0.5 ml) prior to injection of therapy, serum separated by centrifugation, and stored at \(-20^\circ\text{C}\) until analysis. At the end of the treatment phase, ducks were killed with an overdose of sodium pentobarbital, liver samples were collected and stored at \(-70^\circ\text{C}\).

To assess toxicity from the treatment, biochemistry was performed on serum samples from the pre-treatment collection (day -3) and the final post-treatment day. Serum samples were tested for ALT (alanine aminotransferase), total bilirubin, serum albumin, urea and creatinine using a Hitachi 911 analyser (Boehringer Mannheim, Lewes, U.K.). DHBV DNA was quantified by dot blot hybridization for serum levels and Southern blot
hybridization for analysis of viral DNA in liver at the end of treatment, as previously described.

6.2.2 Results

All the ducks survived the treatment period and no overt toxicity was observed. There was marked suppression of serum DHBV DNA levels by dot-blot hybridization in the free-ODN group from the first post-treatment day, with levels remaining low until the end of the study period (Figure 6.1). By contrast, there was no suppression in the liposome-ODN group or the untreated control ducks. Similarly, Southern hybridization of viral DNA in liver at the end of treatment showed loss of replicative intermediates in the free-ODN group, with no reduction in the other 2 groups (Figure 6.2). Fluctuation in serum DHBV DNA levels from day to day was noted in the untreated control ducks.

Table 6.1 shows a comparison of serum pre-treatment biochemical parameters with the same parameters at the end of treatment. Each value shown in the table is the mean change ± SEM of 3 replicates.
Figure 6.1: Dot blot hybridization analysis of serum DHBV DNA from ducks treated with free-ODNs, liposome-entrapped ODNs, and untreated controls. A: autoradiograph of dot blot hybridization, B: DHBV DNA levels in pg/ml 3 days before treatment, just before treatment commenced (day 0), and each day during treatment. Each data point is a mean ± SEM for 3 ducks.
Free ODN | Liposome ODN | Untreated controls | M

RC →
L →
SC →
SS →

Figure 6.2: Southern blot analysis of intracellular DHBV DNA extracted from duck livers at the end of 5 days treatment. Each lane contains 10 µg total cellular DNA. Relaxed circular (RC), linear (L), supercoiled (SC), and single-stranded (SS) species are indicated (S blot 1 ppt)
Table 6.1: Mean change (± SEM) in serum ALT, total bilirubin, albumin, urea and creatinine before and after treatment in the 3 treatment groups. Minus values indicate a fall in the parameter.

<table>
<thead>
<tr>
<th>Biochemical parameter</th>
<th>Free-ODN</th>
<th>Liposome-ODN</th>
<th>Untreated controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (IU/l)</td>
<td>146 ± 68</td>
<td>5 ± 1</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Total bilirubin (μmol/l)</td>
<td>1.9 ± 0.9</td>
<td>1.6 ± 0.7</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Serum albumin (gm/l)</td>
<td>-2.8 ± 0.6</td>
<td>-1.2 ± 0.3</td>
<td>0.1 ± 0</td>
</tr>
<tr>
<td>Serum Urea (μmol/l)</td>
<td>0.3 ± 0.2</td>
<td>0.07 ± 0.3</td>
<td>-0.3 ± 0.03</td>
</tr>
<tr>
<td>Serum creatinine (μmol/l)</td>
<td>9 ± 1.5</td>
<td>2 ± 1.7</td>
<td>2 ± 1.5</td>
</tr>
</tbody>
</table>

There was a striking rise in serum ALT levels in the free-ODN treated ducks, the same group that showed a dramatic fall in DHBV DNA levels. This flare in ALT was not seen in either of the other 2 groups. There was also a concomitant fall in serum albumin levels and a rise in serum creatinine.

6.2.3 Discussion

This preliminary study confirms that antisense oligodeoxynucleotides against the start of the pre-S region of the DHBV genome are potent inhibitors of DHBV replication in vivo. In the free-ODN treated group, serum DHBV DNA levels fell precipitously after the first dose, and remained almost undetectable throughout the treatment period. This was paralleled by a marked suppression of viral replicative intermediates in the livers of these ducks. Thus, these results confirm the results from the study by Offensperger et al. (1993), who also showed almost complete inhibition of viral replication with the same antisense sequence and dose. In that study, serum DHBV DNA levels were not reported; only viral replication in the liver was determined at the end of 14 days of therapy. Thus the rapidity of the effect was not assessed. The untreated control ducklings showed a persistent viraemia. However, there was a notable fluctuation in the serum levels of DHBV DNA from day to day. This is well known to occur with DHBV DNA levels. The viraemia in experimentally infected Mallard ducklings reaches its highest titre 4 days post-inoculation, and fluctuating and decreased titres are observed thereafter (Lambert et al., 1991).
However, the most surprising finding was the lack of any inhibitory effect with the liposome-entrapped antisense ODNs. The minimum effect that might have been expected was a response equivalent to the free-ODNs. At best, the degree of serum and liver DHBV DNA inhibition should have been much greater, as a result of greater hepatic delivery and uptake. There are several possibilities for the lack of any response to the liposome-entrapped antisense ODNs. It was possible that the ODNs were degraded during liposome entrapment; they could have been degraded in the plasma en route to their intended site of action; the ODNs may not have reached the liver, but diverted to some other site; they could have been preferentially taken up and degraded by the Kupffer cells instead of the hepatocytes, or the injected dose may not have been comparable or similar to that of the free-ODN. Another possibility was that the cationic liposome may have interacted strongly with the polyanionic PS-ODN, and the ionic interaction may have affected the activity of the ODN. Alternately, the ODNs may have localised in the wrong cellular compartment; they may have localised to the nucleus as seen with Lipofectin in the in vitro studies, whilst their site of action may be the cytoplasm.

It was unlikely that the ODNs were degraded during liposome entrapment, as this had been tested previously, and the molecules were intact on a 20% PAGE/7M urea gel. The stability of the ODNs in duck plasma had also been confirmed in vivo, in vivo the PS-ODN used in the biodistribution study was sufficiently intact in plasma to be detected by hybridization up to 30 min after injection. Attempts were made to determine the integrity of the ODN in the liver and urine, but this could not be assessed because of the labelling method used in that study. However, radioactivity could easily be detected in the liver, and this was greater in the liposome-treated ducks than the free-ODN group.

Normal values for the serum biochemical parameters measured to determine toxicity are not known, especially in growing ducklings; this information would have been useful in this study. Although all the ducks survived the treatment without any obvious adverse effects, there was a sharp rise in ALT in the ducks which responded to the therapy, which was not observed in the other ducks. This effect is also seen with successful interferon-α therapy for chronic HBV, and indicates a satisfactory response with immune lysis of infected hepatocytes (Hoofnagle & DiBisceglie 1997). Not much is known about
how antisense ODNs work to inhibit viral replication, or whether an immune mechanism is involved in addition to sequence-specific inhibition of translation. Thus, with the current state of our knowledge concerning these molecules and their actions, it is difficult to decide whether this is a toxic or a desirable effect with a favourable outcome. However, the fall in serum albumin and the rise in serum creatinine is cause for concern, as this may indicate non-specific toxicity.

6.3 Investigation of the reasons for a lack of response with Liposome-ODN

Before proceeding to further explore the value of liposomes as a delivery vehicle for antisense therapy, it was necessary to investigate the reasons for the failure of liposome-ODNs to inhibit or suppress DHBV DNA replication in this experiment. Some of the possible reasons for failure have been answered in the studies of stability and biodistribution (chapter 4). Other possibilities still required investigation.

6.3.1 Detection of oligodeoxynucleotides in the liver

This was performed to determine whether the liposome-ODN reached the liver. If the ODN reached the hepatocytes, it should be detectable by hybridization with a complementary ODN probe; the free-ODN did cause inhibition of DHBV replication in the liver and this would therefore serve as a positive control. Twenty µg of extracted cellular DNA form the liver of each duck was electrophoresed on a 2% agarose gel. Known quantities of pure ODN used in the in vivo study was electrophoresed on the same gel as standards, to determine the level of sensitivity of detection. Ten standards were run in adjacent lanes ranging from 50 ng to 50 pg of pure ODN. Nucleic acids from the gel were transferred to a nylon membrane by Southern transfer, and cross linked to the membrane under UV light for 4 min.

An ODN complementary to the antisense ODN was end-labelled with [γ-32P]ATP to a specific activity of 36 x 10^7 cpm/µmol using T4 polynucleotide kinase. The nylon membrane was prehybridized in 6 x SSC, 10 x Denhardt's solution, 50 µg/ml denatured salmon sperm DNA and 0.1% SDS for 1 hour at 45°C, followed by overnight hybridization with the probe at the same temperature. The membrane was then washed thrice at 45°C for 5 min each, followed by one wash at 50°C for 5 min in 6 x
SSC/0.1%SDS. Thereafter, the membrane was exposed overnight for autoradiography at -70°C.

The autoradiograph of the Southern blot for ODNs in duck liver is shown in Figure 6.3. A strong signal was obtained from the livers of ducks treated with free-ODN, no signal from those treated with liposome-ODN, and as expected, no signal from the untreated control lanes (negative controls). The bands were the same size as the pure ODN standards. Pure ODNs were detected in lanes containing 50 ng, 25 ng and 12.5 ng ODN, but not in those containing 6.25 ng or less. Thus the sensitivity of detection was between 6.25 and 12.5 ng ODN.

![Autoradiograph](image)

**Figure 6.3:** Southern blot analysis of intracellular antisense ODN extracted from livers and probed with a complementary ODN probe at the end of 5 days treatment. Autoradiograph after 48 hours exposure. Each lane contains 20 μg total cellular DNA. Standard amounts of ODN (lanes A-J) are from 50 ng to 50 pg.

It can be concluded that there was insufficient antisense ODN in the livers of ducks treated with liposome-entrapped ODN to inhibit DHBV replication. This could be as a result of the ODNs not reaching the liver, or an insufficient quantity reaching the liver. However, this would be contrary to the findings in the biodistribution study.

### 6.3.2 Fractionation of uptake between hepatocytes and Kupffer cells

In order to determine whether the liposome-ODNs were reaching sites other than the hepatocyte, such as Kupffer cells, preferentially, hepatocytes and sinusoidal cells were separated and the uptake to each cell type measured. For this purpose, collagenase
perfusion of duck livers and separation of hepatocytes and sinusoidal cells was performed by centrifugation on a percoll gradient.

6.3.2.1 Validation of anti-duck Kupffer cell antibody

A mouse monoclonal anti-duck Kupffer cell antibody (IgG subtype) (a gift from Dr John Pugh, Fox Chase Centre, Philadelphia) was used to identify duck Kupffer cells. This was first validated by immuno-peroxidase staining of duck liver tissue. Five µm sections of frozen normal duck liver and heart were cut on a cryostat, picked up on glass microscope slides and air-dried for 10 minutes. The sections were then fixed in acetone at -20°C for 4 min following which they were dried in air for 5 min before washing in PBS. Sections of heart were used as a negative control, as tissue macrophages are not found in heart muscle. Endogenous peroxidase activity was quenched by incubation with 3% H₂O₂ for 15 min. Sections were incubated with undiluted primary antibody for 2 hr at room temperature in 100% humidity. Sections were then washed 3 times in PBS for a total of 10 min, followed by incubation with a 1:400 dilution of goat anti-mouse/rabbit-IgG biotinylated antibody for 60 min at room temperature in 100% humidity. The diluent here was normal duck serum (NDS), diluted to 10% in PBS to remove cross-reacting elements from the developing antibody. The antibody was washed off in PBS as previously.

The sections were then overlaid with a strepavidin/biotinylated horse radish peroxidase complex (ABC) (Dako duet, CA, USA), diluted to 1:400 with 10% NDS/PBS as above, for 60 min in a 100% humid atmosphere. Slides were rewashed in PBS as previously. Colour development for subsequent visualisation of bound antibody was achieved using a horse-radish peroxidase-mediated polymerisation of the substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB). One 10 mg DAB tablet (Sigma), was dissolved in Tris/HCl buffer 0.05 M pH 7.6, prepared fresh just before use and also contained 166 µl 1% H₂O₂. Sections were left in this solution for 5 min and the reaction stopped by washing sections in several changes of distilled water. Sections were then counterstained in Mayer’s Haemalun, 60 seconds, excess stain being washed off with distilled water. Specific and positive staining was indicated by the presence of dark brown colour on microscopic examination which was not present in negative control sections. To check the specificity of staining, negative control sections were also used, where the anti-Kupffer cell
antibody was omitted, 0.1% Bovine Serum Albumin in PBS being substituted instead. All further stages including the addition of the developing antibody and ABC were performed as above.

Figure 6.4 is a series of photomicrographs of sections of duck liver and heart immunostained with the anti-duck Kupffer cell antibody and the negative controls. Cells with the morphological appearance of Kupffer cells, staining positively (dark brown) with the antibody are apparent in the liver. These cells are not obvious in the heart, an organ where Kupffer cells have not been described, and there is an absence of any positive brown staining, implying that the antibody is binding to duck liver elements. Negative control sections of duck liver, where the 1st antibody was omitted, are also negative and the reasonable conclusion that may be drawn from these results is that the antibody is binding specifically to a population of cells, with the morphological appearance of Kupffer cells found in duck liver.

6.3.2.2 Collagenase perfusion and Percoll gradient centrifugation

Material and Methods

Percoll (Sigma Chemical Co. Ltd, Poole, UK) was filtered through a 0.2 μm filter and prepared as 90% stock with 10% 10 x PBS. Collagenase, crude type IA (Sigma Chemical Co. Ltd, Poole, UK) was aliquoted into stock solutions of 100 mg/ml and stored at -20°C until use. PS-ODNs were labelled with 125I-dCTP using TdT as previously described for monitoring. ODNs were entrapped into liposomes by the same procedure and using the same lipid composition as the \textit{in vivo} antiviral study.

One uninfected Mallard duck each was injected with ~1 mg of either free- or liposome-entrapped ODN labelled with 125I. Thirty minutes later the ducks were killed with an overdose of sodium pentobarbital, the peritoneal cavity and chest wall opened immediately, and the portal vein infused with prewarmed DMEM containing collagenase in a final concentration of 1 mg/ml through a peristaltic pump. Prior to infusion of collagenase, sterile PBS was infused to flush the blood from the hepatic vasculature. The perfusate containing collagenase was returned to the reservoir and recirculated. It was not possible to perfuse the liver in situ, as the liver is positioned high up in the chest cavity in ducks. Therefore, perfusion was commenced immediately after killing the duck. The liver tissue turned a pale colour after infusion with collagenase.
Figure 6.4: Light microscopic sections of duck liver and heart stained with a monoclonal antibody against duck Kupffer cells. A: duck liver incubated with 1° and 2° antibody; B: duck liver incubated with 2° antibody only; C: duck heart incubated with 1° and 2° antibody. (X 25 magnification).
Thereafter, the liver was immediately transferred to a laminar flow cabinet, where all procedures were carried out aseptically. The liver was chopped finely in a petri dish and incubated with 1 mg/ml collagenase for 2.5 hours at 37°C; after 1 hour, tissue pieces were further diced to aid release of cells. Cells were harvested by washing through a cell dissociation sieve. The cell digest was washed twice with DMEM by centrifugation at 2000 rpm for 10 min and the final pellet was resuspended in 32 ml DMEM. The cell digest (4 ml) was gently overlaid on preparations (x 8) of 3 ml of 33% Percoll underlaid by 3 ml of 77% Percoll (stock Percoll was 90% in 10 x PBS).

After centrifugation at 2000 rpm for 30 min, 3 layers were observed (Figure 6.5): a layer of hepatocytes below the medium, the sinusoidal fraction in the middle layer formed at the 77º/33º Percoll differential density gradient, and a red cell pellet at the bottom. The hepatocyte and sinusoidal cell layers were harvested from each tube and pooled, washed twice in DMEM and resuspended. The hepatocytes were kept at 37°C, while the sinusoidal cells were suspended in 0.5 ml DMEM. Mouse anti-duck Kupffer cell antibody (75 µl) was added to the sinusoidal cells, and the cells incubated at 37°C for 30 min, with shaking after 15 min. DMEM was added to a total of 10 ml, and the cells centrifuged at 2000 rpm for 10 min. The supernatant was discarded and the cells were resuspended in 500 µl chilled PBS, before incubation with 10^7 sheep anti-mouse IgG1 Fc coated Dynabeads™ (Dynabeads M-450, Dynal, Oslo, Norway) for 30 min at room temperature. The Dynabead™ associated Kupffer cells were collected using a magnetic particle concentrator. Kupffer cells were resuspended in DMEM.

The cells were counted in a Neubauer Improved haemocytometer and viability was tested using the trypan blue exclusion test. The cell yield was equal to the number of viable cells x 10^4 x total volume of cell suspension. The radioactivity per million cells was counted in a gamma counter for hepatocytes and Kupffer cells.

**Results**

The uptake to hepatocytes and Kupffer cells, expressed as cpm per million cells, is shown in Table 6.2. In both cases, the uptake was greater in the hepatocytes than in the Kupffer cells. Therefore, liposome-ODNs were not preferentially targeted to Kupffer cells in the liver; this was unlikely to be the reason for the non-response in the liposome-treated ducks. This, coupled with the fact that there are far more hepatocytes...
than Kupffer cells in the liver, imply that preferential Kupffer cell uptake would not pose a serious problem with liposome-entrapped antisense therapy.

**Figure 6.5:** Separation of the liver cell digest on a Percoll gradient after centrifugation. The layer of hepatocytes is below the medium, the sinusoidal fraction in the middle layer formed at the 77%/33% percoll differential density gradient, and the red cell pellet at the bottom.
Table 6.2: Uptake of $^{125}$I-labelled ODN in hepatocytes and Kupffer cells in counts per min/10^6 cells

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Hepatocytes cpm/10^6 cells</th>
<th>Kupffer cells cpm/10^6 cells</th>
<th>Hepatocyte:Kupffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free-ODN</td>
<td>1307</td>
<td>563</td>
<td>2.3</td>
</tr>
<tr>
<td>Liposome-ODN</td>
<td>111</td>
<td>32</td>
<td>3.5</td>
</tr>
</tbody>
</table>

6.3.3 Quantification of injected dose of liposome-ODNs

To determine whether the dose of liposome-ODN injected in the DHBV antiviral study was the same as the free-ODN dose, the ODN content of the liposomes was determined. The ODNs were released from the liposomes and the quantity entrapped compared to known standards of pure ODN by hybridization. The method used was the same as that in section 4.2.7 for detecting ODNs in duck plasma in the plasma clearance study.

Liposome-ODN was incubated with 1% Triton X-100 (v/v) at 37°C for one hour to disrupt the lipid membrane of the vesicle. The amount of liposome-ODN was based on the concentration used for dosing (stock solution of 5 mg/ml). Known standards of the pure antisense ODN were prepared from 1000 to 1 ng. Comparable amounts of ODN were prepared from the liposome-ODN based on the concentration in the stock solution, after disruption of the vesicles. All the ODNs were prepared to a total volume of 200 μl with distilled water, before spotting onto a nylon membrane with a slot blot manifold. The procedure for spotting, prehybridization and hybridization was as described in section 4.2.7. The membrane was probed with a $^{32}$P-labelled ODN complementary to the antisense sequence used. ODNs extracted from liposomes were spotted adjacent to the same dose of pure ODN for comparison.
Figure 6.6: Hybridization of ODNs used in the *in vivo* study with a complementary ODN probe to compare the dose of liposome-ODN to free-ODN injected. The amount of liposome-ODN spotted was based on the stock solution used in the *in vivo* experiment. Autoradiograph after 24 hours exposure.

Figure 6.6 shows the autoradiograph from the slot blot hybridization. For the free-ODN, a strong signal could be obtained from the top 3 standards (1000, 500 and 100 ng ODN), a weak signal from the next standard (50 ng), but none from the lower 2 (10, 1 ng). For the liposome-ODN, a signal was only obtained from the 2 highest amounts (1000 and 500 ng), the strongest signal was clearly weaker than the 100 ng standard. Thus the amount of liposome-ODN injected in the antiviral study was only between 5 and 10% of that injected in the free form. Therefore, the level of liposome-entrapment was probably overestimated at the time of the procedure. This would explain the lack of a therapeutic effect seen with liposome-ODNs. However, this method had not been used previously by other workers to measure the amount of ODN entrapped within liposomes, and thus has not been validated.
6.3.4 Discussion

Investigations for the reasons for a non-response to the high-dose liposome-ODN therapy showed that no ODN sequences could be detected in the livers of these ducks by probing with a complementary ODN sequence, as could be done in the livers of ducks treated with an equivalent dose of free-ODN. This could be relatively quantified by probing known quantities of ODN electrophoresed on the same gel. The untreated control ducks were used as a negative control. Yet previous results had shown that the PS-ODNs were stable in plasma \textit{in vitro} and \textit{in vivo}, and that the ODNs reached the liver rapidly after intravenous injection. This was inferred from the observation that the label attached to the ODNs (${}^{125}$I) could be detected in large quantities in the livers of ducks killed at one hour.

Liposomes are known to be taken up by the mononuclear phagocytic system (Juliano & Akhtar 1992); macrophages are especially abundant in the liver, spleen and lymphoid organs, and thus contribute to the tendency of liposomes to accumulate at these sites. However, the biodistribution study had shown that the hepatic uptake was far greater than the splenic uptake, partly because the hepatic mass is much greater than the splenic mass in ducks. Autoradiography had demonstrated a uniform distribution of silver grains on sections of liver from liposome-ODN injected ducks. Separation of hepatocytes and Kupffer cells by Percoll gradient centrifugation showed that a greater proportion of injected liposome-ODN, and free-ODN, was reaching the hepatocytes. It would have been preferable to infuse the collagenase \textit{in situ} rather than immediately after killing the ducks, as is done in studies using rat models; however, because of anatomical constraints, this was not feasible.

These results suggested the distinct possibility that the amount of liposome-ODN injected was too little to produce an effect comparable to that of the free-ODN. Therefore, it was decided to measure the amount injected from the remaining liposome preparation. This confirmed the suspicion that the estimate of the amount entrapped was probably inaccurate. Using solid phase hybridization with an ODN probe was a useful way to quantify ODNs; this method was first described by Temsamani \textit{et al} (1993) and adapted previously in this study. However, it has not previously been used to quantify ODNs entrapped within liposomes.
6.4 Dose response experiment with free antisense and a random sequence control ODN

It was decided to try inhibiting DHBV replication using lower doses of antisense ODN in the free form in order to determine the lowest dose necessary to provide a favourable response. Further experiments could then be performed using this lower dose. It was also necessary to check the effect of a random sequence ODN on DHBV replication.

6.4.1 Materials and methods

Neonatal Mallard ducklings were inoculated with DHBV infectious serum and bled 14 days later to confirm infection by dot blot hybridization. Ten infected ducklings were used for testing of antiviral efficacy.

A random sequence ODN was acquired commercially for use in the study. This was a scrambled sequence of the 18-mer antisense ODN; thus it had the same nucleotide length, molecular weight, base composition and Tm. The sequence of this ODN was: 5’-ATC-TGA-ATC-TCG-ACC-TAA-3’ (The antisense sequence was 5’-AAA-TAC-TAC-CCC-GTT-GTA-3’). Two ducklings per treatment group were injected intravenously daily for 5 days, and 2 were used as untreated controls. The dose of ODN was adjusted daily to account for the change in weight.

The groups were as follows:

- Antisense ODN 5 µg/gm bw
- Antisense ODN 10 µg/gm bw
- Antisense ODN 15 µg/gm bw
- Random sequence ODN 20 µg/gm bw
- Untreated controls

Although the random sequence ODN was used in a higher dose than any of the antisense ODNs in this experiment, it was important to determine whether the response observed in the previous experiment with a high dose antisense ODN was sequence-specific. Therefore, the same dose of random sequence (20 µg/gm bw) was used as the previous experiment. Blood samples for measurement of DHBV DNA levels and liver for the detection of viral replicative intermediates were taken at the same times as the previous in vivo antiviral experiment. DHBV DNA analysis was as described previously.
Figure 6.7: Dot blot hybridization analysis of serum DHBV DNA from ducks treated with 15, 10, and 5 μg/gm bw free antisense ODN, 20 μg/gm bw random sequence ODN, and untreated controls. A. autoradiograph of dot blot hybridization, B. DHBV DNA levels in pg/ml 3 days before treatment, just before treatment commenced (day 0), and each day during treatment. Each data point is a mean for 2 ducks (SEM is not shown).
6.4.2 Results

Dot blot analysis of serum DHBV DNA (Fig. 6.7) showed a significant variation in the response between the 2 ducks from each of the treatment groups, as shown in Fig 6.7A. A fall in serum levels was noticed in all the antisense ODN-treated groups; this was greater in the ducklings treated with the highest dose. The untreated controls also showed a gradual decline in serum levels, although the pre-treatment levels were lower and a positive signal could be detected on all samples by dot blot. In one of the 2 ducks treated with the random sequence ODN, there was marked suppression of DHBV DNA to undetectable levels; the other duckling had a more gradual decline in the levels. The serum viral DNA response also appeared to be dependent on the pre-treatment levels. Southern blot analysis of liver DNA (Figure 6.8) showed a marked loss of replicative intermediates in all treated ducklings, including those treated with the random sequence ODN. However, when Southern blot analysis was performed together with DNA from ducklings treated with 20 μg/gm bw of free antisense ODN from the previous experiment, the suppression was the greatest with this group (data not shown for 20 μg/gm bw).

6.4.3 Discussion

The results of this experiment showed inhibition of DHBV replication with all doses of free antisense ODNs used. The main difference was in the time taken to reach undetectable levels of DHBV DNA, which was shorter with higher doses (15 and 20 μg/gm bw) (Fig. 6.7B). It also revealed marked fluctuation in the response between ducks to the same dose of therapy (Fig 6.7A). This makes the data difficult to interpret, especially with the small numbers of ducks treated in each group.
Figure 6.8 Southern blot analysis of intracellular DHBV DNA from liver at the end of 5 days treatment. Each lane contains 10 μg total cellular DNA. Relaxed circular (RC), linear (L), supercoiled (SC), and single-stranded (SS) species are indicated. The doses of antisense ODNs used are shown above the lanes. C = untreated controls, R = random sequence ODN 20 μg/gm bw; M = 1 kb size marker.
A striking feature was the fall in serum DHBV DNA levels and inhibition of viral replication in the liver with the random sequence ODN. Most of the available evidence for non-specificity stems from studies performed in vitro, in which cells are more susceptible to toxic effects, rather than studies in whole organisms. These results indicate that the effect may also occur when used for the treatment of viral infections in whole animals, thus having serious implications for the potential clinical use of antisense therapy in humans. It was not clear whether this non-specific effect was a result of detrimental binding of the PS-ODN to viral or cellular proteins, partly due to its highly anionic charge, or non-antisense hybridization to the target mRNA. It was decided to investigate whether non-antisense hybridization with the random sequence ODN can occur in vitro.

6.5 Assessment of non-specific hybridization

It was known from earlier experiments performed that the antisense ODN can hybridize to a probe made of the sense sequence. This was used to detect ODNs in duck plasma during the plasma clearance studies (section 4.2.7) and to quantify liposome-entrapped ODN (section 6.3.3) by solid phase hybridization. However, it would be relevant to know the specificity of this reaction, and to determine whether the sense ODN could hybridize to the random sequence ODN used in the previous experiment. This would provide valuable information about the mechanism of non-specific actions with random or non-antisense ODNs. It would also tell us whether hybridization occurred to the same extent as with the antisense ODN, by using varying amounts of random sequence ODN.

Known quantities of antisense ODN, ranging from 1000 ng to 1 ng, were spotted onto a nylon membrane using a slot blot apparatus. All ODNs were prepared to a final volume of 200 μl. Adjacent to this, the random sequence ODN used in the previous in vivo experiment was spotted in the same quantities. The membrane was probed with a $^{32}$P-labelled ODN probe that was complementary to the antisense ODN. The procedure for prehybridization, hybridization, probe preparation and washing was as described previously. The membrane was exposed overnight for autoradiography at -70°C.
Antisense Random

ODN    ODN

1000 ng
500 ng
100 ng
50 ng
10 ng
1 ng

Figure 6.9: Hybridization of antisense and random sequence ODNs used in the
*in vivo* study with a *[^3]P*-labelled ODN probe complementary to the antisense
ODN to determine the specificity of hybridization.

The results of the slot blot hybridization are shown in Figure 6.9. The 1000, 500 and 100
ng of antisense ODN were easily detectable by hybridization; no signal was obtained
from any of the slots with random sequence ODN. This indicates that, at least *in vitro*,
there was no cross hybridization between the sense sequence probe and the random
sequence ODN, which had the same base composition as the antisense ODN. However,
hybridization between the sense probe and its antisense ODN was distinct and
reproducible. Thus it is more likely that the effect of the random sequence ODN on the
inhibition of viral replication was not through non-specific hybridization with the target
mRNA. It may occur through inhibition of a number of proteins or enzymes, including
many involved in nucleic acid metabolism, or by blocking viral adsorption to cells.
However, the sense ODN was not used to probe DHBV DNA, which would also help to
indicate whether non-specific hybridization was occurring.
6.6 Liposome-entrapped, free-, and random sequence ODN inhibition of DHBV replication

Finally it was decided to compare the effect of free and liposome-entrapped antisense ODNs to inhibit DHBV replication, using the liposome-ODN in a high and a low dose. In addition, the random sequence ODN was used as a control to whether the unexpected results from the previous experiment were reproducible. Thus, the effect of the high dose free antisense against the high dose random sequence against the high dose liposome-entrapped antisense ODN would be tested. Also, the low dose entrapped antisense ODN could be compared to the high dose free antisense ODN, which had been confirmed to be a potent inhibitor of viral replication. Since the entrapment into positively-charged liposomes was unreliable previously, neutral liposomes were used for this antiviral experiment.

6.6.1 Materials and Methods

Twenty neonatal Mallard hatchlings were inoculated with infectious serum containing DHBV particles. Five days later the ducklings were bled to confirm the presence of infection by dot blot hybridization; all the ducklings were infected. Fourteen birds with high levels of DHBV DNA were chosen for the study. Antisense ODNs were entrapped into neutral liposomes composed of phosphatidylcholine (PC), DOPE and cholesterol in a molar ratio of 1: 1: 2, respectively, by the dehydration-rehydration method. The average size of the vesicles was 210-226 nm. To determine the specificity of the antisense sequence a random sequence ODN was used as a control; this was the same ODN that was used previously (5'-ATC-TGA-ATC-TCG-ACC-TAA-3').

Smaller and younger ducklings were used in this study in order to economise; also, from previous studies (Offensperger et al 1996; Omata et al, 1984), it has been shown that when the ducklings were inoculated within the first 24 hours after birth, all developed chronic infection. This experience was the same as in this study in earlier experiments performed. A 5-day course of intravenous antiviral therapy was commenced when the ducklings were 7 days old. The final dose was made up to a total volume of 1 ml with PBS (pH 7.4). Three ducklings were treated with 20 μg/gm body weight (bw) free antisense ODN, 3 with the same dose of random sequence free ODN, 2 with 20 μg/gm bw liposome-entrapped antisense ODN, 2 with 5 μg/gm bw liposome-entrapped
antisense ODN, and 4 ducklings were used as untreated controls. The injected dose of the ODN was adjusted daily to match the increase in body weight. Serum samples were taken before treatment, daily prior to dosing, and 24 hours after the final dose, when the ducklings were killed with an overdose of sodium pentobarbital. Liver tissue was stored frozen for detection of DHBV DNA.

Detection of viral antigens in serum

The effect of the antisense ODN on target proteins was monitored in serum by detecting DHBV surface antigen (DHBsAg) prior to treatment and at the end of treatment. Mouse ascitic fluid from duck anti-preS (1H.1) and anti-S (7C.12) (a gift from Dr. John Pugh) was used for immunoblotting. Fifteen µl of duck serum was electrophoresed on an SDS-12% polyacrylamide gel. The gel was semi-dry blotted onto a nitrocellulose membrane and soaked in PBS plus 1% (wt/vol) bovine serum albumin plus 0.05% (vol/vol) Tween overnight at 4°C. The membrane was incubated with a 1:500 dilution of a 1:1 mixture of mouse monoclonal antibodies 1H.1 and 7C.12 for 2 hours at room temperature. After washing 4 times in PBS/0.05% Tween, the membrane was incubated with 5 µCi ¹²⁵I-labelled sheep anti-mouse immunoglobulin (Amersham Int., UK) in PBS/0.05% Tween for 1 hour at room temperature with shaking. The membrane was washed again in PBS/0.05% Tween, dried for 10 min on filter paper, and exposed for autoradiography overnight.

The viral antigens were quantified by scanning densitometry (BioRad GS-670 Imaging Densitometer), and the intensity of the pre-treatment signal (expressed as optical density/mm²) was compared to the post-treatment signal to determine the relative change in the concentration of serum DHBsAg. As a control protein, serum albumin concentration was also measured on the same samples pre- and post-treatment to determine the specificity of the antisense ODN on the target protein (DHBsAg). This was performed on a Hitachi 911 analyser (Boehringer Mannheim, Lewes, UK).
6.6.2 Results

6.6.2.1 DHBV DNA response to antiviral therapy

Mean pre-treatment levels of serum DHBV DNA were in the region of \(10^4\) to \(10^5\) pg/ml. All ducklings survived the therapy with no observed adverse events; the expected increase in weight during the treatment phase was observed. There was a rapid and marked decline in serum DHBV DNA levels in all treated ducklings from the first post-treatment day, which was sustained throughout the treatment period (Figure 6.10). Levels in the ducks treated with the high doses (20 \(\mu\)g/gm bw) of free antisense and random sequence ODN were undetectable from the second post-treatment day. The random sequence ODN appeared to be as efficient as the free antisense ODN in suppressing viraemia. The ducklings treated with liposome-entrapped antisense ODN at both dosages also showed a marked decline in serum levels, although low levels were still detectable on days 4 and 5. In the 4 untreated ducklings, a gradual decline in serum DHBV DNA levels was noted, although levels were still approximately 3 logs higher than the treated groups (Figure 6.10). There was a 5 log reduction in serum DHBV DNA levels in ducklings treated with the free antisense-ODN and random sequence-ODN, a 4 log reduction in all ducklings treated with liposome-entrapped antisense ODN, and a less than 2 log reduction in the untreated control ducklings.

Southern blot analysis for viral forms in the liver showed a marked suppression of viral replicative intermediates in the livers of all treated ducklings, especially in those treated with 20 \(\mu\)g/gm bw of free antisense and random sequence ODN (Figure 6.11); almost a total loss of episomal DNA was observed in these ducklings. There was also a decline in the 2 ducklings treated with 5 \(\mu\)g/gm bw of liposome-entrapped antisense ODN, although episomal DNA was still detected in the livers of these ducklings. However, in none of the treated ducklings was there a loss of relaxed circular or supercoiled forms of DHBV DNA. The control ducklings showed all replicative forms of viral DNA.
Figure 6.10: Serum DHBV DNA response to the different treatment regimens. Days -2 and 0 are pre-treatment samples. A: autoradiograph of dot blot hybridization; B: DHBV DNA levels in pg/ml before and during treatment. Each data point is a mean for 2 or 3 ducks.
Using gel electrophoresis and Southern blot hybridisation, which does not exclude the possibility of detection of supercoiled DHBV DNA, signals were found on the autoradiograph at the correct size of migration of supercoiled DHBV DNA, and these molecules have been labelled as such. Supercoiled DHBV DNA is present in very small amounts, and the enrichment techniques used by Summers et al (1991) would be essential for accurate quantitation. However, in that quantitation of supercoiled DHBV
DNA was not the primary aim of these experiments, this was not addressed further. The Southern hybridisations were not used to quantitate the antiviral effect, this was performed using dot blot hybridisation. Southern blots were performed in order to determine whether intra-hepatic levels of DHBV DNA (including replicative intermediates) were reduced, as was observed by dot blot hybridisation, in serum.

6.6.2.2 Serum DHBsAg response to therapy

Immunoblotting and autoradiography revealed a 17 kDa major band corresponding to the S surface polypeptide of DHBV, and 2 minor bands at 27 and 33 kDa that were interpreted as pre-S2 polypeptides (Figure 6.12) (Pugh et al, 1995; Qiao et al, 1990). Table 6.3 shows the percentage reduction in post-treatment DHBsAg from pre-treatment values, quantified by scanning densitometry. The untreated controls showed some reduction in post-treatment DHBsAg levels (mean 51.0%); however this corresponded to a gradual fall in serum DHBV DNA levels. All ducklings treated with the high dose of free antisense and random sequence ODN showed a marked reduction in DHBsAg levels, with levels becoming undetectable in 2 of 3 ducklings in each group. However, the reduction in levels in the ducklings treated with high and low doses of liposome-antisense ODN was inconsistent, with a marked reduction in one duckling per group but only a moderate reduction in the remaining duckling per group.

In contrast to the fall in serum DHBsAg levels, the serum albumin rose from the pre-treatment to the post-treatment period in almost all the ducklings, suggesting little or no effect of the ODN on this non-targeted protein (Table 6.3). The difference between the treatment groups in the reduction in DHBsAg levels was not statistically significant (p = 1.048; Kruskal-Wallis test), similarly, the change in serum albumin levels between the treatment groups was also not significant (p = 0.389; Kruskal-Wallis test).
Figure 6.12: Autoradiograph of immunoblot showing the 17 kDa major band corresponding to the S surface polypeptide, and 2 minor bands at 27 and 33 kDa. Each pair of lanes shows the pre-treatment serum DHBsAg bands followed by post-treatment bands from the same duckling in the next lane.
Table 6.3: Percent reduction in DHBsAg from pre-treatment values, and change in serum albumin (gm/l) from pre-treatment to post-treatment values. Minus signs indicate a fall in the value.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Duck No.</th>
<th>% reduction from pre-treatment DHBsAg</th>
<th>Change in serum albumin (gm/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td>507</td>
<td>43.7</td>
<td>+8</td>
</tr>
<tr>
<td></td>
<td>506</td>
<td>29.3</td>
<td>-4</td>
</tr>
<tr>
<td></td>
<td>504</td>
<td>46.3</td>
<td>+4</td>
</tr>
<tr>
<td></td>
<td>501</td>
<td>76.5</td>
<td>+1</td>
</tr>
<tr>
<td>Free antisense ODN</td>
<td>518</td>
<td>80.6</td>
<td>+4</td>
</tr>
<tr>
<td>(20 µg/gm bw)</td>
<td>505</td>
<td>100</td>
<td>+1</td>
</tr>
<tr>
<td></td>
<td>521</td>
<td>100</td>
<td>+3</td>
</tr>
<tr>
<td>Random sequence ODN</td>
<td>513</td>
<td>100</td>
<td>-1</td>
</tr>
<tr>
<td>(20 µg/gm bw)</td>
<td>514</td>
<td>100</td>
<td>+2</td>
</tr>
<tr>
<td></td>
<td>502</td>
<td>92.7</td>
<td>+1</td>
</tr>
<tr>
<td>Liposome-antisense ODN</td>
<td>519</td>
<td>69.9</td>
<td>+3</td>
</tr>
<tr>
<td>(20 µg/gm bw)</td>
<td>511</td>
<td>100</td>
<td>+5</td>
</tr>
<tr>
<td>Liposome-antisense ODN</td>
<td>515</td>
<td>40.6</td>
<td>+3</td>
</tr>
<tr>
<td>(5 µg/gm bw)</td>
<td>520</td>
<td>93.5</td>
<td>+8</td>
</tr>
</tbody>
</table>

6.6.2.3 Changes in serum biochemistry

Serum biochemical parameters were measured from the first pre-treatment sample and the final post-treatment sample. The mean change in serum ALT, bilirubin, urea, creatinine and albumin is shown in Table 6.4.

A rise in serum ALT was noticed once again in the ducks treated with the higher doses of PS-ODNs, although not to the same extent as the first high dose in vivo experiment. No overt toxic effect was seen in the serum bilirubin, albumin, urea or creatinine. The fall in serum DHBsAg levels on immunoblotting was not paralleled by a fall in serum albumin, which continued to rise during the treatment period.
Table 6.4: Mean change (± SEM) in serum ALT, total bilirubin, albumin, urea and creatinine before and after treatment in the 3 treatment groups. Minus signs indicate a fall in the value.

<table>
<thead>
<tr>
<th>Biochemical parameter</th>
<th>Free antisense ODN</th>
<th>Random sequence ODN</th>
<th>Liposome-ODN (20 µg/gm)</th>
<th>Liposome-ODN (5 µg/gm)</th>
<th>Untreated controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (IU/l)</td>
<td>23±12</td>
<td>51±33</td>
<td>34±8</td>
<td>7.0±0</td>
<td>-32±42</td>
</tr>
<tr>
<td>Bilirubin (µmol/l)</td>
<td>-1.7±0.9</td>
<td>0.3±0.3</td>
<td>1±3</td>
<td>0±0</td>
<td>-2.0±0</td>
</tr>
<tr>
<td>Albumin (gm/l)</td>
<td>2.7±0.9</td>
<td>0.7±0.9</td>
<td>4±1</td>
<td>7±1</td>
<td>2.8±2.5</td>
</tr>
<tr>
<td>Urea (µmol/l)</td>
<td>-0.5±0.2</td>
<td>-0.3±0.2</td>
<td>-0.1±0.3</td>
<td>-0.9±0.6</td>
<td>-0.3±0.2</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>1.3±1.7</td>
<td>3.3±2.2</td>
<td>3.0±2.0</td>
<td>2.0±2.0</td>
<td>3.0±1.4</td>
</tr>
</tbody>
</table>

6.6.3 Discussion

Use of the antisense oligodeoxynucleotides to inhibit viral replication in chronically infected ducklings again showed that these agents were potent in vivo inhibitors of viral replication, with a reduction in circulating viral DNA detectable from the first day of treatment. A reduction in replicative forms and circulating target protein was also confirmed. Although the reduction in target protein between the treated groups was not statistically significant, the results appear to be clinically relevant, and the small number of ducks used did not allow sufficient power for a statistical difference to be observed. A previous study has demonstrated that this antisense sequence inhibited viral replication in chronic DHBV infection nearly completely (Offensperger et al, 1993), but daily measurements of serum DHBV DNA were not reported to show the rapidity of the antiviral effect.
Using liposomes to deliver the ODN also caused a fall in serum levels of DHBV DNA and hepatic replication of the virus, implying delivery of the entrapped agents to the liver and a biological effect at the target site. However, the effect was not dramatic, and use of a lower dose (25% of the free dose) in the entrapped form did not produce the equivalent, or enhanced, inhibition of viral replication. However, empty liposomes should also have been used as a negative control in the in vivo studies, as in the in vitro studies with Lipofectin. This would demonstrate whether the lipid vesicles had any effect on viral replication. Lipofectin alone had no effect on HBsAg secretion into the medium in the in vitro experiments, suggesting that empty liposomes also would not have affected DHBV replication. Cationic liposomes used in the high dose in vivo antiviral study showed no effect on viral replication.

The spontaneous decline in levels of serum DHBV DNA seen in the untreated control ducklings has been observed by others. The viraemia in experimentally infected Mallard ducklings reaches its highest titre 4 days post-inoculation, and fluctuating and decreased titres are observed thereafter (Lambert et al., 1991). This natural variation in DHBV DNA levels implies that larger numbers of ducks need to be used in order to reduce the variability in the results. Small sample sizes thus make the results more difficult to interpret, as was observed in the dose-response experiment. However, the high cost of large quantities of PS-ODNs limited the number of ducklings that could be used.

Another limiting factor was the size of the birds; experiments had to be planned such that ODNs were synthesised in large scale, ducklings were available and infected, and the ODNs were entrapped into liposomes before the birds were too large, resulting in the use of much larger doses.

A surprising finding was the marked effect on viral replication seen with the random sequence ODN used in a high dose; this was also seen in the previous experiment with the same PS-ODN. In fact, the random sequence ODN-treated ducklings lost viraemia as efficiently as the ducklings treated with antisense-ODNs at the same dose. No sequence homology between the random sequence ODN and any region of the DHBV genome could be found. This effect seen with phosphorothioates has been a cause for much debate and discussion in in vitro studies (Stein 1995). Although non-sequence-specific effects were observed in our study, a control protein (albumin) measured pre- and post-treatment showed no reduction in serum levels, and even an increase in 9 of the 10
treated ducklings. If the non-specific effects were due to binding to serum proteins, one would have expected a reduction in serum albumin in the groups treated with high doses of oligodeoxynucleotides. It was confirmed earlier that, at least in vitro, there was no cross hybridization between the random sequence ODN and a sense ODN. Whether this takes place in vivo has not been documented.

If the random sequence ODN had an effect on DHBV replication at a high dose, it is likely that the antisense ODN, used at the same dose, also had effects other than the inhibition of DHBV replication. Thus, although inhibition of viral replication and a fall in the target protein was observed in this study, we cannot be certain that this was through an antisense mechanism. It has been suggested that ODNs delivered to cells in low concentrations by liposomes may reduce the amount of nonspecific effects caused by binding of ODNs to serum proteins (Wagner 1995). This would be an interesting aspect for further study.

Thus, the in vivo studies confirmed that antisense PS-ODNs can cause a significant inhibition of viral replication. In the liposome-entrapped form, they do reach their target sites in the liver and produce a biological effect; degradation by Kupffer cells in the liver does not pose a major obstacle for this route of delivery. However, using liposomes as a delivery vehicle does not enhance their efficacy significantly. Nonetheless, short courses of ODNs, in the free or entrapped form, appear to be safe, although their long-term effects remain unknown. The mechanism of action of these ODNs remains unclear and the effects of the non-antisense PS-ODNs requires further study; sequence-specific inhibition does not appear to be the only way that viral replication is inhibited.
Chapter 7

GENERAL DISCUSSION AND CONCLUSIONS

This is the first study investigating the use of liposomes to deliver antisense oligodeoxynucleotides to the liver in an *in vivo* model. Other studies have shown its efficacy in cell culture. A pilot study in ducks chronically infected with DHBV showed no antiviral efficacy with a small dose of free or liposome-entrapped antisense ODN. Subsequent experiments explored the possible reasons for this. Sequencing of the target region of the DHBV genome confirmed that the antisense sequence was correct. Plasma clearance and uptake studies revealed that the PS-ODN and its liposome-entrapped form were stable in plasma, and that delivery to the liver was satisfactory, with the liposome-ODN reaching the liver in far greater quantities than free-ODNs. The use of a high dose of antisense ODN in infected ducks showed marked antiviral efficacy with the free ODN, confirming that a subtherapeutic dose had been used in the pilot study.

However, no antiviral efficacy was observed with the liposome-ODN in the same high dose experiment. Investigation of the reasons for this showed that no ODN sequences could be detected in the livers of these ducks, as could be done in the livers of ducks treated with an equivalent dose of free-ODN. Yet previous results had shown that the PS-ODNs were stable in plasma *in vitro* and *in vivo*, and that the ODNs reached the liver rapidly after intravenous injection. PS-ODNs were not degraded during the liposome entrapment procedure. Separation of hepatocytes and Kupffer cells by Percoll gradient centrifugation showed that a greater proportion of injected liposome-ODNs, and free-ODNs, was reaching the hepatocytes than the Kupffer cells. Autoradiography had demonstrated a uniform distribution of silver grains on sections of liver from ducks injected with liposome-entrapped $^{125\text{I}}$-labelling ODNs. Evaluation of the amount injected from the remaining liposome preparation confirmed the suspicion that the estimate of the amount entrapped was lower than expected. This was confirmed by a rapid antiviral response, together with a fall in the target protein, when the experiment was repeated using the same dose of entrapped antisense ODN in the final experiment.
During these antiviral studies, an inhibition of viral replication was also observed with a control non-antisense PS-ODN with the same base composition but different base sequence. *In vitro* experiments to inhibit HBsAg gene expression in the PLC/PRF/5 cell line demonstrated marked inhibition with one of the antisense ODNs. However, the effect with liposomes and Lipofectin was disappointing. These studies also demonstrated the sensitivity of these cells to the non-specific effects of ODNs that had not been purified by HPLC; the use of HPLC-purified ODNs minimised these effects. Cellular uptake and nuclear concentration was markedly enhanced when fluorescein-labelling ODNs were transfected complexed with Lipofectin than when transfected in the free form.

The ability to synthesise PS-ODNs in large quantities with automated techniques, and their resistance to nuclease degradation, make these molecules attractive agents for the blockage of gene expression and the treatment of viral infections such as hepatitis B. The use of an efficient delivery system to concentrate them at the target site, whilst reducing their delivery to unwanted sites, would lower the cost of therapy and reduce the potential for adverse effects. However, if the ODNs need to be purified by HPLC prior to *in vivo* or clinical use, this would increase the costs and reduce the yield substantially. Several clinical trials using antisense ODNs have already begun (Gura 1995); a preliminary trial using GEM 91 in AIDS patients has reported a drop in viral load a few days after commencing treatment.

Of great concern in this study was the effect of the random sequence PS-ODN on viral replication *in vivo*. Most previous reports of this effect are from *in vitro* studies. In the ideal strategy, binding of the antisense ODN inactivates the intended mRNA and prevents its translation into protein, while leaving all other RNAs unaffected. Several factors make it difficult to develop clinical applications of the classical antisense model (Branch 1996). First, conventional antisense nucleic acids are too large and highly charged to readily pass into the nucleus and cytoplasm of cells. Second, they are complex molecules that interact with a wide variety of unintended cellular and microbial components. Third, many of the techniques used to obtain specific binding *in vitro*, such as increasing the temperature, cannot be used in living cells, making it difficult to design antisense approaches that discriminate between closely related RNAs. Finally, in many
diseases, continuous degradation of the target RNA is needed to counteract transcription, which soon replenishes the supply of the undesirable RNA.

Despite the initial concept that the extreme target-specificity of antisense ODNs would preclude adverse effects, decreased blood clotting and white cell count as well as changes in blood pressure and heart rate related to antisense ODNs are now reported in animal models. These effects may be explained by their propensity to bind to both intra- and extracellular proteins (Gura 1995; Stein 1995), particularly with phosphorothioate ODNs (Brown et al., 1994; Weidner et al., 1995). Furthermore, antisense ODNs mimic bacterial DNA in triggering a potent response by mammalian immune cells. Experiments have shown that DNA fragments containing the two-base sequence CpG activate mammalian B cells and natural killer cells in culture (Gura 1995).

Protein synthesis is prevented because the target RNAs are cut by ribonuclease H, enzymes that cleave the RNA component of RNA-DNA hybrids. Even very short hybrid regions are recognised by RNase H. In vitro, an RNA-DNA hybrid containing only 4 bases can be cleaved by RNase H (Branch 1996). Only interventions capable of selecting unique sequences can eliminate individual RNAs. Because the haploid human genome contains about $3 \times 10^9$ bases, statistically there is a good chance that any 17-base long sequence will occur only once. Statistical considerations indicate that conventional ODNs cannot perform as perfect magic bullets because RNase H-mediated cleavage does not require a sufficiently long recognition sequence. Based on studies performed in *Xenopus* oocytes, Woolf et al. (1992) concluded that it is probably not possible to obtain cleavage of an intended target RNA without also causing at least partial destruction of many nontargeted RNAs. They reported that “the dose of antisense oligomer required for efficient RNA cleavage in *Xenopus* embryos is normally close to the toxic dose, and we suspect that at least some of this toxicity is due to fortuitous antisense effects.” Thus fortuitous RNase-H-mediated cleavages can be an important source of nonantisense effects.

It is also known that PS-ODNs bind to proteins more avidly than standard ODNs (Stein 1996). They inhibit a number of enzymes, including many involved in nucleic acid metabolism, such as HIV-1 reverse transcriptase and human DNA polymerases. Although a PS-ODN complementary to the HIV-1 *rev* gene has been shown to reduce
p24 gag protein production in chronically-infected CD4+ lymphoid cells, PS-ODNs of almost any sequence and sufficient chain length (>15-mer) can potentially inhibit HIV replication (Stein 1992). In addition, they can block viral adsorption to cells, penetration or uncoating (Wagner 1994). The hepatitis B virus also uses reverse transcriptase to replicate through an RNA intermediate. It has also been shown that phosphorothioate oligonucleotides bind to multiple nuclear proteins in vitro, but no detectable protein binding was found to phosphodiester oligonucleotides (Weidner et al, 1995). The protein with the strongest binding was shown to be nucleolar C23/nucleolin, a 110 kDa protein; very low level binding was found for cytoplasmic proteins.

Nucleosides and nucleotides, the degradation products of ODNs can affect cell proliferation and differentiation. In addition, PS-ODNs may also exhibit nonantisense effects that may be sequence selective, for example ODNs that contain four contiguous guanosine residues (the G-quartet) (Stein & Krieg 1994) may be antiproliferative in a sequence-independent manner. Our control ODN did not contain a G-quartet, however, so this is unlikely to be the reason for the non-specificity in our study. It may also be possible that the ODNs (antisense and random) may be activating macrophages or cytotoxic T lymphocytes to release TNF-alpha or interferon-gamma that inhibit viral replication on an immune basis (Guidotti et al, 1996).

What happens to viral replication after therapy is stopped in the ducks which showed a good response was not determined in this study. Other studies have also not established this. However, it is likely that viraemia recurs, as the supercoiled forms of DHBV were still detectable in the liver after the 5-day course of therapy. This is a major problem with antiviral agents for HBV. Antiviral therapy to eliminate infection and prevent progression of liver disease may never be possible with existing technologies in most individuals that are not already mounting a strong cytotoxic immune response to infected hepatocytes.

Hepadnaviruses have a highly efficient mechanism for sustaining chronic productive infection of individual cells without the production of cytopathic effects. Such infection is maintained by multiple (10-30) copies of episomal, covalently-closed, circular (CCC), viral DNA in the nucleus of the infected cell (Mason 1993). In non-dividing cells, these DNA molecules are made by the viral DNA polymerase and are apparently, once synthesised, beyond the reach of conventional antiviral agents. Given this situation and
the putative long life of a hepatocyte, it is not surprising that deoxynucleoside analogues and other agents that act as inhibitors of viral DNA synthesis have not been very useful as short-term treatments of patients. Hepatocytes are thought to have a definite, programmed life-time exceeding 6 months, and the immune system of chronic HBV carriers could accelerate hepatocyte turnover (Rensen et al., 1996). Though virus production by infected hepatocytes can be blocked or reduced during antiviral therapy, it almost inevitably rebounds when therapy is withdrawn. This observation is consistent with the hypothesis that viral CCC-DNA remains in the nucleus of the infected hepatocytes during therapy and directs the rebound of virus replication in the hepatocytes, which are still infected when therapy is withdrawn. Nevertheless, future studies should establish whether viral replication can be eradicated completely with a prolonged course of antisense therapy, especially using liposomes with a long circulation time.

The use of liposomes as a delivery vehicle to increase the antiviral efficacy in this study was disappointing, even though studies of biodistribution showed increased hepatic uptake. Although antiviral efficacy was seen with a lower dose of liposome-entrapped antisense ODNs, the efficacy was insufficient to justify their use because of the cost and effort related to the entrapment procedure. Charge, lipid composition, and size (ranging from 20 to 10,000 nm) of liposomes can be varied and such factors may strongly affect the elimination from the circulation (Meijer & Molema, 1995). Repeated extrusion through polycarbonate filters with appropriate pore size and microfluidizing techniques can yield very small liposomes (<100 nm) while most of the entrapped solute is maintained. Sialic acid groups or other polar moieties such as polyethyleneglycol and certain gangliosides at the liposomal surface may decrease phagocytic uptake and thereby increase circulation time of liposomes (Wu & Zern, 1996). By manipulating the size, charge, and membrane lipid composition of liposomes, their availability for the infected cells can, to at least some extent, be improved. In a study using nucleoside analogues, when liposome-encapsulated phosphatidyl-dideoxycytidine (DOP-ddC) was administered intraperitoneally to mice, drug levels in the liver were 40 times greater than ddC when expressed as area under the curve (Hostetler et al., 1994). Targeting of HBV-specific antisense ODNs is, therefore, mainly aimed at improving their pharmacokinetic profile, including prevention of urinary loss and increased hepatic disposition, resulting in
sufficient concentrations within the hepatocyte following administration of a relatively low (cost-effective) dosage.

A more selective type of targeting could be achieved by including a target device at the external surface of the carrier. Examples of such targeting moieties are tissue-specific antibodies, glycoproteins, and glycolipids (Meijer & Molema 1995). An increased tissue specificity can also be obtained by coupling epidermal growth factor or asialoglycoproteins to the liposomes. Asialoglycoprotein receptors are highly specific for hepatocyte membranes, thus permitting liver-specific targeting (Wu & Wu 1988). With immunoliposomes, the attached monoclonal antibody reacts specifically with its antigen on the surface of its target cell and thereby bring the intraliposomal drug to the vicinity of the particular cell. Yet, only local release of the drug may then occur because the liposome generally will not fuse with the cell nor will it be endocytosed. Apart from problems of extravasation, such immunoliposomes may be opsonized with circulating antigens leading to clearance by Fc receptors on macrophages. In addition, \textit{in vivo} studies may exhibit major uptake by cells of the macrophage-phagocyte system. For the hepatitis B virus, antibody to hepatitis B core antigen may be a suitable ligand to attach to the liposome. Although uptake by Kupffer cells is a potential problem with liposomes, they make up only approximately 2.1% of the liver volume compared to hepatocytes which constitute 78% of the liver volume in humans (Meijer & Molema 1995).

The choice of a suitable hepatocyte-specific drug-carrier complex is subject to certain requirements, as recently summarised by Meijer and Molema (1995): (i) passage of the complex through 100 to 105 nm sized fenestrae that are formed by the liver endothelial layer; (ii) sufficient drug loading; (iii) target-specificity within the whole organism, including prevention of uptake by cells of the reticulo-endothelial system (RES) such as the liver macrophages (Kupffer cells); (iv) absence of immunogenic reactions; (v) biodegradability and absence of toxicity; (vi) release and retention of the active drug in the cytosol; and (vii) presence of the particular target receptor under pathological conditions.

Recently, new nucleoside analogues with potent activity against HBV have been developed which can produce undetectable levels of serum HBV DNA during treatment (Dienstag \textit{et al}, 1995; Grellier \textit{et al}, 1996). The practical application of antisense
oligonucleotide therapy has to be measured against the efficacy of these new agents such as famciclovir and 3'-thiacytidine, which are given orally and have minimal toxicity. However, the emergence of mutants resistant to these agents may mean that combined antiviral therapy to inhibit HBV replication will be an advantage (Ling et al., 1996). The combination of nucleoside analogues with antisense ODNs may be an area of interest. A recent study in woodchucks infected with woodchuck hepatitis virus showed that intraperitoneal injection of the lipid prodrug 1,2-Dipalmitoylphosphatidyl-dideoxyguanosine (DPP-ddG) was substantially more effective than free ddG in reducing WHV-DNA levels in serum (Korba et al., 1996). However, when treatment with the liponucleotide was stopped, serum WHV DNA levels returned to baseline levels; only two woodchucks were used in each treatment group.

It is likely that antisense ODNs may also need to be administered regularly to inhibit viral replication, similar to nucleoside analogues. An interesting new concept is the use of antisense RNAs (Branch 1996). Strategies involving effector RNAs generally differ from those involving DNA. Antisense DNA molecules are ready for action once they reach the appropriate cellular compartment: no new nucleic acid synthesis is required. In contrast, effector RNAs are rarely directly applied to cells, although it is possible to do this, especially when employing RNA molecules that have been chemically modified to enhance stability. More commonly, cells are transfected by vectors that contain the DNA sequence able to transcribe the effector RNA. When successfully implemented, this approach provides a permanent supply of the “transgene.” Ongoing production of the antisense molecules would be desirable in many situations and is an attractive feature of antisense RNA compared to antisense DNA. However, progress in developing clinical applications of antisense RNA has been delayed by the need to improve techniques for introducing vectors into animal cells.

Another new approach in blocking gene expression is the use of ribozymes, which are molecules that contain a catalytic active site composed of RNA (Branch 1996; Kiehntopf et al., 1995). As potential therapeutic agents, ribozymes have an important advantage over conventional antisense molecules: they are capable of catalytic cleavage. A single ribozyme molecule can cleave many target RNA molecules. In vitro studies to inhibit HBV replication have already commenced, with some success (Wands et al., 1993).
In conclusion, this study has shown that antisense oligodeoxynucleotides are potent inhibitors of gene expression and viral replication; using liposomes as a delivery vehicle does get the molecules to their target sites in the liver and hepatocytes. The antisense molecules are stable during liposome entrapment and transport to the target site. However, liposome delivery did not significantly improve the efficacy of ODNs in this study. Manipulating the design of the liposomes to increase the circulation time and reduce their size may enhance their potential as a non-viral delivery vehicle.

Nonantisense effects remain a major stumbling block to their use in humans with chronic hepatitis B virus infection. Antisense RNAs probably have greater potential if problems facing their cellular delivery can be overcome, as these may provide a continuous in vivo source of antisense molecules. Liposome delivery of antisense RNA to the liver may also be a useful avenue for future research.
PUBLICATIONS

Published abstracts

PN Soni, D Brown, R Saffie, D Moore, G Gregoriadis, GM Dusheiko.
Pharmacokinetics of free and liposome-entrapped phosphorothioate antisense oligonucleotides in ducks: A model for antisense therapy of hepatitis B.

PN Soni, D Brown, R Saffie, D Moore, K Savage, G Gregoriadis, GM Dusheiko.
Plasma clearance, organ uptake, urine excretion and hepatic distribution of free and liposome-entrapped antisense oligonucleotides in ducks.

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Inhibition of hepatitis B virus gene expression in a human hepatoma cell line by antisense oligodeoxynucleotides complexed with lipofectin.
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Antiviral efficacy of liposome-entrapped antisense oligodeoxynucleotides for the in vivo inhibition of duck hepatitis B virus infection.
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Published papers

PN Soni, D Brown, R Saffie, K Savage, D Moore, G Gregoriadis, GM Dusheiko.
Biodistribution, stability and antiviral efficacy of liposome-entrapped phosphorothioate antisense oligodeoxynucleotides in ducks for the treatment of chronic duck hepatitis B virus infection.
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Biodistribution, Stability, and Antiviral Efficacy of Liposome-Entrapped Phosphorothioate Antisense Oligodeoxynucleotides in Ducks for the Treatment of Chronic Duck Hepatitis B Virus Infection

PARESH N. SONI, DAVID BROWN, ROGGIEH SAFFIE, KAY SAVAGE, DUNCAN MOORE, GREGORY GREGORIADIS, AND GEOFFREY M. DUSHEIKO

This study investigated the feasibility of using liposomes to increase the hepatic delivery and antiviral efficacy of phosphorothioate antisense oligodeoxynucleotides (PS-ODN) for the in vivo treatment of hepatitis B virus (HBV) infection. Ducks infected with duck hepatitis B virus (DHBV) were used as the model. We studied the stability of an antisense PS-ODN in duck plasma, its integrity during the process of liposome entrapment, its in vivo biodistribution, plasma clearance, and excretion. In addition, the intrahepatic distribution of a labeled free and liposome-entrapped ODN was also investigated. The results of our studies show that: 1) phosphorothioate ODN remain stable during the process of liposome entrapment; 2) are stable in duck plasma for many hours; 3) are rapidly cleared from the plasma when injected intravenously; 4) intravenous injection of antisense ODNs entrapped within liposomes enhances delivery of the ODN to the liver; and 5) inhibit DHBV replication. Serum DHBV DNA levels fell rapidly, with a corresponding decrease in intrahepatic viral replicative intermediates at the end of the 5-day study period. Although inhibition of viral replication and a fall in the target protein was observed, a marked inhibition of viral replication was entirely through an antisense mechanism. Therefore, liposomes may be effective vehicles to improve the delivery of antisense oligonucleotides to the liver for the therapy of hepatotropic viruses. (Hepatology 1998;28: 1402-1410.)

Interferon alfa remains the only licensed therapy for chronic hepatitis B infection. However, only 25% to 40% of patients respond. Antisense oligodeoxynucleotides (ODN) are synthetic DNA molecules that can inhibit gene expression within cells by their ability to bind a complementary messenger RNA sequence and prevent translation of the mRNA. Antisense oligonucleotides may also activate ribonuclease H (RNase H). The results of in vitro studies, in which drug concentrations and metabolism may or may not mimic in vivo conditions, demonstrate that antisense oligonucleotides have significant antiviral activity. Although antisense therapy was first proposed by Zamecnik and Stephenson in 1978, few in vivo studies have been reported. Clinical trials are now in progress to evaluate the efficacy of antisense ODNs in several human diseases, including malignancies and viral infections.

For antisense oligonucleotides to be effective, appropriate concentrations at the requisite site of action must be attained and maintained in vivo, and their effectiveness therefore depends on adequate delivery to the target site. Their use as therapeutic agents has several other potential limitations: large quantities could be required, and they may be degraded by exo- and endonucleases in plasma and in the intracellular environment. Previous studies have shown that naturally occurring phosphodiester oligonucleotides have limited therapeutic utility because of their rapid degradation in vivo.

Nuclease-resistant backbones, such as phosphorothioate (PS) molecules, confer resistance to degradation so that a biologically active drug can reach the intended site of action as an active substance. The use of specialized delivery methods in cell culture studies appears to be essential if the biological potential of an antisense agent is to be fully realized. One potential means of delivering antisense oligonucleotides, therefore, would be to entrap the molecules within liposomes.

Duck hepatitis B virus (DHBV) infection is a useful in vivo model for the screening of antiviral drugs for hepatitis B virus (HBV). The virus shares a common genomic size, organization, and mode of replication to the human HBV, and chronic infection can be readily induced by neonatal infection. Several studies have now shown that antisense ODN are capable of suppressing HBV replication in vitro and in vivo, but further experimentation is required to explore methods for protecting antisense oligonucleotides against degradation, and for improving their delivery into the cell interior. The aim of this study, therefore, was to assess the
value of liposomes as a delivery vehicle for antisense agents in ducks by studying the stability, plasma clearance, fate, and antiviral efficacy of a free and liposome-entrapped PS-ODN.

**MATERIALS AND METHODS**

**Oligonucleotide Synthesis and Labeling.** An 18-mer PS-ODN sequence previously shown to inhibit DHBV replication in vitro and in vivo was used to assess stability, biodistribution, and antiviral efficacy. The base sequence used (nucleotides 795-812) was 5'-AAA-TAC-TAC-CCC-GTT-GTA-3', which is complementary to the pre-S region of DHBV; in preliminary experiments, the presence of the target sequence was confirmed by amplification, cloning, and sequencing of this region in our viral isolates. In vivo inhibition of DHBV replication using this antisense sequence was also confirmed.

The ODN were synthesized by the solid-phase phosphoramidite method (Oswel DNA Service, Edinburgh, Scotland, and Cruachem, Glasgow, Scotland). After deprotection and cleavage, the ODN were purified by reverse-phase high-performance liquid chromatography. ODNs were trace-labeled with \(^{125}\)I-dCTP using terminal deoxytransferase by incubation at 37°C for 3 hours for plasma clearance and biodistribution studies. Unincorporated label was removed by Sephadex G-50 gel filtration, and labeled ODN were mixed with unlabeled ODN to give a specific activity of 2.4 \(\times 10^4\) cpm/\(\mu\)mol. To assess the in vivo stability of ODN in normal duck plasma, PS-ODN were labeled with \(^{32}\)P]adenosine triphosphate (ATP) using T4 polynucleotide kinase and incubated at 37°C for 2 hours. To test antiviral efficacy, ODN were end-labeled with \([\alpha-^{32}]\)dATP to monitor entrapment within liposomes.

**Liposomal Entrapment of ODN.** ODN were entrapped into positively charged liposomes composed of phosphatidylcholine, dioleoyl phosphatidylethanolamine, and stearylamine in a molar ratio of 2:2:1 by the dehydration-rehydration method. Briefly, phospholipid-choline (16 \(\mu\)mol), dioleoyl phosphatidylethanolamine (16 \(\mu\)mol), and stearylamine (8 \(\mu\)mol) were dissolved in chloroform and placed in a round-bottomed flask. The chloroform solvent was evaporated to dryness, and the pre-S region of DHBV; in preliminary experiments, the presence of the target sequence was confirmed by amplification, cloning, and sequencing of this region in our viral isolates. In vivo inhibition of DHBV replication using this antisense sequence was also confirmed.

The ODN were synthesized by the solid-phase phosphoramidite method (Oswel DNA Service, Edinburgh, Scotland, and Cruachem, Glasgow, Scotland). After deprotection and cleavage, the ODN were purified by reverse-phase high-performance liquid chromatography. ODNs were trace-labeled with \(^{125}\)I-dCTP using terminal deoxytransferase by incubation at 37°C for 3 hours for plasma clearance and biodistribution studies. Unincorporated label was removed by Sephadex G-50 gel filtration, and labeled ODN were mixed with unlabeled ODN to give a specific activity of 2.4 \(\times 10^4\) cpm/\(\mu\)mol. To assess the in vivo stability of ODN in normal duck plasma, PS-ODN were labeled with \(^{32}\)P]adenosine triphosphate (ATP) using T4 polynucleotide kinase and incubated at 37°C for 2 hours. To test antiviral efficacy, ODN were end-labeled with \([\alpha-^{32}]\)dATP to monitor entrapment within liposomes.

**Analysis of Integrity of ODN During Liposome Entrapment.** To assess whether the ODN were degraded during the process of liposome entrapment, ODN were labeled with \(^{32}\)P]ATP and T4 polynucleotide kinase as described. Free label was separated from labeled ODN by Sephadex G-50 gel filtration. Labeled ODN were then entrapped into positively charged SUV as described above. Before, at each step, during, and after entrapment, samples were stored for analysis of ODN size and degradation. ODN were extracted from liposomes by incubation with 1% Triton X-100 at 37°C for 1 hour and electrophoresed on a 20% polyacrylamide gel electrophoresis (PAGE)/7-mol/L urea gel. This was compared with a labeled oligonucleotide size marker (4 to 22 bases). The gel was fixed in 10% acetic acid/50% methanol/10% glycerol solution, and then dried before autoradiography at -70°C.

**In Vivo Plasma Stability.** Twenty-five picomoles of PS-ODN were labeled with \(^{32}\)P]ATP and T4 polynucleotide kinase to a specific activity of 4 \(\times 10^4\) cpm/\(\mu\)mol. Twenty microliters of \(^{32}\)P-labeled PS-ODN was incubated in 250 \(\mu\)L of normal duck plasma at 37°C for 24 hours with gentle shaking. Two microliters of labeled ODN was stored as a control before addition to plasma, and 10-\(\mu\)L samples were taken at various times after exposure to the plasma and diluted in 50 \(\mu\)L of PBS for analysis. Samples were analyzed by electrophoresis on a 20% polyacrylamide gel containing 7 mol/L urea, and compared with a \(^{32}\)P-labeled oligonucleotide size marker (GibCo BRL).

**In Vivo Kinetic and Tissue Distribution Studies.** Ten 12-day-old uninfected Mallard ducklings (204 ± 17 g) were used. Animal use and care were in accordance with the Animals (Scientific Procedures) Act 1986 as stated by the Home Office, London, and approval for all the in vivo studies was obtained from this office. Animals were injected intravenously via a neck vein with a single bolus intravenous injection of approximately 1 mg of ODN made up to 1 mL with PBS. Four were injected with liposome-ODN, 4 control ducks were injected with free ODN, and 2 were injected with 1 mL of PBS. After intravenous injection, each animal was placed in a cage and fed a commercial diet and water ad libitum. Plasma samples were taken at 2, 15, 30, and 60 minutes, and 4, 6, and 24 hours into ethylenediaminetetraacetic acid–containing tubes. Urine and feces were collected between 0 to 1 hour, 1 to 4 hours, 4 to 6 hours, and 6 to 24 hours.

One duckling from each of the ODN-treated groups was killed at 1 and 4 hours, and the remaining 6 ducklings (2 free-ODN treated, 2 liposome-ODN treated, and 2 PBS-treated) were killed at 24 hours. Ducklings were killed with an overdose of sodium pentobarbital anesthesia, and the tissues/organisms were collected from each bird. Organs were weighed and stored at -70°C until further analyses. Plasma was separated by centrifugation, and 100 \(\mu\)L was used for gamma counting. Radioactivity was measured in plasma samples, tissue samples, urine/feces, and aliquots of the injected ODN using a gamma counter (Rigagamma 1271, LKB Wallac, Turku, Finland). Total radioactivity in the ducks' blood was calculated assuming the body volume to be 10% of the body weight. Twenty microliters of \(^{32}\)P-labeled PS-ODN was incubated in 250 \(\mu\)L of normal duck plasma at 37°C for 24 hours with gentle shaking. Two microliters of labeled ODN was stored as a control before addition to plasma, and 10-\(\mu\)L samples were taken at various times after exposure to the plasma and diluted in 50 \(\mu\)L of PBS for analysis. Samples were analyzed by electrophoresis on a 20% polyacrylamide gel containing 7 mol/L urea, and compared with a \(^{32}\)P-labeled oligonucleotide size marker (GibCo BRL).

**Intrahepatic Distribution of ODN.** To assess the intrahepatic distribution of injected ODN, autoradiography of frozen sections of liver was performed. Briefly, after killing the ducklings, liver samples were immediately fixed in 4% paraformaldehyde for 4 hours, washed in PBS, and dehydrated overnight in 15% sucrose/PBS. Thereafter, the tissue was embedded in OCT compound, frozen rapidly in isopentane/liquid nitrogen, and stored at -70°C. Five-micrometer cryostat sections were cut and placed on glass slides precoated with poly-L-lysine, which were then air-dried, wrapped in cling film, and stored at -20°C until use.

The cryostat sections were brought to room temperature. Slides were dipped in prewarmed photographic emulsion (Hypercoat emulsion, Amersham, Bucks, UK) in a dark room, dried, and stored in a light-tight box at 4°C for varying periods of exposure. Slides were developed for 3 minutes (Kodak Microphen), washed, and fixed in 1:4 Hypam fixer for 5 minutes (Ilford), counterstained with hematoxylin and bluing solution, differentiated in acid alcohol, before...
dehydration with 100% alcohol and mounting with DPX. Sections were then examined by light microscopy for the presence and distribution of silver grains. Sections from ducklings injected with PBS were used as negative controls.

**Detection of ODN in Plasma by Hybridization.** Plasma samples from ducks killed at 1 and 4 hours were also used to detect the presence of ODN in the plasma by a hybridization method, modified from Temsamani. Briefly, 250 µL of plasma was incubated with 250 µL of DNA extraction buffer (0.5% sodium dodecyl sulfate [SDS], 10 mmol/L NaCl, 20 mmol/L Tris HCl [pH 7.6], 10 mmol/L ethylenediaminetetraacetic acid) and proteinase K (1 mg/mL final concentration) for 90 minutes at 60°C. After incubation, 200 µL of distilled water was added, and the samples were extracted twice with 500 µL of phenol/chloroform/isoamyl alcohol (25:24:1), and once with 500 µL of chloroform. Before blotting, the samples were heated at 90°C for 5 minutes and mixed with 40 µL of 20X SSC buffer (3 mol/L NaCl, 0.3 mol/L sodium citrate [pH 7.0]). A nylon membrane (pore size, 0.45 mm) and Whatman 3 MM paper were soaked in 1X SSC.

The membrane was placed in a slot-blot apparatus (Minifold II, Shleifer and Schuell, Dassel, Germany), and a vacuum was applied. The wells were first rinsed with 100 µL of 20X SSC buffer, the samples were loaded, and the wells were then rinsed again with 100 µL of 20X SSC. Known quantities of injected ODN were also loaded as standards. The membrane was then removed and cross-linked (0.75 kgerjol) for 10 minutes.

An 18-mer oligonucleotide complementary to the injected ODN was 5’-end-labeled with [y-32P]ATP and T4 polynucleotide kinase. The specific activity of the probe was 87.9 mCi/mmol. The membrane was prehybridized for 1 hour at 45°C in hybridization buffer (6X SSC, 10X Denhardt's solution, 50 mg/mL salmon sperm DNA, 0.1% SDS), and hybridization was performed overnight at 45°C. The membrane was washed three times in 6X SSC/0.1% SDS for 5 minutes each time at the hybridization temperature, and exposed for autoradiography at −70°C.

**RESULTS**

**Plasma Stability of ODN.** Ten-microliter samples were taken at 2, 15, 30, and 60 minutes and 4, 6, and 24 hours after incubation in vitro in normal duck plasma. Electrophoresis on a 20% PAGE/7-mol/L urea gel of the PS-ODN from plasma showed a single band at all time points sampled, indicating that the ODN remained intact over 24 hours with no evidence of degradation (Fig. 1).

**Analysis of Integrity of ODNs During Liposome Entrapment.** ODNs were not degraded during any of the stages of liposome entrapment, as shown by a single 18-base band on the 20% PAGE/7-mol/L urea gel (Fig. 2).

**In Vivo Plasma Clearance.** Uninfected ducklings were used in these studies because the fate of the injected ODN may have been altered if viral sequences complementary to the ODN were present in the duck body fluids such as plasma. Plasma levels of PS-ODN were rapidly depleted after intravenous administration in both treatment groups, with a half-life of approximately 1 to 2 minutes. Figure 3 shows the rapid clearance of the ODN from plasma, as a percentage of the ODN injected, with a little more liposome than free ODN remaining in the circulation at the end of the 24-hour period.

**Biodistribution.** The percentage of the injected dose detected in each of the organs sampled is shown in Table 1. The
Fig. 1. Assessment of the in vitro stability of the $^{32}$P-labeled ODN on exposure to duck plasma from 2 minutes to 24 hours on a 20% PAGE/7-mol/L urea gel. ODN were labeled with $[\gamma-^{32}P]ATP$ and T4 polynucleotide kinase. Twenty microliters of $^{32}$P-labeled PS-ODN was incubated in 250 µL of normal duck plasma at 37°C for 24 hours with gentle shaking. Lane 0, Control labeled ODN before addition to plasma; lanes 1 to 7, 2 minutes, 15 minutes, 30 minutes, 60 minutes, 4 hours, 6 hours, and 24 hours after exposure to duck plasma.

greatest proportion of either injected free- or liposomal-entrapped ODN was present in the liver. However, the percentage of injected liposome-ODN retained in the liver at 1, 4, and 24 hours was 82.8%, 67.3%, and 25.8% (mean of two ducks at 24 hours) compared with 13.9%, 10.8%, and 2.7% for free ODN. Thus, the hepatic uptake and retention of liposome-ODN was far greater than for free-ODN at all time points measured. The ratio of liposome-ODN to free-ODN uptake in the liver for paired ducks measured at corresponding times ranged from 6.0 to 9.6 (Table 1). The amounts present in other organs was far less, and similar for both treatment groups, with the exception of the airsacs, which retained a greater proportion of liposome-ODN than free-ODN at 1 and 4 hours. The amount of ODN present in the brain was the lowest in both groups. Percentage of splenic uptake was much lower than hepatic uptake, even for the liposome-ODN. However, the mean weight of the liver in all ducklings was 18.8 ± 1.5 g compared with 0.3 ± 0.1 g for the spleen.

Excretion of ODN. Ducks excrete urine and feces through the cloacae; therefore, it is not possible to differentiate urinary from fecal excretion. The cumulative excretion of the free-ODN after 1, 4, 6, and 24 hours was 8.7% ± 1.5%, 16.5% ± 0.8%, 17.2% ± 0.9%, and 18.6% ± 2.1%, respectively. For the liposome-ODN, the excretion was 10.2% ± 2.4%, 19.8% ± 2.1%, 25.9% ± 1.4%, and 28.9% ± 1.6%, respectively. Thus, a large proportion of the injected ODN was excreted within 24 hours in both treatment groups.

Intrahepatic Distribution. Hepatic tissue sections exposed to photographic emulsion were developed after 2, 5, 12, and 28 days for estimation of the distribution of silver grains. The strongest signal was seen after 28 days exposure, as shown in Fig. 4. This shows sections from the ducklings killed at 1 hour after injection with either free- or liposome-ODN and a control section from a duckling injected with PBS and killed after 24 hours. Silver grains were readily detectable in the duckling injected with liposome-ODN, but only background signal was detected in the ducks injected with either PBS or free-ODN. This finding was consistent with the much greater proportion of injected ODN present in the liver of liposome-ODN-injected ducks by gamma counting, as shown in Table 1. The distribution of the silver grains was uniform and even, rather than confined to specific cells. However, cell morphology was not adequate to differentiate Kupffer cell uptake from uptake in other intrahepatic cell types.

**ODN Detection by Hybridization.** Known quantities (ranging from 0 to 1,000 ng of ODN) were spotted onto the nylon membrane to determine the sensitivity and specificity of ODN detection in plasma. The sensitivity was between 0 and 3.1 ng of ODN (Fig. 5). ODN were detectable in plasma up to 30 minutes after injection, and in three of the four ducks at 15 minutes. The amount of ODN spotted onto the membrane was the total amount extracted from 250 µL of plasma. Therefore, if required, the total quantity in the circulation can be calculated by correcting for the total blood volume, and producing a standard curve after densitometry.

**DHBV DNA Response to Antiviral Therapy.** Mean pretreatment levels of serum DHBV DNA ranged from $10^3$ to $10^5$ pg/mL. All ducklings survived the therapy with no observed adverse events; the expected increase in weight during the treatment phase was observed. We observed a rapid and marked decline in serum DHBV DNA levels in all treated ducklings from the first posttreatment day, which was sustained throughout the treatment period (Fig. 6). Levels in the ducks treated with the high doses (20 µg/g body weight) of free antisense and

Fig. 2. Assessment of the integrity of the $^{32}$P-labeled ODN during liposome entrapment. Labeled ODN were entrapped into positively charge SUV as described in the text. ODN were extracted from liposomes by incubation with 1% Triton X-100 at 37°C for 1 hour and electrophoresed on 20% PAGE/7-mol/L urea gel. Lane 1, before addition to SUV; lane 2, before freeze-drying; lane 3, after rehydration; lane 4, after centrifugation; lane 5, after microfluidization; lane 6, after separation of unentrapped ODN; lane 7, entrapped ODN; lane M, Oligo(dT)$_{22}$ ladder.
random-sequence ODN were undetectable after the second posttreatment day. The random-sequence ODN appeared to be as efficient as the free antisense ODN in suppressing viremia.

The ducklings treated with liposome-entrapped antisense ODN at both dosages also showed a marked decline in serum levels, although low levels were still detectable on days 4 and 5. In the four untreated ducklings, a gradual decline in serum DHBV DNA levels was noted (Fig. 6). There was a 5-log reduction in serum DHBV DNA levels in ducklings treated with the free antisense ODN and random-sequence ODN, a 4-log reduction in all ducklings treated with liposome-entrapped antisense ODN, and less than a 2-log reduction in the untreated control ducklings.

Using gel electrophoresis and Southern blot hybridization, which does not exclude the possibility of detection of supercoiled DHBV DNA, we found signals on the autoradiograph at the correct size of migration of supercoiled DHBV DNA. Supercoiled DHBV DNA is present in very small amounts, and the enrichment techniques used by Summers et al. would be essential for accurate quantitation. However, because quantitation of supercoiled DHBV DNA was not the primary aim of this experiment, we did not address this further. Southern blot analysis for intrahepatic DHBV DNA showed a marked suppression of viral replicative intermediates in the livers of all treated ducklings, especially in those treated with 20 μg/g body weight of free antisense and random-sequence ODN (Fig. 7); almost a total loss of episomal DNA was observed in these ducklings. There was also a decline in the two ducklings treated with 5 μg/g body weight of liposome-entrapped antisense ODN, although episomal DNA could still be detected in the livers of these ducklings. However, in none of the treated ducklings was there a loss of higher-molecular-weight DHBV DNA, possibly representing covalently closed or supercoiled forms of DHBV DNA. All replicative forms of viral DNA were found in the untreated ducklings.

Serum DHBsAg Response to Therapy. Immunoblotting and autoradiography revealed a 17-kd major band corresponding to the S surface polypeptide of DHBV, and two minor bands at 27 and 33 kd that were interpreted as pre-S2 polypeptides. Table 2 shows the percentage reduction in posttreatment DHBsAg from pretreatment values, quantified by scanning densitometry, and changes in serum albumin (g/L) from pretreatment to posttreatment values. The untreated controls showed some reduction in posttreatment DHBsAg levels (mean, 51.0%); however, this corresponded with a gradual fall in serum DHBV DNA levels. All ducklings treated with the higher dose of free antisense and random-sequence ODN showed a marked reduction in DHBsAg levels (mean, 51.0%); however, this corresponded with a gradual fall in serum DHBV DNA levels. All ducklings treated with the higher dose of free antisense and random-sequence ODN showed a marked reduction in DHBsAg levels, with the levels becoming undetectable in two of three ducklings in each group. However, the reduction in the ducklings treated with high and low doses of liposome-antisense ODN was inconsistent, with a marked reduction in one duckling per group, but only a moderate reduction in the remaining duckling per group. In contrast, the serum albumin levels rose from the pretreatment to the posttreatment period in almost all the ducklings, suggesting little or no effect of the ODN on serum albumin. The difference between the treatment groups in the

### Table 1. Percentage of Injected ODN Retained in Each Organ at Different Times

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidneys</th>
<th>Airsacs</th>
<th>Heart</th>
<th>Brain</th>
<th>Liposome/Free Uptake in Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>Liposome</td>
<td>82.8</td>
<td>1.13</td>
<td>2.9</td>
<td>8.8</td>
<td>1.22</td>
<td>0.16</td>
<td>6.0</td>
</tr>
<tr>
<td>Free</td>
<td>13.9</td>
<td>0.58</td>
<td>2.5</td>
<td>1.9</td>
<td>0.6</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>Liposome</td>
<td>67.3</td>
<td>0.77</td>
<td>0.8</td>
<td>10</td>
<td>0.29</td>
<td>0.1</td>
<td>6.2</td>
</tr>
<tr>
<td>Free</td>
<td>10.8</td>
<td>0.6</td>
<td>0.74</td>
<td>0.52</td>
<td>0.28</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>Liposome</td>
<td>25.8</td>
<td>0.38</td>
<td>0.3</td>
<td>0.37</td>
<td>0.14</td>
<td>0.08</td>
<td>9.6</td>
</tr>
<tr>
<td>Free</td>
<td>2.7</td>
<td>0.39</td>
<td>0.17</td>
<td>0.18</td>
<td>0.03</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE. One- and 4-hour values are for individual ducks, and 24-hour values are for a mean of two ducks.
Bm M

Fig. 4. Intrahepatic distribution of \(^{131}I\)-labeled ODN on autoradiography after 28 days of exposure. Five-micrometer cryostat sections of fixed liver tissue were cut onto glass slides and dipped in prewarmed photographic emulsion. After exposure in a light-tight box at 4°C for 28 days, slides were developed and counterstained. Sections were examined by light microscopy for the presence and distribution of silver grains. (A) Duck injected with PBS killed at 24 hours. (B) Duck injected with free-ODN killed at 60 minutes. (C) Duck injected with liposome-ODN killed at 60 minutes. (Original magnification \(\times 100\).)

reduction in DHBsAg levels was not statistically significant \(P = 1.048\), Kruskal-Wallis test); similarly, the change in serum albumin levels between the treatment groups was also not significant \(P = 389\), Kruskal-Wallis test).

DISCUSSION

The results of our studies show that: 1) PS-ODN remain stable during the process of liposome entrapment; 2) are stable in duck plasma for many hours; 3) are rapidly cleared from the plasma when injected intravenously; 4) intravenous injection of antisense ODN entrapped within liposomes enhances delivery of the ODN to the liver; and 5) inhibit DHBV replication. Thus, liposomes may be effective vehicles to improve the delivery of oligonucleotides to the liver for the therapy of hepatotropic virus infections.

Our findings indicate that elimination of ODN was largely by renal clearance and urinary excretion, in keeping with their water-soluble properties.\(^{26-28}\) Importantly, we ruled out degradation of ODN to smaller molecules during liposomal entrapment, and in vitro, there was no degradation of the ODN by plasma exo- or endonucleases, after 24 hours at 37°C, which is a property of phosphorothioate-modified ODN. Naturally occurring phosphodiester molecules can be degraded in vivo within 5 minutes in some animal species.\(^{29}\) Notably, there was markedly increased hepatic uptake of the liposome- versus free-ODNs. Our data thus suggests that liposomes may be an attractive nonviral vector to increase the delivery of these molecules to the liver. It is known that liposomes are preferentially targeted to the reticuloendothelial system. Thus, they would be adsorbed largely by the spleen and Kupffer cells of the liver. However, the absolute amounts taken up by the spleen (a relatively small organ in ducks) was far less partly a result of the greater mass of the liver.

Being particles rather than molecules, liposomes can only leave the circulation at sites where there are relatively large gaps or "fenestrations" between the endothelial cells lining the blood vessels. Such a fenestrated endothelium is characteristic of the hepatic and splenic sinusoids.\(^{30}\) We have examined thin sections of the liver by microautoradiography to determine whether the liposome-ODN were preferentially targeted to any specific cell type. Kupffer cells could not be identified by light microscopy; however, the distribution within the liver was uniform and not concentrated around

Fig. 5. Quantification of plasma ODN by hybridization on a slot-blot manifold. Plasma samples from ducks killed at 1 and 4 hours were used to detect the presence of ODN in the circulation by a hybridization method. DNA was extracted from plasma, spotted onto a nylon membrane, and hybridized with an 18-mer oligonucleotide probe complementary to the injected ODN that was labeled with \(\gamma^{32}P\)-ATP and T4 polynucleotide kinase. The membrane was then exposed for autoradiography at \(-70°C\). Standards are (from top to bottom): 1,000, 100, 50, 25, 12.5, 6.25, 3.1, and 0 ng of ODN. F and L indicate plasma from ducks injected with free- or liposome-ODN. Ducks F1 and L1 were killed at 240 minutes; ducks F2 and L2 were killed at 60 minutes.
any particular cells. In future studies, this important question could perhaps be resolved by specifically identifying relative uptake within duck Kupffer cells and hepatocytes, for example by using a monoclonal antibody, with or without electron microscopy, to duck reticuloendothelial cells. A preliminary experiment using two ducks injected with free or liposome-entrapped $^{125}$I-labeled ODN and separating sinusoidal cells from hepatocytes showed that a greater proportion of ODN was taken up by the hepatocytes (data not shown).

In this study, an enzymatic method was used for labeling the ODN, allowing only small quantities to be labeled. Analysis of urine, liver, and plasma samples on a 20% PAGE/7-mol/L urea gel soon after the birds were killed showed no additional radioactive bands (data not shown). Thus, it is not possible to exclude partial degradation in tissues. The small number of ducks used did not permit detailed pharmacokinetic analysis of mean distribution and elimination half-life for the two forms of delivery. Because the results of the stability and biodistribution studies were encouraging, we proceeded to test the antiviral efficacy of liposome-entrapped antisense ODN in chronically infected ducklings.

Our data indicate that oligonucleotides were potent inhibitors of DHBV viral replication in vivo. Daily measurements of serum DHBV DNA allowed us to demonstrate the rapidity of this inhibition. We were able to show that serum levels of viremia were lower in untreated ducks, albeit

![Fig. 7](image_url) **Fig. 7.** Southern blot analysis of intracellular DHBV DNA from liver separated on a 1% agarose gel at the end of treatment. Each lane contains 10 µg total cellular DNA. *Lane 1*, free antisense ODN 20 µg/gm body weight; *lane 2*, free random-sequence ODN 20 µg/gm body weight; *lane 3*, liposome-entrapped antisense ODN 20 µg/gm body weight; *lane 4*, liposome-entrapped antisense ODN 5 µg/gm body weight; *lane 5*, untreated controls; *lane M*, 1-kb DNA ladder.

### Table 2. Percent Reduction in DHBsAg From Pretreatment Values, and Change in Serum Albumin From Pretreatment to Posttreatment Values

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Duck No.</th>
<th>% Reduction from Pretreatment DHBsAg</th>
<th>Change in Serum Albumin (gm/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td>507</td>
<td>43.7</td>
<td>+8</td>
</tr>
<tr>
<td></td>
<td>506</td>
<td>29.3</td>
<td>-4</td>
</tr>
<tr>
<td></td>
<td>504</td>
<td>46.3</td>
<td>+4</td>
</tr>
<tr>
<td></td>
<td>501</td>
<td>76.5</td>
<td>+1</td>
</tr>
<tr>
<td>Free antisense ODN</td>
<td>518</td>
<td>80.6</td>
<td>+4</td>
</tr>
<tr>
<td>(20 µg/gm bw)</td>
<td>505</td>
<td>100</td>
<td>+1</td>
</tr>
<tr>
<td></td>
<td>521</td>
<td>100</td>
<td>+3</td>
</tr>
<tr>
<td>Random-sequence ODN</td>
<td>513</td>
<td>100</td>
<td>-1</td>
</tr>
<tr>
<td>(20 µg/gm bw)</td>
<td>514</td>
<td>100</td>
<td>+2</td>
</tr>
<tr>
<td></td>
<td>502</td>
<td>92.7</td>
<td>+1</td>
</tr>
<tr>
<td>Liposome-antisense ODN</td>
<td>519</td>
<td>69.9</td>
<td>+3</td>
</tr>
<tr>
<td>(20 µg/gm bw)</td>
<td>511</td>
<td>100</td>
<td>+5</td>
</tr>
<tr>
<td>Liposome-antisense ODN</td>
<td>515</td>
<td>40.6</td>
<td>+3</td>
</tr>
<tr>
<td>(5 µg/gm bw)</td>
<td>520</td>
<td>93.5</td>
<td>+8</td>
</tr>
</tbody>
</table>
spontaneous fluctuations occur. A dose response may be noticeable, but the number of treated ducks was small.

A surprising finding was the marked effect on viral replication seen with high doses of random-sequence ODN. In fact, the random-sequence ODN-treated ducklings lost viremia as efficiently as the ducklings treated with antisense ODN at the same dose. No sequence homology between the random-sequence ODN and any region of the DHBV genome could be found. Many studies have used a sense ODN complementary to the antisense sequence; however, this was not used in this study, because it has recently been stated that the best control sequences are those that contain the same base composition as the antisense sequence. Although non-sequence-specific effects were observed in our study, a control protein measured pre- and posttreatment showed no reduction in serum levels. If the nonspecific effects were caused by binding to serum proteins, one would have expected a reduction in serum albumin in the groups treated with high doses of ODN.

The non-sequence-specific effects of PS-ODN require further explanation. Sequence-specific and nonspecific binding of PS-ODN, which are polyanions, to small molecules and proteins have been described. Although a PS-ODN complementary to the human immunodeficiency virus-1 reverse transcriptase has been shown to reduce p24 gag protein production in chronically infected CD4+ lymphoid cells, PS-ODN of almost any sequence and sufficient chain length (>$15$-mer) can potentially inhibit human immunodeficiency virus replication. Interference with reverse transcriptase may be a possible mechanism; HBV also uses a reverse transcriptase to replicate. ODN may also inhibit viral infection by other nonantisense mechanisms that are not fully understood, but which may include interference with adsorption, penetration, or uncoating.

Nucleosides and nucleotides, the degradation products of ODN, can affect cell proliferation and differentiation. In addition, PS-ODN may also exhibit nonantisense effects that may be sequence-selective, for example, ODN that contain four contiguous guanosine residues (the G-quartet) may be antiproliferative in a sequence-independent manner. Our control ODN did not contain a G-quartet, however, so this is unlikely to be the reason for the nonspecificity in our study. It may also be possible that the ODN (antisense and random) may be activating macrophages or cytotoxic T lymphocytes to release tumor necrosis factor α or interferon gamma that inhibit viral replication on an immune basis.

With antisense therapy, protein synthesis is prevented because the target RNAs are cut by RNase H, enzymes that cleave the RNA component of RNA-DNA hybrids. Even very short hybrid regions are recognized by RNase H. In vitro, an RNA-DNA hybrid containing only four bases can be cleaved by RNase H. Only interventions capable of selecting unique sequences can eliminate individual RNAs. Because the haploid human genome contains about $3 \times 10^9$ bases, statistically, there is a good chance that any 17-base-long sequence will occur only once. Statistical considerations indicate that conventional ODN cannot perform as perfect magic bullets because RNase H-mediated cleavage does not require a sufficiently long recognition sequence. Based on studies performed in Xenopus oocytes, Woolf et al. concluded that it is probably not possible to obtain cleavage of an intended target RNA without also causing at least partial destruction of many nontargeted RNAs. They reported that the dose of antisense oligomer required for efficient RNA cleavage in Xenopus embryos was normally close to the toxic dose, and suspected that at least some of this toxicity is caused by fortuitous antisense effects. Thus, fortuitous RNase H-mediated cleavages can be an important source of nontargeted effects.

Thus, although inhibition of viral replication and a fall in the target protein was observed in this study, we cannot be certain that this was entirely through an antisense mechanism. Although the reduction in target protein between the treated groups was not statistically significant, the results appear to be clinically relevant, and the small number of ducks used does not allow sufficient power for a statistical difference to be observed. It has been suggested that liposomally entrapped ODN may reduce the amount of nonspecific effects caused by binding of ODN to serum proteins. This would be an interesting aspect for further study.

Our experiments were designed to model the feasibility of liposomal antisense ODN treatment of chronic hepatitis B. Our study demonstrated that liposome-entrapped antisense oligonucleotides are delivered to their target site and indeed inhibit DHBV replication. There are potential advantages for treatment of human hepatitis B infection: first, lower doses can be used, thus reducing the high production costs of these synthetic agents; second, there may be fewer unwanted side effects because of dose reduction and specific targeting; hepatocytes and splenic lymphocytes are known to be infected with hepatitis B. Thus, there seems to be a reasonable possibility for the application of liposome technology for the delivery of antisense oligonucleotide treatment of HBV. The practical application of antisense oligonucleotide therapy must be measured against the efficacy of new nucleoside analogues active against hepatitis B. However, the emergence of mutants resistant to these agents may mean that combined antiviral therapy to inhibit HBV replication will be an advantage.

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REFERENCES


