HIGH-YIELD INCORPORATION OF PLASMID DNA INTO LIPOSOMES:
CHARACTERISATION AND TRANSECTION EFFICIENCY STUDIES

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2001
To my parents

You brought me into this world and
gave me all the love and happiness that any child dreams about.
You were there when I was ill and when I was sad.
You were there when I started my first day at school.
You were there whenever I needed you.
When you were old and needed me,
you let me go unselfishly
to make a better future for a better world.
This is the token I have collected for you
during our painful separation.
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The aim of the thesis was to investigate the potential use of liposomes as a carrier for DNA delivery \textit{in vitro} and \textit{in vivo}.

In the first stage, we examined the incorporation of plasmid DNA pGL2 encoding luciferase and pRc/CMV HBS plasmid DNA encoding HBsAg (S region; ayw subtype) into liposomes prepared by the mild method of dehydration-rehydration (DRV liposomes). The prepared systems were characterised in terms of vesicle size, lipid composition and surface charge [e.g. vesicles composed of PC and DOPE alone or supplemented with anionic (PS or PG) or cationic lipids (DOTMA, SA, DC-Chol or DOTAP)], and stability. The ability of DRV liposomes and microfluidised DRV liposomes to protect their nucleic acid content from deoxyribonuclease attack was also analysed and compared with naked DNA or DNA complexed with preformed cationic liposomes. Furthermore, the transfection activity was assessed by using pGL2 as model DNA incorporated into various types of liposomes.

Next, the effect of various DRV liposomal formulations incorporating plasmid DNA pRc/CMV HBS on both humoral and cellular immunity responses to the encoded antigen was determined and compared with responses from naked DNA or DNA complexed with preformed DRV. DNA immunisation dose response studies were carried out intramuscularly using Balb/c mice. In the final part of the thesis, we assessed the effect of various routes of injection on eliciting immune responses
to the antigen encoded by pRc/CMV-HBS, using both inbred (Balb/c) and outbred (T/O) mice.

Liposomal plasmid DNA was found to retain its structural integrity and to transfect COS-7 cells in vitro in relation to the size and surface charge of the vesicles. Moreover, our studies suggest that plasmid DNA incorporated into cationic DRV liposomes under the conditions described is more effective than plasmid DNA in saline formulation in inducing both humoral and cellular immunity.
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**D) ABBREVIATIONS**

- AAV: Adeno-associated virus
- AEC: Anionic exchange chromatography
- Ag: Antigen
- APC: Antigen presenting cells
- BisHOP: 1,2-Bis(Hexadecylcloxy)-3-trimethyl aminopropane
- BSA: Bovine serum albumin
- cDNA: Complementary deoxyribonucleic acid
- CMI: Cell mediated immunity
- CMV: Cytomegalovirus
- DC: Dendritic cells
- DNA: Deoxyribonucleic acid
- DNase: Deoxyribonuclease
- DOPE: Dioleoyl phosphatidylethanolamine
- DOTAP: 1,2-Dioleoyloxy-3-(trimethylammonium)propane
- DOTMA: N(1-(2,3-dioleloxy)propyl)-N,N,N-triethylammonium
- DC-CHOL: 3β-(N,N-dimethylaminoethane)carbamyl cholesterol
- DRV: Dehydration-rehydration vesicles
- dsDNA: Double strand deoxyribonucleic acid
- DSPC: Distearoyl phosphatidylcholine
- DTH: Delayed type hypersensitivity
- ELISA: Enzyme-linked immunosorbent assay
- FCA: Freud's complete adjuvant
- FITC: Fluorescein isothiocyanate
- gDNA: Genomic DNA
- GM-CSF: Granulocyte macrophage colony-stimulating factor
- GV: Giant vesicles
- HBsAg: Hepatitis B surface antigen
- HBV: Hepatitis B virus
- HDL: High density lipoproteins
- HI: Humoral immunity
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<td>HVJ</td>
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<td>IFN-γ</td>
<td>Interferon-γ</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>IM</td>
<td>Intramuscular</td>
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<tr>
<td>IP</td>
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<td>ISCOM</td>
<td>Immunostimulating complex</td>
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<td>KC</td>
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I. GENERAL INTRODUCTION

I.1 VACCINES

The main aim of vaccination is the protection of the individual against infection. Vaccine represents the most impressive contribution of medical research to society. It is also one of the most cost-effective forms of medicine. The incidence of severe virus induced diseases has been dramatically reduced since the advent of widespread antiviral vaccination approximately 40 years ago. Smallpox has now been eradicated, and the poliovirus is well on its way to becoming extinct in nature.

The current types of vaccines are:

- Killed whole organisms - in this relatively crude approach, the vaccine is made from the entire organism, killed to make it harmless (e.g., typhoid).
- Attenuated organisms - in this technique the organism has been cultured so as to reduce its pathogenicity, while still retaining some of the antigens of the virulent form (e.g., tuberculosis).
- Purified microbial component vaccines - in some diseases, of which diphtheria and tetanus are notorious examples, it is not the growth of the bacterium that is dangerous but the protein toxin that it releases. Treating the toxin with, for example, formaldehyde, denatures the protein so that it is no
longer dangerous, but retains some epitopes on the molecule that elicits protective antibodies (e.g., tetanus toxoid).

- **Surface molecules** - Antibodies are most likely to be protective if they bind to the surface of the invading pathogen triggering its destruction. Several vaccines employ purified surface molecules, such as the influenza vaccine, which contains purified hemagglutinating from the viruses currently in circulation.

- **Inactivated viruses** - like killed bacterial vaccines, these vaccines contain whole virus particles that have been treated (again, often with formaldehyde) so that they cannot infect the host’s cells but retain some unaltered epitopes (e.g. Salk vaccine for polio).

- **Attenuated viruses** - in these vaccines, the virus is still infectious but has been so weakened that it is no longer dangerous (e.g. Measles and Sabin oral polio vaccine).

However, vaccination is not always an overwhelming success in terms of efficacy, safety and cost-effectiveness. Adverse reactions, or side effects, have been reported almost for all existing vaccines. These reactions may range from a sore arm at the injection site to mild fever, joint pains, rash, or nausea, normally lasting only a short time. In some cases, however, side effects can be quite serious for some individuals. For example, live attenuated vaccine viruses may be immunosuppressive (Babiuk et al, 1989; Harland et al, 1992) or may cause clinical disease if not attenuated sufficiently (McKee et al, 1987). Therefore, these vaccines should not be given to individuals whose immune system is already weakened because of malnutrition, old
age, genetically determined immune deficiencies, AIDS, or other immunosuppressive factors. Even if attenuated to some extent, the attenuation of live vaccine viruses can limit their ability to generate immunity (Levine et al, 1990). Another major concern regarding the use of live vaccines is the possibility, however remote, of their reversion to a major virulent phenotype (Ogra et al, 1991; Weeks Levy et al, 1991). In some individuals, attenuated vaccines may cause side effects similar to the effects caused by infections with the actual virus. For example, a small percentage of recipients of the measles vaccine develop postvaccine encephalitis. Oral polio vaccines, in which live virus is used, are known to cause in some cases mild to crippling paralysis. Killed vaccines are often unable to generate a protective level of immunity for reasons of antigen load and loss of important epitopes during inactivation (Van Drunen Little, van den Hurk et al, 1990). In many cases repeated immunisations are necessary to achieve an effective level of immunity. Furthermore, because killed viral vaccines do not provide endogenously synthesised proteins, they are, in general, unable to induce cytotoxic T cells (Monaco, 1992), possibly a required component of a truly effective vaccine (Leung & Ada, 1982; Taylor & Askonas, 1986). Moreover, killed viral vaccines can induce side effects caused by allergic reactions to vaccine components or by toxic contaminations in vaccine preparations. For example, a small proportion of recipients of the pertussis vaccine develop encephalitis type reactions.

Drug companies may be reluctant to produce vaccines because of the threat of lawsuits from those who may be harmed by these side effects. Technical problems and high research costs have also hindered the development of new vaccines.
Many existing vaccines cannot be widely used in developing countries because of storage and administration problems. Clearly, the need for better vaccines exists. One obvious example for such need is the hepatitis B disease.

### 1.1.1 Hepatitis B Virus

General Information (Molinari et al, 1992):

**Genome:** partial dsDNA

**Family:** Hepadnaviridae

**Related Viruses:** Duck Hepatitis B Virus, Ground Squirrel Hepatitis Virus, Snow Goose Hepatitis B Virus, Woodchuck Hepatitis Virus,

**Transmission:** Blood and sexual contact

**Acute Attack:** Mild or severe

**Serum Diagnosis:** anti-HBc, anti HBs or HBsAg

**Treatment:** Symptomatic

**Prevention:** Vaccination with recombinant hepatitis B surface antigens, hepatitis B immunoglobulin post-exposure prophylaxis.
The disease known as hepatitis B is caused by the infectious hepatitis B virus (HBV). HBV alone is estimated to have infected 400 million people in the world, making HBV one of the most common human pathogens. Hepatocellular carcinomas (HCC), one of the most common cancers afflicting humans, is primarily caused by chronic HBV infection. In the past few decades, the correlation between HBV and the development of HCC has been well established (Beasley & Hwang, 1984).

Viral hepatitis is the term reserved for infections of the liver by one or more of the distinct hepatitis viruses. The terms, hepatitis A and hepatitis B, were first introduced by MacCallum in 1947 in order to categorise infectious (epidemic) and serum hepatitis. These terms were eventually adopted by the World Health Organisation Committee on Viral Hepatitis.

In 1963, when searching for polymorphic serum proteins, Blumberg discovered a previously unknown protein in the blood of an Australian aborigine. This protein was denoted as the Australia (AU) antigen. It became apparent that this protein was related to type B hepatitis. By 1968, other investigators, notably Prince, had established that the AU antigen (now known as the hepatitis B surface antigen) was only found in the serum of type B hepatitis infected patients (Blumberg, 1967, Prince, 1968). In 1970, Dane found virus-like particles in the serum of patients suffering from type B hepatitis (Dane et al, 1970). These particles were designed as the hepatitis B virus (HBV). Non-related hepatitis viruses were discovered later, but the hepatitis B virus retained its name.
During HBV infection in humans, virus particles are present in very large quantities in the blood. In some forms of chronic infection, the serum contains only empty viral envelopes. Occasionally, it also contains complete virions, the empty envelope always remaining in large excess (Tiollais et al, 1985). The presence of complete virions in the serum is indicative of an active viral multiplication in the liver. The hepatitis B virion consists of a surface and a core. The DNA structure is double stranded and circular (Kaplan, 1973; Robinson & Greenman, 1974). There are four major polypeptide reading frames (genes): the S (surface), the C (core), the P (polymerase) and the X (transcriptional transactivating). The S gene consists of three regions, the pre-S1, pre-S2 and encodes the surface proteins (HBsAg).

The HBsAg particles are antigenically complex. The antigenic determinants have been identified as one single common determinant designated "a" and four major subdeterminants designated "d, y, w, and r". The four major determinants are therefore: adw, adr, ayw, and ayr (Tiollais et al, 1985). The envelope of HBV consists of proteins, carbohydrates and lipids. Glycoproteins are anchored in a lipid bilayer. The envelope of the virion contains three proteins called the major, middle and the large proteins (Fig I.3).

Compared with more conventional antiviral vaccines, the hepatitis B vaccine is unique in that HBV cannot be propagated in tissue culture (Tiollais et al, 1985; Tiollais & Buendia, 1991). Though the hepatitis B surface antigen (HBsAg) from HBV has been detected in other primates, humans remain its primary host. The
vaccine is obtained from plasma of healthy chronic HBV carriers and consists of purified defective 22-nm HBsAg particles formulated in alum adjuvant. Vaccination strategies differ according to the level of endemicity of the affected population. Purified HBsAg from the blood of chronic carriers has been used as a vaccine since 1981 (Hepatavax-B) and continues to be used in some areas of the world.

Recombinant HBsAg vaccines produced in yeast have been available since 1986 and are now most widely used. The two yeast-derived vaccines licensed in most countries are Engerix-B (SmithKline Beecham) and Recombivax HB (Merck & Co.). Both products are structurally and chemically similar with less than 2% yeast protein remaining in solution (Cottone, 1990). Expression of this protein by yeast results in SHBs particle formation. However, the particles are not secreted by the yeast. Disruption of yeast cells is performed in order to release the produced spheres into solution. These particles are then purified through clarification, ultrafiltration, chromatography, and ultracentrifugation. The purified particles are then adsorbed onto aluminium hydroxide to which thimerosal is added to preserve the solution (Cottone, 1990; Molinari et al, 1992). Recombivax HB is treated with formaldehyde before its adsorption onto alum. The major difference between the products is the number of steps used in recovery and purification of the antigen from the yeast cultures, thus resulting in different dosage amounts (see Table I.1).

The effectiveness and safety of the present hepatitis B vaccine have been demonstrated in both high-risk adult populations and new-born infants (Crosnier
et al, 1981; Laplanche et al, 1982; Maupas et al, 1981; Szmuness, 1982; Lee, 1983; Prozesky et al, 1983; Cottone, 1990). Nevertheless, alternative methods for vaccine production are needed, because the availability of human serum is limited, and because the current vaccine requires elaborate purification and inactivation procedures. In addition, freezing the vaccine results in lower immune response. Furthermore, plasma derived vaccines are very expensive and therefore beyond the financial possibilities of many countries in need of infant vaccination programmes. Moreover, there is the risk that the treatment may influence antigenicity and there is lot-to-lot variability in the preparation.

One of the most promising new approaches creates vaccines from genetic material, either DNA or RNA. In the past 10 years such vaccines have progressed from a maligned idea to entities being studied intensively in both academic and industrial laboratories and in early human trials. The merits of genetic immunisation become most apparent when the actions of traditional vaccines are understood. Prior to looking at the present successes and hurdles of DNA vaccination, I would therefore like to give a brief overview of the immune system mechanisms.

1.1.2 Immune effector mechanisms

Knowledge of the immune effector mechanisms and the immunogenicity of the antigenic determinants present on the surface of the envelope is a crucial factor for any successful vaccine design. Fortunately, our understanding of the human immune system has increased significantly since the days of Edward Jenner's
small pox vaccine. With this increased knowledge, vaccine design can be focused on those areas of the immune system that are most effective against the pathogens in question. With many viruses, the attainment of protective immunity usually means that an effective response of cytotoxic T lymphocytes must be induced (Doherty et al, 1992; Schmid & Rouse, 1992). Such responses occur only if antigens are appropriately processed by antigen-presenting cells (APC), and this may fail to occur with certain types of vaccines, in particular with those that are inactivated (Brodsky & Guagliardi, 1991; Knight & Stagg, 1993; Steinman, 1991).

The immune system defends the body from attack by invaders recognised as foreign. It is an extraordinarily complex system, which relies on an elaborate and dynamic communications network that exists among the many different kinds of immune system cells that patrol the body. At the heart of the system is the ability to recognise substances called antigens and to determine whether they are infectious agents or part of the body (self-antigens). Most immune system cells are white blood cells, of which many types exist. Lymphocytes are one type of white blood cell, and two major classes of lymphocytes are T cells and B cells (Jerne, 1973). T cells are formed in the bone marrow, as are B cells. However, the difference between the two is that T cells travel to the thymus gland to mature, while B cells mature in the bone marrow. Another important difference between the two types of cells is the way in which they attack antigens (Mosmann et al, 1986, 1989a). Humoral immunity is the consequence of antibodies produced by activated B lymphocytes, whereas cell-mediated immunity results directly (e.g.
CD8^ cytolytic T lymphocytes (CTL)) or indirectly (e.g. via macrophages, dendritic cells) from the activity of specific T lymphocytes (Mosmann et al, 1986, 1989b). The induction of both humoral and cell-mediated immunity, including the effector responses mediated by CD8^ CTL, requires the participation of activated CD4^ T cells. Murine CD4^ T cells have been divided into at least two different subsets (Th1 and Th2), based on the cytokine profiles that they secrete upon antigen stimulation (Mosmann et al, 1989a; Powrie et al, 1993). Th1 cells characteristically secrete mainly interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumour necrosis factors (TNF), which are responsible for the promotion of delayed type hypersensitivity (DTH) reactions that are characteristic for cell mediated immune responses (O'Garra & Murphy, 1994). Th2 lymphocytes, on the other hand, typically produce IL-4, IL-5, IL-6, and IL-10 to support humoral immunity via these mediators (Mosmann et al, 1986; Seder & Paul, 1994). CD4^ T cells with an intermediate cytokine profile known as Th0, capable of producing a combination of Th1 and Th2 cytokines, have also been described (Sher & Coffman, 1992). It therefore appears that the immune responses to a particular antigen could be the consequence of the type of activated CD4^ cell. The differential capacity of distinct antigen-presenting cell populations to present antigen to different CD4^ T cell subsets and co-stimulate them has been extensively studied in vitro. While the studies of Gajewski et al (1991) and Abbas et al (1991) suggest that macrophages may preferentially stimulate the expansion of Th1 cells and B cells preferentially stimulate the Th2 cells, contradictory results have been reported from other in vitro studies (Greenbaum et al, 1988; Bottomly, 1988; Chang et al, 1990).
It should be taken into consideration that the two populations of lymphocytes, B and T cells, recognise different kinds of epitopes. B cells can recognise intact antigen by its shape; whereas the activation of T cells, either Th cells or CTL, is triggered by a precise tripartite interaction between the processed antigen peptide, the T cell, and the major histocompatibility complex (MHC).

The major histocompatibility complex (MHC) molecules are so called because they were originally recognized as the cell-surface products of a complex of genes that determines the recognition of foreign tissue grafts (histocompatibility means tissue compatibility) and causes rejection of unmatched tissue. It has since become clear that their physiological function is to sample internal compartments of cells for degraded products (usually peptides) of intracellular and internalized extracellular molecules and carry them to the cell surface for recognition by T cells. The molecular basis of antigen recognition by T cells is well understood. The TcR recognizes short antigen-derived peptide sequences presented in association with MHC class I or MHC class II molecules at the surface of an antigen presenting cell (APC). T cell recognition, therefore, involves direct cell-cell contact between the antigen-specific TcR on the T lymphocyte and an MHC compatible cell which presents the processed antigen in association with surface MHC molecules (Cresswell, 1994). However, for the T cell to respond to a foreign antigen on the MHC, another molecule on the antigen-presenting cell must send a second signal to the T cell (Morris et al, 1994). A corresponding molecule on the surface of the T cells recognises the second signal. These two secondary molecules of the antigen-presenting cell and the T cell are called co-stimulatory
molecules. There are several different sets of co-stimulatory molecules that can participate in the interaction of an antigen-presenting cell with a T cell. Of these regulatory molecules, CD28 and B7 have attracted considerable interest (Powell et al, 1998; Lane et al, 1994; Greenfield et al, 1998). CD28 and B7 (B7-1 and B7-2) are transmembrane glycoproteins of helper T cells and antigen-presenting cells, respectively. They are part of a complex termed the "immunologic synapse", which contains adhesion molecules, the immunoreceptor, plus the peptide-MHC pair and the CD28-B7 pair. This complex regulates and conveys to the T cell the signals generated by ligated immunoreceptors and co-stimulatory molecules. The main effect of these signals is to induce the T cell to produce interleukin-2, a cytokine that activates the T cell, enabling it to proliferate and interact with B cells and cytotoxic T cells (Dustin & Shaw, 1999).

The orchestrator of these events is the antigen-presenting cell (APC). Antigen-presenting cells surround foreign substances and digest them in a process called phagocytosis (Bevan, 1987; Hoffman, 1992). Through phagocytosis, APC engulf foreign substances and break them up into smaller pieces. The cells then present the fragments, which include antigen pieces, to nearby T cells. Presenting the fragments involves moving them to the surface of the APC. There, the T cells can come into contact with the fragments. In some cases, an immune response will then be triggered (Townsend et al, 1985; Morrison et al, 1988; Germain, 1994; Morris et al, 1994). Basically, APC contain segregated pathways to process and present antigen and to influence the specificity of an immune response. Intracellular antigen or endogenous antigen, cut into peptides in the cytosol of the APC
(Goldeberg & Rock, 1992), bind to MHC class I molecules are recognised by T cells after being exported to the cell surface (Townsend et al, 1985; Morrison et al, 1988). In contrast, extracellular antigen or exogenous antigen that has entered the endocytic pathway of the APC are processed there and loaded onto the MHC class II molecules, and are subsequently displayed on the cell surface for CD4^+ helper T cell recognition (Cresswell et al, 1987; Harding & Unanue, 1989). It is important to point out that the class I pathway is not restricted to endogenously synthesised antigen. Exogenous antigen gains access to the class I-restricted presentation pathway if it can be introduced into the cytosol of APC (Moore et al, 1988; Reddy et al, 1991; Harding et al, 1991). Therefore, differential admittance of an antigen to the class I or class II pathway is mostly determined by the intracellular location of the antigen. It is widely agreed that the chief antigen-presenting cells are B lymphocytes, dendritic cells, and macrophages. Yet, the proposed mechanisms of antigen capture employed by different APC and their consequences for presentation of antigen on MHC class I and class II molecules are still controversial. For many years, macrophages were thought to be the predominant antigen-presenting cells. Although many of these cells have a phagocytic function, increasing evidence suggests that other cells such as dendritic cells (DC) are also involved in antigen presentation and may play, at least in some experiments, a more important role than macrophages (Steinman, 1991; Crowley et al, 1990, Van Rooijen & Sanders, 1994; Allen, 1994). In vitro studies have shown that macrophages present class I-restricted peptides after endocytosis of particulate or soluble proteins by phagocytosis or macropinocytosis (Rock et al, 1990;
Schirmbeck et al, 1994; Norbury et al, 1995), but other bone marrow-derived APC (such as dendritic cells) may be involved in the cross-priming phenomenon seen in vivo (Bevan, 1976; Huang et al, 1994). It is believed that dendritic cells capture antigen or take it to the lymphoid organs where an immune response is initiated (Steinman, 1991), and that they are in fact more efficient antigen-presenting cells than macrophages. However, a paucity of markers for DC, the difficulty to distinguish DC from monocytes/macrophages, and the problems involved in purifying DC have made for slow progress. Nonetheless, several laboratories have persisted in their investigations, leading to the current view that DC represent discrete leukocyte populations of specialist or "professional" APC, with an extraordinary capacity for initiating primary (and secondary) T lymphocyte responses (Steinman, 1991; Crowley et al, 1990; Levine & Cahin, 1992; Sallusto & Lanzavecchia, 1994; Hart & Calder, 1994; Cella et al, 1997; Austyn, 1996; Banchereau & Steinman, 1998; Lane & Brocker, 1999). Dendritic cells originate in the bone marrow, migrate through the blood to the skin and interstitial tissues and then migrate through afferent lymphatics to the T-dependent areas of lymph nodes, where they are termed interdigitating cells. In 1973, Steinman and Cohn using the spleen of mice succeeded in separating some properties of spleen DC from macrophages. Spleen DC were distinguished from macrophages on the basis of cytochemical reactions, a lack of phagocytic activity, and an apparent lack of Fc and complement C receptors (Steinman et al, 1974), features which may be present in less mature DC. Although DCs lack substantial phagocytic activity, this relative deficiency has been over-emphasised. The lack of
phagocytic activity is a feature of more differentiated or mature DC and less differentiated or immature DC have selective phagocytic activity (Banchereau & Steinman, 1998).

Recent data from various laboratories (Cella et al, 1997; Hart, 1997; Shortman & Caux, 1997) have shown that DC are not a single cell type, but a heterogeneous collection of cells arising from distinct, bone marrow-derived, hematopoietic lineages. Subsequent studies (reviewed by Steinman, 1991) showed that DC express MHC molecules, particularly class II products, in high density (mouse macrophages have low-density MHC class II) and are exceptionally effective stimulators of primary T-lymphocyte responses. To date, at least three different pathways have been described. The emerging concepts are that each pathway develops from unique progenitors, that particular cytokine combinations drive developmental events within each pathway, and that cells developing within a particular pathway exhibit distinct specialised functions (Austyn, 1998).

DC presentation of exogenous antigens as peptide-MHC class II complexes favours the activation of CD4\(^+\) T cells, many of which are helper T cells. Presentation of endogenous antigens as peptide-class I complexes favours the activation of CD8\(^+\) T cells, many of which are cytotoxic T cells (Austyn, 1996). As mentioned above, DC possess a heterogeneous lineage in that subsets from different tissues have been shown to possess a differential phenotype and function. This may be the reason for diverting views about the mechanisms of endocytic activity of DC. Mechanisms of antigen processing by dendritic cells are
of critical importance, because these cells are initiators of adaptive immune responses. In fact, the immunological dominance of certain determinants in an immune response may be caused in large part by their preferential processing and presentation by dendritic cells. What makes DC stand out from other professional APC is their seemingly unique ability to present antigen to T lymphocytes, which have had no previous contact with antigen. This gives dendritic cells a central role in the initiation of immune responses and creates opportunities for their exploitation in the development of therapeutic strategies against tumours and other diseases. For the rational design of new vaccines, precise understanding of the basic mechanisms, such as those leading to the preferential activation of particular CD4⁺ T cells or to the presentation of peptides in the context of either class I or II molecules, is therefore vital. The study of dendritic cells has seen a rapid expansion in recent years, and their importance within the immune system is now widely recognised.

The type of experimental system, antigen-presenting cells, and cytokine environment, as well as the specific major histocompatibility complex/peptide density on the surface of the APC have all been shown to play a role in the immune effector mechanisms (Hsieh et al, 1992; Macatonia et al, 1993; Seder & Paul, 1994; Constant & Bottomly, 1997). It is general consensus that any method that confers cytosolic delivery is potentially useful for delivering a protein antigen to the class I presentation pathway (Zhou & Huang, 1995). By understanding these molecular control points, it is becoming possible to manipulate immune responses more effectively with regard to vaccination strategies.
DNA immunisation has been shown to successfully induce both cellular and humoral immunity in several antigen systems. Using this technology, protective immunity in several virus and cancer disease models has been demonstrated in animal systems. The exact mechanism by which injected or particle-coated DNA leads to antigen presentation capable of eliciting a T cell immune response has yet to be fully defined. Each of the three arms of the immune system needs to encounter antigen in a different context: antibodies usually bind soluble antigen; CD4⁺ T cells primarily recognize peptide-MHC class II complexes on the surface of APC that have endocytosed and processed exogenous antigens; and CD8⁺ T cells are generally restricted to peptide-MHC class I complexes derived from endogenously produced protein that has undergone proteosome-dependent intracellular processing. How then are DNA vaccines able to activate all three arms of the immune system?

In the past few years, the cellular mechanisms by which the antigen encoded by a bacterial plasmid is processed and presented to the immune system have been researched intensely by both immunologists and biologists. However, the scientific views diverge to such an extent that a definitive explanation of these mechanisms is not yet possible. The presently available evidence suggests the following explanations for the cellular responses elicited by DNA vaccination: (1) direct priming by somatic cells (eg. myocytes), (2) direct transfection of professional APC (eg. DC), and (3) cross-priming.
As for the first concept, a number of studies showing that DNA vaccination can lead to protein expression and induction of CTL responses have been performed by injecting the DNA directly into the muscle (Wolf et al, 1990, Ulmer et al, 1993 & 1996). Ulmer et al (1996) provided conclusive evidence that the expression of viral protein by muscle cells *in vivo* is sufficient for CTL-mediated protection. The problem with this concept is that myocytes lack important costimulatory molecules necessary for priming naive CD8\(^+\) T cells for cytotoxic responses, and are thus unlikely to function as effective APC (Pardoll & Beckerleg, 1995). Furthermore, in a study by the Torres group (1997), the removal of the muscle immediately after intramuscular immunisation with DNA did not alter the subsequent immune response.

The other two proposed mechanisms involve bone marrow-derived 'professional' APC such as dendritic cells or monocyte/macrophages. These cells express high levels of MHC class I and II molecules, as well as costimulatory molecules such as B7.1 and B7.2, and are highly efficient presenters of antigen to T lymphocytes. One proposed mechanism is based on the presumption that a small number of these professional APC are directly transfected with the injected DNA. This view was corroborated by Casares et al (1997); Porgador et al (1996). They found separately that small numbers of DC were transfected following DNA vaccination and that isolated DC but not B cells were able to efficiently present antigen to T cell lines *in vitro*. These cells would then traffic to regional lymphoid tissue, where they can activate CD4\(^+\) T cells, B cells, and CD8\(^+\) T cells (Pardoll & Beckerleg, 1995; Doe et al 1996). This mechanism seems conceivable in epidermal
immunisation, because the skin is known to contain a relatively high proportion of Langerhans cells, but appears less likely in intramuscular immunisation, as dendritic cells and macrophages are thought to be scarce in muscle (Hohlfeld & Engel, 1994). In the other proposed mechanism, antigen produced by transfected myocytes (in the case of intramuscular inoculation) is transferred to bone marrow-derived APC that have infiltrated the muscle as part of an inflammatory response to the immunisation procedure. With epidermal immunisation, it is the transfected keratinocytes that would be producing the antigen and transfer it to DC located in the skin. The transferred protein would then cross over into the MHC class I-restricted processing pathway, allowing the APC to prime CTL responses (Corr et al, 1996, Fu et al, 1997; Akbari et al, 1999). Though this would seem to contradict the general belief that only endogenously produced proteins can enter the MHC class I pathway, it would be consistent with the recent reports by Bevan and colleagues, Rock and colleagues, Srivastava and colleagues, and others (Carbone & Bevan, 1990; Huang et al, 1994; Pardoll & Beckerleg, 1995; Kovacsovics-Bankowski & Rock, 1995), who have described how exogenously produced protein, particularly in particle form, can be taken up by APC and then presented in the context of MHC class I molecules. It is possible that both mechanisms play a role in the generation of immunity through genetic immunisation, and likely that as-yet-unimagined mechanisms may also be contributing.
1.2 DEOXYRIBONUCLEIC ACID (DNA)

Friedrich Miescher discovered DNA in 1869 as a novel organic phosphate compound in the nuclei of eukaryotic cells. The original material was later shown to be a complex of DNA and protein. Initially, it was believed that the nuclear proteins were the genetic material (Mirsky, 1968). Evidence that DNA, not protein, was the genetic material accumulated gradually. Indeed, until the experiments of Avery in 1944 demonstrated that the transformation of virulent Diplococcus pneumoniae by a heat-killed suspension was due to uptake of DNA, it was assumed genes were made of protein. Later, the structure of DNA was elucidated, the genetic code cracked, and the processes of transcription and translation were described.

The recent observation that protective immunity can be induced following genetic immunisation with DNA opens a new approach to vaccination. The procedures for introducing the transforming material range from simple injection of DNA to administration of DNA via specially designed vesicles. Although the development of both nucleic acid drugs and carrier systems suitable for in vivo delivery has made rapid progress, to date no technology has emerged that is highly efficient, reproducible, and applicable to a wide range of target cells and tissues. Irrespective of the delivery method, it is crucial to transfer DNA into target cells in a concentration high enough to be effective in modifying the disease. DNA must be delivered to the desired cell population in an intact state so that it can be efficiently transcribed and ultimately translated. The method of gene transfer must
be highly efficient, non-toxic, and the delivery system must be relatively easy to prepare and administer.

Any understanding of the obstacles to gene therapy and vaccination requires a basic understanding of what genes do and what attempts at gene therapy are currently being carried out. The following sections therefore aim to provide an introduction to DNA and to liposomes as a carrier for vaccine delivery. The first part deals with the structure of DNA and gives an overview of techniques currently used in DNA vaccination. The second part presents a brief theoretical description of the origin of liposomes, of their use for drug delivery, and of the possible improvement of DNA vaccination by using DRV liposomes.

1.2.1 Structure of DNA

Hydrolytic degradation of DNA has shown that DNA consists of three essential components: (1) the pentose sugar 2-deoxy-D-ribose, (2) phosphoric acid which confers on DNA its acidic properties, and (3) nitrogenous bases. The nitrogenous bases in DNA are the two substituted purines, adenine (A) and guanine (G), and the two substituted pyrimidines, thymine (T) and cytosine (C). The sugar molecule can combine with any of the four bases to form a nucleoside; attachment of a phosphate group to the sugar of the nucleoside yields a nucleotide. Chemical analysis has shown that DNA is a polynucleotide. The sugar residues of the nucleotides are linked by their phosphate groups so that the C3' position of one sugar is joined to the C5' position of the next. The particular order of the bases
arranged along the sugar-phosphate backbone is called the DNA sequence. The sequence specifies the exact genetic instructions required to create a particular organism with its own unique traits (Fig.1.1). Each time a cell divides into two daughter cells, its full genome is duplicated; in humans and other complex organisms, this duplication occurs in the nucleus. During cell division the DNA molecule unwinds and the weak bonds between the base pairs break, allowing the strands to separate. Each strand directs the synthesis of complementary bases on each of the separated strands. Each daughter cell receives one old and one new DNA strand. The cells' adherence to these minimises the incidence of errors (mutations) that could greatly affect the resulting organism or its offspring. For cell division, DNA does not remain loose, long and thin but is packed up tightly. The packing process is called DNA condensation. However, when a cell is not dividing, it needs to be able to access its genetic code. Therefore, after the cell has divided it has to unpack its DNA back into chromatin form. This process is called DNA decondensation.

DNA holds genetic information according to the sequence of bases. A sequence of three bases (a codon or triplet) is required for each character of the "genetic code". Given four different bases, 64 characters are possible within the code (see Fig.1.1), more than sufficient to specify both the order of the 20 amino acids found in proteins and the other signals required for the control of DNA functions and protein synthesis (Crick, 1966). Genetic information is conserved by replication of DNA, which contains this information, and by transcription and translation, which via RNA results in the synthesis of the encoded proteins. Transcription is a
process in which a particular RNA complementary to the DNA template is synthesised from nucleotides with the help of enzymes and energy from ATP. In the translation process, aminoacids are linked to proteins in ribosomes with the help of enzymes and in accordance with the RNA code. In many genes, the DNA sequence that codes for proteins (exons) may be interrupted by stretches of non-coding DNA, called "introns". In the cell nucleus, the DNA that includes all the exons and introns of the gene is first transcribed into a complementary RNA copy called "nuclear RNA", or "nRNA". In a second step, introns are removed from nRNA by a process called RNA splicing. The edited sequence is called "messenger RNA", or "mRNA". The mRNA is able to leave the nucleus for the cytoplasm where it encounters cellular bodies called ribosomes. The mRNA, which carries the gene's instructions, dictates the production of proteins by the ribosomes.

In the laboratory, the mRNA molecule can be isolated and used as a template to synthesis a complementary DNA (cDNA) strand, which can then be used to locate the corresponding genes on a chromosome map. The value of the genetic map is that an inherited disease can be located on the map by following the inheritance of a DNA marker present in affected individuals, even though neither the molecular basis of the disease may be fully understood nor the responsible gene may have been identified. Genetic maps have been used to find the exact chromosomal location of several important disease genes, such as cystic fibrosis, sickle cell disease and myotonic dystrophy.
In contrast to RNA, DNA molecules are chemically rather stable. However, DNA (especially longer chain molecules) can be physically damaged by pipetting, vigorous shaking, agitation, and freeze-thawing. In biological samples one must be careful to inhibit DNA degradative enzymes, with respect to temperature stability, oxidation and reduction (Templeton & Lasic, 1996).

In gene therapy, the major interest is in cDNA (i.e. sequences of DNA encoding particular proteins). These genes are inserted into plasmids containing appropriate enhancers+promoters, introns, and other sequences to increase gene expression, as schematically shown in Fig. I.2. Successful in vitro or in vivo studies require carefully designed DNA vectors. Points to consider for achieving expression of a cDNA of interest include the required level of expression, regulated or inducible expression, and the cell types or tissues to be transfected. Correct protein function depends on the folding and the structure the proteins assume in biological environments. Understanding the protein structure is therefore essential in determining the gene function. In the case of hepatitis B, DNA of HBV has a large open reading frame encompassing 1167 or 1200 base pairs. The structural gene encoding the HBV envelope proteins that carry the surface antigen determinants (HBsAg) has a single open reading frame containing three in-frame ATG (adenine, thymine, guanine) start codons that divide the gene into three coding regions known as preS1, preS2 and S (proceeding in a 5' to 3' direction) (see Fig. I.3). The major forms of the HBsAg carrying protein (the S protein, or SHBs) contain all the topological information needed for particle assembly (Whalen et al, 1995a; Davis et al, 1993a,b, & 1996). Such particles form and are
secreted when the gene for the HBsAg carrying protein is expressed in a variety of eukaryotic cell types. The surface antigen epitopes are conformational since they can only be induced by correctly formed particles (Whalen et al, 1995b, 1995c).

1.2.2 Gene therapy

Gene therapy is the insertion of a functioning gene into the cells of a patient to correct an inborn error of metabolism (i.e. genetic abnormality or birth defect) or to provide a new function in a cell (e.g. insertion of immunostimulatory gene into infected cells to vaccinate a patient against a disease).

Depending on the cell types affected, gene therapy can be broadly classified into two categories: germ-line gene therapy and somatic cell gene therapy. In germ-line therapy the germ cells (reproductive cells) are altered, with the genetic changes being passed on to the patient's offspring. Somatic cell gene therapy involves the alteration of somatic cells (non-reproductive body cells, like skin, brain, or muscle cells). This genetic manipulation affects only the individual that has received the treatment. Somatic cell gene therapy is the only type that is presently being considered for use in humans.

Successful gene therapy requires not only the identification of an appropriate therapeutic gene for treating the disease, but also a delivery system by which that gene can be delivered to the desired cell type both efficiently and accurately (Miller & Vile, 1995). However, delivering DNA to the cells faces two major challenges: 1) DNA is relatively large and negatively charged resulting in poor
membrane transport and inefficient cellular uptake (Mahato et al, 1997) the extremely low pH and enzymes within endosomes and lysosomes usually lead to rapid degradation of the DNA. Vectors are the means by which DNA is delivered to the target cell. DNA delivery lies at the heart of gene therapy and its future success depends on the continued development of gene delivery vectors. Broadly speaking, vectors can be divided into two groups: those that use viruses and those that don't (see Table 1.2).
I.3 DNA DELIVERY SYSTEMS

I.3.1 Viral delivery systems

Viruses infecting mammalian cells have developed sophisticated and specific mechanisms for cell attachment, penetration, survival and replication. The most widely used viruses for gene transfer are retroviruses. They are single-strand RNA viruses, which after entering the cell nucleus incorporate the gene directly into the chromosome. This may give cause to safety concerns because of potential carcinogenicity and infectivity although normally several viral protein codes are deleted. Moreover, contamination with intact retroviruses, which can be highly oncogenic, during preparation cannot be ruled out (Miller et al, 1990; Smith et al, 1993; Felgner & Rhodes, 1991). Adenoviruses are less hazardous. They are double-strand DNA viruses that are able to incorporate larger genes than retroviruses. They efficiently infect dividing and non-dividing cells, such as airway epithelial cells, endothelial cells, hepatocytes, and cancer cells (Trapnell, 1993, Eissa et al, 1994, Yei et al, 1994). Adenoviral vectors do not integrate genes into the host’s genome and thus reduce the risk of malignant transformations (Welsh et al, 1993). The immune reaction is potent, eliciting both humoral and cellular immune responses (Dai et al, 1995; Yan et al, 1994). Despite the advantages of adenoviruses over retroviral vectors, safety concerns with regard to potential toxicity have been raised. Preclinical and clinical studies have demonstrated immunological responses to adenoviral vectors and inflammation in the target tissues (Setoguchi et al, 1994; Yang et al, 1995; Felgner, 1997). It is
possible that the target cell contains factors that might trigger the synthesis of adenoviral proteins, leading to an immune response (Verma & Somia, 1997). Another type of viral delivery vector is the adeno-associated viruse (AAV), a simple, non-pathogenic, single-stranded DNA virus. Its two genes (cap and rep) are sandwiched between inverted terminal repeats that define the beginning and the end of the virus and contain the packaging sequence (Verma & Somia, 1997). They cannot replicate without adenoviruses or herpes viruses. They insert themselves into a specific location on chromosome 19 and do not require dividing cell for expression (Berkner, 1988). However, a major limitation of currently available adeno-associated viruses is the difficulty of developing packaging cell lines to produce sufficient titers of the virus for clinical use without the presence of a helper virus (Kay et al, 1994). AAV vectors can accommodate only 3.5-4.0 Kb of foreign DNA, thus excluding large genes (Lasic, 1997).

1.3.2 Non-viral delivery systems

Many drawbacks of viral gene delivery agents can be overcome by non-viral systems. The ultimate goal is to imitate desirable features of viral vectors whilst avoiding their inherent limitations. Recent developments in recombinant DNA technology and human genome characterisation have led to the identification of the molecular origins of many genetic and acquired diseases and have facilitated the construction of appropriate plasmids containing desired genes. Plasmid DNA constructs containing suitable controlling elements are technically easier and less time-consuming to prepare and test than viral constructs. Scale-up manufacturing is
more complex for potentially infectious viral materials than for non-infectious plasmids, which are produced in conventional fermentation facilities in the same way as recombinant proteins. Non-viral techniques could be especially suitable for repeated use, because they do not elicit immune responses against antigenic coat protein. They have the potential for wide clinical application and offer cost-effective, safe and convenient methods for in vivo delivery of therapeutic proteins (Fraley et al, 1987; Felgner et al, 1990; Wolff et al, 1990; Jiao et al, 1992; Legendre & Szoka, 1992; Davis et al, 1993; Rolland et al, 1994).

Non-viral gene medicines are composed of three elements: (I) a gene encoding a therapeutic protein, (II) a gene delivery system that controls the location of the gene in the body, and (III) a plasmid-based gene expression system that controls both the function of a gene in the target cell and the secretion of the produced therapeutic protein (Rolland, 1998). Non-viral gene therapy can be broadly divided into truly non-viral methods, such as direct plasmid injection, DNA-coated particle bombardment, or polymers, polypeptides or cationic lipid-base carriers, and virally enhanced modes of delivery, such as the inactivated hemagglutinating virus of Japan (HVJ) complexed with neutral liposomes.

1.3.2.1 Direct plasmid injection

The method of direct plasmid DNA injection is a simple, inexpensive, and non-toxic procedure when compared to viral delivery. Plasmids are colloidal systems of specific hydrodynamic size, as determined by several experimental techniques such
as light scattering. They have a highly negative surface charge owing to the phosphate group of each nucleotide. These colloidal and surface properties of plasmids determine their biological distribution, cellular uptake, intracellular trafficking, and nuclear translocation. Southern blot DNA analysis suggests that the DNA exists as a non-replicating closed circular episome (Schofield & Caskey, 1995). In the early 1990s, the observation that plasmid DNA can directly transfect animal cells \textit{in vivo} initiated animal trials aimed at inducing immune responses by direct injection of DNA encoding antigenic proteins (Lin et al, 1990; Nabel et al, 1990; Wolff et al, 1990; Acsadi et al, 1991a,b). This method, termed DNA-based immunisation, has now been used to elicit protective antibody and cell-mediated immune responses in a large variety of pre-clinical animal models for viral, bacterial and parasitic diseases (Wolff et al, 1991; Cox et al, 1993; Ulmer et al, 1993, 1994; Wang et al, 1993; Sedegah et al, 1994). In contrast to vaccines that employ recombinant bacteria or viruses, genetic vaccines consist only of DNA (as plasmid), or RNA (mRNA), which is taken up by cells, where it is transcribed and then translated into protein. DNA vaccines employ genes encoding proteins of pathogens or tumours, rather than using the proteins themselves, a live replicating vector, or an attenuated version of the pathogen itself. DNA vaccine consists of a bacterial plasmid DNA with a strong viral promoter, the relevant gene, and a polyadenylation/transcriptional termination sequence (Davis et al, 1993, 1995). The plasmid is grown in bacteria (E.coli), purified, dissolved in a saline solution, and then simply injected into the host (Whalen et al, 1995c). Most plasmids used for vaccination purposes share the basic attributes of vectors developed for \textit{in vitro}
expression of genes in transfected cell lines. These include: 1) an origin of replication (ori) suitable for producing high yields of plasmid in E.coli; 2) an antibiotic resistance gene to confer antibiotic-selected growth in E.coli; 3) a strong enhancer/promoter and an mRNA transcript termination/polyadenylation sequence for directing expression in mammalian cells (Fig.1.3).

The new technology offers a unique method of immunisation that may overcome many of the deficits of traditional antigen-based vaccines. The potential of DNA immunisation is to partially mimic viral vaccines promising the efficacy of live attenuated vaccines without the risk of inadvertent infection. Furthermore, it is easy to design expression vectors including sequences chosen to induce a desired immune response. Although the in vivo transfection efficiency is typically low, expression of the exogenous gene in muscle cells could be observed over several months (Davis et al, 1995). Expressing the antigen in its native form may also offer optimal processing and presentation to the immune system. Direct DNA immunisation has the additional advantage of producing internal proteins that may differ from surface antigens and may otherwise be difficult to generate. Studies with influenza vaccines also suggest that the DNA inoculation approach may be more effective than inactivated virus or subvirion vaccines in protection against different strains of viruses.

And yet, despite the many potential advantages of DNA immunisation, it still faces several difficulties. Plasmid DNA degrades rapidly in vivo. Kawabata et al (1995) showed that in experiments where mice were injected intravenously with
plasmid DNA, half-life was about 5-10 min after injection. The same authors also demonstrated that plasmid DNA degrades faster \textit{in vivo} than \textit{in vitro}, which indicates that plasmid DNA undergoes degradation not only by plasma DNase but also by the enzymes of other body components. These drawbacks force the use of relatively large quantities of material and/or special immunity enhancing conditions (e.g. regeneration of muscle), with the potential risk that repeated administration of high doses may lead to the production of anti-DNA antibodies (Zhu et al, 1996). Given that DNA translation and transcription occur intracellularly, the delivery of DNA plasmid to the cytosol represents one of the greatest challenges for novel DNA vaccines.

\subsection{1.3.2.2 Particle bombardment}

Another non-viral approach is the gene gun delivery system, which has been used to improve the efficacy of plasmid DNA transfection. In this system, plasmid DNA coupled with gold particles is administered through the skin by bombardment with a gene gun under helium pressure. Characterisation of the nature of the \textit{in vivo} transfected cells after gene gun immunisation has been performed using DNA encoded with reporter genes such as the green fluorescence protein (GFP) whose expression in the transfected cells can be easily detected (Condon et al, 1997). Gene-gun immunisation with DNA results in \textit{in vivo} transfection of both resident keratinocytes (KC) and also of dendritic cells (DC), which are highly enriched in the skin. DNA immunisation using the gene-gun induced a predominantly IgG1 (Th2) response with IL-4 producing cells, while intramuscular injection of naked DNA
induced IgG₂a (Th1) responses (Fyana et al, 1993; Cardoso et al, 1998) with expansion of IFN-γ producing CD4⁺ T cells and CD8⁺ CTL. The basis for these divergent responses may reflect the different gene transfer methodologies. Indeed, DNA coated onto gold beads for ballistic immunisation requires 100 times less DNA than saline formulations for raising immune responses (Fyana et al, 1993). However, these advantages are counterbalanced by the requirement for gold beads and a bombarding device connected to a large tank of pressurised gas to drive it. Although the system is now on the way to refinement, the gene gun is still too costly for large-scale vaccination programmes, especially in third-world countries.

I.3.2.3 Polymer-based gene delivery

Another widely used delivery system are cationic polymers, such as polyberne and DEAE-dextran. Due to relative low efficiency, cytotoxicity, or non-biodegradability, however, their application has been solely restricted to in vitro gene transfer. Consequently, novel non-linear cationic polymers, such as polyamidoamine cascade dendrimers, have been investigated for in vitro and in vivo gene transfection (Haensler & Szoka, 1993). Dendrimers can condense plasmids through electrostatic interaction of their terminal primary amines with the DNA phosphate groups. The effect of colloidal and surface characteristics of plasmid/dendrimer complexes on gene delivery efficiency has been examined. A variety of cell types has been efficiently transfected using dendrimer-based complexes (Tomalia et al, 1990; Tomlinson & Rolland, 1996). It appears that in order to obtain high transfection efficiency, fifth and sixth generations of
dendrimers are needed (Haensler & Szoka, 1993). However, the high molecular weight dendrimers have shown significant cytotoxicity.

Cationic polypeptides, such as poly-L-lysine (PLL) and poly-L-ornithine have also been used to condense plasmids through ionic interactions and facilitate in vitro cellular uptake of plasmids via non-specific adsorptive mechanisms. PLL has been covalently modified with a number of targeting ligands, such as asialoglycoprotein, transferrin, EGF, insulin, folic acid, antibodies and carbohydrates, to enhance plasmid delivery via receptor-mediated endocytosis (Wu et al, 1991). The delivery of plasmids to hepatocytes in vivo has been reported using poly-L-lysine covalently coupled to galactosyl residue as targeting ligands (Trubetskoy et al, 1992). Plasmid/transferrin-PLL complexes have also shown effective gene delivery into various cell types in vitro, including T cells and the pulmonary epithelium (Harris et al, 1993). All these approaches, though successful in obtaining expression of therapeutic gene in animal models, have limitations associated with the use of polypeptides such as PLL, which is known to be toxic and to have variable quality. The use of proteins such as asialoglycoprotein as a targeting ligand also presents the risk of inducing an immune response.

1.3.3 Liposomes in DNA delivery

The most widely investigated gene delivery system in both laboratories and clinical studies are liposomes. Recent attempts to deliver genetic material have created
additional interest in lipid-based gene delivery systems. (Soriano et al, 1983; Bruch & Mahan, 1991; Thierry & Dritschilo, 1992; Smith, J.G., et al, 1993). Liposomes are easily biodegradable, they are not or only weakly immunogenic (Van Rooijen & Van Nieuwmegen, 1980) and possess limited intrinsic toxicity (Campbell, 1983). Homogeneous populations of liposomes can be prepared and are preferable to polydispersed systems as drug carriers (Zumbuehl & Weder, 1981; Olson et al, 1979). Liposomes can interact with target cells in various ways and are therefore able to promote the intracellular delivery of drug molecules that in their free form (eg. DNA molecules) would not be able to enter the cellular interior due to unfavourable physicochemical characteristics.

I.3.3.1 General structure and properties of liposomes

In the early 1960s, Bangham and his collaborators at Babraham, Cambridgeshire, made the observation that hydration of dry phospholipids in water results in the arrangement of phospholipid molecules in bilayers to form closed spherical vesicles consisting of internal aqueous compartments, which came to be known as liposomes. Several years earlier, Friedman (1957) had shown how to facilitate the elimination of cholesterol from the body. However, the enormous boost in liposome literature that we have seen in more recent years did not start before the early 1970s, when Gregoriadis and Ryman (1972) proposed the enzyme-loaded liposome as an approach to the treatment of (lysosomal) storage diseases. Since then the interest in liposomes as a drug carrier system has continually increased, culminating over the
past few years in a large number of reports on clinical trials with liposome-based applications.

Liposomes are microscopic and submicroscopic vesicles with a size ranging from 25nm to 20μm. They are composed of one or several lipid bilayers enclosing aqueous compartments. When phospholipids are hydrated, they spontaneously form lipid spheres enclosing the aqueous medium and the solute. The particulate nature of liposomes causes them to be distributed within the body pattern significantly different from that of free drugs. Liposomes have been used experimentally in virtually every area of medicine and have been administered through every traditional route.

Liposomes can be prepared from a variety of lipids (usually phospholipids) with the possible addition of sterols, glycolipids, organic bases or acids, or even of macromolecules such as membrane proteins or artificial polymers (Fig. I.4-7). The physical characteristics of the membrane lipid are highly dependent on the temperature, the ionic strength and the pH value (New, 1990). The most commonly used phospholipid molecules and their gel to liquid crystalline transition temperatures (Tc) are presented in Table I.3. As the temperature is raised, the fatty acyl side-chains of the membrane phospholipids progress from a rather closely packed form known as "solid" liposomes to a more loosely rotational motion known as "fluid" liposomes. The Tc is a function of the acyl chain length. In phospholipids composed of the same acyl chain in both positions, the Tc increases by about 14-17°C with every 2-methylene unit increase in chain length (New, 1990). The
presence of unsaturated acyl chains, branched chains, or those carrying bulky side groups leads to decreased transition temperatures. Apart from this property of phospholipids, liposomes can be prepared with a positive, negative, or neutral surface charge depending on the lipid composition and chemical components such as DOTAP or PS, which can provide positive or negative charge, respectively.

Typically, natural lipids are zwitterionic, neutral or anionic (Fig.1.5). Nature has synthesised several thousand different lipids. They are a combination of several polar heads (choline, ethanolamine, glycerol, serine and other sugars), backbone molecules (glycerol, sphingosine), and about a dozen of fatty acids (either saturated such as myristic acid or unsaturated such as oleic acid). The latter can be attached, mostly two at a time, to all three positions on the backbone (Lasic, 1993). Lecithin (phosphatidylcholine) is one of the most widespread natural lipids. It contains the choline head group attached via glycerol to two fatty acid chains. At physiological pH values it is zwitterionic. Another example are the phosphatidylethanolamines, which have relatively small polar heads and therefore tend to undergo phase transition, which may play an important role in transfection. Anionic lipids contain charged groups either on the polar head (phosphatidylserine) or on the phosphate group (phosphatidylglycerol). Inclusion of anionic lipids, especially phosphatidylserines, in liposomes enhances their accumulation in the cells' reticuloendothelial system upon systemic application (Lasic, 1996). Whether this is also true for complexes of such liposomes with DNA has not been demonstrated yet.
Cationic lipids are extremely rare in nature. Before the explosion in lipid synthesis in the early 1990s, several cationic detergents were used for preparation of liposomes with positive charge. The most popular single-chain amphiphiles are chloride and bromide salts of the alkyl trimethyl ammonium surfactants. The most widely used have either a dodecyl (C\(_{12}\)) or a hexadecyl (cetyl, C\(_{16}\)) chain. To impose positive charge on liposomes, such detergents were incorporated in the lipid bilayer (Lasic, 1997). In the beginning, DODAB/C and DOTAP were basically only two families of two chained amphiphiles used for cationic liposomes. Since it was realised that cationic liposomes can complex DNA (Behr, 1986) and these complexes can transfect DNA, as was shown by Felgner et al (1987) for liposomes containing DOTMA, numerous new lipids have been synthesised to improve gene transfer efficiency and to decrease their toxicity. Natural cations, such as spermine\(^{4+}\) and spermidine\(^{3+}\), can be also coupled to fatty acids. These lipids (eg. DOSPA and DOGS) form micellar rather than vesicular structures (Behr et al, 1989). Positive charge can also be associated to the sterol backbone, with DC-Chol being the best-known example (Gao & Huang, 1991). Cationic liposomes are avidly interacting with anions and anionic polyelectrolytes. Simple anions decrease liposomes stability because of the reduction of electrostatic shielding of the surface charge by anions in the solution. This interaction follows in general the Dejaguin-Landau-Verwey-Overbeek (DLVO) model while anionic polyelectrolytes, like polyglutamic acids and most notably DNA, adsorb and can induce bridging and/or possibly lipid phase change. This surface potential is an important parameter for the physical stability of
liposomes, i.e. the aggregation or deaggregation (floculation or defloculation) of
the liposome particles (Kaler et al, 1989). Cationic liposomes are currently the
most widely used lipid transfection system.

1.3.3.2 Liposome preparation techniques

At a New York Academy of Sciences meeting held in 1978, liposomes were
classified by several three letter acronyms: multilamellar vesicles (MLV),
unilamellar vesicles of a small size of less than 100nm in diameter (SUV), generally
produced by sonication of MLV, and large unilamellar vesicles (LUV). Since this
initial classification various other terms have been introduced to describe liposomes
produced by specific methods. These methods have been extensively discussed by
Gregoriadis (1984; Gregoriadis et al, 1992); New (1990). The common step of all
these methods consists in evaporating the organic solvent in which the lipids are
generally dissolved and stored. The dried lipids are then dispersed in an aqueous
buffer solution. Preparation methods differ with regard to the method by which the
lipids are dispersed. These methods can be classified as follows:

• thin lipid film hydration;
• mechanical methods using preformed liposomes (sonication, extrusion
  through polycarbonate membranes, microfluidisation);
• dispersion of lyophilised or frozen liposomes;
• dispersion of an organic solution of phospholipids (water miscible or non-
  miscible solvent); and
mixed micelles dispersion.

Although some of the preparation methods for liposomes are highly efficient, most of them have crucial drawbacks for the preparation of suitable drug delivery vehicles. They are uneconomical, applicable only to drugs of low molecular weight (thus excluding vaccines and other proteins), or require detergents, sonication or organic solvents (Gregoriadis, 1984), which may be detrimental to the structural activity of certain drugs, such as enzymes or DNA.

In contrast, liposomes prepared by the method of dehydration-rehydration (DRV) do not show any of those problems. They are able to encapsulate relatively large amounts of material, and their preparation does not expose the solutes to toxic organic solvents, detergents and mechanical forces (Kirby & Gregoriadis, 1984; Gregoriadis, 1984). Furthermore, because the DRV technique requires only a limited number of preparation steps (Fig.1.8), the sterility of the starting materials can easily be maintained by using aseptic techniques.

I.3.3.3 Liposome-cell interaction

The general objective of using liposomes as drug carriers is to achieve specific interaction of the carrier with a particular type of cell or tissue followed by internalisation of the drug or the drug/carrier complex by the cells. Whether or not the drug, after this has been achieved, will reach its intended intracellular target site, depends to a large degree on the mechanism of internalisation and on the
specific characterisation of the drug as well as of the carrier. Liposomes can interact with cells via four major mechanisms, namely (1) lipid exchange, (2) adsorption to the cell surface, (3) fusion with the cell plasma membrane, and (4) endocytosis by the cell (Gregoriadis et al, 1981) (Fig. 1.9).

The first type of interaction is lipid exchange. As lipids themselves have low aqueous solubility, liposome and cell membrane can exchange lipid molecules. In biological systems several proteins can increase this exchange significantly. Such exchange is higher for liposomes in liquid crystalline phase and for lipids with shorter hydrocarbon chains (Lasic, 1997). Exchange of phospholipids with cells is thought to occur possibly via the intermediary of a specific cell surface exchange protein, since an exchange only occurs with certain phospholipids (PC or PE) and since the process is slowed down after trypsin treatment (Sendra & Pagano, 1979).

The second type of interaction is the adsorption of liposomes to the cell surface, which can occur with little or no internalisation of either aqueous or lipid components. It may take place either as a result of physical attractive forces, or as a result of binding by specific receptors to ligands on the vesicle membrane (Pagano & Takeichi, 1977). If adsorbed liposomes leak, parts of the encapsulated compound can enter the cell by diffusion (Lasic, 1997).

The third mechanism of interaction is the fusion of vesicles and cell membrane via an intermediate, resulting in the release of liposomal contents into the cytoplasm. In the case of multilamellar vesicles, this involves the introduction of
the internal membrane lamellae of the liposome into the cytoplasm intact so that interactions between liposomes and subcellular organelles, such as those described above, can take place. *In vitro*, fusion may be brought about quite readily by incorporation of fusogens, such as lysolecithin, detergents, surfactants, PE, oleic acid, positively charged lipids, or a Sendai virus fusion protein, into the membrane (Felgner et al, 1987).

The most important mechanism of interaction is endocytosis in which the cell engulfs adsorbed or bound vesicles into vacuoles (endosomes). After fusion with lysosomes, which deliver lytic agents (enzymes, proton-lower pH), the liposome and possibly its contents are digested. During the process of breakdown of liposomes into lysosomes, the contents of the aqueous compartment are released, after which they either remain sequestered in the lysosomes until exocytosis or slowly leak out of the lysosome and gain access to the rest of the cell (Scherphof et al, 1978; New et al, 1990; Lasic, 1992).

An understanding of the exact mechanism of liposome uptake by cells is still emerging. While it is not yet known which factors determine the uptake of liposomes by various cells, the different degree of binding for a given liposome composition by different types of cells suggests that the binding itself may be the crucial step (Miller et al, 1998).

Numerous studies on the *in vivo* fate of liposomes and liposome-encapsulated drugs and enzymes have been published. Studies on the mechanisms of
liposomes, mainly by the groups of Gregoriadis (Gregoriadis, 1976; Kirby & Gregoriadis, 1980, 1981 & 1984; Senior & Gregoriadis, 1982; Senior et al, 1983), Allen (Allen & Cleland, 1980; Allen, 1981; Allen & Everest, 1983), and Scherphof (Scherphof et al, 1979; Damen et al, 1982), have revealed details of the behaviour of liposomes (and entrapped drugs) within the biological milieu, and of ways of controlling it. Of particular relevance to the drug delivery concept are:

a) the effect of components of biological fluids (with which injected liposomes first come into contact) on the structural integrity of liposomes, and

b) the rates at which liposomes are cleared from the site of administration and distributed in tissues, mostly the reticuloendothelial system.

In both cases, the behaviour of liposomes is dictated by their structural characteristics (Gregoriadis, 1988). Stable liposomes containing high amounts of cholesterol or composed of high melting phospholipids have longer circulation half-lives in the blood stream due to reduced opsonin adsorption on such vesicles (Patel, 1992). In the case of proteins, for example, plasma high-density lipoproteins (HDL) remove phospholipid molecules from the bilayers of intravenously injected liposomes made of egg phosphatidylcholine (PC) alone. These will then eventually disintegrate and release their drug contents from the liposomal bilayers and thus destabilise them (Senior & Gregoriadis, 1982). This HDL attack and destabilisation can be avoided by the incorporation of excess amounts of cholesterol and/or by using phospholipids with a high Tc in the
liposome structure (Kirby & Gregoriadis, 1981, 1983). Sphingomyelin also renders liposomes resistant to HDL attack, but it is known to be toxic in vivo (Weereratne et al, 1983). Furthermore, half-life is extended with reduced vesicle size and a neutral surface charge (Gregoriadis, 1983 & 1985). Although the presence of negative or positive surface charge is generally associated with accelerated clearance rates, some negatively charged lipid molecules can actually cause the opposite effect. PS (phosphatidylserine), for instance, accelerates liposome clearance, but the presence of GM1ganglioside in the bilayers reverses this phenomenon by a steric conformation effect preventing interaction of plasma proteins (opsonins) with the carboxyl group of the sialic acid moiety (Gabizon & Papahadjopoulos, 1992). Inclusion of certain synthetic diacyl lipids with bulky polyethylene glycol (PEG) head groups also results in the prolongation of blood circulation times (Lasic et al, 1991). Such liposomes, known as Stealth liposomes, exhibit in addition to long half-lives dramatically altered pharmacokinetics and biodistribution due to the presence of surface-attached polymer chains. These prevent vesicle-plasma protein interactions and reduce the adsorption of the liposomes to cells and blood vessel walls, thus enhancing transcapillary passage of the vesicles (Senior et al, 1991; Allen, 1994; Maruyama et al, 1994; Working et al, 1994).

The transition from basic research to clinical practice has relied on technical breakthroughs in the control of liposome stability and reactivity. Enormous efforts have been undertaken to retain an encapsulated drug for a sufficiently long time after its administration, in order to appropriately alter the pharmacokinetics of the
drug. It is well documented that the three major factors for an extended retention in the circulating blood are the lipid composition, the vesicle charge, and the size of liposomes (Gregoriadis, 1985).

However, conventional liposomes (neutral and/or negatively charged and/or cholesterol liposomes) are generally characterised by a relatively short blood circulation time (Lasic, 1997). When administrated in vivo by a variety of parenteral routes, they display a strong tendency to accumulate rapidly in the phagocytic cells of the mononuclear phagocyte system (MPS), also referred to as the reticuloendothelial system (RES). The major organs of accumulation in terms of total uptake and uptake per gram of tissue are the liver and the spleen. An abundance of MPS macrophages and a rich blood supply are the primary reasons for the preponderance of particles in the liver and the spleen (Storm & Crommelin, 1998). A logical therapeutic translation of this MPS-directed distribution behaviour is that conventional liposomes may be attractive candidates for drug delivery to RES.
1.4 LIPOSOMES AS VACCINE CARRIERS

Modern technology has allowed mass production of safe synthetic or recombinant antigens for vaccine development. However, many of these purified proteins or peptides have proved poor immunogens. These poorly immunogenic antigens can be combined in vaccine formulations with a variety of immunostimulatory materials to enhance their immunogenicity. Immunostimulatory materials can be classified conveniently into three basic categories: adjuvants, carriers for antigens, and vehicles (Edelman & Tacket, 1990; Allison, 1997). Adjuvants, carriers and delivery systems are required to enhance the low immunogenicity of viral subunits, recombinant proteins and synthetic peptides through appropriate and optimal antigen presentation, non-specific stimulation of host defences and, particularly in immunosuppressed hosts, direction or re-direction of the immune response (Jennings et al, 1998).

An immunological adjuvant may be defined as any substance that when incorporated into a vaccine formulation acts generally to accelerate, prolong, or enhance the quality of specific immune responses to vaccine antigens. The word adjuvant is derived from the Latin word *adjuvare*, which means "to help" or "aid". Immunological adjuvants act directly or indirectly on antigen-presenting cells such as macrophages and dendritic cells (Wu et al, 1994; Kovacsovics-Bankowski & Rock, 1994). Although many immunological adjuvants are capable of inducing immune responses to vaccine antigens, most of them are toxic, such as lipopolysaccharide and lipid A, and/or cause inflammation at the site of injection,
such as Freund's complete adjuvant (Gregoriadis et al, 1993; Gluck, 1994). Alum has been the only immunological adjuvant licensed for the use in humans for the past 70 years. Alum adjuvants are used with protein antigens in two ways: a) as alum-precipitated vaccines and b) as alum-adsorbed vaccines. Commercially available Al (OH)3 can be used to adsorb proteins in a ratio of 50-200g protein/mg aluminum hydroxide. Adsorption of protein is dependent on the pI (isoelectrical pH) of protein and the pH of the medium. Salt adjuvants are most frequently used as adjuvants for vaccine antigen delivery. They are generally weaker than emulsion adjuvants and are best used with strongly immunogenic antigens.

However, alum is far from ideal, because it fails to induce significant cell-mediated immunity, generally causes mild inflammatory reactions, and is often unsuitable for a variety of antigens. Efforts are therefore being made to develop safe and effective adjuvants to meet the challenges of new generation vaccines. The emulsion-based adjuvant MF59 has recently been shown to be internalised by dendritic cells (Dupuis et al, 1998). Certain novel adjuvants such as purified saponins, immunostimulating complexes (ISCOM), and liposomes have been shown to greatly improve the induction of MHC class-I-restricted CD8+ CTL responses compared to those induced by the same antigen given alone or in combination with standard alum adjuvants (Takahashi et al, 1990, Newman et al, 1992; Shahum & Therien, 1995). The induction of CTL by these adjuvants may be caused by the delivery of antigen directly to the cytosol for presentation with MHC class I molecules (Kovacsovics-Bankowski & Rock, 1995). Cytosolic
antigen delivery by membrane-active adjuvants could mimic antigen presentation that occurs during viral infection or immunisation with live-attenuated vaccines. Antigen presented to the cytosol could bypass endosomal antigen delivery and subsequent processing by MHC class II molecules, which occurs when antigen is delivered alone or in alum and induces primarily antibody responses via presentation to CD4\(^+\) T-helper lymphocytes (Audibert & Lise, 1993).

As mentioned further above, two of the most widely used novel adjuvants are saponins and ISCOMs. Saponins are triterpene glycosides, which are derived from the bark of the Quillaja saponaria tree and have detergent and adjuvant properties. Due to their haemolytic activity, saponins should not be injected intraperitoneally or intravenously but only subcutaneously or intramuscularly. ISCOMs are prepared from QuilA (purified saponins), cholesterol and phospholipids. The antigen to be inserted into ISCOMs must be amphipathic (Dalsgaard et al, 1995). ISCOMs can be recommended for viral vaccines, because they can deliver the antigen to the cytosolic compartment of antigen processing/antigen-presenting cells. Indeed, ISCOM-based influenza virus vaccine is currently undergoing clinical trial (Jennings et al, 1998). ISCOM-associated antigen molecules do not form a depot site but are transported to the draining lymph nodes.

Various cytokines induced by adjuvants act on lymphocytes to promote predominately Th1 or Th2 immune responses (Audibert & Lise, 1993; Kovacsovics-Bankowski & Rock, 1995; Xu-Amano et al, 1993). Adjuvants that enhance Th1 immune responses through the induction of IFN-\(\gamma\) and delayed-type
hypersensitivity (DTH) also elicit the production of IgG subclasses that fix complement and bind with high affinity to Fc \( \gamma \) I receptors (e.g., IgG\(_{2a} \) in mice and IgG\(_1 \) in humans) (Allison, 1998; Phillips & Emili, 1992; Unkeless et al, 1988). Several cytokines are under evaluation as vaccine adjuvants, including IL-2, interferon gamma and IL-12 (Nunberg et al, 1989, Pardoll, 1995; Luis et al, 1994).

Interestingly, recent evidence discussed by Pisetsky et al (1995) suggests that the chemical nature of DNA may also play a role as an adjuvant. The mitogenic effects of bacterial DNA has been observed with both ssDNA and dsDNA (Tokunaga et al, 1984; Messina et al, 1991 & 1993). Since mitogenicity appears common among bacterial DNA, it can be attributed to structural characteristics of these nucleic acids. Studies by Kreig et al (1995) suggest that CpG motifs are the source of mitogenicity, consistent with their content and pattern of methylation in bacterial DNA. The importance of these motifs is supported by studies showing that methylation of bacterial DNA eliminates mitogenicity. Because bacterial DNA is a mitogen, its effect in the setting of immunisation would be to enhance responsiveness to any protein encoded on the construct. This adjuvant effect is likely to be beneficial and may be one of the reasons DNA vaccination is so effective (Pisetsky et al, 1995). It is apparently due to the high frequency of CG (i.e. cytosine-guanine) sequences in plasmids. Plasmid DNA, derived from bacteria, has a greater frequency of CG sequences than the DNA in vertebrates. Moreover, the CG units in bacterial plasmids tend to have no methyl group attached, whereas those in vertebrates are generally methylated (Kreig et al,
Animal studies suggest that DNA can play a role in host defence, both by stimulating B cells for antibody production and by inducing cytokines. Its resemblance to other bacterial macromolecules triggers nonspecific immune cell activation (Pisetsky et al, 1995). Whilst the role of bacterial DNA in human immunity is at present unknown, it is of interest and relevance for DNA vaccination.

Adjuvants have diverse mechanisms of action and must be selected with regard to the immune responses (e.g., antibody, mucosal, CTL) contributing to the induction of protective immunity.

Adjuvants are thought to exert their effect by one or more of the following mechanisms (Gupta et al, 1993):

- formation of an antigen depot at the site of injection while allowing slow antigen release;
- presentation of antigen to immunocompartment cells, and induction via cytokine production.

Antigen can also be targeted to macrophages or dendritic cells by particulate adjuvants such as liposomes, and APC can be stimulated by adjuvants to secrete immunomodulatory cytokines. Since the first demonstration of the use of liposomes as immunological adjuvants (Allison & Gregoriadis, 1974), numerous studies have been carried out demonstrating the ability of these carriers to enhance both humoral and cell-mediated immune responses (reviewed by Gregoriadis, 1990).
The way in which liposomes induce immune responses to antigens is not clear, but has been attributed to a depot mechanism and to the ability of vesicles and antigen content to migrate to regional lymph nodes following local injection (Gregoriadis, 1990; Alving, 1991; Frézard, 1999). Liposomes have the well-known ability to channel protein and peptide antigens into the MHC class II pathway of phagocytic antigen-presenting cells and thereby enhance the induction of antibodies and antigen specific T cell proliferation responses. Liposomes also serve as an efficient delivery system for entry of exogenous protein and peptide antigens into the MHC class I pathway and are therefore very efficient inducers of cytotoxic T cell responses (Rouse et al, 1986; Warren et al, 1986; Gregoriadis, 1990). Several hypotheses have been postulated to explain the adjuvant character of liposomes (Edelman, 1980; Roderdink et al, 1982; Gregoriadis, 1984, 1990; Alving, 1991; Frézard, 1999). It is known that \textit{in vivo} liposomes are naturally targeted to macrophages of the RES by which they are phagocytosed and degraded (Gregoriadis, 1984). However, before their dispersion through phagocytosis by macrophages, liposomes circulate in the blood where they encounter all the different types of cells that actively participate in the elaboration of an immune response. Locally injected liposomes are known to be taken up avidly by antigen-presenting cells (APC) infiltrating the site of injection or in the lymphatics, an event that is probably implicated in their immunoadjuvant activity (Walden et al, 1986; Gregoriadis, 1990; Wagner et al, 1991).

The two major possible mechanisms, by which liposomes could conceivably be accommodated by a cell following its interaction with the cell surface, are fusion
with plasma membrane or by the endocytic pathway (Weinstein & Leserman, 1984). Both routes require cell membrane destabilisation at some point. It has been demonstrated that liposome-encapsulated antigens are taken up by cells via receptor-mediated or non-receptor-mediated endocytosis (Huang, 1983). The ingested liposomes are usually not degraded until they are incorporated into the lysosomes (Collins & Haung, 1989). Antigen fragments generated in the lysosomes can somehow be rescued from complete digestion and bind to class II molecules during transit through the endocytic pathway (Harding et al, 1991). In recent years, however, several investigators have reported significant delivery of liposome-encapsulated substances to the cytoplasm of various cell types in vitro by addition of fusogenic lipids (pH-sensitive and cationic liposomes) or by inserting naturally fusogenic components, such as fusion proteins of membrane viruses, into the liposomal membrane (virosomes).

The current presumed mode of action of liposomes (Fig 1.9) has been proposed by Frézard (1999), according to which the enhancement of HI to antigens by means of their incorporation into liposomes can be attributed to the generation of a depot at the site of injection, which prolongs the release and interaction of free or liposome-associated antigens with antigen-presenting cells. Amongst APC, macrophages have been shown to play a major part, because of their unique ability to phagocytose liposomes. It is unclear, however, whether macrophages process liposomal antigens and present the fragments directly to T cells or cooperate with other antigen-presenting cells in the microenvironment so that these accomplish the presentation task (Szoka, 1992). It has been proposed (Rao et al,
that other antigen-presenting cells such as immature dendritic cells play roles by which tailored liposomes exert potent activity as vehicles for vaccines.

Since the lipid composition is known to influence the \textit{in vivo} behaviour of liposomes, it may also influence their adjuvant action. Bilayer fluidity, number of lamellae, vesicle size, and the mode of antigen localisation within the liposomes all seem to have an effect on liposomal adjuvanticity (Gregoriadis, 1990). For example, the presence of phospholipids with high Tc values (above 37°C) improved antibody responses to hydrophobic antigens, but not to soluble antigens (Davis & Gregoriadis, 1989). A variety of factors, including an appropriate lipid to antigen mass ratio (Gregoriadis et al, 1987), are known or suspected to influence \textit{in vivo} the balance of a number of mechanisms of liposomal antigen supply to APC leading to optimal adjuvanticity. Such a balance is expected to reflect primarily the structural profile of a given liposomal vaccine formulation (Gregoriadis, 1995). The differences in the structural characteristics of antigens, in their spatial arrangement within liposomes, and in the way they interact with liposomes may also influence the events determining liposomal adjuvanticity. Further improvement of adjuvanticity has been achieved by the use of co-adjuvants such as positively charged lipids and interleukin-2, and also by ligand-mediated targeting to antigen-presenting cells (Gregoriadis, 1990; Alving, 1991).

The advantages of using liposomes as vaccine carriers and immunological adjuvants can be summarised as follows:
• Liposomes are able to convert a non-immunogenic molecule to an immunogenic one.

• They have the ability to accommodate both hydrophilic and hydrophobic antigen.

• They can improve antibody responses with even lower doses than the free antigen.

• They can accommodate multiple antigens in the same vesicle.

• They allow the entrapment of antigen together with a co-adjuvant.

• They augment humoral responses to several antigens.

• They can induce cell-mediated immunity to antigens.

• Certain liposome formulations are able to induce cytotoxic T cells.

• Liposomes reduce the toxicity of an antigen or a co-adjuvant, increase their half-lives in blood and alter the biodistribution.

• They allow ligand-mediated targeting to antigen-presenting cells.

• They offer a choice for the spatial distribution of the antigen or the co-adjuvant within the same vesicle (i.e. adsorption, covalent coupling to the vesicle surface, or entrapment).

• Liposomally associated antigens and co-adjuvants can be kept in freeze-dried form without substantial loss of activity after reconstitution.
I.5 TYPES OF LIPOSOMES

pH-sensitive liposomes and proteoliposomes (also named virosomes or chimerasomes) have been used to enhance the efficiency of gene delivery by controlling the intracellular trafficking of plasmids after endocytosis of the liposomal plasmids by the target cells (Wang & Huang, 1987; Tikchonenko et al, 1988; Gould-Fogerite et al, 1989; Kato et al, 1991). Such pH-sensitive liposomes have been designed to fuse with lipid membranes in an acidic environment, such as the endosomal compartment, thus facilitating the specific release of plasmids from the endosomes into the cytoplasm of transfected cells (Legendre & Szoka, 1992). A major drawback of pH-sensitive liposomes is their poor stability in vivo. Although their stability can be improved by using small liposomes of the same composition, this leads to loss of pH sensitivity.

More recently, some of the advantages of viral delivery vectors have been combined with the safety and 'simplicity' of the liposome to produce fusogenic virosomes (Tikchonenko et al, 1988; Kaneda et al, 1989). Virosomes have been engineered by complexing the membrane fusion proteins of the hemagglutinating virus of Japan (HVJ, also known as Sendai virus) with either liposomes that already encapsulate plasmid DNA or oligodeoxynucleotides (ODN) for antisense applications (Kato et al, 1991; Wu et al, 1995). The inherent ability of the viral proteins in virosomes to cause fusion with cell membranes means that these hybrid vectors can be very efficient in introducing their nucleic acid to the target cell, resulting in good gene expression. While the size of transgene that can be
incorporated into viral vectors is limited, no such limit exists for virosome or liposome technology. Genes of up to 100 kilobase pairs have been delivered by fusigenic virosomes to cells both *ex vivo* and *in vivo*. However, the transient nature of gene expression is a major disadvantage of the current HVJ-liposome system. Since the system is a hybrid between viral and nonviral vectors, the safety of the liposome and the immunogenicity of the HVJ-liposome complex must be taken into consideration (Dzau et al, 1996).

Another type of liposomes with interesting properties for gene delivery is cationic liposomes. Because of its convenience and efficacy, cationic lipid-mediated gene delivery technology has become a standard transfection technique for cultured cells (Felgner & Ringold, 1989, 1990; Duzgune & Felgner, 1993). In particular, the formation of complexes of DNA with cationic liposomes has led to promising results (Felgner et al, 1987; Behr et al, 1989; Yoshimura et al, 1992; Legendre & Szoka, 1992; Nabel et al, 1993; Caplen et al, 1995). Given that all biological surfaces carry a net negative charge, cationic lipids are preferable, because they interact spontaneously with the cell surface and deliver the associated polynucleotide to the cell's interior (Felgner et al, 1987; Bertling et al, 1991; Rose et al, 1991; Legendre & Szoka, 1992; Zhou & Huang, 1994). Cationic lipids are synthetic in nature and vary from monocation head groups to polycation head groups (Lasic, 1996).

While the use of liposomes, in particular pH-sensitive, proteoliposomes and cationic liposomes, is a promising new area of gene therapy research, its success
depends to a large degree on technological improvements in the formation of vesicles of various sizes and properties. The encapsulation of gene expression systems into liposomes presents several limitations, such as low yield of encapsulation, and can vary widely with the conditions employed during encapsulation (Rolland, 1998). Furthermore, most procedures for drug incorporation with liposomes expose the components to organic solvents and/or sonication, which may be detrimental to DNA integrity (Schieren et al, 1978; Mimms et al, 1981; Gregoriadis, 1984). Moreover, the risk of cell toxicity limits the amount of cationic lipids that can be offered to the cells (Raz et al, 1994a). Another downside is that most cationic liposomes complexed with DNA have been shown to be unable to protect the DNA from enzymatic attack (Gao & Huang, 1996). These problems clearly restrict the applicability of liposome-mediated transfection in vivo.

A simple encapsulation method reported by this laboratory (Kirby & Gregoriadis, 1984; Gregoriadis, 1985; Gregoriadis et al, 1987; see also section 1.3.3.2) may offer a solution to the above problems. Based on the fusion of preformed phospholipid vesicles by dehydration followed by rehydration, it produces multilamellar liposomes (dehydration-rehydration vesicles, DRV), which, in the process of formation, entrap up to 80% of the added solute without employing potentially damaging conditions (Kirby & Gregoriadis, 1984).

The advantages of the DRV liposomal approach in vaccination are widely documented in the literature. Previous work with DRV liposomes has shown high
entraption values for a number of small molecular weight drugs and macromolecules, including proteins (Kirby & Gregoriadis, 1984; Norley et al, 1986), and it is expected that the method will be similarly successful for a variety of antigens relevant to human and animal immunisation (Gregoriadis et al, 1987). When appropriately designed (in terms of size, surface charge and lipid composition), DRV liposomes offer true encapsulation of content, thus protecting the content from enzyme attack in the blood plasma (Gregoriadis, 1995). The dehydration-rehydration method avoids damaging conditions during entrapment of antigens and other sensitive agents and it is encouraging that DRV liposomes have proved to elicit immune responses to the entrapped antigens (Gregoriadis et al, 1987).

Here, for the first time, we have examined liposomes produced by the dehydration-rehydration technique as a carrier for DNA vaccination to establish whether liposomes can be used as a suitable delivery tool for gene therapy and vaccination.
### Table I.1 Properties of two commercially marketed hepatitis B vaccines

#### Recombivax HB® vs. Engerix-B® production differences

<table>
<thead>
<tr>
<th>Action</th>
<th>Recombivax HB</th>
<th>Engerix-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiocyanate conversion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antigen folding</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Formalin treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inactivation Step:</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Stabilisation of antigen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alum adjuvant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increase immunogenicity</td>
<td>in situ formation</td>
<td>Alum preformed</td>
</tr>
<tr>
<td>Purify yeast protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increase immunogenicity</td>
<td>&gt;99%</td>
<td>&gt;97%</td>
</tr>
</tbody>
</table>

The two yeast-derived vaccines licensed in most countries are Engerix-B and Recombivax HB. Both products are structurally and chemically similar with less than 2% yeast protein remaining in solution.

The major difference between the products is the number of steps used in recovery and purification of the antigen from the yeast cultures, thus resulting in different dosage amounts (Molinari, 1992).
Table I.2 Comparison of properties of various vector system for gene delivery

<table>
<thead>
<tr>
<th>Features</th>
<th>Retroviral</th>
<th>Adenoviral</th>
<th>AAV</th>
<th>Naked/lipid-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum insert size</td>
<td>7-7.5 kb</td>
<td>30 kb</td>
<td>3.5-4.0 kb</td>
<td>Unlimited size</td>
</tr>
<tr>
<td>Concentrations (viral particles per ml)</td>
<td>&gt;10⁸</td>
<td>&gt;10¹¹</td>
<td>&gt;10¹²</td>
<td>No limitation</td>
</tr>
<tr>
<td>Route of gene delivery</td>
<td>Ex/in vivo</td>
<td>Ex/in vivo</td>
<td>Ex/in vivo</td>
<td>Ex/in vivo</td>
</tr>
<tr>
<td>Integration</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes/No</td>
<td>Very poor</td>
</tr>
<tr>
<td>Duration of expression in vivo</td>
<td>Short</td>
<td>Short</td>
<td>Long</td>
<td>Short</td>
</tr>
<tr>
<td>Stability</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
<td>Very good</td>
</tr>
<tr>
<td>Ease of preparation (scale up)</td>
<td>Pilot scale up, up to 20-50l</td>
<td>Easy to scale up</td>
<td>Difficult to purify, difficult to scale up</td>
<td>Easy to scale up</td>
</tr>
<tr>
<td>Immunological problems</td>
<td>Few</td>
<td>Extensive</td>
<td>Not known</td>
<td>None</td>
</tr>
<tr>
<td>Pre-existing host immunity</td>
<td>Unlikely</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Safety problems</td>
<td>Insertional mutagenesis?</td>
<td>Inflammatory response, toxicity</td>
<td>Inflammatory response, toxicity</td>
<td>None</td>
</tr>
</tbody>
</table>

Table present the most promising delivery systems used for gene delivery. Their properties, their limitations and their advantages for using in this field is quite interestingly summarised (adopted from Verma, IM and Somia, N Gene therapy-promises, problems and prospects. Nature, 1997)
Table 1.3 Most commonly used phospholipids in liposome preparations and their gel to liquid crystalline transition temperatures (adapted from Weiner, 1990)

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Abbreviation</th>
<th>transition temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg phosphatidylcholine</td>
<td>EPC</td>
<td>-15 to -7</td>
</tr>
<tr>
<td>Dilauryloyl phosphatidylcholine</td>
<td>DLPC</td>
<td>-1.80</td>
</tr>
<tr>
<td>Dimyristoyl phosphatidylcholine</td>
<td>DMPC</td>
<td>23.00</td>
</tr>
<tr>
<td>Diaplmitoyl phosphatidylcholine</td>
<td>DPPC</td>
<td>41.00</td>
</tr>
<tr>
<td>Distearoyl phosphatidylcholine</td>
<td>DSPC</td>
<td>55.00</td>
</tr>
<tr>
<td>Dioleoyl phosphatidylcholine</td>
<td>DOPC</td>
<td>-22.00</td>
</tr>
<tr>
<td>Dilauryloyl phosphatidylglycerol</td>
<td>DLPG</td>
<td>4.00</td>
</tr>
<tr>
<td>Dimyristoyl phosphatidylglycerol</td>
<td>DMPG</td>
<td>23.00</td>
</tr>
<tr>
<td>Dipalmityl phosphatidylglycerol</td>
<td>DPPG</td>
<td>41.00</td>
</tr>
<tr>
<td>Dioleoyl phosphatidylglycerol</td>
<td>DSPG</td>
<td>55.00</td>
</tr>
<tr>
<td>Distearoyl phosphatidylglycerol</td>
<td>DOPG</td>
<td>-18.00</td>
</tr>
<tr>
<td>Dimyristoyl phosphatidylethanolamine</td>
<td>DMPE</td>
<td>50.00</td>
</tr>
<tr>
<td>Dipalmitylphosphatidylethanolamine</td>
<td>DPPE</td>
<td>66.00</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>PS</td>
<td>7.00</td>
</tr>
<tr>
<td>Dimyristoyl phosphatidylserine</td>
<td>DMPS</td>
<td>38.00</td>
</tr>
<tr>
<td>Dipalmityl phosphatidylserine</td>
<td>DPPS</td>
<td>51.00</td>
</tr>
<tr>
<td>Dimyristoyl phosphatidic acid</td>
<td>DMPA</td>
<td>51 (pH 6.0)</td>
</tr>
<tr>
<td>Dipalmityl phosphatidic acid</td>
<td>DPPA</td>
<td>67 (pH 6.5)</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>SPH</td>
<td>32.00</td>
</tr>
<tr>
<td>Dipalmityl sphingomyelin</td>
<td>DPSPH</td>
<td>41.00</td>
</tr>
<tr>
<td>Distearoyl Sphingomyelin</td>
<td>DSSPH</td>
<td>57.00</td>
</tr>
</tbody>
</table>
Figure 1.1  DNA in schematic and chemical structure presentation
Figure 1.2  Schematic presentation of plasmid DNA with sequences needed for effective expression and replication
Figure 1.3  Map of HBsAG-expressing plasmid pRc/CMV-HBs(S)
(adopted from Whalen)
Figure 1.4 Structure of HBV envelope proteins and the plasmid expression vectors for DNA-mediated immunisation (adopted from Whalen, 1995)

(A) A linear representation of the S, M, and L envelope polypeptides. The N- and C-terminal ends of the polypeptides are indicated, as are the preS1, preS2 and S sequences and their size (in amino acids). (B) The plasmid vectors containing the HBV envelope coding sequences are designated pCMV-S, pCMV-S2.S and pCMV-S1.S2.S.
Figure I.5  Chemical formulae of various neutral and anionic lipids

Dioleoyl phosphatidylcholine (DOPE)

Phosphatidylcholine (PC)

Phosphatidylserine (PG)

Phosphatidyl glycerol (PS)
Figure I.6 Chemical formulae of various cationic lipids

3β-(N,N-dimethylaminoethane)carbamyl cholesterol (DC-Chol)

N[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA)

1,2-dioleyloxy-3-(trimethylammonium)propane (DOTAP)
Figure 1.7  Chemical formulae of various fluorinated lipids

DF6C7PC

\[
\begin{align*}
C_6F_{13}(CH_2)_{6} & \xrightarrow{\text{O}} \xrightarrow{\text{O}} \\
C_6F_{13}(CH_2)_{6} & \xrightarrow{\text{O}} \xrightarrow{\text{O}} \\
& \xrightarrow{\text{O}} \xrightarrow{\text{O}} \xrightarrow{\text{N}} \\
\end{align*}
\]

F8C2PC

\[
\begin{align*}
C_8F_{17}(CH_2)_{2} & \xrightarrow{\text{O}} \xrightarrow{\text{O}} \xrightarrow{\text{N}} \\
\end{align*}
\]

F8C11PC

\[
\begin{align*}
C_8F_{17}(CH_2)_{11} & \xrightarrow{\text{O}} \xrightarrow{\text{O}} \xrightarrow{\text{N}} \\
\end{align*}
\]

F6H11GlyLact

\[
\begin{align*}
\text{OH} & \xrightarrow{\text{OH}} \xrightarrow{\text{OH}} \xrightarrow{\text{OH}} \\
\text{OH} & \xrightarrow{\text{OH}} \xrightarrow{\text{OH}} \xrightarrow{\text{OH}} \\
(\text{CH}_2)_n C_6F_{13} & \xrightarrow{\text{N}} \xrightarrow{\text{N}} \xrightarrow{\text{N}} \\
\end{align*}
\]
Figure 1.8  Preparation of liposomes by dehydration-rehydration technique
(schematic presentation adopted from Gregoriadis, 1995)

A solution containing the drug is mixed with a suspension of empty liposomes and the mixture is freeze-dried. The inset shows intimate contact of flattened liposomal membrane structures and of drug molecules in a dry environment. DRV liposomes formed upon controlled rehydration contain much of the original drug in an entrapped form.
Proposed modes of cellular uptake of liposomes
(adopted from Juliano and Leyton, 1989)

Liposome-cell interaction:
(1) Adsorption
(2) Fusion
(3) Endocytosis
(4) Lipid exchange
Figure 1.10  Schematic presentation of proposed immunoadjuvant action of liposomes
(adopted from Frezard, 1999)
II MATERIALS AND METHODS

II.1 MATERIALS

The following chemicals were purchased from Sigma Chemical Co., Poole, Dorset, UK: dioleoyl phosphatidylcholine (DOPE), phosphatidylserine (PS), stearylamine (SA), cholesterol (CHOL), deoxyribonuclease I (bovine pancreas, type II; specific activity: 2500 Kunitz unit mg−1 protein), gelatine, bovine serum albumin, chloramine-T, RPMI-1640 medium, and o-phenylenediamine.

Egg phosphatidylcholine (PC), 1,2-bis (hexadecylcycloxy)-3-trimethylammonopropylamine (BisHOP), and phosphatidyl glycerol (PG) were from Lipid Products, Nutfield, Surrey. N[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA) was a gift from GeneMedicine (Houston, Texas, USA). 1,2-Dioleoyloxy-3-(trimethylammonium) propane (DOTAP) was from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and 3b-(N,N-dimethylaminoethane)carbamyl cholesterol (DC-Chol) was a gift from Dr. C. Kirby. Fluorinated double-chain amphiphiles (DF6C7PC, F8C11PC, F8C2PC and F6H11GLY GLY LACT) were from ATTA (Nice, France). The cationic lipofectAMINE was obtained from Gibco BRL (Paisly, UK).

The eukaryotic expression vector pGL2-control (3.99×10^6 Daltons) expressing the luciferase reporter gene from a SV40 promoter was purchased from Promega (Southampton, UK). Plasmid pRc/CMV-HBS expressing sequences coding for the S
protein of hepatitis B virus (HBsAg, subtype ayw) was cloned by Dr. Robert Whalen using pRc/CMV (in vitro) as a vector backbone. Recombinant hepatitis B surface antigen (HBsAg) (S region; subtype ayw) was purchased from Genzyme, West Mailing, Kent, UK. Oligodeoxyonucleotide was a gift from Dr. Pierri Soni, The Royal Free Hospital, London, UK. Horseradish peroxidase conjugated goat anti mouse IgG1, IgG2a, IgG2b, and foetal calf serum were from Sera-Lab Ltd., Crawley Down, Sussex, UK. Ninety-six well flat bottom microtiter plates (Immunolon I) for ELISA plates were from Dynatech Labs, Billingshurst, W. Sussex, UK. Primary monoclonal antibodies against IFN-γ (R46A2) and IL-4 (11B11), secondary biotinylated anti-mouse IL-4 (BVD6-24G2) and anti-mouse IFN-γ (XMG1.2) monoclonal antibodies, as well as recombinant interferon-γ and interleukin-4 standards were from Pharmingen (USA). Streptavidin peroxidase came from Dako (Denmark). Standard capture ELISAs used with monoclonal antibody pairs and Maxisorp plates were from NUNC (UK). Carrier-free 35S-dATP (37 kBq) was purchased from ICN Flow, Thames (UK). All the column materials used including Sephadex G-25, Sepharose CL 4B and Sepharose 6-B were from Pharmacia Biotech, St Albans, Herts, UK. All other reagents used were of analytical grade.
II.2 METHODS

II.2.1 Batch to batch investigation

From the pharmaceutical point of view, it is crucial that liposomal preparations are easy to prepare and reproducible. Data were therefore considered valid only if they proved repeatable in at least four batches (i.e. each preparation repeated at least four times). Prior to each in vivo experiment, the formulations were usually prepared twice, i.e. as two batches, and were used for in vitro and in vivo experiments only if they were found reproducible. If there was any sign of aggregation of lipid or liposomes (which may happen during the storage or preparation procedure, especially with large number of preparations), the sample was discarded and a fresh sample was produced. Experimental protocols and interpretation of results followed throughout our studies for this thesis are based on both the valid standard protocols obtained from reference works, such as 'Molecular Cloning. A Laboratory Manual' (Sambrook et al, 1989), 'Introduction to Biostatistics' (Sokal & Rohlf, 1973), 'Liposomes: a Practical Approach' (New, R, 1990), and/or internal protocols used by experts in the field [Prof. Gregory Gregoriadis (for liposome preparation and immunisation), Dr Robert Whalen (for plasmid DNA pRc/CMV-HBS), Dr Stephen Hart (for transfection and gel electrophoresis) and Dr Brian De Souza (for spleen removal time and the cytokine assays)].
II.2.2 Preparation of liposomes

A method of liposome preparation has been developed, which is simple to use, employs mild conditions, and facilitates the entrapment of relatively large amounts of material (Kirby & Gregoriadis, 1984). The procedure, called dehydration-rehydration method, is based on the induction of fusion of preformed empty small unilamellar vesicles (SUV) by means of dehydration and controlled rehydration eventually yielding vesicles of multilamellar type (dehydration-rehydration vesicles; DRV). The processes during the dehydration-rehydration cycles are outlined below.

II.2.2.1 Preparation of multilamellar liposomes (MLV)

MLV were prepared from egg phosphatidylcholine (PC) as such or with dioleyl phosphatidylcholine (DOPE), and in the experiments where negatively or positively charged liposomes were required, phosphatidylserine (PS), phosphatidyl glycerol (PG), stearylamine (SA), N[1-(2,3-dioleoyloxy)propyl]-N,N,N-triethyl-ammonium(DOTMA), 1,2-bis(hexadecyloxy)cycloxy)-3-trimethyl aminopropane (BisHOP), 1,2-Dioleoxyloxy-3-(trimethylammonium) propane (DOTAP) and 3b-(N,N-dimethylaminoethane)carbamyl cholesterol (DC-CHOL) were used.

The lipid mixture in chloroform was placed in a 50ml round bottom spherical Quick-fit flask and dried to a thin film at 37°C by evaporation of the solvent at a low speed in a rotary evaporator (Buchi) connected to a running tap water pump. In order to
ensure complete solvent removal and to replace air, the lipid film obtained was left under a stream of nitrogen for 15 min. Rehydration and formation of MLV was effected by the addition of 2 ml double distilled water (depending on the amount of lipids used) and by disruption of the lipid film with glass beads. Liposomes were allowed to anneal at ambient temperature, Ta, for about 1 h.

II.2.2.2 Preparation of small unilamellar vesicles (SUV)

MLV prepared as described above were sonicated using an MSE sonicator (titanium probe of 19 mm diameter) in an ice bath for 5-10 cycles (depending on the amount of lipids used), each of which lasted 60 sec, with 30 sec rest intervals in between to obtain a clear suspension. The SUV produced were then centrifuged at 1000 g for 5 min to spin down titanium particles shed from the probe of the sonicator. The sonicated suspension of the SUV was allowed to rest at room temperature (RT) for 30 min.

II.2.2.3 Preparation of dehydration-rehydration vesicles (DRV)

DRV preparation was carried out according to the method of Kirby and Gregoriadis (1984). SUV prepared as above in the presence of distilled water were mixed with 10-100 µl of the nucleic acid (DNA or oligonucleotide) to be encapsulated, together with corresponding radiolabelled tracers or fluorescence marker when available. They were frozen at minus 70°C and freeze-dried overnight in a Christ freeze-drier until a powder was obtained. The powder was rehydrated with 0.1-0.3 ml of distilled
water and swirled vigorously, and then incubated at RT for 45 min. At the end of this time, 0.1M sodium phosphate buffer, pH 7.4, containing 0.9% NaCl (PBS) was added and the mixture was incubated for another 30 min. The suspension composed of liposome-entrapped and free drug was diluted with 6ml PBS and centrifuged at 40,000 g for 30 min using a Sorvall Combi Plus Du Pont ultracentrifuge. The liposomal pellet was washed twice with 6ml PBS and the final pellet was suspended in PBS for further use.

II.2.2.4 Incorporation of fluorinated amphiphiles into DRV

The dehydration-rehydration procedure was used for the incorporation of nucleic acid into liposomes composed of fluorinated amphiphiles. In short, the fluorinated DF6C7PC, F8C11PC, F8C2PC and F6H11GLY GLY LACT phospholipids were hydrated above their respective phase transition temperature (Tc=51°C for DF6C7PC, 69°C for F8C2PC and F8C11PC, and 75°C for F6H11GLY GLY LACT). Small unilamellar vesicles (SUV) were prepared by probe sonication of dispersed fluorinated/lipid mixture in 2ml double distilled water. Nucleic acid was added to fluorinated SUV and placed in a freezer. After freezing, the preparation was lyophilised over night in a freeze-drier at a vacuum of 0.1 Torr. The preparation was rehydrated with 0.1-0.3ml of distilled water. After 30 min the fluorinated vesicles were reconstituted to PBS buffer (pH 7.4) followed by addition of 6ml of the same buffer, and were centrifuged to separate free from entrapped nucleic acids.
II.2.2.5 Size reduction of DRV liposomes by microfluidisation

DRV were prepared as described above in mixture with free, non-incorporated drug (i.e. before centrifugation) and were passed for 1, 2, 3, 5 and 10 cycles through a Microfluidiser M110S (Microfluidics, Newton, MA, USA). The pressure gauge was set at 60 psi throughout the procedure to give a flow rate of 35ml per min. Separation of the free from the incorporated drug in microfluidised liposomes was carried out by centrifugation as above (1, 2 and 3 cycles) for 40 min at 50,000 g or by molecular sieve chromatography (5 and 10 cycles) using a Sepharose 4B CL column (Gregoriadis et al, 1990). In some experiments, preformed DRV liposomes were mixed with nucleic acids and incubated at RT for 1 h and were microfluidised for 3 cycles. In this case, the liposomes were centrifuged as above to separate absorbed from non-absorbed DNA or oligonucleotide.

II.2.3 Column preparation (molecular sieve chromatography)

A column (1.5 x 25cm) was placed on a stable surface in a vertical position, far from draughts and sunlight to prevent changes in temperature and the formation of air bubbles in the pack column. The Sepharose CL 4B gel suspension was poured into a vertical column over a glass rod and the flow of solvent was started as soon as the column had been filled. After the column had been packed with gel, the dead space under the net and the cell wall of the column was checked for air bubbles. To allow the bed to be equilibrated and to ensure adequate packing, the gel material was washed with 100ml PBS buffer. Since the method requires a drained bed surface for sample application, the top air vent was closed and the
outlet opened to remove excess saline solution from the column. Finally, the outlet was closed and the remaining eluant on the top of the bed was removed with a glass Pasteur pipette. 1-2ml of the sample were applied immediately on the top of the gel bed (under no circumstances the surface of the bed left run dry). The outlet was opened and the sample was allowed to drain into the top of the bed. The sample remaining on the bed surface and the column wall was washed with a small amount of buffer. The column was refilled with eluant and the fractions (1ml each) were collected.

II.2.4 Estimation of nucleic acids entrapped into liposomes

The extent of nucleic acid (oligonucleotide and plasmid DNA) entrapment into DRV liposomes or microfluidised DRV was estimated according to the following equation:

Entrapment efficiency (\%) = \( \frac{D_e}{D_i} \times 100 \).

\( D_e \) is the amount of DNA entrapped by liposomes (suspended pellet) and \( D_i \) is that of DNA applied initially (total DNA i.e entrapped and non-entrapped materials).

To ensure that the initial concentration of nucleic acid used for entrapment into liposomes was not reduced during the preparation procedure (i.e. freeze-drying, centrifugation, and in some cases microfluidisation), the percentage recovery of the initial concentration was estimated for every single experiment by using the following equation:
% recovery = D_e + D_f/D_t × 100.

D_e is the amount of DNA entrapped by liposomes (suspended pellet), D_f is the amount of free DNA in supernatants and D_t is that of DNA applied initially (total DNA i.e entrapped and non-entrapped materials).

To determine the amount of nucleic acid entrapped into those liposomes that were microfluidised for more than 3 cycles (i.e. vesicle size smaller than 300nm), molecular sieve chromatography was used. At first, the microfluidised sample (volume about 10ml) was placed in a dialysis tube, which was covered in a flat container with polyethylene glycol 6000 to remove excess of water and non-entrapped content (nucleic acid). When the required volume (1-2 ml) had been reached, the sample was treated for the separation of entrapped from remaining non-entrapped nucleic acid. This was carried out by molecular sieve chromatography using a Sepharose CL 4B column, in which liposomes containing nucleic acids were eluted at the end of the void volume. The extent to which unwashed DRV retained their solute after microfluidisation was estimated as described in section II.2.3-II.2.4 and was expressed as a percentage of nucleic acid in the original DRV preparation. Because the samples were microfluidised following the preparation of DRV (i.e. before the separation of non-entrapped nucleic acid and the estimation of entrapped nucleic acid), a small portion of the sample to be microfluidised (as total) was kept aside for the estimation of drug entrapment. The entrapment value was monitored either by radio assay of β or γ emission or fluorimetric measurements. When nucleic acids were labelled with
fluorescent material, a sample of liposome suspension was mixed with Triton X-100 (up to 5% final concentration) and incubated at 37°C for about 10 min to release the content. Fluorescence values were determined in a Perkin-Elmer LS-3 fluorescence spectrometer with excitation and emission lengths at 360 and 465 nm, respectively (New, 1990).

II.2.5 Measurement of vesicle size

We have used photon correlation spectroscopy (PCS) for measuring the size of liposomes. Depending upon the power of the laser, it is possible to measure particles in the range of about 30 nm to about 3 μm. The z-average mean diameter and polydispersity index of liposomes were measured by PCS using a Malvern Model 4700 apparatus (Malvern Instruments Ltd., Malvern, UK). A laser light source (helium-neon) was focused on the contents of a glass cuvette (square cross section). The cuvette was housed within a thermostatically controlled goniometer cell. To minimise random convection currents superimposed on the Brownian movement, which could lead to substantial errors in particle size measurement, the temperature of the goniometer cell was kept within ±0.1°C. The sample under examination was suspended in media that matched the refractive index of the dispersion fluid, to eliminate potential light flare at the surfaces through which the laser beam passes. Once the signal had been recorded in terms of a series of photomultiplier bursts over a period of time, a mathematical process called "correlation" was carried out.
II.2.6 Transmission electron microscopy (TEM)

The formation of liposomes was observed by transmission electron microscopy according to the negative staining method. A drop of the lipid dispersion was placed on the grid for 1 min and the excess was then removed with filter paper. The sample was coloured by depositing a drop of phosphotungstic acid (2%, pH adjusted to 7) for 1 min, and the excess was removed using a filter paper. The grid was then dried at 60°C for 1 h. The sample was examined using a Phillip microscope (CM/2 model) at 80KV. (TEM done by Dr L. Zarif at the University de Nice Sophia-Antipolis, France).

II.2.7 Stability Protocol

In order to determine the stability of liposomes containing nucleic acids, we investigated the leakage of nucleic acids from these vesicles during incubation within various media and storage at different temperatures, according to the procedure of Kirby and Gregoriadis (1981 & 1984).

II.2.7.1 Storage experiments

Each liposomal system was divided into two parts, which were kept at 4°C for 14 days, and at room temperature (20-25°C) for 24 h. At prefixed time intervals, 100μl aliquots of liposomes maintained at different temperatures were removed and placed into the tubes, then diluted with PBS buffer to 4.0ml and finally centrifuged at 36000 g for 25 min to separate nucleic acids entrapped into liposomes from the free. The
pellets were then resuspended in 0.5ml PBS and the release of the solutes from the liposomes was measured by the amount of radioactivity found in pellets and supernatants. The initial amount of radioactive material present in the samples (immediately after preparation of the pellet, i.e. encapsulated material was analysed) was used as a control.

II.2.7.2 Plasma and buffer experiments

Male mouse (Balb/c and T/O) fresh blood plasma (2ml) or PBS (2ml) samples pre-warmed at 37°C were mixed with 0.5ml of liposomal pellet containing radiolabelled nucleic acids or fluorescence marker incubated at 37°C for 1 h. The samples (100μl) were removed at time intervals and after dilution to 4 ml with PBS, centrifuged at 36000 g for 25 min at 4°C to separate liposomes containing solute (nucleic acids) from the incubation medium. The pellet was resuspended in 0.5ml PBS and together with the supernatants, assayed for amount of release after incubation in various media. The initial amount of radioactive material present in the samples (immediately after preparation of the pellet, i.e. encapsulated material was analysed) was used as a control.

II.2.8 Incubation of liposomes with deoxyribonuclease

Plasmid DNA (pGL2, pRc/CMV) in saline (form of free), mixed with preformed liposomes or entrapped into liposomes was incubated at 37°C for 10 min in a buffer containing 50mM Tris Cl (pH 7.5). Samples and tracer were mixed with 100
units deoxyribonuclease I, and incubated at 37°C for 10-30 min. The reaction was stopped with 1 ml of 0.5 M EDTA (pH 8.0) and the mixtures were centrifuged or, in the case of free plasmid DNA and microfluidised DRV (5 and 10 cycles), molecular sieve chromatography was used to separate digested DNA from non-digested liposomal DNA. In other experiments, samples of similar liposomes containing 20 µg plasmid DNA were diluted to 100 µl with a buffer containing 50 mM Tris Cl (pH 7.5), 10 mM MgSO4, 0.1 mM dithiothreitol, and 50 µg/ml bovine serum albumin (fraction V; Sigma Chemical Co.). The samples were mixed with 10 units of RQ1 deoxyribonuclease (Promega) and incubated at 37°C for 30 min. Digestion was terminated by the addition of 1 ml 0.5 M EDTA (pH 8.0).

II.2.9 Extraction and analysis of liposome-entrapped DNA

100 µl samples of liposome suspension were mixed with an equal volume of phenol/chloroform (1:1) mixture and vortexed for 30 sec. Samples were spun in a centrifuge (12000 g) for 10 min and the supernatant was collected. An equal volume of chloroform/isoamylalcohol (24:1) was added and vortexed for 30 sec. The centrifugation was repeated and the supernatant was collected. DNA was precipitated after addition of one-tenth volume of 3 M sodium acetate, pH 5, and two volumes of 90% ethanol and was kept at minus 20°C for 30 min. DNA was centrifuged at 12000 g for 20 min, then the pellet was washed with 70% ethanol and was spun in a centrifuge for 10 min. The pellet was resuspended in 100 µl of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8). DNA was analysed by agarose gel
electrophoresis (0.8% gel in TE buffer), stained with ethidium bromide (10μg/ml) and was photographed under UV light.

II.2.9.1 Gel electrophoresis

Agarose is a natural product (a polysaccharide) made from agar-agar, which is isolated from algae. It has large pores and is generally used for the separation of relatively large DNA molecules. Agarose gel tends to have high mechanical strength and biological inertness.

The edge of a clean, dry glass plate was sealed with autoclave tape so as to form a mould. The electrophoresis tank was filled with TE buffer (the buffer occupying about 50% of the tank volume). Powder agarose was dissolved in TE buffer (0.8% w/v) by heating the mixture to 80°C. When the mixture was completely dissolved, the solution was left to cool to 55°C, and as soon as the comb was positioned above the plate (0.5-1.0mm), the gel was poured onto the plate immediately. After approximately 35 min the gel was set, the comb and the autoclave tape were carefully removed and the gel was mounted into the electrophoresis tank (since small differences in ionic strength or pH create front in the gel that can greatly affect the mobility of DNA fragments, we used the same buffer in both the electrophoresis tank and the gel). Electrophoresis buffer was added until it covered the gel to a depth of about 1mm. The samples were slowly loaded into the slots of the submerged gel using a disposable micropipette. The lid of the gel tank was closed and electrical leads were attached to the tank. Naked DNA and DNA entrapped into liposomes
(0.1μg equivalent weight of DNA in each line) were run at constant voltage (100V). The gel was then stained for 15 min in ethidium bromide (10μg/ml) and photographed under UV light.

II.2.10 Transfection experiments

Reporter genes are widely used in cell biology for the study of gene expression and related cellular events (Alan & Cook, 1990). Luciferase is the reporter gene of choice because the luciferase assay is extremely sensitive, rapid, easy to perform and relatively inexpensive (Ow, et al, 1986). Because luciferase is a monomeric protein (61kDa) that does not require post-translational processing for enzymatic activity, it can function as a genetic reporter immediately upon translation. Furthermore, light production by luciferase has the highest quantum efficiency of any known chemiluminescent reaction (Seliger & McElory, 1960).

Monkey kidney COS-7 epithelial cells maintained in Dulbecco's modified Eagle medium (DMEM) with 200mM GLUTAMAX I (Gibco BRL) containing 10% foetal calf serum, were harvested by trypsinisation, seeded in 24-well plates at a concentration of 0.5×10⁴ cells per well and incubated over night at 37⁰C with 5% CO₂. Liposomes (100μl) containing entrapped pGL2 plasmid DNA encoding luciferase were diluted with 400μl of optiMEM buffer. The wells were washed with PBS buffer and liposome suspension was added to the wells and incubated at 37⁰C. Each sample of different liposomes was analysed in duplicates. After 3 h incubation,
the liposomes suspensions were removed from the wells and replaced with DMEM/10% FCS and incubated for a further 48 h to allow gene expression.

The wells were washed twice with PBS, before 100μl of Reporter Lysis Buffer (Promega) were added. After the wells had been rocked gently for 20 min, the cells were scrape from the dish and transferred to a microfuge tube. The tubes were frozen at minus 70°C, then thawed, briefly vortexed and centrifuged to remove cellular debris. The clear cell-free supernatant was removed to a fresh tube. The luciferase assay was performed with the Luciferase Assay Kit (Promega). 100μl of luciferin substrate in the supplied reagent buffer was added to 20μl of the cell free supernatant, then immediately placed in a luminometer (LKB Wallach 1251) and total RLUs values was recorded for 60 s. RLU stand for "Relative Light Unit". This term is used because luminometers typically do not yield a measurement directly in units of photons. The protein concentration in each of the lysates was measured by the method of Bradford's (1976) using Bio-Rad protein assay solution.

II.2.11 Cell cytotoxicity of cationic liposomes

The MTT assay is one of the numerous in vitro methods, which have been proposed as alternatives to in vivo assays for determining the irritative potential of new compounds. The basis of this cytotoxicity assay is that MTT or (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) bromide, when reduced by mitochondrial dehydrogenases to the corresponding formazan, precipitates in coloured crystals. These crystals dissolved in dimethylsulfoxide present an
absorption peak at 540nm. Therefore, the measure of absorbency is directly proportional to the number of viable cells. The B16F10 cell is a murine melanoma and is an adherent cell line, i.e. it grows as monolayer. B16F10 cells were seeded at a density of $1 \times 10^5$ cells per ml ($1 \times 10^4$ cells per well) in a 96 well flat bottomed microtitre plate (Costar) in RPMI 1640 tissue culture media (Gibco) supplemented with 10% foetal calf serum (FCS) (Gibco). All cellular growth and cytotoxicity incubations were carried out in a cell incubator at 37°C and 5% CO$_2$ atmosphere. Cell density was assessed using an improved neubauer haemocytometer (Sigma). The cells were washed twice with PBS, fresh RPMI media (supplemented with FCS) was added, and then the cells were seeded in a microtitre plate. The cells were left for 24 h to recover and re-adhere. All liposomes and controls were dissolved in RPMI media (supplemented with FCS) and then sterilised through a 0.5mm sterile filter (acrodisk), the first few microlitres of the solution being discarded in the case of adherence of the liposomes to the filter membrane. Then liposomes and control were added in increasing concentrations to the cells in the microtitre plate. Some cells were left in the RPMI media to act as controls. The control cells (without liposomes) were treated with the equivalent amount of sterile water. While the cells were left in the incubator for 72 h, they were occasionally checked for yeast or bacterial contamination. 5 h before the end of the incubation time, at 67 h, 20µl tetrazolium (colorimetric) dye (MTT) were added. At the end of the incubation time the cellular medium was removed, 100µl of optical grade DMSO (Sigma) were added, and the MTT crystals were dissolved. The plates were read in a Titereck plate reader and the results (OD) were expressed as a
percentage of the OD seen in the cell wells containing no liposomes (i.e control cells).

II.2.12 Preparation of samples for scanning electron microscopy

Samples from the cell density line were fixed in 0.25% glutaraldehyde for 24 h followed by 1% osmium tetroxide for 1 h. Then all samples were dehydrated by exposing them for 5 min to a gradually increasing concentration of ethanol (50, 60, 70, 80, 90, and 100% in PBS). Following the exposure to absolute alcohol and acetone, the samples were exposed to HMDS and left to dry. They were then gold-coated (emtech gold coat-20μA) and viewed. [Electron microscopy analysis was performed by David McCarthy at the Electron Microscopy Unit, School of Pharmacy].

II.2.13 Immunisation protocol

The procedures used in all animal immunisation experiments, including the sample collection from the tail vein, the volume of injection during immunisation, and the plasma collection were according to the established internal protocol in our laboratory. In all immunisation experiments, male Balb/c and outbred T/O mice (20-25g body weight) were used (in groups of four or five). The mice were bled from the tail vein 1-2 days before one or several injections of 0.1ml volume of samples. Blood samples (50μl) from the tail vein were collected with heparinised capillaries at time intervals after the first injection and transferred into appendorf
tubes containing PBS (0.45ml), which were then centrifuged at 1000 g for 10 min to obtain the plasma. All plasma samples were stored at minus 20°C until further use.

II.2.13.1 Enzyme-linked immunosorbent assay (ELISA)

96 well flat-bottom ELISA plates were coated with the antigen (1-2μg/ml hepatitis B surface antigen; S region, ayw subtype, 60μl/well) in 0.05M sodium carbonate buffer of pH 9.6. The plates were incubated at 37°C (oven) for 1 h (or overnight at 4°C) and washed three times with 1/10 diluted washing buffer composed of 200g NaCl, 36.25g Na2HPO4.2H2O, 5g KCL, 12.5 ml Tween-20 in 2.5L total volume for a 10×buffer. After the plates had been dried, gelatine (0.5% in washing buffer, 60μl/well) was added to block the non-specific binding sites and was incubated at 37°C (oven) for 30 min and removed. Plasma (60-90μl) diluted according to the strength of antibody response assessed in preliminary work was added to the top well of each column. The contents of the top wells (plasma and gelatine) were mixed and 60μl samples were transferred to the second row of wells and mixed. This procedure was continued to the eighth row of wells and the final 60μl samples from these wells were discarded. The plates were incubated at 37°C for 2-3 h (or over night at 4°C), and after having been washed three times as above the plates were dried and peroxidase-conjugated goat anti-mouse IgG (one of the subclasses) was added to the wells (50μl/well). The plates were then again incubated at 37°C for 2-3 h (or over night at 4°C), followed by three times washing and drying. Substrate (200μl/well) was added to the wells and incubation was carried out as above for 30
min. The reaction was stopped by the addition of H$_2$SO$_4$ (25µl/well, 1.5M). The colour developed was measured at 492nm using a Titertek multiscan MCC/340 ELISA reader. Immune response was expressed as the log$_{10}$ of the reciprocal of serum dilution required for the OD to reach a reading of about 0.200 (end point dilution). Sera from untreated mice gave log$_{10}$ values of less than 2.0. End-point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of nonimmune serum with a cut-off value of 0.05.

II.2.14 Cytokine protocol

II.2.14.1 Preparation of organ extracts

Endogenous levels of IFN-γ and IL-4 in the spleen were determined using the method of Nakane et al (1992) as previously modified (De Souza et al, 1996). Extracts of spleen homogenate were prepared as follows: The spleens were aseptically removed from the mice and suspended in RPMI 1640 medium (Sigma). Individual spleens were weighed and homogenised in ice-cold RPMI containing 1%(wt/vol) 3-[(cholamidopropyl) dimethylammonia]-1-propanesulfonate (CHAPS; Sigma), and 10%(wt/vol) homogenates were prepared with a Dounce tissue grinder. After the homogenates had been left for 1 h on ice, the insoluble debris was clarified by centrifugation at 2,000 g for 20 min. The organ extracts (clear supernatant) were stored at minus 70°C until the cytokine assay.
II.2.14.2 Cytokine assays

IFN-γ and IL-4 were quantified by a double-sandwich enzyme-linked immunosorbent assay (ELISA). Maxisorp (NUNC, UK) plates were coated (50μl/well) with the captured antibodies, IFN-γ (R46A2), and IL-4 (11B11) at 2μg/ml in 0.1M carbonate buffer (pH 9.6) and were incubated over night at 4°C in a box with damp tissue.

The plates were washed three times with a PBS/Tween mixture (250μl of Tween in 500ml of PBS). After they had been dried, uncoupled binding sites in the wells were blocked by addition of 1% Marvel (Casein) and 0.3% gelatine mixed in PBS (200μl/well) and the plates were incubated at 37°C for 2 h. Subsequently, the plates were washed three times. They were dried and 100μl PBS/Tween was added to row "A" on the plate as blank. The remaining wells were incubated (100μl/well) with duplicates of serially diluted standards of IFN-γ (10ng/ml) or IL-4 (25ng/ml) and of neat or diluted (1/5) samples at 37°C for 1 h. The plates were again washed and dried as described before and the wells were coated (50μl/well) with 1mg/ml of secondary biotinylated anti-mouse IL-4 (BVD6-24G2) or anti-mouse IFN-γ (XMG1.2) monoclonal antibodies and were then incubated at 37°C for 1 h. After removal of excess second antibodies by washes with PBS/Tween buffer, 50μl of a streptavidin peroxidase conjugate (1:1000 dilution) were added to the plates. A 45 min incubation at 37°C was followed by the washing and drying steps. A substrate (100μl/well) was added to the wells and the plate was kept in dark for 5-10min to allow the colour to develop. The colour developed was measured at 405nm using
an ELISA plate reader (ELISA auto reader, model EL310, Bio-Tek Instruments, Burlington, VT). The concentration of cytokines in the samples was determined from a standard curve. Results (mean ±S.D.) were expressed as ng/spleen from at least 4 mice. Each experiment was repeated twice and accepted as valid only when both sets showed similar results.

II.2.15 Statistical analysis

The student un-pair $t$-test was used to examine the difference between two groups (Sokal & Rohlf, 1973). The test is suitable when sample sizes are unequal and $n_1$ or $n_2$ or both sizes are small (3<30). Degrees of freedom was calculated (d.f) = n (n-1) and data were considered statistically significant if $P$ values were < 0.05. The Stat Microsoft program was used for calculating the mean, SD and $p$ value.
III LIPOSOME-ENTRAPPED PLASMID DNA:
CHARACTERISATION AND TRANSFECTION EFFICIENCY

IN VITRO

III.1 INTRODUCTION

The limitations of viral vectors have led to the development of non-viral models of DNA delivery. Non-viral systems potentially offer many advantages over viral systems, such as ease of manufacture, safety, lack of vector size limitations, and low immunogenicity (Fraley et al, 1987; Felgner et al, 1990; Wolff et al, 1990; Jiao et al, 1992; Legendre & Szoka, 1992; Rolland et al, 1994).

The colloidal nature, hydrophilicity, and high negative surface charge of plasmids results in poor transport across biological barriers such as plasma membranes and continuous endothelia. Their diffusion through extracellular matrices, such as connective tissue in skeletal muscle, is also very limited. Moreover, insufficient protection of plasmid DNA against enzymatic attack reduces its intended biological activity (Levy et al, 1996; Lasic, 1997; Mahato et al, 1997). A simple encapsulation method reported by this laboratory (Kirby and Gregoriadis, 1984; Gregoriadis, 1985; Gregoriadis et al, 1987) may offer a solution to the above problems. Based on the fusion of preformed phospholipid vesicles by dehydration followed by rehydration, it produces multilamellar liposomes (dehydration-rehydration vesicles, DRV), which, in the process of formation, entrap up to 80% of the added
solute without employing potentially damaging conditions (Kirby & Gregoriadis, 1984).

The apparent virtues of the DRV technique have led us to study its potential use for DNA delivery. To this end, we have carried out *in vitro* experiments to investigate:

- the incorporation of nucleic acids with various lipid compositions of DRV liposomes;
- the mean particle size of DRV liposomes.
- the effect of lipid composition and vesicle size on the stability of DRV liposomes containing nucleic acids under storage conditions and in the biological milieu;
- the ability of various lipid compositions of DRV liposomes to protect their contents from nuclease attack;
- the transfection efficiency of DNA incorporated with various lipid compositions of DRV liposomes;
- the cytotoxicity of cationic DRV liposomes.
III.2 METHODS

The dehydration-rehydration procedure (Kirby & Gregoriadis, 1984) was used for the incorporation of nucleic acids into liposomes. Liposomes were composed of PC (16μmoles); PC (16μmoles) and DOPE (molar ratio 1:1 or 1:0.5); PC (16μmoles), DOPE, PS or PG (molar ratios 1:1:0.5 or 1:1:0.25; negatively charged); PC (16μmoles), DOPE, SA, or BisHOP (molar ratios 1:1:0.5 or 1:1:0.25; positively charged); PC (16μmoles), DOPE and DOTMA, or DOTAP, or DC-Chol (molar ratios 1:1:0.25 or 1:0.5:0.25; positively charged); or DOPE (16μmoles) and DOTMA or DOTAP (molar ratios 1:0.5 or 1:0.25; positively charged). The liposomes were prepared in form of DRV and microfluidised DRV as described in section II.2.2.

The various amounts and combinations of SUV liposomes were mixed with 10-100μg pGL2 or pRc/CMV HBV plasmid DNA, to which tracer $^{32}$P or $^{35}$S-labelled plasmid DNA ($6-7 \times 10^4$ dpm) had been added, and were freeze-dried over night (in experiments where oligodeoxynucleotides were entrapped into liposomes, $^{125}$I isotope or fluorescence marker was used). Nucleic acid incorporated into liposomes was separated from free nucleic acid by ultracentrifugation of the DRV (see section II.2.2) or by molecular sieve chromatography using a Sepharose CL 4B column for 5 and 10 cycles microfluidised liposomes (see section II.2.3). The yield of the incorporated DNA after the preparation of either liposome-encapsulated DNA or liposome-DNA complexes was estimated on the basis of radioactivity or fluorescent dye recovered in the suspended pellets (see section II.2.4). The z-average mean size
of non-microfluidised and microfluidised liposomes was measured in a Malvern Autosizer IIc (see section II.2.5). The morphology of plasmid DNA encapsulated into DRV liposomes was examined by negative staining electron microscopy (see section II.2.6).

The stability of the nucleic acids in the liposomes was examined in two independent experiments following the protocol used in our laboratory (Kirby et al, 1981) as described in detail in section II.2.7. To assess the storage stability, DNA encapsulated with neutral, anionic and cationic DRV liposomes before and after microfluidisation, was stored at 4°C or 20-25°C for 14 days or 24h respectively. 100μl aliquots of liposomes were placed into the tube diluted with PBS buffer to 4.0ml and were subjected after an equilibration time of 10 min to centrifugation or column sieve chromatography (liposome size smaller than 250nm), in order to separate the liposomal DNA from free DNA. The size of the vesicles was measured with a Malvern Autosizer and the percentage of the encapsulation was measured on the basis of the radioactive release. To monitor leakage in vitro, liposomal DNA was prepared as described above against PBS and mouse plasma at 37°C for 1h. At a prefixed time 100μl aliquots of liposomes were placed into the tube, diluted with PBS buffer (pH 7.4) to 4.0ml and centrifuged to separate the liposomes containing nucleic acid from the incubation medium (for full protocol see section II.2.7). The pellet was then resuspended in 0.5ml PBS and, together with the supernatants, assayed for radioactivity or fluorescence to estimate the release. In both cases (storage stability and plasma stability) the release of plasmid DNA was calculated by dividing the radioactive DNA incorporated found in pellet/supernatant at each
point by that total radioactive plasmid DNA in liposomes before subjection to storage or plasma.

To determine whether liposomes (non-microfluidised or microfluidised DRV) with incorporated pGL2 or pRc/CMV plasmid DNA could protect the content against enzyme attack, the DNA (in form of free, mixed with preformed vesicles, or entrapped into liposomes) was digested with DNase type I. Subsequently, the samples were exposed to deoxyribonuclease and subjected to agarose gel electrophoresis (see sections II.2.8 and II.2.9). The transfection efficiency of the DNA-incorporating liposomes (see section II.2.10) was monitored by entrapping pGL2 plasmid DNA into six different formulations of non-microfluidised DRV liposomes (see legend to Fig. III.9), and into microfluidised PC:DOPE:DOTMA DRV (3 cycles). LipofectAMINE (cationic liposomes transfection kit) complexes with plasmid DNA were used as a positive control in our transfection study. Cells were incubated for 48h, lysed and then centrifuged to obtain clear supernatants. Theses were assayed in triplicates for luciferase activity with the luciferase assay system kit using an LKB 1251 luminometer with total light emission being recorded over 60 s. Cytotoxicity of liposomes containing plasmid DNA was estimated by cell viability, which was measured by the dye-uptake method (MTT assay). The control cells (without liposomes) were treated with the equivalent amount of sterile water (see sections II.2.11 and II.2.12).
III.3 RESULTS

III.3.1 Incorporation of plasmid DNA into liposomes

Tables III.1-5 presents incorporation values of nucleic acid into neutral, negatively and positively charged DRV or microfluidised DRV, and neutral, negatively and positively charged preformed DRV or microfluidised preformed DRV mixed with plasmid DNA.

It has been previously shown (Kirby & Gregoriadis, 1981, 1984; Gregoriadis et al, 1987; Gregoriadis, 1991) that a wide range of solutes such as small drugs, peptides and proteins can be quantitatively incorporated under mild conditions into the aqueous phase of multilamellar liposomes by the dehydration-rehydration procedure. Our results demonstrate that plasmid DNA [pGL2 encoding luciferase and pRc/CMV-HBS encoding HBsAg (S region; ayw subtype)] with a molecular weight of around 3.99x10^6 Daltons can be quantitatively incorporated into neutral DRV liposomes (482.16±63.04nm diameter; Table III.5) composed of PC and DOPE and into similar DRV liposomes supplemented with negatively (472.64±4.89nm; Table III.5) or positively (568.6±8.83nm; Table III.5) charged amphiphiles. Charged vesicle bilayer surfaces are known to contribute to larger aqueous spaces between the bilayers (New, 1990) and consequently to greater solute entrapment.

Tables III.1-4 show high yield incorporation of nucleic acids into various DRV liposomes. With neutral or anionic DRV, encapsulation values ranged from 39% to
59% and 45% to 61% of the amount used (10-100 µg) respectively. With neutral and anionic preformed (empty) DRV mixed with plasmid DNA, on the other hand, only a modest proportion of DNA (10-12%) was recovered. This suggests that most of the plasmid DNA was entrapped within the vesicles rather than adsorbed onto the liposomal surface.

As can be seen in Tables III.1, III.2 and III.5, microfluidisation of similar DNA incorporating neutral or negatively charged DRV liposomes in the presence of non-incorporated DNA (free) resulted in smaller vesicles (120-329nm diameter; depending on the number of microfluidisation cycles) with a DNA content considerably reduced to 10-20% for neutral and 32-41% for negatively charged liposomes. Again, very little DNA was recovered (6-10%) when preformed liposomes were microfluidised in the presence of free DNA. Although neutral or anionic preformed DRV liposomes presented very low recovery of nucleic acid before and after microfluidisation, preformed cationic DRV liposomes mixed with DNA retained as much as 40-85% of the DNA, depending on the type and amount of cationic lipid. In the case of nucleic acids entrapped into cationic DRV liposomes, the incorporation values of nucleic acid in cationic SA, BisHOP, DOTMA, DOTAP and DC-Chol DRV (62-92%) remained high after microfluidisation (60-83%) (Tables III.3 and III.4).

Photon correlation spectrometry showed no significant difference in the vesicle size of DRV liposomes before (empty vesicles) or after incorporation with plasmid DNA. In contrast, the vesicle size of preformed cationic liposomes
increased from 450±9nm (empty vesicles) to 992.4±51.65nm after complexion with plasmid DNA (Table III.5).

We also encapsulated nucleic acid in fluorinated compounds (DF6C7PC, F8C11PC, F6C11GlyLact) using the DRV method. The amount of entrapped nucleic acid in these vesicles varied (20 to 44%) depending on the type of fluorinated compound and the amount of materials used (Table III.1). The main difference between the fluorinated and non-fluorinated DRV was that the particle size obtained from fluorinated DRV was around 300nm, whereas the smallest size of non-fluorinated DRV liposomes was around 450nm. Given the novelty of such a property, we decided to do further tests on fluorinated compounds.

III.3.2 Electron microscopy

Electron microscopy observations of DNA encapsulated into DRV liposomes revealed a mixture of homogeneous and heterogeneous vesicles. Some of the samples (Fig. III.1) were heterogeneous in size (200nm to 3μm) and often large elliptical or cylindrical crystal shaped structures were observed. Although the micrographs showed the vesicles as close multilayered structures, i.e. with the bilayers being packed in an onion-like structure (Fig. III.1, III.3), the size determination of the population was difficult. Electron microscopy pictures failed to reveal in-depth information. In most cases we observed disc-like micelles and SUV with some large MLV, although the vast majority of the vesicles being less than 1μm with a few round and unusual shapes, which may have been an artefact.
of impurities (Fig. III.1). It is worthwhile to note that the negative staining and freeze-fracture methods are prone to artefacts due to changes during sample preparation (i.e. fixation and/or dehydration passage) and it is difficult to estimate the size of collapsed dry vesicles in the negative staining procedure (Lasic, 1997).

Determining the size distribution of heterogeneous populations of vesicles such as MLV or LUV is generally rather difficult and has in most cases been utilised to examine the gross size distribution of large vesicles produced from single chain amphiphiles (Hargreaves & Deamer, 1978). The contribution of electron microscopy to our understanding of the structural organisation of liposomal DNA was merely confirmatory. Nonetheless, some features such as shape and bilayers of the vesicles could be observed by electron microscopy, which are not detectable by photon correlation spectroscopy, because electron microscopy is a physical technique providing direct visualisation of macromolecular structures. For our purposes, i.e. examination of the vesicles formation, it was an appropriate tool.

III.3.3 Physical stability of liposomes

While for all our studies we used freshly prepared liposomes within 1-2 days, we also investigated the vesicle stability of DRV liposomes (in terms of nucleic acid leakage) over a period of 14 days. Tables III.6-7 summarise the stability results for various compositions of DRV liposomes at storage temperatures of 4°C and 20°C. Data obtained from this set of experiments clearly showed the importance of helper
lipid, DOPE, in the formulation. At 4°C, all liposome compositions appeared to retain quantitatively the nucleic acids over a period of 14 days except the neutral charge liposomes prepared with PC alone and PC:Dope in molar ratio of 1:0.25. Based on the release of radioactivity in the supernatants, plasmid DNA encapsulated into MLV liposomes composed of PC exhibited rapid and high content release in storage. The stability of microfluidised DRV liposomes (SUV) was lower than that of the non-microfluidised DRV liposomes, especially with PC:Dope. Microfluidised neutral liposomes composed of PC (16μmoles):DOPE (molar ratio 1:0.25) showed less stability than the similar composition of liposomes with molar ratio 1:0.5. Nonetheless, even the latter proved to be less stable than neutral non-microfluidised DRV liposomes in both temperatures. Our results indicate that the lamellarity of the liposomes, the presence of helper lipid (DOPE), and the amount of the lipid (DOPE) can influence the physical stability of the vesicles.

Cationic and anionic microfluidised DRV liposomes (SUV) appeared to be more stable than neutral liposomes during storage at 4°C (Table III.7). Neglecting chemical, thermodynamic and biological instability of lipids and liposomes, aggregation and fusion render the liposome suspension colloidal unstable with respect to size distribution. Both positive and negative charges seem to have a stabilising effect. In the case of anionic liposomes, a negative surface charge may increase the electrostatic repulsive forces between liposomes and reduce aggregation (New, 1990). By using lipids with low aqueous solubility, liposomes can be stabilised electrostatically.
The introduction of an amphiphile, such as DOTAP, which imparts a positive surface charge to liposomes, also increases the stability of the vesicles in the presence of different forces. This is due to the high surface charge and low ionic strength of cationic liposomes. According to the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, the ubiquitous attractive force between liposomes is van der Waals attraction while the long-range repulsive force is due to electrostatic repulsion. The balance between the two determines colloidal stability (Lasic, 1996).

As for the size of the vesicles during storage, we found that negatively charged liposomes did not produce any significant differences in size, whereas cationic vesicles showed a small enhancement in particle size after 2 weeks storage at 4°C.

In the case of fluorinated vesicles, DF6C7PC (32 µmoles) and F6H11GlyLact vesicles exhibited a relatively good stability in various storage conditions (Table III.6). This could be due to the fluorocarbon/hydrocarbon mixed amphiphiles or to the synthetic glycolipids, which are equipped with a peptide link providing for good storage stability.

III.3.4 Stability in plasma

In these experiments, we tried to establish to what degree the lamellarity and charge of different types of liposomes could affect their stability in the presence of plasma and PBS. We also investigated the effect of the molecular weight of nucleic acids on the stability of liposomes by using oligonucleotide with a molecular weight of
around 8,000 Daltons and plasmid DNA pGL2 with a molecular weight of around $3.9 \times 10^6$ Daltons.

Fig. III.4 and III.5 show the leakage rate of nucleic acids in neutral, negatively, and positively charged DRV and microfluidised DRV (SUV) liposomes during prolonged incubation with mouse plasma and PBS at 37°C. (Although the final amount of encapsulated nucleic acids was different in various types of liposomes, in both conditions the liposomes were prepared with the same concentration of lipid and a similar initial concentration of nucleic acid.) During incubation with plasma, the plasmid DNA was most efficiently retained by cationic and anionic DRV liposomes, with more than 76% of DNA remaining entrapped at the end of a 1h period. This presumably reflects the surface charge of the DRV, since neutral DRV liposomes showed an increasing leakage of both nucleic acids (i.e. oligonucleotide and plasmid DNA) at the end of the same period of time. The microfluidised DRV experiment showed that after incubation of liposomal nucleic acids with plasma as much as 60% of nucleic acids was released from microfluidised DRV liposomes. (Since there was no significant difference between retention of DNA and oligonucleotide, we are only presenting the results from plasmid DNA).

Our data indicate that multilamellarity of vesicles as well as surface charge has the advantage of decreasing the rate of loss of diffusible entrapped nucleic acids in the presence of plasma. Results obtained from oligonucleotide and plasmid DNA proved that the molecular weight of nucleic acids has no relevance for the stability
of liposomes, while in both cases charged liposomes presented a slightly higher ability to retain solutes than neutral liposomes. Nonetheless, all preparations presented high stability (92-99%) when incubated in the presence of PBS at pH 7.4 without any significant differences between various types of liposomes.

Based on the data obtained it appears that supplementing phosphatidylcholine bilayers with a small amount of DOPE:DOTAP or DOPE:PS increased significantly (p<0.01) the retention of plasmid DNA in the presence of plasma. This may be due to the formation of more compact vesicles or the forming of bilayers which would protect the DNA. It is possible that stronger bilayers present a better support for DNA adhesion and that the structure formed is more stable, i.e. more resistant to interaction with plasma. Crommelin (1984) suggested that cationic lipids form hydrogen bridges with the lipid through its terminal amine groups, which extends the retention of the nucleic acids.

The biological tests performed on fluorinated DRV liposomes showed 76-87% recovery in plasma and almost 94% in PBS after incubation in these media (plasma and PBS) for the same duration as the DRV liposomes. However, in the case of F8C11PC a rapid release of DNA was observed after incubation in mouse plasma (Fig. III.6).

### III.3.5 Incubation of liposomal DNA with deoxyribonuclease

As shown in Tables III.1-4, a high percentage of the DNA incorporated into neutral (45-72%), anionic (58-69%), or cationic (68-86%) liposomes was not degraded by
DNase. On the other hand, about 80% of DNA was digested by DNase in neutral and negatively charged preformed DRV mixed with DNA (Tables III.1 & III.2). However, with DNA adsorbed onto the surface of cationic liposomes, a considerable proportion of the DNA was protected from deoxyribonuclease attack (Table III.3). The higher resistance of cationic complexed DNA to the enzyme compared to neutral or anionic complexed DNA could be attributed to its condensed state (Legendre & Szoka, 1995). The condensation of DNA, i.e., the collapse of an extended worm-like random coil DNA molecule into a compact ordered state, has received considerable attention in diverse areas of science. In biology, it represents a process by which genetic information is packaged and protected. In medicine, it provides a promising means for gene therapy applications through new forms of condensed genes to be delivered to target cells.

As Fig. III.7 shows, cationic DRV liposomes microfluidised in 1 or 3 cycles (420nm, 310nm and 230nm respectively) were still able to protect the DNA from nuclease attack. However, increasing the microfluidisation to 5 cycles (180-120nm) resulted in the digestion of most of the DNA by enzymes (Fig. III.7). We also incubated free plasmid DNA with the same amount of enzyme used for the liposomes and passed the mixture through the Sepharose CL 4B column. Fig. III.8 shows a curve shift between DNA before DNase treatment and DNA exposed to DNase, resulting in the complete degradation of DNA in the presence of deoxyribonuclease.
DF6C7PC and F6H11GlyGlyLact fluorinated compounds displayed a retention of nucleic acids in the presence of deoxyribonuclease (Table III.1). This stability is possibly due to an enhanced hydrophobic character of the fluorinated membrane.

### III.3.6 Agarose gel electrophoresis

Our results of liposomal DNA vulnerability to DNase were largely confirmed by experiments where samples of non-microfluidised or microfluidised DNA-incorporating DRV as well as naked DNA with or without RQ1 deoxyribonuclease were subjected to agarose gel electrophoresis as described in section II.2.6. (Fig. III.9 & III.10). The intensity of staining and the smearing pattern show that DNA associated with cationic liposomes was highly protected (Fig. III.9a, lanes 3-10, & Fig. III.9b, lanes 3&4), whereas naked DNA was completely digested (Fig. III.9a/b; lanes 1&2). DNA in neutral and negatively charged DRV, on the other hand, was less well protected as demonstrated by the lighter bands in the DNase digested samples compared to the undigested ones (Fig. III.9a, lanes 11-14). Experiments where pRc/CMV DNA was encapsulated into various cationic DRV liposomes led to similar observations as the experiments with cationic DRV liposomal pGL2 plasmid DNA. For both pGL2 and pRc/CMV-HBS plasmid DNA encapsulated into cationic DRV liposomes no smearing was observed. This suggests that the entrapment procedure was mild enough to preserve the integrity of the molecules. Furthermore, Fig. III.9 and III.10 show that DNA encapsulated into cationic DRV liposomes was protected to
a high degree from degradation by DNase whereas free DNA was in both cases (PGL2 and pRc/CMV-HBS) fully digested.

**III.3.7 Transfection with liposomal DNA *in vitro***

Gene therapy and/or gene based vaccination are mediated through the delivery of DNA containing selected genes into cells by a process known as transfection. Transfection agents are normally positively charged and are surface active. A common class of materials used for transfection are the cationic lipids. This type of lipid forms a micelle or liposomal type of structure when complexed with DNA and can associate with and alter the integrity of a cell membrane, thus increasing the uptake of the complexed DNA.

The ability of various DRV liposomes to enhance transfection was studied using a DNA plasmid pGL2 and COS-7 cells. A commercial cationic lipid based transfection agent, LipofectAMINE, was used as active control (section II.2.7). Fig. III.11 shows the level of luciferase activity with each of the DRV formulations. When the plasmid was encapsulated into cationic DRV (DOPE:DOTMA, PC: DOPE:DOTMA, and PC:DOPE:SA), the luciferase activity was approximately 10 fold higher than the activity obtained with neutral (PC:DOPE) and negatively charged (PC:DOPE:PS) liposomes or with PC:DOPE:BisHOP liposomes.

We also investigated the effect of the vesicle size on the transfection efficiency, by microfluidising DNA-incorporating DRV liposomes for 3, 5, or 10 cycles to
produce smaller size vesicles (319, 194 or 123nm z-average diameter respectively). The results show that DNA incorporated into microfluidised DRV (3 cycles) improved the transfection efficiency (Fig.III.11). On the other hand, when liposomes of the same combination (PC:DOPE:DOTMA) were subjected to 5 or 10 cycles microfluidisation, they failed to show enhanced luciferase activity. This could be explained by the microfluidisation-induced progressive smearing of DNA (Fig.III.9, lane 11&12).

In a separate set of experiments, COS-7 cells were exposed to pGL2 incorporated into fluorinated vesicles (DF6C7PC, F8C11PC, F8C2PC and F6H11GLYGLY). Surprisingly, no light emission was observed with any of the fluorinated compounds, which means that the transfection efficiency was very low to zero. This observation was confirmed by two repeats of the experiment. When fluorinated liposomes were applied for transfection, we observed that at the end of the procedure most of the cells were heavily aggregated. This could result from the toxicity of the vesicles during their interaction with the cells.

### III.3.8 Cytotoxicity of cationic liposomes

To assess the cytotoxicity of different cationic liposomes, we examined their effect on the cell viability of melanoma cells by using three different cationic liposomes composed of PC (16μmoles), DOPE and SA (molar ratio 1:0.5:0.5 or 1:0.5:0.25); PC (16μmoles), DOPE and DOTAP (molar ratio 1:0.5:0.5 or 1:0.5:0.25) and of PC (16μmoles), DOPE and DC-Chol (molar ratio 1:0.5:0.5 or
1:0.5:0.25) incorporated within 50 μg plasmid DNA carrying the HBsAg reporter gene. Our data from this experiment showed that none of the cationic liposomes produced any toxicity in the cells (Fig. III.12). We can therefore conclude that the amount of cationic lipids used for incorporating nucleic acids is high enough to produce high yield entrapment, while it is low enough to prevent toxicity in the cells.

In repeated experiments this result was corroborated by electron microscopy observation, which revealed no aggregation and fatal damage of the cells after 24 h (Fig. III.12a, b, c).
III.4 CONCLUSIONS

The challenge of delivering DNA in vivo is not significantly different from the problem of delivering conventional drugs or biological products to the body in a controlled manner (Ledley, 1994). In both cases, the success depends on efficient delivery and expression of the therapeutic agent. Content incorporation and retention, plasma stability, immunogenicity, toxicity, and vascular permeability are issues that can be addressed by proper formulation of liposomal drug carriers (Cullis et al, 1997; Jain et al, 1989; Gangadharam et al, 1995). Plasmids have been incorporated in various types of lipid-based carrier systems by a number of procedures that would often be unrealistic for scale-up production due to low encapsulation efficiency, low stability and poor protection of content from enzymatic attack. As a first step towards the establishment of a more practical strategy for gene delivery systems, the potential of plasmid DNA alone or mixed with preformed liposomes as non-viral carrier was investigated and compared with liposomes produced by the dehydration-rehydration technique (Tables III.1-5). Prior to animal studies, we performed stability tests (Tables III.6-7 & Figs. III.4-7), transfection and cell viability experiments (Figs. III.11-12), as well as gel electrophoresis (Figs. III.9-10), to optimise the types of liposomes suitable for the incorporation of DNA for in vivo delivery. The results in Tables III.1-5 show high yield incorporation of nucleic acids into uncharged or charged DRV and microfluidised DRV liposomes. Furthermore, visual observation of the vesicles showed no phase separation and according to data obtained by photon correlation
spectroscopy, the size of the vesicles did not appear to change under different storage conditions. While according to our data most of the nucleic acids used were incorporated quantitatively with all the liposomes tested, the best incorporation was exhibited by nucleic acid encapsulated into cationic DRV liposomes. This can be attributed to additional DNA binding to the positively charged (inner and outer) bilayers.

According to electron microscopy observations, DRV are mainly oligo- and multilamellar, though it is possible that the morphology is influenced by experimental conditions and by the nature of the lipid and the encapsulated material. Electron microscopy also revealed that the cationic DRV liposomes incorporated with DNA are packed in an onion-like structure (Fig. III.3) and that some fingerprint-like pattern across multilamellar lipid-DNA, which can be attributed to DNA condensed as parallel helices between the lipid bilayers (Figs. III.1). We believe that the procedures used for sample preparation, such as fixation and dehydration passage, can partly damage the vesicles and distort the observation results. Detailed studies using a variety of techniques have each led to a different picture (Xu et al, 1995; Felgner et al, 1996; Sternberg et al, 1994). For example, cryoelectron microscopy has revealed a variety of shapes of different cationic liposomes. Surprisingly, even SUV are often not spherical, but show dumbbell or oval structures (Lasic, 1997). It is possible that different methods visualise different sub-populations of complexes in a particular sample.
The first evidence of true entrapment of nucleic acid encapsulated into liposomes using the mild dehydration-rehydration procedure was observed in the case of neutral and negatively charged liposomes. While here the incorporation values were relatively high (30-50% of the amount used), only a small proportion of nucleic acid was recovered (6-10%) when mixed with preformed "empty" neutral or negatively charged DRV.

For cationic liposomes, the differences between the incorporation of DNA by encapsulation and by complex formation were not as noticeable as for neutral or anionic DRV. However, one difference between the two preparation techniques was revealed by exposure to deoxyribonuclease. In our DNase experiments a greater percentage of the entrapped DNA remained intact than of the complexed DNA.

Another clear difference between the two preparation techniques could be observed when the vesicle size was measured. Regardless of the initial amount of nucleic acid used for preparation, the size of the cationic liposomes remained almost unaltered upon encapsulation. In contrast, the vesicle size of preformed cationic DRV liposomes increased significantly upon complex formation, presumably due to aggregation. One way to explain such aggregation is that mixing liposomes with an aqueous solution of DNA results in heterogeneous colloidal dispersion. Two-phase colloid dispersions are thermodynamically unstable and any form of heterogeneity associated with the dispersion will presumably accelerate instability. It is plausible to conclude that in the case of encapsulation the nucleic acid is entrapped within the liposomes rather than adsorbed onto their surface, resulting in greater stability.
Understanding the behaviour of liposomes in the biological milieu is essential to their rational design and effective use in drug targeting (Gregoriadis, 1979). Previous work (Gregoriadis, 1973) revealed that, after intravenous injection, circulating liposomes lose much of their solute content. This was subsequently attributed (Krupp et al, 1976; Scherphorf et al, 1978) to the destabilising action of plasma high density lipoproteins which remove phospholipid molecules from the bilayers. A considerable shortcoming of liposomes is the risk of vesicle aggregation and fusion owing to their relatively low physical stability. The control and prediction of liposome stability is therefore crucial. Based on the data obtained in experiments with plasma it appears that supplementing phosphatidylcholine bilayers with a small amount of DOPE:DOTAP or DOPE:PS increased significantly the retention of plasmid DNA in the presence of plasma. This may be due to the formation of more compact vesicles or the forming of bilayers which would protect the DNA. It is possible that stronger bilayers present a better support for DNA adhesion and that the structure formed is more stable, i.e. more resistant to interaction with plasma.

With regard to industrial usability, we have also examined the storage stability of DRV liposomes. Multilamellarity clearly increased the storage stability (Tables III.6-7 & Fig. III.4-5). Apart from this, our results indicate that also the surface charge plays an important role for the stability of liposomes. Our findings clearly demonstrated that the storage stability of liposome depends largely on the lipid composition, type of liposomes (MLV or SUV), method of loading, and the surface charge on the composition.
The presence of deoxyribonuclease revealed further differences between the three preparations (plasmid alone, mixed with preformed vesicles, or entrapped into DRV liposomes). When treated with DNase, plasmid DNA showed complete degradation. In the case of DNA mixed with neutral or negatively charged preformed DRV liposomes, the recovery of DNA adsorbed to the surface was very low after exposure to the enzyme. In contrast, most of the nucleic acids encapsulated into neutral or anionic DRV liposomes were resistant to degradation by deoxyribonuclease, indicating a high level of true entrapment. Preformed cationic DRV liposomes mixed with plasmid DNA demonstrated higher protection of DNA in the presence of DNase than neutral or anionic preformed liposomes. The higher resistance of cationic complexed DNA to the enzyme compared to neutral or anionic complexed DNA could be attributed to its condensed state. Positively charged liposomes bind the negatively charged phosphate molecules on the DNA backbone through electrostatic interactions, forming a complex between the liposomes and the DNA. Such complex formation may provide an efficient way to associate DNA with a liposome, thus protecting a significant amount of DNA from enzymatic attack. However, such multivalent binding reaction typically results in aggregation of the components, which is extremely difficult to control as observed in our work and by others (Sen & Crothers, 1986; Mahato et al, 1995). Other laboratories have found that vesicle aggregation is not a crucial factor in vitro (Caplen et al, 1995). However, it has proved to create difficulties in vivo and in clinical trials where a high concentration of the complex is used (Nabel et al, 1993, Caplen et al, 1995; Lasic, 1997). For example, coalescence or fusion of liposomes
may occur and result in the leakage of the entrapped nucleic acid. Furthermore, the increase in size may lead to an alteration of the *in vivo* clearance of the liposomes (Juliano & Stamp, 1975).

In this part of the experiment we also observed that the level of DNA protection was dependent on the type of the cationic lipid used in the preformed cationic liposomal formulation, while in the case of encapsulation into cationic DRV the lipid type had no significant effect on the protection from deoxyribonuclease attack (Table III.4). The true entrapment protects the content from enzymatic attack and prevents aggregation on the surface.

With the exception of uncharged liposomes, microfluidisation of DRV liposomes (3cycles, 230nm) retained much of the original content of nucleic acid, which was still significantly inaccessible to the enzyme. When the DNA recovery after DNase treatment was examined by agarose gel electrophoresis, intact DNA could be detected in the case of encapsulation into DRV liposomes, especially cationic DRV liposomes. In contrast, DNA alone or mixed with neutral or anionic DRV exhibited a smeared pattern.

Results obtained from transfection experiments demonstrated the levels of luciferase activity for all the DRV preparations tested. As anticipated by others, our data show that cationic vesicles offer better transfection activity than uncharged or anionic liposomes.
It appears (Fig. III.11) that positively charged vesicles provide for a 10-fold higher transfection efficiency than neutral or negatively charged liposomes, and that this can be even further improved when cationic DRV are microfluidised to a size of 210-380nm diameter (extensive microfluidisation of DRV to a size of 100nm results in DNA damage as agarose gel electrophoresis has shown). According to Felgner et al (1987), Bertling (1991), and Mahato et al (1995b), the fusion between cationic vesicles and cell surfaces results in the delivery of the nucleic acids directly across the membrane, thus preventing lysosomal degradation processes, which occur when anionic or neutral liposomes are used, and leads to a reduced efficiency of these vesicles as DNA carriers.

Although the data from this set of experiments demonstrate the suitability of DRV liposomes for DNA transfection, the commercial cationic liposomes, LipofectAMINE, proved more effective, as can be seen from the higher luciferase activity. One possible explanation for the superior transfection efficiency of SUV-plasmid DNA complexes to any of our other preparations could be that in SUV complexes the adsorption of nucleic acids onto the surface is higher than their entrapment within the vesicles (as shown in the experiments with DNase digestion). As a result, the SUV-plasmid DNA complex associates rapidly and extensively to the cell, while the uptake of DNA encapsulated into cationic liposomes is slower and consequently lower.

Sternberg and co-workers (1994) have been reported also freeze-fracture electron micrographs depicting the lipid morphology arising when cationic liposomes
interact with DNA. They proposed a model for the structure that they termed the
"spaghetti (DNA helix inside a lipid tube) and meatball (aggregated liposomes)
model. DNA and cationic liposomes in fact undergo a radical topological transition
whereby the liposomes rupture and fuse into condensed multilamellar structures.
Although it is well known that the judicial choice of cationic lipids (Legendre &
Szoka, 1995) and vesicle size (Liu et al, 1997) plays an important role for the
transfection activity of DNA \textit{in vitro}, recent work by Koltover et al (1998) has shed
further light on the mechanism of cationic liposomes complexed DNA. Using
synchroton small-angle x-ray scattering and optical microscopy, Koltover et al
demonstrated that the mixing of plasmid DNA with SUV cationic liposomes leads
to a transition from the lamellar phase to a columnar inverted hexagonal liquid
crystalline state. The authors suggest that the lamellar structure of cationic
liposome-DNA complexes binds solidly to the cellular membrane, whereas the
more transfectant hexagonal complexes are unstable and rapidly fuse and release
DNA upon adhering to the cellular membrane phase of cationic liposome-DNA.

While the exact mechanism of transfection is not known, many reports suggest that
high amounts of cationic lipid are necessary for successful transfection activity. It
seems that the transfection efficiency increases with increasing cationic lipid
concentration (Mahato et al, 1995a; Felgner et al, 1987). Yet the use of high
amounts of cationic lipids results in high cell toxicity (Hofland et al, 1996). While it
is generally agreed that the intracellular delivery of DNA, RNA, and antisense
oligonucleotides can be improved significantly by the use of cationic liposomes, the
toxicity of the cationic lipid cell presents the major drawback of the technology.
(Yoshihara & Nakae, 1986; Felgner et al 1987; Hannun & Bell, 1989; Leventis & Silvius, 1990). Nearly any substance can of course be toxic if administered in high amounts. It is therefore one of the main challenges to avoid using high amounts of a substance and still benefit from its properties. Our toxicity tests with three cationic DRV liposomes (SA, DOTAP, DC-Chol) have exhibited no sign of toxicity in the cells. When the effects of cationic lipid concentration in various liposomes on cells were studied, all formulations showed similar trends, although the absolute values were different. This is greatly due to the low amount of cationic lipids required by the DRV method to produce surface charge and to condense DNA. Optimal dosing often depends on the balance between transfection activity and cytotoxicity. In our case, at a fixed DNA:lipid ratio, a high transfection efficiency requires more DNA and low cytotoxicity requires less cationic lipid. As mentioned above, an excess of positive charge has been reported to be necessary for successful transfection. If we assume the average molecular weight of many of the cationic lipids is around 750g/mol, then charge neutrality would occur at a ratio of approximately 1:3 w/w (DNA/cationic lipid) for 15mg of DNA complexed with cationic SUV. This shows why most other researchers have used molar ratios of 1:7, 1:10, or 1:23 in order to achieve transfection activity \textit{in vitro} or \textit{in vivo}. In these cases, cell toxicity might arise from direct interaction of the SUV-DNA complexes with external or internal cellular membranes at the site of injection. In contrast, the molar ratios of cationic lipid:DNA used in our DRV preparations were 1:1, 1:2, and 1:2.5 and no apparent relationship was observed between amount of DNA (20-100μg) and values of incorporation for the compositions and lipid mass used.
This is an important achievement, since saturation of the cells with carrier lipid and lipid toxicity are less likely to occur, which is a distinct advantage where transfection *in vivo* is considered. Moreover, a high ratio of entrapped material:lipid would be economical with respect to both liposomal lipids and nucleic acid.

In order to establish if there are any changes in the cells after treatment with cationic liposomes, which may not be determined by the MTT assay, we also used light microscopy to examine the cell morphology. Cells treated with DOTAP, DC-Chol and SA at a concentration of 2μM or 4μM of cationic lipid showed no morphology change. The pictures obtained from microscopy showed no toxic effects such as cell shrinkage, or vacuolisation of the cytoplasm (see Fig. III.12 a,b,c). The cells continued to appear normal, to spread out, and none of the cells were apoptotic.

Finally, the highly hydrophobic and lipophobic character of a fluorinated liposomal membrane was expected to have an impact on its interactions with serum components and other biological compounds (Frézard et al, 1994; Guedj et al, 1994). In fact, the most important finding in the experiments with fluorinated vesicles was that the presence of a fluorinated core (fluorinated membrane of variable lipophilic/lipophobic character) inside the liposomal membrane greatly increases the storage stability as well as the stability of these vesicles towards plasma. The fluorinated compounds produced a suitable vesicle size in DRV form (about 300nm), making microfluidisation or other size reduction techniques
superfluous. In terms of biological tolerance, the stability of the fluorinated liposomes whose membrane was in the semi-gel state (i.e. DF6C7PC) displayed a significantly greater stability than the other formulations. Unfortunately, the behaviour of fluorinated vesicles in transfection experiments was unsatisfactory. This could be due to chemical degradation of the vesicles in the presence of the cells and/or due to the high stability of the fluorinated core, which would limit the interaction of the vesicles with the cells and therefore lead to increased toxicity on the cell surface. The stability results obtained with fluorinated compounds make the latter explanation more plausible. However, the encouraging outcome of the entrapment and stability studies is deemed to warrant further studies. From our point of view, fluorinated vesicles may be suitable delivery vehicles for non-medical rather than medical applications. However, fluorinated vesicles might also be used for medical delivery purposes, provided some basic problems, such as the risk of chemical degradation and the balance between encapsulation and release of the solute, could be overcome. Improving the vesicle-shell packing by investigating the structural parameters, and studying the behaviour of fluorinated compounds on cell cultures, their haemolytic activity and toxicity in vitro and in vivo might guide the development of neat mixed fluorocarbon-hydrocarbon vesicles for medical applications.

In collaboration with the Royal Free Hospital’s School of Medicine, we also assessed the value of our liposomal preparation technique for delivery of antisense agents in ducks. To this end, oligodeoxynucleotides (ODN) were entrapped into DRV liposomes. This led to similar results as those presented here. Furthermore,
the studies carried out at the Royal Free Hospital showed no degradation of ODN to smaller molecules during entrapment into DRV liposomes. *In vitro*, no degradation of ODN by plasma exo- or endonucleases was observed after 24 hours at 37°C, which is a property of phosphorothioate-modified ODN (Soni et al, 1998).

In summary, our *in vitro* studies have provided evidence that the DRV method offers a number of crucial advantages compared with those previously used for incorporation of DNA in liposomes (Rose et al, 1991; Legendre & Szoka; 1992; Zhou & Huang, 1994).

Our data indicate true, high yield entrapment (80%) of nucleic acids into DRV vesicles and within the vesicle bilayers, high storage stability and high protection of the DNA from degradation enzymes. Liposomal plasmid DNA was found to retain its structural integrity and to transfect cells *in vitro* in relation to the size and surface charge of the vesicles.

The following chapters will show whether such DNA incorporating liposome constructs could be effective for plasmid DNA expression *in vivo*. 
### Table III.1  Amount of nucleic acids encapsulated or mixed with neutral DRV liposomes

<table>
<thead>
<tr>
<th>Nucleic acids incorporated in PC:DOPE</th>
<th>Amount used (µg)</th>
<th>% incorporation into DRV (% retained DNA)</th>
<th>% incorporation into microfluidized DRV (% retained DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA PGL2</td>
<td>10</td>
<td>40.4 (71.9)</td>
<td>12.5 (49.8)</td>
</tr>
<tr>
<td>Plasmid DNA PGL2</td>
<td>20</td>
<td>43.8 (62.0)</td>
<td>17.3 (48.1)</td>
</tr>
<tr>
<td>Plasmid DNA PGL2</td>
<td>25</td>
<td>41.4 (67.1)</td>
<td>16.9 (48.0)</td>
</tr>
<tr>
<td>Plasmid DNA PGL2</td>
<td>30</td>
<td>39.5 (60.0)</td>
<td>20.6 (42.4)</td>
</tr>
<tr>
<td>Plasmid DNA PGL2</td>
<td>50</td>
<td>41.3 (45.3)</td>
<td>10.1 (44.0)</td>
</tr>
<tr>
<td>Plasmid DNA PGL2</td>
<td>100</td>
<td>55.4</td>
<td>-</td>
</tr>
<tr>
<td>Plasmid DNA mixed with liposomes</td>
<td>10</td>
<td>12.1 (17.8)</td>
<td>6.8 (10.2)</td>
</tr>
<tr>
<td>Plasmid DNA mixed with liposomes</td>
<td>50</td>
<td>10.8 (12.9)</td>
<td>5.9</td>
</tr>
<tr>
<td>Plasmid DNA mixed with liposomes</td>
<td>100</td>
<td>14.1 (10.0)</td>
<td>-</td>
</tr>
<tr>
<td>Plasmid DNA pRc/CMV HBS</td>
<td>50</td>
<td>43.9 (51.9)</td>
<td>14.7 (49.0)</td>
</tr>
<tr>
<td>Plasmid DNA pRc/CMV HBS</td>
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<td>40.9 (60.8)</td>
<td>14.3 (52.8)</td>
</tr>
<tr>
<td>Plasmid DNA pRc/CMV HBS</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid DNA pRc/CMV HBS</td>
<td>100</td>
<td>10.9 (8.9)</td>
<td>19.8 (54.8)</td>
</tr>
<tr>
<td>Plasmid DNA pRc/CMV HBS</td>
<td>100</td>
<td>13.7 (11.1)</td>
<td>21.0 (48.6)</td>
</tr>
<tr>
<td>Plasmid DNA pRc/CMV HBS</td>
<td>50</td>
<td>14.1 (10.0)</td>
<td>-</td>
</tr>
<tr>
<td>Plasmid DNA pRc/CMV HBS</td>
<td>100</td>
<td>14.1 (10.0)</td>
<td>-</td>
</tr>
<tr>
<td>Plasmid DNA pRc/CMV HBS</td>
<td>225*</td>
<td>59.8 (61.8)</td>
<td>19.9 (41.8)</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>225</td>
<td>50.4 (60.1)</td>
<td>6.6</td>
</tr>
<tr>
<td>Hepatitis B mixed with liposomes</td>
<td>225</td>
<td>43.7 (54.4)</td>
<td>6.9</td>
</tr>
<tr>
<td>Hepatitis B mixed with liposomes</td>
<td>500*</td>
<td>41.9 (50.9)</td>
<td>10.1 (16.1)</td>
</tr>
<tr>
<td>Oligonucleotide Hepatitis B mixed</td>
<td>225</td>
<td>10.8 (12.9)</td>
<td></td>
</tr>
<tr>
<td>Oligonucleotide Hepatitis B mixed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGL2 encapsulated:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF6C7PC (16µmole)</td>
<td>50</td>
<td>29.8</td>
<td></td>
</tr>
<tr>
<td>DF6C7PC (32µmole)</td>
<td>50</td>
<td>44.5</td>
<td></td>
</tr>
<tr>
<td>F8C11PC (16µmole)</td>
<td>50</td>
<td>20.1</td>
<td></td>
</tr>
<tr>
<td>F8C11PC (32µmole)</td>
<td>50</td>
<td>30.0</td>
<td></td>
</tr>
<tr>
<td>F6H11GLYGLY</td>
<td>50</td>
<td>20.5</td>
<td></td>
</tr>
<tr>
<td>F6H11GLYGLY</td>
<td>50</td>
<td>31.9</td>
<td></td>
</tr>
</tbody>
</table>

$^{35}$S-labelled plasmid DNA or $^{125}$I-labelled oligonucleotide was incorporated into neutral DRV liposomes composed of PC (16µmole) and DOPE in molar ratio of 1:0.5 and similar microfluidized (3cycles) DRV. Numbers in parentheses denote % of DNA or oligonucleotide retained by liposomes after exposure to deoxyribonuclease I for 10 min at 37°C. (*) Present oligonucleotide incorporated into similar neutral liposomes composition with molar ratio 1:1. Plasmid DNA pGL2 encapsulated into different fluorinated vesicles composed of DF6C7PC, F8C11PC or F6H11GlyGlyLact.
Table III.2  **Amount of nucleic acids encapsulated or mixed with anionic DRV liposomes**

<table>
<thead>
<tr>
<th>Nucleic acids incorporated with PC:DOPE:PS</th>
<th>Amount used (μg)</th>
<th>% incorporation into DRV (% retained DNA)</th>
<th>% incorporation into microfluidized DRV (% retained DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA PGL2 encapsulated into liposomes</td>
<td>10</td>
<td>55.8 (69.1)</td>
<td>44.6 (67.8)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>61.2 (60.9)</td>
<td>40.9 (66.1)</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>61.0 (58.2)</td>
<td>42.3 (62.6)</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>45.5 (58.0)</td>
<td>37.0 (67.1)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>63.0</td>
<td>51.0</td>
</tr>
<tr>
<td>Plasmid DNA PGL2 mixed with liposomes</td>
<td>10</td>
<td>12.0 (17.6)</td>
<td>10.2 (9.8)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>13.3</td>
<td>10.8</td>
</tr>
<tr>
<td>Plasmid DNA pRc/CMV HBS encapsulated into liposomes</td>
<td>50</td>
<td>49.9 (58.9)</td>
<td>42.8 (69.1)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>55.9 (62.0)</td>
<td>44.8</td>
</tr>
<tr>
<td>Plasmid DN pRc/CMV HBS mixed with liposomes</td>
<td>50</td>
<td>14.1</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>10.9 (11.0)</td>
<td>11.2 (5.3)</td>
</tr>
<tr>
<td>Oligonucleotide Hepatitis B encapsulated into liposomes</td>
<td>225 *</td>
<td>59.9 (67.0)</td>
<td>48.1 (51.0)</td>
</tr>
<tr>
<td></td>
<td>225</td>
<td>51.1 (60.9)</td>
<td>45.0 (61.2)</td>
</tr>
<tr>
<td></td>
<td>500 *</td>
<td>49.8 (65.9)</td>
<td>41.5</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>52.8 (59.8)</td>
<td>46.9</td>
</tr>
<tr>
<td>Oligonucleotide Hepatitis B mixed with liposomes</td>
<td>225 *</td>
<td>10.2</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>225</td>
<td>11.0 (4.4)</td>
<td>6.9</td>
</tr>
</tbody>
</table>

$^{35}$S-labelled plasmid DNA or $^{125}$I-labelled oligonucleotide was incorporated into negatively charged DRV liposomes composed of PC (16μmoles) and DOPE:PS in molar ratio of 1:0.5:0.25 and similar microfluidised (3cycles) DRV. Numbers in parentheses denote % of DNA or oligonucleotide retained by liposomes after exposure to deoxyribonuclease I for 10 min at 37°C. (*) Present oligonucleotide was incorporated into similar neutral liposomes composition in molar ratio 1:1:0.5
### Table III.3  
Amount of nucleic acids encapsulated or mixed with cationic  
DRV liposomes

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>Amount used (µg)</th>
<th>% incorporation into DRV (% retained DNA)</th>
<th>% incorporation into microfluidized DRV (% retained DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC:DOPE:SA</td>
<td>10</td>
<td>64.1 (78.1)</td>
<td>50.3 (63.1)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>71.9 (75.0)</td>
<td>65.5 (59.9)</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>82.3 (75.2)</td>
<td>64.7 (58.2)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>74.8 (70.1)</td>
<td>56.2 (58.0)</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>71.2 (67.9)</td>
<td>50.9 (57.4)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>84.4 (70.9)</td>
<td>60.1</td>
</tr>
<tr>
<td>PC:DOPE:SA complexed</td>
<td>10</td>
<td>59.7 (41.1)</td>
<td>31.6 (40.2)</td>
</tr>
<tr>
<td>With pGL2 plasmid DNA</td>
<td>25</td>
<td>45.1 (58.5)</td>
<td>12.9 (41.2)</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>40.3</td>
<td>16.3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>40.0 (49.9)</td>
<td>19.9 (46.3)</td>
</tr>
<tr>
<td>PC:DOPE:BISHOP</td>
<td>10</td>
<td>68.4 (75.3)</td>
<td>51.9 (63.1)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>70.8 (70.2)</td>
<td>55.5 (64.0)</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>62.3 (69.9)</td>
<td>52.2 (78.2)</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>75.6</td>
<td>62.1</td>
</tr>
<tr>
<td>PC:DOPE:DOTMA</td>
<td>50</td>
<td>81.0 (85.9)</td>
<td>76.3 (79.1)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>92.6</td>
<td>83.2</td>
</tr>
<tr>
<td>PC:DOPE:SA encapsulated</td>
<td>225 *</td>
<td>89.9 (81.6)</td>
<td>85.6 (65.9)</td>
</tr>
<tr>
<td>Oligonucleotide Hepatitis B</td>
<td>225</td>
<td>94.0 (79.0)</td>
<td>89.9 (71.6)</td>
</tr>
<tr>
<td></td>
<td>500 *</td>
<td>85.1 (69.2)</td>
<td>71.2 (69.5)</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>80.3 (70.1)</td>
<td>71.0 (61.9)</td>
</tr>
<tr>
<td>PC:DOPE:SA complexed</td>
<td>225 *</td>
<td>90.0 (65.9)</td>
<td>68.1 (50.2)</td>
</tr>
<tr>
<td>Oligonucleotide Hepatitis B</td>
<td>225</td>
<td>82.1</td>
<td>54.9</td>
</tr>
</tbody>
</table>

*[^S]-labelled plasmid DNA pGL2 or [^35]I-labelled oligonucleotide was incorporated into positively charged DRV liposomes composed of PC (16µmoles) and DOPE:SA, BiSHOP or DOTMA in molar ratio of 1:0.5:0.25 and similar microfluidised (3cycles) DRV. Numbers in parentheses denote % of DNA or oligonucleotide retained by liposomes after exposure to deoxyribonuclease I for 10 min at 37°C. (*) Present oligonucleotide was incorporated into similar neutral liposomes composition in molar ratio 1:1:0.5
Table III.4  Amount of pRc/CMV-HBS encapsulated or mixed with cationic DRV liposomes

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>Amount used (μg)</th>
<th>% incorporation into DRV (% retained DNA)</th>
<th>% incorporation into microfluidized DRV (% retained DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC:DOPE:SA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(encapsulated)</td>
<td>50</td>
<td>70.1 (79.8)</td>
<td>60.1 (59.7)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>86.9 (70.9)</td>
<td>61.9</td>
</tr>
<tr>
<td>(complexed)</td>
<td>50</td>
<td>60.9 (51.0)</td>
<td>35.9 (41.9)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>65.6 (52.9)</td>
<td>41.2 (40.1)</td>
</tr>
<tr>
<td>PC:DOPE:DOTAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(encapsulated)</td>
<td>25</td>
<td>89.9 (81.9)</td>
<td>64.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>79.0 (85.1)</td>
<td>62.0 (71.4)</td>
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<td></td>
<td>100</td>
<td>85.2 (70.1)</td>
<td>76.9</td>
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<tr>
<td>(complexed)</td>
<td>25</td>
<td>88.1 (60.9)</td>
<td>56.2 (49.3)</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>89.1 (65.4)</td>
<td>50.9 (50.0)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>73.0</td>
<td>68.9</td>
</tr>
<tr>
<td>PC:DOPE:DC-Chol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(encapsulated)</td>
<td>25</td>
<td>90.2 (73.9)</td>
<td>79.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>82.8 (80.5)</td>
<td>73.4 (69.9)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>80.9 (74.9)</td>
<td>70.0</td>
</tr>
<tr>
<td>(complexed)</td>
<td>25</td>
<td>80.9 (71.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>80.0 (62.9)</td>
<td>60.1 (49.9)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>77.9 (60.2)</td>
<td>50.1</td>
</tr>
</tbody>
</table>

$^{35}$S-labelled plasmid DNA pRc/CMV-HBS was incorporated into positively charged DRV liposomes composed of PC (16μmoles) and DOPE:SA, DOTAP or DC-Chol in molar ratio of 1:0.5:0.25 and similar microfluidised (3cycles) DRV. Numbers in parentheses denote % of DNA retained by liposomes after exposure to deoxyribonuclease I for 10 min at $37^\circ C$. 

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Table III.5  Incorporation efficiency of plasmid DNA with different liposomes

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>DNA</th>
<th>Efficiency (%)</th>
<th>Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cationic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRV</td>
<td>Encapsulated</td>
<td>80.56±9.097</td>
<td>568.6±8.83</td>
</tr>
<tr>
<td>DRV</td>
<td>Complexed</td>
<td>73.42±5.63</td>
<td>992.4±51.65</td>
</tr>
<tr>
<td>SUV</td>
<td>Encapsulated</td>
<td>69.7±9.87</td>
<td>236.5±44.20</td>
</tr>
<tr>
<td>SUV</td>
<td>Complexed</td>
<td>62.48±6.46</td>
<td>429.8±70.98</td>
</tr>
<tr>
<td>Anionic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRV</td>
<td>Encapsulated</td>
<td>41.88±8.9</td>
<td>472.6±4.89</td>
</tr>
<tr>
<td>DRV</td>
<td>Complexed</td>
<td>10.3±3.54</td>
<td>418.3±73.80</td>
</tr>
<tr>
<td>SUV</td>
<td>Encapsulated</td>
<td>33.63±4.71</td>
<td>235.9±39.43</td>
</tr>
<tr>
<td>SUV</td>
<td>Complexed</td>
<td>9.42±1.66</td>
<td>240.4±48.13</td>
</tr>
<tr>
<td>Neutral</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRV</td>
<td>Encapsulated</td>
<td>39.16±4.12</td>
<td>482.16±63.04</td>
</tr>
<tr>
<td>DRV</td>
<td>Complexed</td>
<td>11.38±1.22</td>
<td>414.0±20.73</td>
</tr>
<tr>
<td>SUV</td>
<td>Encapsulated</td>
<td>15.26±3.82</td>
<td>235.3±40.02</td>
</tr>
<tr>
<td>SUV</td>
<td>complexed</td>
<td>8.84±2.53</td>
<td>246.2±53.58</td>
</tr>
</tbody>
</table>

35S-labelled plasmid DNA was encapsulated in or mixed with neutral composed of PC (16µmoles) and DOPE in molar ratio 1:0.5; anionic composed of PC (16µmoles):DOPE and PS in molar ratios 1:0.5:0.25 or cationic composed of PC(16µmoles):DOPE and SA, DOTAP, Dc-Chol and DOTMA in molar ratio 1:0.5:0.25. SUV present the DRV liposomal DNA after 3 cycles microfluidisation of similar composition of lipids. Values shown are mean of values obtained from 10-15 experiments at different time.
Table III.6 Stability of DNA encapsulated neutral, anionic, cationic, and fluorinated DRV liposomes at 4°C and 20°C

<table>
<thead>
<tr>
<th>Liposomes composition</th>
<th>Storage</th>
<th>% of retention</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>PC</td>
<td>4°C</td>
<td>85.2</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>51.3</td>
</tr>
<tr>
<td>PC:DOPE(a)</td>
<td>4°C</td>
<td>98.1</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>70.3</td>
</tr>
<tr>
<td>PC:DOPE(b)</td>
<td>4°C</td>
<td>96.2</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>79.5</td>
</tr>
<tr>
<td>DF6C7PC(c)</td>
<td>4°C</td>
<td>95.0</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>91.8</td>
</tr>
<tr>
<td>DF6C7PC(d)</td>
<td>4°C</td>
<td>74.3</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>59.6</td>
</tr>
<tr>
<td>F6H11GlyGly</td>
<td>4°C</td>
<td>86.9</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>74.9</td>
</tr>
<tr>
<td>PC:DOPE:PS</td>
<td>4°C</td>
<td>98.9</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>97.3</td>
</tr>
<tr>
<td>PC:DOPE:SA</td>
<td>4°C</td>
<td>95.6</td>
</tr>
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<td></td>
<td>20°C</td>
<td>94.1</td>
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<tr>
<td>PC:DOPE:DOTAP</td>
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<td>96.3</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>95.1</td>
</tr>
</tbody>
</table>

a) PC (16μmoles):DOPE (molar ratio 1:0.5); b) PC(16μmoles):DOPE (molar ratio 1:0.25); c) DF6C7PC (32μmoles); d) DF6C7PC(16μmoles); PC(16μmoles):DOPE and PS (molar ratio 1:0.5:0.25); PC(16μmoles):DOPE and SA (molar ratio 1:0.5:0.25) and PC(16μmoles):DOPE and DOTAP (molar ratio 1:0.5:0.25).
Table III.7  

Stability of DNA encapsulated into neutral, anionic, cationic, and microfluidised DRV liposomes at 4°C and 20°C

<table>
<thead>
<tr>
<th>Liposomes composition</th>
<th>Storage</th>
<th>% of retention</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>PC:DOPE(a)</td>
<td>4°C</td>
<td>88.1</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>60.2</td>
</tr>
<tr>
<td>PC:DOPE(b)</td>
<td>4°C</td>
<td>94.9</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>71.6</td>
</tr>
<tr>
<td>PC:DOPE:PS</td>
<td>4°C</td>
<td>92.6</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>90.6</td>
</tr>
<tr>
<td>PC:DOPE:SA</td>
<td>4°C</td>
<td>91.0</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>85.5</td>
</tr>
<tr>
<td>PC:DOPE:DOTAP</td>
<td>4°C</td>
<td>92.3</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>90.3</td>
</tr>
</tbody>
</table>

a) PC (16µmoles) and DOPE (molar ratio 1:0.5); b) PC(16µmoles) and DOPE (molar ratio 1:0.25); PC(16µmoles):DOPE and PS (molar ratio 1:0.5:0.25); PC(16µmoles):DOPE and SA (molar ratio 1:0.5:0.25); PC(16µmoles):DOPE and DOTAP (molar ratio 1:0.5:0.25).
100μg of DNA was incorporated into cationic DRV liposomes composed of PC:Dope:DOTAP (1:0.5:0.25 molar ratio). Fingerprint-like patterns (black arrow) on the surface of the vesicles indicate the compact formation of DNA with liposomes. There are also some artefactual aggregates of liposomes, which make assessment of the particle size distribution very difficult. The formation of liposomes was observed by transmission electron microscopy according to the negative staining method. The sample was examined using a Philips microscope, CM2 model, at 80kV at 280X magnification. Average size of the liposomal DNA measured by dynamic light scattering.
100μg of DNA was incorporated into cationic DRV liposomes composed of PC:Dope:DOTAP (1:0.5:0.25 molar ratio) after microfluidisation. Vesicles appear to be packed in monolayer form as expected from SUV liposomes. The formation of liposomes was observed by transmission electron microscopy according to the negative staining method. The sample was examined using a Philips microscope, CM/2 model, at 80kV at 750X magnification. Average size of the macrofluidised liposomal DNA measured by dynamic light scattering.
Appearance of 'multilamellar' structures in liposomes prepared by DRV method. Electron microscopy revealed that the DRV liposomes are packed in an onion-like structure. The formation of liposomes was observed by transmission electron microscopy after negative staining with 1% phosphotungstic acid solution at 750X magnification. Average size of the liposomes also measured by dynamic light scattering.
Different types of DRV liposomes were prepared containing 50μg of pGL2 plasmid DNA. The liposomes were composed of PC (16 μmoles) and DOPE in molar ratios 1:0.25 or 1:0.5, or PC:DOPE and DOTAP, or SA or PS in molar ratios 1:0.5:0.25. The final amount of DNA encapsulated in all liposomal preparations was adjusted equally before the preparations were mixed with mouse plasma or PBS for assay. Each preparation (0.5ml) was mixed with 2ml of heparinised fresh mouse plasma and incubated at 37°C. At various time intervals samples were removed, diluted with PBS, and radioactivity was measured for calculation of DNA release. Each value is a mean ± SD of three independent experiments. The initial amount of radioactive material present in the samples at zero time (immediately after preparation of the pellet, i.e. encapsulated material) was used as a control.

Significant release of plasmid DNA was observed where encapsulated into neutral liposomes (p<0.001-0.0008). There were no significant differences between all DRV liposomal DNA preparations where incubated in PBS at all time.
Various types of microfluidised DRV liposomes were prepared containing 50µg of pGL2 plasmid DNA. Liposomes were composed of PC (16 µmoles) and DOPE in molar ratio 1:0.5, or PC:DOPE and DOTAP, or SA or PS in molar ratios 1:0.5:0.25. The final amount of DNA encapsulated in all liposomal preparations was adjusted equally before the preparations were mixed with mouse plasma or PBS for assay. Each preparation (0.5ml) was mixed with 2ml of heparinised fresh mouse plasma and incubated at 37°C. At various time intervals samples were removed, diluted with PBS, and radioactivity was measured for calculation of DNA release. Each value is a mean ± SD of three independent experiments. The initial amount of radioactive material present in the samples at zero time (immediately after preparation of the pellet, i.e. encapsulated material) was used as a control.

All preparations showed significant release (p<0.01-0.0001) of plasmid DNA at the end of incubation in plasma. Although microfluidised cationic DRV liposomes (PC:DOPE:DOTAP) showed higher stability in the first 30 min, rapid release of plasmid was observed for the following time.
Figure III.6  Retention of plasmid DNA encapsulated into fluorinated liposomes in the presence of mouse plasma

DF6C7PC (32 μmoles), F6H11GlyLact (32 μmoles) and F8C11PC (32 μmoles) fluorinated DRV liposomes were prepared containing 50μg of pGL2 plasmid DNA. The final amount of DNA encapsulated in all fluorinated liposomal preparations was adjusted equally before the preparations were mixed with mouse plasma or PBS for assay. Each preparation (0.5ml) was mixed with 2ml of heparinised fresh mouse plasma and incubated at 37°C. At various time intervals samples were removed, diluted with PBS, and radioactivity was measured for calculation of DNA release. Each value is a mean ± SD of three independent experiments. The initial amount of radioactive material present in the samples at zero time (immediately after preparation of the pellet, i.e. encapsulated material) was used as a control. Significant release of plasmid DNA was observed where encapsulated into F8C11PC liposomes (p<0.001). There were no significant differences between all DRV liposomal DNA preparations when incubated in PBS at all time.
Figure III.7  Effect of deoxyribonuclease on DNA incorporated into microfluidised cationic DRV liposomes

Molecular sieving on Sepharose CL 4B of $^{35}$S-plasmid DNA pGL2 encapsulated into microfluidised cationic DRV liposomes (PC:DOPE:DOTAP) after 1, 3, and 5 cycles. Fractions (1ml) were collected from the column and radioactivity was measured by liquid scintillation.
Molecular sieving on Sepharose CL 4B of $^{35}$S-plasmid DNA pGL2 (100μg/ml) before and after incubation in (a) mouse plasma or (b) 100 units of deoxyribonuclease I for 30 min. Fractions (1ml) were collected from the bottom of the column and radioactivity was measured by liquid scintillation. For further details see Methods and Materials, Chapter II.
Figure III.9  Agarose gel electrophoresis of plasmid DNA pGL2

(a) Plasmid DNA pGL2 samples extracted from six different formulations of non-microfluidised DRV liposomes. Lanes 1,2: naked pGL2; lanes 3,4: PC:DOPE:DOTMA; lanes 5,6: PC:DOPE:BisHOP; lanes 7,8: DOPE:DOTMA; lanes 9,10: PC:DOPE:SA; lanes 11,12: PC:DOPE; lanes 13,14: PC:DOPE:PS. Samples in lanes 2, 4, 6, 8, 10, 12, 14 were pre-incubated with DNase. (b) Plasmid DNA pGL2 extracted from non-microfluidised and microfluidised (1, 3, or 5 cycles) PC:DOPE:DOTMA DRV. Samples 2, 4, 6, 8, 10, 12 were pre-incubated with DNase. Lanes 1, 2, naked pGL2; lanes 3, 4, non-microfluidised DRV; lanes 5, 6,DRV microfluidised (1 cycle); lanes 7, 8 (2 cycles); lanes 9, 10 (3 cycles) and lanes 11, 12 (5 cycles).
All liposomal samples composed of PC (16 μmoles):DOPE and SA, DOTAP, Dc-Chol in molar ratio 1:0.5:0.25 contained 100 μg of pRe/CMV-HBS. Plasmid DNA pRe/CMV-HBS samples were extracted from three different cationic DRV liposomes and recovered by the standard method of phenol extraction and ethanol precipitation (Sambrook et al, 1989). Lanes 1, 5: naked pRe/CMV-HBS; lanes 2, 6: PC:DOPE:SA; lanes 3, 7: PC:DOPE:DOTAP; and lanes 4, 8: PC:DOPE:Dc-Chol. Samples in lanes 5, 6, 7, 8 were pre-incubated with DNase. For further details see Methods and Materials, Chapter II.
Figure III.11 Effect of the charge and the size of DRV liposomes incorporating DNA on transfection.

COS-7 cells were transfected with pGL2 incorporated in DRV liposomal formulations. LipofectAMINE (first bar) was used as a positive control for assay. Bar 1 represents the DRV liposomes composed of DOPE (16μmoles):DOTMA in molar ratio 1:0.5. Bars 2, 3, 4, 5, 6 represent DRV liposomes composed of PC (16μmoles):DOPE alone in molar ratio 1:0.5 or supplemented with BisHOP, DOTMA, SA or PS respectively in molar ratio 1:0.5:0.25. The final bar represents the microfluidised DRV liposomes (3 cycles) composed of PC:DOPE:DOTMA in similar ratio as non-microfluidised liposomes. Luciferase activity is expressed as relative light units (RLU) per mg of protein. The number in y-axis is the exponent applied to the base "E". The constant "E" equals 2.71828, the base of the natural logarithm.
Figure III.12  Cytotoxicity of different cationic DRV liposomes

Cell viability of different liposomes at two different concentrations before and after encapsulation of 100µg plasmid DNA pRc/CMV-HBS was measured by the dye-uptake method and was expressed as percent of the control. Plates were read on a multi-well scanning spectrophotometer using a wavelength of 570nm. DRV liposomes composed of PC:DOPE and SA, DOTAP or Dc-Chol were prepared in two different concentrations of cationic lipid: (a) 16:8:2 pmoles or (b) 16:8:4 pmoles. Cells treated with water in absence of liposomes used as a control. For assay procedure see Methods and Materials, Chapter II.
Figure III.12a  Morphology of cells after exposure to cationic DRV liposomes (PC:DOPE:SA)

a) 1:0.5:0.5 molar ratio  
b) 1:0.5:0.25 molar ratio
Figure III.12b  Morphology of cells after exposure to cationic DRV liposomes (PC:DOPE:DOTAP)

a) 1:0.5:0.5 molar ratio  
b) 1:0.5:0.25 molar ratio
Figure III.12c  Morphology of cells after exposure to cationic DRV liposomes (PC:DOPE:DC-Chol)

a) 1:0.5:0.5 molar ratio  
b) 1:0.5:0.25 molar ratio
IV LIPOSOMES AS A CARRIER FOR DNA VACCINES

IV.1 INTRODUCTION

Although the genetic vaccination approach has been applied to the development of DNA vaccines against many different pathogens, the efficacy of DNA vaccines has varied largely. Some DNA vaccines are incapable of inducing specific immune responses even after several inoculations of large amounts of plasmid DNA (Parronchi et al, 1991; Romagnani, 1991; Sedegah et al, 1994). In many systems, the efficacy of genetic vaccines has not proved satisfactory, leading some to conclude that genetic vaccines are not a viable alternative to conventional vaccines (Manickan et al, 1997).

The highly variable levels of gene expression after intramuscular injection of plasmid have been proposed to be a result of various factors. Plasmid formulated in saline has poor bioavailability due to the inability of the system to circumvent barriers encountered by plasmid on its route from the administration site to the nucleus of the target cell, such as rapid DNA degradation in blood or tissues by nuclease, poor translocation of DNA across periplasmic or nuclear membrane, and inefficient uptake by target cells (Mahato et al, 1997; Ledley & Ledley, 1998). Improvement of vaccine efficacy has therefore become a critical issue for the acceptance of DNA vaccines as a standard vaccination technology.
We therefore set out to study the efficacy of DRV liposomes as DNA delivery vehicles \textit{in vivo}. To this end, we injected plasmid DNA pRc/CMV-HBS (expressing hepatitis B surface antigen under control of the CMV immediate-early promoter) intramuscularly once or several times into Balb/c mice.

The plasmid DNA was injected in the form of free plasmid DNA, mixed with preformed cationic DRV, or encapsulated into DRV liposomes. Three issues relevant to DNA vaccination were to be examined:

- the ability of liposomal DNA to induce immunity;
- the effect of lipid composition and liposomes preparation techniques.
IV.2 METHODS

IV.2.1 Incorporation of plasmid DNA pRc/CMV-HBS into liposomes

100μg of pRc/CMV (encoding the S region of hepatitis B antigen; HBsAg), mixed with $^{35}$S-labelled tracer, were entrapped into neutral, negatively and positively charged DRV liposomes as previously described (see section II.2.2). In some experiments, preformed cationic liposomes were mixed with 100μg of pRc/CMV-HBS and incubated for 1 h at 20°C, and then the free DNA was separated by centrifugation from the DNA incorporated with the vesicles, as described in section III.2.2. Liposomes were prepared from 16μmol PC and 8μmol DOPE for neutral, or with the addition of 4μmol PS for anionic, or 4μmol of DOTMA, SA, DOTAP or DC-Chol for cationic liposomes. In the case of preformed cationic DRV, liposomes were prepared from 16μmol PC, 8μmol DOPE and 4μmol DOTAP. Unless otherwise stated, these molar ratios and lipid compositions were used in all animal experiments. Percentage of entrapment calculation was based on $^{35}$S-labelled tracer of plasmid DNA (see section II.2.4). The vesicles were then diluted accordingly in PBS so that each mouse (in-groups of 4-5) received a dose of 0.1-10μg in a total volume of 0.1ml.

IV.2.2 DNA immunisation

Male Balb/c mice, 4-8 weeks old, were bled from the tail vein 1-2 days before a single or several intramuscular (hind leg) injections of 0.1-10μg of naked,
complexed or liposome-entrapped pRc/CMV-HBS. Blood serum samples taken at time intervals were then tested for anti-HBsAg (S region) IgG1, IgG2a, and IgG2b subclasses by enzyme-linked immunosorbent assay (ELISA), using the same antigen to coat the plates (for details see sections II.2.13). In all experiments, plasma of naïve mice (non-immunised) and plasma of the mice before injection were used as control.

**IV.2.3 Cytokine production**

Extracts of spleen homogenates were prepared according to Nakane et al (1992), as described in section II.2.14. In various experiments, the spleens of the mice were removed aseptically a few weeks after the final injection. In several separate experiments, spleen cells from vaccinated and unvaccinated mice were removed and subjected to cytokine assay (see sections II.2.13.1 and II.2.13.2). In all cases, the spleens were suspended in RPMI 1640 medium (CHAPS) and 10% (wt/vol) homogenates were prepared with a Dounce grinder. After the homogenates had been left for 1 h on ice, they were clarified by centrifugation at 2,000g for 20 min. The organ extracts (100μl) were applied to the well with duplicates of serially diluted samples (1/5, 1/2 or neat). In all experiments, the spleens of native mice (non-immunised) were used as controls. IFN-γ (Th1) and IL-4 (Th2) cytokine secretion was measured in the spleen cells from Balb/c mice vaccinated with plasmid DNA encoding pRc/CMV (free, complexed, or entrapped into liposomes).
IV.3 RESULTS

IV.3.1 Immunisation with low-dose plasmid DNA

IV.3.1.1 IgG responses after two injections of pRe/CMV-HBS as such or encapsulated into neutral and cationic DRV liposomes

A dose of 0.1, 1 or 10μg/mouse was used for each formulation in order to prepare a dose-response curve. Intramuscular injection of a dose of 0.1μg or 1μg of pRe/CMV-HBS in form of naked DNA (plasmid DNA in saline) or encapsulated into neutral DRV liposome formulations did not show any humoral responses even after the second injection (booster). Groups of mice injected with 1μg doses of pRe/CMV-HBS encapsulated into cationic DRV liposomes presented low IgG1 responses after the second injection and no IgG2a responses.

Fig. IV.1 shows the immunisation results of a 10μg dose injection of DNA (free or encapsulated into neutral or cationic DRV liposomes). Judging from the antibody assay, mice injected with naked DNA or DNA entrapped into neutral liposomes elevated anti-HBsAg IgG1 responses. While during the first three weeks after the first injection the two groups of mice did not show major differences in the anti-HBsAg IgG (all three subclasses) responses, this changed after the second injection. The sera from the group of mice injected with neutral liposomes showed higher IgG1 responses after four weeks and higher IgG2a and IgG2b responses after five weeks, compared to the group of mice injected with naked DNA. However, the immunisation with 10μg of DNA entrapped into
cationic liposomes boosted the IgG\textsubscript{1} titres to significantly higher levels after the second injection (P<0.001-0.05) than immunisation with the same doses of DNA in free form or entrapped into neutral liposomes. The second injection, which was applied 3 weeks after the first one, further augmented the IgG\textsubscript{2a} and IgG\textsubscript{2b} titers (p<0.001 and p<0.05, respectively). It is too early to conclude whether this augmentation of anti-HBsAg IgG responses is due to the second injection (boosting) or to the ability of the formulation to produce higher levels of antibodies after a certain period of time. Nonetheless, one can say from the data obtained in this experiment that cationic DRV liposomes were superior for vaccination with 10\mu g of plasmid DNA than were the other two formulations (plasmid DNA as such or encapsulated into neutral liposomes).

IV.3.1.2 IgG responses after a single injection of pRc/CMV-HBS as such, complexed with cationic DRV liposomes, or encapsulated into DRV liposomes

In the previous experiment, we have demonstrated that the lowest dose of DNA for inducing antibody response in mice is two injections of 1\mu g. We have also shown that, with varying efficiency, all the groups of mice that received 10\mu g of DNA presented anti-HBsAg IgG\textsubscript{1}, IgG\textsubscript{2a} and IgG\textsubscript{2b} responses. Although the group of mice injected with DNA encapsulated into cationic liposomes induced antibody responses already after the first injection, the augmentation of IgG\textsubscript{1} response was higher after 4 or 5 weeks, i.e. after the second injection. As mentioned above, it is not clear to what extent this augmentation is due to the booster injection or to the fact
that the formulation induces stronger humoral immunity only after a certain period of time. We therefore decided to determine the importance of the second injection in another experiment using only one injection of 2 and 10µg of plasmid DNA in various formulations.

Davis and her collaborators (1993a) have shown that the intramuscular injection of a single dose of naked pRc/CMV-HBS plasmid DNA into regenerated muscles induces humoral responses to the encoded antigen. They could detect an antibody response two weeks after injection. We therefore attempted to examine the effect of a single dose injection of DRV liposomal DNA on inducing humoral responses in non-regenerated muscle (normal muscle) and to compare it to the effect of naked DNA. Apart from this, a number of earlier reports have demonstrated a high transfection efficiency of plasmid DNA complexed with preformed cationic liposomes *in vitro* (Felgner et al., 1987). This has been confirmed by our own results (section III.3.8). In a study by Sedegah et al (1994), on the other hand, cationic liposomes (lipofectin) complexed with antigen encoding plasmid DNA did not show any antibody responses. Therefore, we also decided to examine the transfection ability of DNA complexed with cationic DRV liposomes *in vivo*.

To this end, Balb/c mice in groups of five were injected intramuscularly with a single dose of 2 or 10µg of pRc/CMV-HBS in form of free, complexed with preformed cationic liposomes, or encapsulated into neutral, anionic (10µg), and cationic DRV liposomes. Fig. VI.2 shows that anti-HBsAg IgG<sub>1</sub> responses from all groups of mice injected with 2µg of DNA were barely detectable even after 8
weeks. Although the mice injected with 2µg doses of DNA encapsulated into cationic DRV liposomes induced higher antibody responses than the other groups, the result was not statistically significant.

However, in the experiment with 10µg doses the DNA encapsulated into cationic DRV liposomes elicited significantly higher IgG_{1} antibodies to pRc/CMV-HBS than the other formulations at all time points (p<0.01). A delayed, but better response than with the 2µg dose was detected for the groups of mice that received 10µg DNA entrapped into neutral or anionic liposomes or complexed with preformed cationic liposomes (Fig. IV.2). However, the groups of mice injected with 10µg doses of naked DNA showed no improvement in eliciting antibody levels, compared to animals injected with 2µg naked DNA. It appears that a single dose injection of DNA encapsulated into cationic DRV liposomes can induce antibodies for encoded protein with much lower doses (10µg) than those (up to 100µg) normally used for naked pRc/CMV-HBS (identical to the plasmid used here) vaccination. Despite the good responses obtained with DNA encapsulated into cationic DRV liposomes, the long-term responses for this formulation were disappointing. When the antibody responses were checked two months after injection, all titers dropped to levels obtained with the other two liposomal formulations.
IV.3.2 Immunisation with high-dose plasmid DNA

IV.3.2.1 IgG responses after multiple injections of pRc/CMV-HBS as such, complexed with cationic DRV liposomes, or encapsulated into neutral, anionic, and cationic liposomes

Balb/c mice received five intramuscular injections of 1 or 10μg doses of pRc/CMV-HBS over a period of 28 days. Fig IV.3 to IV.7 show the results from injections of 1μg and 10μg DNA in form of free, complexed with preformed cationic liposomes, or encapsulated into neutral or positively charged liposomes (see legend to Fig. IV.3-7 for lipid compositions). While after five injections of 1μg DNA only low IgG1 responses could be observed from all groups of mice, plasmid DNA encapsulated into cationic DRV liposomes elicited higher levels of IgG1 antibodies than the other formulations.

The differences between the various formulations appeared clearly after the multiple injections of 10μg DNA. Based on the antibody assay, we observed that all liposomal formulations induced better responses than naked DNA. This could be explained by the presence of phospholipids in the application. Fig. IV.7 shows that sera from the groups of mice injected with DNA encapsulated into cationic liposomes elevated significantly higher IgG1, IgG2a, and IgG2b titers (p<0.0001- p<0.005) at all time points than sera from the other groups of mice. As anticipated by other researchers (Sedegah et al, 1994), DNA complexed with preformed cationic liposomes failed to show a similar in vivo transfection efficiency as in vitro. The difference in liposomal activity could be due to differences in organ
localisation and/or more efficient uptake by the cells especially in the case of
cationic DRV liposomes.

IV.3.2.2 IgG responses after multiple injections of pRc/CMV-HBS as such
or encapsulated into various cationic DRV liposomes

In the previous experiments, we found that cationic DRV liposomes under the
conditions used are the best preparation for pRc/CMV-HBS vaccination. We now
studied the effect of different types of cationic lipid used in DRV liposomes,
compared to naked DNA. Balb/c mice were injected intramuscularly on days 0, 7,
14, 21, and 28 with 5 or 10μg pRc/CMV-HBS in form of naked DNA or entrapped
into four different types of cationic DRV liposomes (for lipid composition see
legend to Fig. IV.8 to 13). Judging from the antibody assay, mice injected with five
doses of 10μg of naked DNA showed slightly higher elicitation of immune
responses against the encoded antigen, compared to the injection of five doses of
5μg or to the previous experiments with two doses of 1 to 10μg. Nonetheless, mice
injected with any of the cationic liposomes showed significantly higher IgG1, IgG2a
and IgG2b antibodies to pRc/CMV-HBS than any of the mice treated with naked
DNA at all time points. However, we could observe slight differences between the
results for the four different types of cationic DRV liposomes. The group of mice
treated with DNA encapsulated into liposomes composed of PC:DOPE:SA
presented lower antibody titers than those groups treated with the other three
formulations (i.e. PC:DOPE:DOTMA, PC:DOPE:DC-Chol, or PC:DOPE:DOTAP).
It therefore appears that DNA encapsulated into certain types of cationic lipid may
enhance the immune responses better than others. However, given that all four liposomal formulations elicited significantly high immune responses, the type of the cationic lipid in the formulation of DRV liposomes is probably not a crucial factor, as opposed to the formulation of preformed cationic DRV liposomes complexed with DNA.

Antibodies to HBsAg from groups injected with cationic DRV liposomes were first detected 20 days after day 0 and increased to peak ELISA antibody titers of >10^4 by 6 weeks after the first injection. Although some of the animals vaccinated with the 10μg doses of liposomal DNA had titers that were initially one fold higher than those of animals vaccinated with 5μg, overall no significant differences were observed between the two doses.

IV.3.3 Cytokine production

In order to establish to what extent the humoral responses were reflected in cytokine levels, the total concentration of IFN-γ and IL-4, calculated in nanograms per spleen, was measured as indicator of Th1 and Th2 subset T-cell activation at various time intervals after the final injection.

In the first cytokine experiment, mice received a single 10μg dose injection of pRc/CMV-HBS in form of free, complexed with preformed cationic liposomes, or entrapped into neutral or cationic DRV liposomes and the levels of IFN-γ and IL-4 in the spleens were measured. Fig. IV.14 and IV.15 show that the spleen cells
from mice immunised with DNA encapsulated into cationic liposomes had significantly higher IFN-γ and IL-4 responses than the spleen cells from all other groups of mice injected with similar doses of DNA (p<0.01 and P<0.05 respectively). Nonetheless, after a single injection of 10µg pRc/CMV-HBS the activity levels of IL-4 and IFN-γ were low in all groups of mice.

In another experiment, mice received multiple injections of 10µg doses of DNA in form of free, complexed with preformed cationic liposomes, or encapsulated into neutral or cationic DRV liposomes. They were tested for cytokine responses 2 weeks after the final immunisation. Although the spleen weights varied between groups that had received different treatments, the variations within the groups were minimal. Normal (standard deviation) spleen weights were 0.108±0.004g. Fig. IV.16 and IV.17 show that the spleen cells from groups of Balb/c mice immunised with DNA encapsulated into cationic liposomes provided higher levels of IL-4 and IFN-γ responses than those in the group injected with naked plasmid DNA (P<0.001-0.05). There were modest differences between the cytokine activity in the spleen cells isolated from mice injected with naked DNA, complexed DNA, or DNA encapsulated into neutral liposomes.

In the following experiment, mice received multiple injections of 5 or 10µg doses of DNA encapsulated into various cationic DRV liposomal preparations or in form of free DNA and their spleens were subjected to cytokine assay 3 weeks after the final immunisation. Data in Fig. IV.18 and IV.19 show that again the activation of both IFN-γ (Th1) and IL-4 (Th2) subsets was greater with cationic
liposomal DNA than with naked DNA. However, the IFN-γ and IL-4 activity varied between the liposomal formulations. The group of mice injected with the cationic DRV liposomes composed of PC:DOPE:SA presented lower cytokine activity than the other two formulations (i.e. PC:DOPE:DC-Chol or PC:DOPE:DOTAP).

All our cytokine experiments confirm the results from our humoral experiments. It appears that DNA encapsulated into cationic DRV liposomes enhances both Th1 and Th2 responses in mice.
IV.4 CONCLUSIONS

Our immunisation experiments have provided the first evidence for the potential of DRV liposomes for DNA vaccination. Balb/c mice immunised by single or multiple intramuscular injections of 10µg of naked plasmid DNA attained only low titers of anti-HBs. In contrast, anti-HBsAg titers were significantly higher (p<0.0001-0.005) in mice immunised with plasmid DNA encapsulated into cationic DRV liposomes.

The first illuminating finding was that pRc/CMV-HBS plasmid DNA encapsulated into cationic DRV liposomes proved effective in inducing both Th1 and Th2 responses. Moreover, our results from all immunisation experiments indicated that pRc/CMV-HBS plasmid DNA encapsulated into cationic DRV liposomes generates significantly higher levels of antibody responses to encoding antigen than naked plasmid DNA or DNA mixed with preformed cationic liposomes. The superiority of DNA encapsulated into cationic DRV liposomes compared to naked plasmid DNA was first observed in the experiments with single dose injection. Even a dose of 2µg pRc/CMV-HBS elicited antibody responses (only IgG1) to encoding antigen when the DNA was encapsulated into cationic DRV liposomes, and the immune responses improved by increasing the dose from 2µg to 10µg of plasmid DNA. Animals injected with naked DNA, on the other hand, produced none to low humoral responses to 2µg antigen encoded plasmid DNA and increasing the dose of plasmid DNA to 10µg did not change the results significantly.
In an attempt to examine the effect of dosage and number of injections on antibody responses to encoding antigen, mice were injected with various doses of plasmid DNA at various times. The results from our in vivo immunisation studies with multiple dose injections of pRc/CMV-HBS plasmid DNA are consistent with the observation by Sedegah et al (1994) and Gardner et al (1996) that the immunisation regime and the number of doses influence the immune responses.

The data from multiple injections, i.e. increased quantities and numbers of doses, differed significantly from the data obtained with single dose injections. The groups of mice treated with five injections of 1 or 5μg of naked plasmid DNA induced similarly low responses as the single injection. In contrast, the groups of mice injected with the same doses of pRc/CMV-HBS encapsulated into DRV liposomes presented increasing immune responses with increasing doses.

Given that in all our immunisation studies we have used a lower dose of DNA than other laboratories, we were surprised by two results. First, we consistently observed that in all experiments DNA encapsulated into cationic liposomes elicits a higher proportion of all three antibodies subclasses (IgG1, IgG2a and IgG2b) compared with the preformed cationic DRV liposomes mixed with plasmid DNA and more significantly with free DNA. Secondly, in contrast to results obtained by other researchers (Davis et al, 1993a, 1996), immunisation with plasmid DNA encapsulated into cationic DRV liposomes gave a mixed Th1/Th2 response, but with more anti-HBs Abs of the IgG1 than the IgG2a isotype.
We also observed that the mice injected twice or five times with plasmid DNA encapsulated into liposomes showed elevated titers of antibody, suggesting that boosting had occurred. Antibody responses in the groups of mice injected with plasmid DNA in saline formulation, however, showed no further enhancement of antibody titers and sustained immune responses.

What could be the explanation for the higher immune activity of pRc/CMV-HBS plasmid DNA encapsulated into cationic DRV liposomes observed as opposed to the low responses of naked plasmid DNA (in saline formulation)?

(1) Efficiency of plasmid DNA uptake by the target cells

Davis et al (1993) have shown that pre-injection of sucrose improves the intramuscular distribution of plasmid and results in reduced variability of gene expression. They demonstrated that the coefficient of variation of expressed luciferase activity was decreased to 25% from 120% by the pre-injection of 25% sucrose 15-30 min prior to injection of plasmid. Davis and her collaborators proposed that hypertonic sucrose solutions help to force muscle fibres apart by increasing hydrostatic pressure within the muscle. However, the mechanism by which hypertonic sucrose solutions enhance gene expression and reduce variability is complicated by the fact that these solutions result in large areas of muscle damage (Wolff et al, 1991; Davis et al, 1993). Pre-treatment with 10mM cardiotoxin has also been shown to lead to a 2- to 10-fold enhancement of gene expression. Bupivacaine and cardiotoxin are known to selectively destroy
myofibres, which then regenerate (Benoit & Belt, 1970, Carlson et al, 1990). The process of regeneration involves revascularisation, cellular infiltration, phagocytosis of necrotic damage muscle, satellite cell proliferation and their fusion into myotubes and re-innervation (Grounds, 1991; Carlson, 1988). It has been proposed that the connective tissue may be less of a barrier to plasmid in regenerating muscle than in normal muscle. Regenerated muscle may allow better diffusion of plasmid due to the smaller diameter of the myofibres and the loss of structure of the connective tissue (Davis et al, 1993). Thus, the poor ability of naked DNA to produce immune responses with the doses used in our animal experiments is partly due to the fact that plasmid DNA formulated into saline has a poor ability to cross the external lamina and access the muscle cells. We can conclude that saline formulation is not suitable for delivery of plasmid DNA, unless the DNA is administered into regenerated muscle cells (Whalen et al, 1995) or the muscles are pre-treated with hypertonic sucrose prior to plasmid DNA injection (Wolf et al, 1990 & 1991; Davis et al, 1993). Issues of safety, acceptability, and compliance make it appear doubtful that either method will be acceptable for industrial production.

In histological studies by the group of Alving the muscle at the site of injection showed predominantly infiltration of macrophages and neutrophils into and between the muscle fibers. The I/M injection of fluorescence-labelled liposomes resulted in intense fluorescence in the muscle even six days after injection of the liposomes and very small amount of fluorescence was observed in the local lymph node. These findings suggest that intramuscular injection of liposomes results in a
"depot" of liposomes that gradually leave the site of injection (Richards et al, 1995). The enhancement of antibodies to antigens by means of DNA encapsulation into liposomes may therefore be attributed to the generation of a depot at the site of injection, which prolongs the release and interaction of free or liposome-associated antigens with APC invading the depot area in response to local inflammation. A fraction of liposomal compound may then migrate to areas in the regional lymph node containing T cells. Induction of CD4\(^+\) T cells requires presentation of antigen by surface class II MHC, which is normally associated only with professional antigen presenting cells, and these molecules are likely to be absent on the surface of transfected muscle fibers. The presence of T-helper response in our immunisation experiments using intramuscular injection of cationic DRV liposomes demonstrate that the APC may be a preferred alternative to skeletal muscle cells as target for DNA expression.

(2) Degradation of plasmid DNA.

In the previous chapter, we demonstrated the complete digestion of plasmid DNA in the presence of DNase. Rapid degradation of a substantial proportion of DNA in the interstitial space by DNase has been shown to markedly reduce the number of intact gene constructs available for uptake by the cells (Wolff et al, 1991; Manthorpe et al, 1993). This suggests that rapid nuclease degradation limits gene transfer of plasmid formulated in saline into muscle cells. The extent of DNA degradation by extracellular deoxyribonuclease is unknown, but depending on the time of its interstitial residence, degradation could be considerable. In fact, in all
immunisation experiments with naked plasmid DNA higher immune responses were observed from the groups of mice injected with five injections of 10μg of pRc/CMV-HBS plasmid DNA. However, even these responses failed to compete with the immune activity achieved by similar doses of plasmid DNA encapsulated into cationic DRV liposomal formulation.

Our data demonstrate that augmentation of cellular and humoral immune responses to genetic immunisation with low amounts of plasmid DNA (20-25μg) is achievable when plasmid DNA is encapsulated into cationic DRV liposomes. This finding suggests that the amount of DNA for genetic immunisation can be reduced without reducing antigen specific cellular and humoral responses. The amount of DNA used for genetic vaccination may present an important safety issue because it can be assumed that the risk of DNA integration into the host's genome depends, at least in part, on the total amount of DNA delivered, even though the risk may appear relatively low (Kurth, 1995).

While it is evident that plasmid DNA formulated into liposomes can circumvent some of the delivery problems of DNA vaccination, the formulation itself may also have an effect. Given that in all our immunisation experiments the highest immune activity was achieved with cationic DRV liposomes as carrier, we studied two different techniques for DNA incorporation with cationic liposomes: 1) complex formation with preformed cationic liposomes, and 2) encapsulation into cationic DRV liposomes. In experiments where groups of mice were injected with a single dose of pRc/CMV-HBS, pronounced anti-HBsAg IgG isotype responses
were observed for DNA entrapped into DRV cationic liposomes. Sera from mice injected with DNA complexed with preformed cationic liposomes, on the other hand, presented significantly less activity in terms of the anti-HBsAg IgG responses. The differences in the immune activity of the two different liposomal preparations became even more significant in the experiment with multiple injections. Although the groups of mice immunised with DNA mixed with preformed cationic liposomes induced better responses than naked DNA, the antibody titers were remarkably higher (p<0.008) in the mice injected with DNA encapsulated into cationic DRV liposomes. We have previously observed a size enhancement of preformed cationic DRV liposomes upon mixture with plasmid DNA (see chapter III), which is a clear sign of aggregation of the complex. Instability of the complex combined with a lower level of protection in the presence of DNase (see section III.3.4) may explain the different outcome of the two formulations in our immunisation studies. Since DNA encodes linear information, any damage in the coding region can inactivate the entire molecule. Fraley et al (1981) have demonstrated that the efficiency of DNA delivery with various liposome preparations depends both on the magnitude of the vesicle binding to the cells and on the ability of the liposomes to prevent cell-induced leakage of contents. Freeze-fracture of cationic liposome/DNA complexes shows that in most complexes, such as DC-Chol/DNA, the DNA molecules are not well condensed (Sternberg et al, 1994), as a result of which they are not able to protect the DNA sufficiently from enzymatic attack or complete deactivation in the presence of serum (Gao & Huang, 1996). In contrast, as previously demonstrated,
the encapsulation into cationic DRV liposomes provides superior protection, as most of the DNA is entrapped within the vesicle bilayers. Therefore, the chance of delivering undamaged, functional DNA into the target cells is higher with encapsulated DNA than with complexed DNA.

The adjuvanticity and type of immune responses generated by liposomal molecules is significantly influenced by the size, surface charge, and composition of the liposomes, as well as the method of liposome preparation (Van Rooijen, 1990). The physical association of antigen via incorporation or surface attachment has been found to be an important factor for adjuvanticity and efficient production of IgG antibodies (Shek & Sabiston, 1981; Therien & Shahum, 1989; Phillips & Emili, 1992).

We also investigated the nature of the antibody and cytokine secreting cells activated by DNA immunisation. It is known that the subsets of Th cells can be distinguished by the pattern of cytokines they produce (Mosmann & Coffman, 1989; Romagnani, 1991). Th1 cells produce IFN-γ and IL-2, and Th2 cells produce IL-4, IL-5, IL-10, and IL-13. Our cytokine assay data suggest that plasmid DNA encapsulated with cationic DRV liposomes induces Th0, which is a mixed Th2 (IL-4) and Th1 (IFN-γ) response.

Th1 cells induce cell-mediated immune responses in addition to antibody production and have been considered important for the protection against certain viral and intracellular infections (Zhou & Huang, 1994). DNA encapsulated into
cationic DRV liposomes can also enhance IFN-γ production, thereby stimulating Th1 type immunity, suggesting raised CMI levels, although the latter was not examined in the present study. The high levels of IgG2a and IgG2b antibodies with cationic liposomes show therefore the superiority of the system in inducing Th1 cell derived CMI, compared to naked DNA or DNA complexed with preformed cationic liposomes. The production of IgG2a became significant only when the mice were subjected to two or more injections.

We also studied the effect of the lipid composition on the immune responses to the encoded antigen using plasmid DNA pRc/CMV-HBS. While some researchers attribute no importance to the effect of the phospholipid composition, others (Phillips et al, 1996) have reported that the level of immune responses generated by liposomes is significantly influenced by the composition of the liposomes. In the case of liposomal DNA vaccination, our data corroborate the latter observation. Comparing sera from mice injected with naked DNA as such and DRV liposomal formulations (neutral, negative and positive charge), we consistently observed that plasmid DNA encapsulated into cationic DRV liposomes elicits a higher IgG1 and IgG2a isotype (p<0.05-0.0001) production than neutral or anionic DRV liposomes when each group is compared with plasmid DNA in saline form. Although plasmid DNA encapsulated into neutral and anionic liposome compositions was able to activate immune responses against the encoded antigen, the presence of cationic lipid in the liposomal formulation resulted in a significantly higher induction of IgG1 and IgG2a immune activity.
This phenomenon was not altered by changing the time intervals between each injection and/or by increasing the dose of plasmid DNA.

One possible explanation is the different mechanism of the intracellular interaction of cationic liposomes. The fraction of positively charged lipid in a liposome is likely to be critical to its effective delivery and cellular uptake (Senior et al, 1991)

An understanding of the mechanism of liposomes uptake by cells and the method of drug delivery by liposomes is still emerging. It is known that soluble antigens which are rendered particulate by encapsulation are presented by macrophages to T cells and cause T cell proliferation (Dal Monte & Szoka Jr, 1989; Verma et al, 1992). However, divergent theories on the exact mechanism of presentation of liposomal antigens have been proposed (Collins & Haung, 1989; Harding et al, 1988 & 1991). It has been suggested that liposomal encapsulated antigen requires processing in endocytic vesicles for activation of antigen-specific T cells. The liposomal antigen would therefore follow the classical intracellular antigen-processing pathway described for soluble protein antigens, in which exogenous protein antigens are internalised by APC and partially degraded to produce immunogenic peptides binding to major histocompatibility complex (MHC) class II molecules. The MHC class II-peptide complex is then expressed on the surface of the APC, where it is recognised by the clonotypic T-cell receptor (Harding et al, 1988). In contrast, other investigators (Walden et al, 1985; Bakouche & Lachman, 1990) have suggested that antigen linked to the liposomes surface can

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be presented directly to T cells without further processing, i.e. in the absence of APC. However, it is not yet evident whether the class I-restricted presentation is mediated by the cytosolic fraction of the delivered antigen or the liposomal antigen itself via other unknown intracellular pathways.

It is now accepted that cationic lipids deliver nucleic acids into the cell predominately via an endocytotic pathway (Nair et al, 1992; Walker et al 1992; Legendre & Szoka Jr, 1992; Gao & Huang, 1993; Zhou & Huang, 1994; Zelphati & Sozka Jr, 1996). It is conceivable that some of the endocytosed DNA escapes the endocytic vacuoles prior to their fusion with lysosomes to enter the cytosol for eventual episomal transfection and presentation of the encoded antigen. How the nucleic acid is released from the cationic lipid once in the endosome is not fully understood. The work by Zelphati and Szoka (1996) using double-label techniques has helped to shed some light on the possible mechanisms involved. According to their proposed scheme, first the cell surface-associated cationic lipid/DNA (complex) is internalised via an endosome. The liposomal nucleic acid initiates a destabilisation of the endosomal membrane that results in a flip-flop of anionic lipids that are predominately located on the cytoplasmic face of the membrane. The anionic lipids laterally diffuse into the complex and form a charged neutralised ion-pair with the cationic lipid. This displaces the nucleic acid from the liposomes and the nucleic acid can diffuse into the cytoplasm. This anionic lipid driven uncoating of the complex is consistent with the lipid asymmetry of the plasma and endosomal membranes, as well as with observations concerning cationic lipid-mediated nucleic acid delivery, such as cell biological

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data showing accessibility of nucleic acids to the cellular translation and transcription machinery (Devaux, 1992; Malone et al, 1989; Rose et al, 1991; Gao & Huang, 1993).

The results of our studies suggest that the entrapment of DNA into cationic liposomes can enhance the potency of DNA vaccines, possibly by facilitating uptake of the plasmid by APC. Our data demonstrate that cellular and humoral immune responses to genetic immunisation can be obtained with low doses of plasmid DNA.
Balb/c mice were injected intramuscularly on days 0 and 21 with 10μg of pRc/CMV-HBS plasmid DNA in form of free, encapsulated into neutral or positively charged DRV liposomes. Sera from animals collected one day before and every week after the first injection and were analysed for anti-HBsAg IgG1 and IgG2a by ELISA. Each bar represents the mean ± S.D. of a group of four mice. End point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of non-immune serum (control animals). **p<0.001, P value are comparisons with the group received naked DNA injection.
pRc/CMV plasmid DNA was injected once intramuscularly with 2μg (white bars) or 10μg (black bars) in form of free, complexed with cationic DRV liposomes or incorporated into neutral, anionic and cationic DRV liposomes. The differences in the log₁₀ values between mice immunised with DNA incorporated into cationic DRV liposomes and mice immunised with DNA in form of free, complexed with cationic DRV liposomes or incorporated into neutral and anionic DRV liposomes were statistically significant (p<0.001-0.02).
Figure IV.3  IgG₁, IgG₂a and IgG₂b responses to pRe/CMV-HBS encapsulated into cationic DRV liposomes

Balb/c mice were injected intramuscularly on days 0, 7, 14, 21 and 28 with 1 or 10μg of pRe/CMV-HBS plasmid DNA incorporated into cationic DRV liposomes composed of PC:DOPE:DOTAP. Sera from animals were collected one day before and every week after the first injection and were analysed for anti-HBsAg IgG₁, IgG₂a and IgG₂b by ELISA. Each bar represents the mean ± S.D. of a group of four mice. End point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of non-immune serum (control animals).
Figure IV.4  IgG₁, IgG₂a and IgG₂b responses to pRc/CMV-HBS complexed with cationic DRV liposomes

Balb/c mice were injected intramuscularly on days 0, 7, 14, 21 and 28 with 1 or 10μg of pRc/CMV-HBS plasmid DNA mixed with preformed cationic DRV liposomes composed of PC:DOPE:DOTAP Sera from animals were collected one day before and every week after the first injection and were analysed for anti-HBsAg IgG₁, IgG₂a and IgG₂b by ELISA. Each bar represents the mean ± S.D. of a group of four mice. End point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of non-immune serum (control animals).
Figure IV.5  
IgG<sub>1</sub>, IgG<sub>2a</sub> and IgG<sub>2b</sub> responses to pRe/CMV-HBS incorporated into neutral DRV liposomes

Balb/c mice were injected intramuscularly on days 0, 7, 14, 21 and 28 with 1 or 10 μg of pRe/CMV-HBS plasmid DNA incorporated into cationic DRV liposomes composed of PC:DOPE. Sera from animals were collected one day before and every week after the first injection and were analysed for anti-HBsAg IgG<sub>1</sub>, IgG<sub>2a</sub> and IgG<sub>2b</sub> by ELISA. Each bar represents the mean ± S.D. of a group of four mice. End point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of non-immune serum (control animals).
Balb/c mice were injected intramuscularly on days 0, 7, 14, 21 and 28 with 1 or 10μg of naked plasmid DNA pRc/CMV-HBS. Sera from animals were collected one day before and every week after the first injection and were analysed for anti-HBsAg IgG1, IgG2a, and IgG2b by ELISA. Each bar represents the mean ± S.D. of a group of four mice. End point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of non-immune serum (control animals).
Figure IV.7  Comparison of IgG₁, IgG₂a and IgG₂b responses to free and liposomal DNA

Balb/c mice in groups of four were injected intramuscularly on days 0, 7, 14, 21 and 28 with 10μg of pRe/CMV-HBS plasmid DNA incorporated into cationic DRV liposomes composed of PC:DOPE:DOTAP (A), incorporated into neutral DRV liposomes composed of PC:DOPE (B), complexed with preformed cationic DRV liposomes composed of PC:DOPE:DOTAP (C), or in form of naked DNA (D). Each bar represents the mean ± S.D. of a group of four mice. ***P<0.0001, **P<0.002. p value are comparisons with the group received naked DNA injection.
Figure IV.8  Comparison of IgG1 responses in mice injected five times with 5μg of free or liposome-encapsulated pRe/CMV-HBS

Comparison of immune responses in mice injected intramuscularly on day 0, 10, 20, 27 and 37 with 5μg of pRe/CMV-HBS plasmid DNA as such or incorporated into various cationic DRV liposomes. The differences in the log_{10} values in mice immunised with liposomal DNA and mice immunised with naked DNA were statistically significant (p<0.0001-0.002). Each bar represents the mean ± S.D. of a group of four-five mice. End-point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of non-immune serum (control group).
Comparison of immune responses in mice injected intramuscularly on day 0, 10, 20, 27 and 37 with 5μg of pRe/CMV-HBS plasmid DNA as such or incorporated into various cationic DRV liposomes. The differences in the log_{10} values in mice immunised with liposomal DNA and mice immunised with naked DNA were statistically significant (p<0.001-0.005). Each bar represents the mean ± S.D. of a group of four-five mice. End-point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of non-immune serum (control group)
Figure IV.10  Comparison of IgG2b responses in mice injected five times with 5μg of free or liposome-encapsulated pRe/CMV-HBS

Comparison of immune responses in mice injected intramuscularly on day 0, 10, 20, 27 and 37 with 5μg of pRe/CMV-HBS plasmid DNA as such or incorporated into various cationic DRV liposomes. The differences in the log_{10} values in mice immunised with liposomal DNA and mice immunised with naked DNA were statistically significant (p<0.001-0.005). Each bar represents the mean ± S.D. of a group of four-five mice. End-point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of non-immune serum (control group).
Comparison of immune responses in mice injected intramuscularly on day 0, 10, 20, 27 and 37 with 10μg of pRc/CMV-HBS plasmid DNA as such or incorporated into various cationic DRV liposomes. The differences in the log_{10} values in mice immunised with liposomal DNA and mice immunised with naked DNA were statistically significant (p<0.0001-0.002). Each bar represents the mean ± S.D. of a group of four-five mice. End-point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of non-immune serum (control group).
Comparison of IgG2a responses in mice injected five times with 10μg of free or liposome-encapsulated pRe/CMV-HBS DNA

Comparison of immune responses in mice injected intramuscularly on day 0, 10, 20, 27 and 37 with 10μg of pRe/CMV-HBS plasmid DNA as such or incorporated into various cationic DRV liposomes. The differences in the log_{10} values in mice immunised with liposomal DNA and mice immunised with naked DNA were statistically significant (p<0.001-0.005). Each bar represents the mean ± S.D. of a group of four-five mice. End-point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of non-immune serum (control group).
Comparison of immune responses in mice injected intramuscularly on day 0, 10, 20, 27 and 37 with 10μg of pRc/CMV-HBS plasmid DNA as such or incorporated into various cationic DRV liposomes. The differences in the log₁₀ values in mice immunised with liposomal DNA and mice immunised with naked DNA were statistically significant (p<0.01-0.02). Each bar represents the mean ± S.D. of a group of four-five mice. End-point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of non-immune serum (control groups).
Effect of immunisation on IL-4 secreting cells in Balb/c mice after single injection. Animals were injected once with 10μg of pRe/CMV-HBS plasmid DNA alone, incorporated into neutral and cationic DRV liposomes, or complexed with preformed cationic DRV liposomes. Each bar represents the mean ± S.D of cytokine concentration 74 days after injection of plasmid DNA in four to five mice/group. The control bar represents the response of normal unvaccinated mice. The IL-4 responses in the group of mice injected with plasmid DNA incorporated into cationic DRV liposomes were higher than in the groups injected with naked DNA, incorporated into neutral or complexed with preformed cationic DRV liposomes (p<0.05). The results for the latter groups of mice showed no significant differences.
Effect of immunisation on IFN-\(\gamma\) secreting cells in Balb/c mice after a single injection. Animals were injected once with 10\(\mu\)g of pRe/CMV-HBS plasmid DNA alone, incorporated into neutral and cationic DRV liposomes, or complexed with preformed cationic DRV liposomes. Each bar represents the mean ± S.D of cytokine concentration 74 days after injection of plasmid DNA in four to five mice/group. The control bar represents the response of normal unvaccinated mice. The IFN-\(\gamma\) responses in the group of mice injected with plasmid DNA incorporated into cationic DRV liposomes were higher than in the groups injected with naked DNA, incorporated into neutral or complexed with preformed cationic DRV liposomes (p<0.01). The results for the latter groups of mice showed no significant differences.
Effect of immunisation on IL-4 secreting cells in Balb/c mice after multiple injections. Animals were injected five times with 10μg of pRe/CMV-HBS plasmid DNA alone, incorporated into neutral and cationic DRV liposomes, or complexed with preformed cationic DRV liposomes. Each bar represents the mean ± S.D of cytokine concentration two weeks after the final injection of plasmid DNA in four to five mice/group. The control bar represents the response of normal unvaccinated mice. The IL-4 responses in the group of mice injected with plasmid DNA incorporated into cationic DRV liposomes were significantly higher than those in the other groups (p<0.006-0.01).
Figure IV.17  Production of IFN-γ after multiple injections of plasmid DNA

Effect of immunisation on IFN-γ secreting cells in Balb/c mice after multiple injections. Animals were injected five times with 10μg of pRe/CMV-HBS plasmid DNA alone, incorporated into neutral and cationic DRV liposomes, or complexed with preformed cationic DRV liposomes. Each bar represents the mean ± S.D of cytokine concentration two weeks after the final injection of plasmid DNA (day 45) in four to five mice/group. The control bar represents the response of normal unvaccinated mice. The IFN-γ responses in the group of mice injected with plasmid DNA incorporated into cationic DRV liposomes were significantly higher than those in the other groups (p<0.001-0.05).
Figure IV.18  Production of IL-4 after multiple injections of plasmid DNA incorporated into various DRV liposomes

In vivo production of cytokine secreting cells in Balb/c mice immunised with 5μg of pRe/CMV-HBS plasmid DNA in form of free or incorporated into various cationic DRV liposomes five times at 5-weeks intervals. Each bar represents the mean ± S.D. of cytokine concentration of four to five mice/group. The control bar represents the responses of normal unvaccinated mice. The cytokine values in mice immunised with any of the cationic liposomal DNA compositions were significantly higher than in the groups of mice injected with naked DNA (P<0.008-0.01).
In vivo production of cytokine secreting cells in Balb/c mice immunised with 5μg of pRc/CMV-HBS plasmid DNA in form of free or incorporated into various cationic DRV liposomes five times at 5-weeks intervals. Each bar represents the mean ± S.D. of cytokine concentration of four to five mice/group. The control bar represents the responses of normal unvaccinated mice. The cytokine values in all mice immunised with any of the cationic liposomal DNA compositions were significantly higher than in the groups of mice injected with naked DNA (P<0.001-0.008).
V EFFECT OF ROUTE OF INJECTION AND STRAIN OF MICE ON IMMUNE RESPONSES TO ANTIGEN ENCODED BY pRC/CMV-HBS PLASMID DNA

V.1 INTRODUCTION

Although the most common route of administration in human vaccines is the intramuscular injection (Wassef et al, 1994), a number of acquired and inherited diseases are treated by repeated intravenous or subcutaneous injections of recombinant or purified proteins. Among these are diabetes mellitus, which is treated with subcutaneous or intravenous injections of insulin, and pituitary dwarfism, which is treated with subcutaneous injection of growth hormones. Therefore, the route of administration and the physiological attributes of the tissue in which one wishes to achieve DNA expression are important parameters.

We have already demonstrated (this thesis; and Gregoriadis et al, 1997a,b) the effectiveness of DRV liposomes-mediated DNA immunisation following intramuscular injection in Balb/c mice. Among the various liposomal DNA formulations, the most effective one for induction of humoral and cellular responses immunity against plasmid DNA encoded antigen was cationic DRV.

Little is known about the pharmacodynamics or pharmacokinetics of gene delivery, processing and expression. Any further understanding will need to consider the cellular processes involved in gene delivery, uptake and expression. For example,
Sikes et al (1994) have clearly demonstrated that the rate of DNA elimination and the time course of gene expression after DNA injection into the thyroid is significantly different from values reported from DNA injection into muscle cells or receptor-mediated targeting of DNA to the liver (Wolff et al, 1992; Manthorpe et al, 1993). These differences in the kinetics of gene and gene production may reflect either tissue specific differences in the degradation or compartmentalisation of DNA, or differences in the replicative state of different cells, or differences in the mechanism of uptake of DNA vectors into these cells. Tissues also vary in the efficiency with which they present antigen to the immune system. Tissues serving as barrier against the entry of pathogens, such as skin, have associated lymphoid tissues that provide high levels of local immune surveillance (Streilein, 1985, 1989; McGhee & Kiyono, 1992). Such tissues also contain cells that are specialised for major histocompatibility class II restricted presentation of antigens to T helper cells. T helper cells produce the lymphokines, which induce growth and differentiation of lymphoid cells.

In view of the above, we attempted to investigate the efficiency of cationic DRV liposomes as a carrier for pRc/CMV-HBS plasmid DNA vaccine using various routes of administration. In the previous chapter, we studied the effect of dosage and number of injections on antibody and cytokine responses with intramuscular administration. The intramuscular administration of $2 \times 10^4 \mu g$ pRc/CMV-HBS plasmid DNA with a gap of 3 weeks between the two injections had proved to raise all three subclass antibodies (IgG1, IgG2a and IgG2b) and T cell subsets. We therefore used the same low dosage and a similar immunisation regime to compare
the delivery of DNA encapsulated into cationic DRV liposomes via four different routes:

- by a route that usually supports efficient transfection (intramuscular/hind leg);
- by routes that support less efficient transfection but are frequently used for the administration of antigen to test animals (intravenous/tail vein, and intraperitoneal/abdomen);
- and by a route that supports less efficient transfection but delivers DNA to tissues with high levels of local immune surveillance (subcutaneous/interscapular).

Furthermore, the effect of the route of injection on DNA vaccination was investigated in both inbred (Balb/c) and outbred (T/O) mice. In both models, the vaccine consisted of pRc/CMV-HBS plasmid DNA designed to express the hepatitis B surface antigen (S region). The rationale behind the use of outbred mice in this set of experiments was to investigate the potential of our formulation in animals of different genetic background. MHC molecules are highly polymorphic both among different species and within the same species. This means that while MHC molecules of a given MHC class are structurally very similar to one another, there is a wide degree of variation in amino-acid sequence among them (McConnell et al, 1982). Mice can easily be inbred. By performing extensive breeding between siblings, back-crossing with parents, and further inbreeding, one can generate an inbred strain of mouse such that all members of the inbred strain are genetically
identical at the H-2 (MHC) locus of the mouse, i.e. they become homozygous. Outbred mice, on the other hand, have differing epitopes on the MHC due to genetic variation. Inbred mice present therefore less variability to vaccines than outbred mice. However, using outbred mice in vaccination experiments can serve to corroborate the reproducibility of results obtained with inbred mice and ensures that the results are not specific for one given strain only.
V.2 METHODS

V.2.1 Incorporation of plasmid DNA into cationic liposomes

Plasmid DNA pRc/CMV-HBS was encapsulated into liposomes composed of 16 μmoles of phosphatidylcholine (PC); dioleoyl phosphatidylcholine (DOPE), and dioleoyl trimethylammonium (DOTAP) with a molar ratio of 1:0.5:0.25 by a modification of the freeze-drying method, as described by Kirby and Gregoriadis (see section II.2.2). The efficiency of DNA encapsulation was measured by entrapping nick-translated $^{35}$S radiolabelled DNA into the liposomes. DNA encapsulated into cationic liposomes was separated from free DNA by centrifugation, and $^{35}$S counts in the two fractions were recorded (see section II.2.4). Vesicles were then diluted accordingly in PBS so that each mouse (in groups of 4) received a dose of 10 μg in a total volume of 0.1 ml.

The light scattering particle analyser was used for measuring the size of the liposome suspension containing plasmid DNA (see section II.2.5 for full procedure).

V.2.2 Schedule of DNA administration

Balb/c or T/O mice were injected with plasmid DNA encapsulated into cationic DRV liposomes or in form of naked DNA by the intramuscular (I/M), subcutaneous (S/C), intravenous (I/V), or intraperitoneal (I/P) route (four mice per route and strain). In both strains of mice and in all four routes of injection two doses of 10 μg
pRc/CMV plasmid DNA were administered, the first at day 0 and the second 3 weeks later. For detection of anti-HBsAg antibodies, blood was drawn from the tail vein using heparin as anticoagulant, one day before the first injection (as a control) and every week after the first immunisation over a period of six weeks, and the anti-HBsAg titers were assessed by ELISA as previously described (see section II.2.13.1). Statistical differences between two groups (naked DNA vs Liposomal DNA) were performed using student t-test. As we compared only two groups at a time the student t-test is valid test for the observation. Student unpair t-test can be performed for a group between 3>30.

Approximately 6 weeks following the primary immunisation, the spleens of all mice were removed and the IL-4 and IFN-γ levels were determined (see section II.2.14). The spleens of the groups of mice (Balb/c and T/O) without DNA vaccination were used as control.
V.3 RESULTS

V.3.1 IgG responses after immunisation of Balb/c or T/O mice by a variety of routes with pRh/CMV-HBS as such or incorporated into cationic DRV liposomes.

The percentages of plasmid DNA associated with each of the cationic liposomes and the size of the vesicles after removing free DNA from DNA encapsulated with liposomes are shown in Table III.5. As expected from our previous studies, the entrapment of the DNA was considerable and reproducible (80-84% of the amount used) at all preparation times. The size of the vesicles was around 500-620nm and similar to the sizes obtained previously.

Fig. V.1 to V.3 present the IgG_1, IgG_2a and IgG_2b anti-HBsAg responses in Balb/c mice after I/M, S/C, I/V and I/P injection of two doses of 10μg of plasmid DNA encapsulated into cationic liposomes, compared with the group of mice that received the same dose of DNA in free form. The data from intramuscular injection in this experiment were very similar to our previous experiment with the similar formulation (chapter IV). Mice injected intramuscularly with liposomal DNA boosted the IgG titers (all subclasses tested) markedly higher than mice injected with free DNA (p<0.001). Sera from all groups of mice collected for six weeks after the first injection showed the earliest significant responses at day 21. The antibody titers peak was at day 38 for the group of mice injected with cationic DRV liposomes. Judging from the antibody assay, similar results were obtained when DNA encapsulated into cationic liposomes was injected intramuscularly in T/O...
mice (Fig. V.4, V.5 and V.6). Although the antibody titers were slightly higher than in the Balb/c group, the data showed overall no statistically significant differences.

Fig. V.1 to V.6 also show the results from subcutaneous and intravenous injections of the two strains of mice with two doses of 10μg of pRc/CMV-HBS encapsulated into cationic liposomes or in form of free DNA. Again, both strains of mice exhibited similar results and the immune responses in the groups of mice treated with the liposomal formulation were comparable to those observed after intramuscular administration, i.e. they were significantly higher than in the groups injected with DNA alone. Relatively increased responses could be observed in Balb/c mice injected subcutaneously with naked DNA however, these responses did not last for a long period of time.

The same experiments as those described for I/M, S/C or I/V were carried out using the intraperitoneal route. As can be seen from Fig. V.1 to V.6, the immune response results for the I/P route were markedly lower than for the other three routes of administration. Even though the mice injected with liposomal DNA were still able to show better responses than the mice treated with naked DNA, the differences between the two formulations were not statistically significant.

The above experiments demonstrated a significant increase in specific antibody titers when mice were injected via the intramuscular, subcutaneous and intravenous route of injection, regardless of the strain of mice.
V.3.2  Cytokine production

Fig. V.7 to V.10 show that the cytokine assay results confirmed the data from our humoral experiments. The spleens of mice were removed two weeks after the final injection and the levels of interferon-γ (IFN-γ) and interleukin-4 (IL-4) were measured as indicators of Th1 and Th2 subset T cell activation. With the exception of the intraperitoneal route, it was evident that regardless of the route of administration and the strains of mice the activation for both IL-4 and IFN-γ cytokine was greater with cationic DRV liposome encapsulated DNA than with naked DNA.
The experiments presented in this chapter were carried out for two reasons. First, we have previously noted that the best immune responses are achieved by pRc/CMV HBS plasmid DNA encapsulated into cationic DRV, but we do not know whether such responses are due to muscle uptake, or to what extent other tissues may be involved in this activity. Second, we wanted to investigate the effect of various routes and strains of mice on the induction of immune responses with the cationic DRV delivery system.

Previous work by Fynan et al (1993) on gene expression from inoculated plasmid DNA using the gene-gun has provided evidence for significant effects of the vehicle and the route of DNA delivery. The present study complements and extends the observations by other laboratories and compares for the first time the ability of pRc/CMV-HBS plasmid DNA in saline form to induce antibodies and cellular immune responses with that of pRc/CMV-HBS plasmid DNA encapsulated into cationic DRV liposomes.

Our results have confirmed that DRV formulated plasmid DNA combines efficient transfection with efficient antigen presentation and recognition. With the exception of the intraperitoneal route, significant (p<0.08-0.001) anti-HBsAg levels in both Balb/c and T/O mice were elicited by all routes of injection, when DNA was encapsulated into cationic DRV liposomes, in contrast to plasmid DNA in saline formulation (Naked DNA).
We have provided the first evidence that plasmid DNA incorporated into cationic DRV liposomes is able to elicit both Th1 and Th2 immune responses via other routes than intramuscular injection. Our results from both strains of mice (Balb/C and T/O) demonstrated that not only the intramuscular, but also the subcutaneous and intravenous routes of administration can be effective for DNA vaccines when the plasmid is formulated with cationic DRV liposomes. Moreover, the IgG1 titers were augmented after the second injection suggesting that boosting had occurred. A boosting injection of liposomal DNA vaccine also resulted in a dramatic increase in the HBs-specific IgG2a response.

Although the intraperitoneal route of immunisation is commonly used to prime immune activity, we noted only modest humoral and cytokine responses in I/P injected mice. A plausible explanation would be that large DRV are unable to reach the lymphatic system or are unstable in the peritoneal environment.

It is well established that in vivo liposomes are avidly taken up by phagocytes of the mononuclear phagocytic system (MPS), including macrophages (Gregoriadis, 1990; Ishioka et al, 1989; Lasic, 1996). I/V injected liposomes are taken up almost exclusively by phagocytes associated with the liver, spleen and bone marrow due to a natural tendency of these cells to take up (opsonin-coated) foreign particles resulting in an increased presentation of antigenic fragments to helper T cells. On the other hand, only a minor part of S/C or I/M injected liposomes is taken up by the organ-associated MPS; rather, they are phagocytosed by APC that are associated with draining lymph nodes or infiltrate the site of injection (O'Hagan et
al, 1992; Velinova et al, 1996). Lymphoid tissue is rich in cells capable of presenting transfected antigens to the T-helper component of the immune system (Streilein, 1983 & 1989). Qussoren and Storm (1997) have shown that after S/C injection of liposomes into the dorsal side of the foot of rats, liposomes are efficiently absorbed via lymphatic capillaries draining the site of injection. Once liposomes have entered the lymphatic capillaries, they pass through the lymphatic system where they can be captured in regional lymph nodes.

It has been suggested that phagocytosis by macrophages is one of the major mechanisms of uptake of liposomes in lymph nodes. For many years it was generally assumed that macrophages are the predominant antigen-presenting cells (Ishioka et al, 1989; O'Hagan et al, 1992; Velinova et al, 1996). However, Van Rooijen and Sanders (1994) have shown that macrophages in lymph nodes and the spleen can be efficiently depleted using a bisphosphonate in liposomes, and that such depletion augments immune responses to soluble and viral protein. This suggests that while macrophages partially digest large organisms for presentation of antigens, other antigen-presenting cells, such as dendritic cells, play a major role. Others (Allen, 1994 Igantius et al 2000) have confirmed the above view by demonstrating that liposomes coated with polyethylene glycol (PEG) oppose macrophage uptake and are taken up by other APC, such as

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immature dendritic cells. Once immature DC pick up antigens, they migrate to the regional lymph nodes (LN). Upon arrival in the LN, they display a mature phenotype and present antigens to lymphocytes, efficiently activating naïve and memory T-cell and B-cell responses. Further investigation of the action of each type of APC could help to understand their functions better and thus improve future vaccine design.

Striated muscle is considered to be the only tissue that takes up and expresses plasmid DNA efficiently in the absence of a viral vector or a physical carrier (Wolff et al, 1990; Davis et al, 1993). However, muscle cells are unlikely to be the site for antigen presentation because they contain few if any dendritic cells, macrophages, and lymphocytes (Raz et al, 1994). Blood, on the other hand, has associated lymphoid tissues that provide specialized and highly active immune surveillance. The success of liposomal DNA immunisation by the intravenous route may therefore reflect efficient antigen presentation to very low numbers of transfected cells, while the success of I/M and S/C injections of liposomal DNA could reflect the combination of efficient transfection with efficient antigen presentation and recognition.

Our results indicate that liposome-mediated DNA immunisation is more effective than the use of naked DNA, and also suggest that the presence of cationic lipid in DRV in conjunction with an appropriate content of fusogenic phosphatidylethanolamine (Dope) might contribute to more effective liposomal DNA vaccines.
Production of anti-HBsAg antibodies after plasmid DNA immunisation. Balb/c mice were injected I/M (A), S/C (B), I/V (C) or I/P (D) on days 0 and 21 with 10 μg of pRe/CMV-HBS encapsulated in positively charged liposomes composed of PC, DOPE and DOTAP or in the naked form. Animals were bled one day before the immunisation and every week after the first injection and sera were tested for IgG1 responses against the encoded antigen hepatitis B surface antigen. Values are mean ± S.D. of four animals/time points. * p<0.005, **p<0.001, P values are comparisons with the group received naked DNA injections.
Figure V.2  Comparison of IgG₂ₐ responses in Balb/c mice injected with plasmid DNA by the I/M, S/C, I/V and I/P routes

Production of anti-HBsAg antibodies after plasmid DNA immunisation. Balb/c mice were injected I/M (A), S/C (B), I/V (C) or I/P (D) on days 0 and 21 with 10 µg of pRe/CMV-HBS encapsulated in positively charged liposomes composed of PC, DOPE and DOTAP or in the naked form. Animals were bled one day before the immunisation and every week after the first injection and sera were tested for IgG₂ₐ responses against the encoded antigen hepatitis B surface antigen. Values are mean ± S.D. of four animals/time points. * p<0.01, **p<0.008. P values are comparisons with the group received naked DNA injection.
Figure V.3  Comparison of IgG2b responses in Balb/c mice injected with plasmid DNA by the I/M, S/C, I/V and I/P routes

Production of anti-HBsAg antibodies after plasmid DNA immunisation. Balb/c mice were injected I/M (A), S/C (B), I/V (C) or I/P (D) on days 0 and 21 with 10 μg of pRe/CMV-HBS encapsulated in positively charged liposomes composed of PC, DOPE and DOTAP or in the naked form. Animals were bled one day before the immunisation and every week after the first injection and sera were tested for IgG2b responses against the encoded antigen hepatitis B surface antigen. Values are mean ± S.D. of four animals/time points. * p<0.05, **p<0.01. P values are comparisons with the group received naked DNA injection.
Production of anti-HBsAg antibodies after plasmid DNA immunisation. T/O mice were injected I/M (A), S/C (B), I/V (C) or I/P (D) on days 0 and 21 with 10μg of pRe/CMV-HBS encapsulated in positively charged liposomes composed of PC, DOPE and DOTAP, or in the naked form. Animals were bled one day before the immunisation and every week after the first injection and sera were tested for IgG1 responses against the encoded antigen hepatitis B surface antigen. Values are mean ± S.D. of four animals/time points. * p<0.005, **p<0.001, ***p<0.0008. P values are comparisons with the group received naked DNA injection.
Figure V.5  Comparison of IgG_{2α} responses in T/O mice injected with plasmid DNA by the I/M, S/C, I/V and I/P routes

Production of anti-HBsAg antibodies after plasmid DNA immunisation. T/O mice were injected I/M (A), S/C (B), I/V (C) or I/P (D) on days 0 and 21 with 10 µg of pRe/CMV-HBS encapsulated in positively charged liposomes composed of PC, DOPE and DOTAP, or in the naked form. Animals were bled one day before the immunisation and every week after the first injection and sera were tested for IgG_{2α} responses against the encoded antigen hepatitis B surface antigen. Values are mean ± S.D. of four animals/time points. * p<0.05, **p<0.001. P values are comparisons with the group received naked DNA injection.
Figure V.6 Comparison of IgG_{2b} responses in T/O mice injected with plasmid DNA by the I/M, S/C, I/V and I/P routes

Production of anti-HBsAg antibodies after plasmid DNA immunisation. T/O mice were injected I/M (A), S/C (B), I/V (C) or I/P (D) on days 0 and 21 with 10μg of pRc/CMV-HBS encapsulated in positively charged liposomes composed of PC, DOPE and DOTAP or in the naked form. Animals were bled one day before the immunisation and every week after the first injection and sera were tested for IgG_{2b} responses against the encoded antigen hepatitis B surface antigen. Values are mean ± S.D. of four animals/time points. * p<0.05, **p<0.001. P values are comparisons with the group received naked DNA injection.
Cytokine levels in the spleens of Balb/c mice which had received 10μg of pRc/CMV-HBS plasmid DNA alone or encapsulated into cationic DRV liposomes intramuscularly, subcutaneously, intravenously or intraperitoneally. Each bar represents the mean ± S.D of cytokine concentration one week after the final injection of plasmid DNA in four mice/group. The control bar represents the response of normal unvaccinated mice. *p<0.01, **p<0.005. P values are comparisons with the group received naked DNA injection.
Figure V.8  Production of IFN-γ in Balb/c mice injected with plasmid DNA by the I/M, S/C, I/V and I/P routes

Cytokine levels in the spleens of Balb/c mice which had received 20μg of pReCMV-HBS plasmid DNA alone or encapsulated into cationic DRV liposomes intramuscularly, subcutaneously, intravenously or intraperitoneally. Each bar represents the mean ± S.D of cytokine concentration one week after the final injection of plasmid DNA in four mice/group. The control bar represents the response of normal unvaccinated mice. *p<0.01, **p<0.001. P values are comparisons with the group received naked DNA injection.
Cytokine levels in the spleens of T/O mice which had received 20μg of pRc/CMV-HBS plasmid DNA alone or encapsulated into cationic DRV liposomes intramuscularly, subcutaneously, intravenously or intraperitoneally. Each bar represents the mean ± S.D of cytokine concentration one week after the final injection of plasmid DNA in four mice/group. The control bar represents the response of normal unvaccinated mice. *p<0.01, **p<0.008. P values are comparisons with the group received naked DNA injection.
Cytokine levels in the spleens of T/O mice which had received 20μg of pRc/CMV-HBS plasmid DNA alone or encapsulated into cationic DRV liposomes intramuscularly, subcutaneously, intravenously or intraperitoneally. Each bar represents the mean ± S.D of cytokine concentration one week after the final injection of plasmid DNA in four mice/group. The control bar represents the response of normal unvaccinated mice. *p<0.01, **p<0.001. P values are comparisons with the group received naked DNA injection.
Advances in molecular biology have resulted in a new concept for the treatment of diseases, the concept of gene therapy. The ability to successfully introduce nucleic acid sequences and DNA into cells has brought to light a new approach for the treatment of many human diseases, such as cancer, inflammatory diseases, infectious diseases, vascular diseases, as well as inheritable genetic abnormalities. Gene therapy requires that genes are effectively delivered to certain cells, transferred across the periplasmic membrane into the body of the cell, and compartmentalised within the nucleus where gene expression can occur.

Gene vaccines, or the use of antigen-encoding DNA for vaccination, may be an attractive approach for the induction of anti-microbial or anti-tumour immunity. Nucleic acid based immunisation involves the induction of immune responses to a protein antigen expressed in vivo subsequent to the introduction of sequences encoding an antigenic polypeptide. In contrast to vaccines that employ recombinant bacteria or viruses, genetic vaccines consist only of DNA (as plasmid) or RNA (as mRNA), which is taken up by the cells and translated into protein (Davis & Whalen, 1995). Uptake of plasmid DNA has been shown to be most efficient in striated muscle cells (i.e. cardiac and skeletal muscle), although other tissues may also be transfected with plasmid DNA, albeit with a much lower efficiency (Davis et al, 1995). Despite the large number of genetic vaccine studies conducted so far, many of the results are difficult to compare and inconsistent. The mechanisms underlying the different efficacies of various DNA vaccines
have not yet been clearly addressed, but they are presumably related to the
efficacy of transfection, the ability of the protein to be appropriately presented to
the immune system, and the expression and antigenic nature of the encoded
antigen.

The substantial quantities of DNA required for successful immunisation and/or the
need for special immunity enhancing techniques, such as the pre-treatment or
regeneration of muscles (Davis et al, 1993a,b), indicate the limitations of cellular
uptake of naked DNA as such. Thus, there is a need for the development of well-
defined, potent carrier systems. Viruses and recombinant viruses are presently the
most efficient systems for delivering genes to cells. Plasmid vectors are available
and interest in developing this technology is a result of concerns about the safety of
viral vectors and of limits in the viral insertion size.

Pharmaceutical experience with advanced drug delivery, especially the
development of particulate drug carriers for drug targeting and controlled release,
provides a foundation for the development of non-viral gene therapies. Certain
methods for controlled delivery of small molecule drugs or proteins may be useful
for delivering genes to cells in vitro and in vivo. These include the use of cationic
lipids, liposomes, targeting ligands, and polymeric carriers. Although in principle
the use of liposomes in gene therapy is a promising new research area, the system
depends to a large degree on technological improvements in the formation of
vesicles of various sizes and properties. We have chosen liposomes as a DNA
carrier because they can be formulated so as to successfully meet the main
challenges of drug delivery, such as efficiency, content incorporation and retention, plasma stability, circulation lifetimes, biodistribution, immunogenicity and vascular permeability (Gregoriadis, 1979; Kirby & Gregoriadis, 1981; Jain, et al, 1989; Gangadharam et al, 1995; Cullis et al, 1997). Our laboratory's experience with using liposomal formulations for vaccine purposes was considered an additional benefit. The dehydration-rehydration method was our chosen preparation method for liposomal DNA vaccine carriers because this technique requires no detergents and organic solvents, offers high yield encapsulation of substances with high protection against enzymatic attack, and is suitable for scale-up.

In this thesis, we focused on the pharmaceutical improvement of DRV liposomal carriers with the aim to enhance the therapeutic activity of plasmid DNA. To this end, we set out to design a carrier system that could:

- protect plasmid DNA from rapid nuclease degradation;
- disperse and retain intact plasmid in the tissue or organ; and
- facilitate the uptake of plasmid DNA by the cells.

Our in vitro studies demonstrated that the encapsulation of DNA into liposomes produced by the dehydration-rehydration method offers a number of potential advantages over other liposomal systems:
1) The ability of DRV liposomes to incorporate quantitatively nucleic acids

In order to achieve optimum efficacy and cost effectiveness for a nucleic acid delivery system, we studied the ability of various types of liposomes to encapsulate plasmid DNA. Data achieved from systematic experiments showed that plasmid DNA can be quantitatively incorporated by the dehydration-rehydration method into multilamellar liposomes composed of PC and DOPE alone or supplemented with anionic or cationic lipids. Significantly, even DRV liposomes microfluidised to smaller vesicles were able to retain much of the original DNA without damaging the structural integrity of the DNA.

Furthermore, the size of DRV liposomes did not alter with the incorporation of plasmid DNA. On the other hand, when the same lipid composition was used for liposomal mixtures with plasmid DNA, the size of the DRV complex formation increased significantly, in particular in the case of liposomes with cationic surface charge. Such aggregation may be due to liposome-DNA-liposome crosslinking. Positively charged liposomes bind to negatively charged phosphate molecules on the DNA backbone through electrostatic interactions, forming a complex between the liposomes and the DNA. Although complex formation provides an efficient way to associate DNA with liposomes, the binding reaction is driven by interactions between two polyvalent surfaces. Such multivalent binding reactions typically result in aggregation of the components (Sen & Crothers, 1986; Mahato et al, 1995). In the case of encapsulation, the nucleic acid is probably entrapped within the liposomes rather than adsorbed onto their surface, an assumption
substantiated by our biological stability tests (see point 3 below). This true entrapment would prevent aggregation on the surface.

2) Liposomal DNA stability during storage in various temperature

From the pharmaceutical point of view, it is important to have a formulation that is stable during storage. Judging from the radioactivity release, there was no substantial loss of DNA during storage of liposomal DNA at $4^\circ$C, and the initial vesicle size remained unaltered. However, microfluidised DRV liposomes containing nucleic acid presented less stability during storage at various temperatures. It appears that multi lamellarity of the vesicles improves the storage stability, at least when nucleic acids are the content.

3) The ability to protect the content from enzymatic attack and structural integrity of DNA encapsulated into DRV liposomes.

An important prerequisite for the effective use of liposomes as a drug carrier is control over their biological stability (Gregoriadis, 1979). Defined here as the extent to which the carrier retains its contents \textit{in vitro} or \textit{in vivo}, stability is influenced not only by the biological environment with which liposomes come into contact, but also by their structural characteristics as well as those of the associated drugs (Kimelberg, 1978; Mayhew et al, 1978; Gregoriadis, 1979). Provided the DNA is successfully incorporated into the vesicles without denaturation, the vesicles should
be able to protect the content from enzymatic attack and degradation within the vesicles and thus retain the originally incorporated content.

In fact, one of the major drawbacks of current cationic lipids mixed with plasmid DNA is their inability to protect the nucleic acids in the presence of plasma and enzymes, i.e. their lack of biological stability, which renders them ineffective *in vivo*. We have therefore compared the stability of plasmid DNA alone, mixed with cationic liposomes, and encapsulated into DRV liposomes in the presence of both plasma and deoxyribonuclease. Again, we found our expectations regarding the virtues of the dehydration-rehydration preparation technique confirmed in that DNA encapsulated into DRV liposomes appeared to be relatively stable in the presence of plasma and was protected from degradation by nuclease.

In this context, we could also determine two important factors for a successful delivery system: the roles of the carrier and of the physical location of the DNA in the carrier. We observed that DNA alone is digested completely in the presence of enzymes and that this may explain the need for using high amounts of DNA for successful transfection *in vivo* with DNA alone. This result was further corroborated by experiments where samples of free and DRV liposomal DNA were exposed to DNase and subjected to agarose gel electrophoresis. In addition, we could clearly demonstrate the significant difference between two liposomal preparation techniques. While our data support results from other research laboratories suggesting that cationic preformed liposomes can quantitatively interact with nucleic acid, DNA complexes with preformed liposomes failed to
protect the content against degradation by nuclease to the same degree as nucleic acid encapsulated into DRV liposomes. This is supposedly due to the fact that cationic vesicles complexed with DNA rely on electrostatic interaction between the positive charge of lipids and the negative charge of nucleic acids rather than on true encapsulation of the DNA. DNA encapsulated into DRV liposomes, on the other hand, appears to be not just bound externally but primarily entrapped within the vesicle bilayers, as a result of which enzymes do not have access to the interior of the liposomes. Although liposomes with a positive surface charge presented higher recovery of DNA than neutral or anionic DRV, this does not abolish the fact that DRV liposomes offer true entrapment regardless of the surface charge.

4) Transfection and cell viability

Our data confirm the observation by other researchers that cationic liposomes represent a highly efficient vehicle for the delivery of DNA molecules into a large variety of eukaryotic cells (Felgner et al, 1987; Ballas et al, 1988; Felgner et al, 1989; Malone et al, 1989). Of all liposomal preparations tested, the highest transfection in vitro was achieved by cationic liposomes. Transfection appears to be greatly facilitated by the presence of cationic lipids. The microfluidisation of cationic DRV liposomes showed further transfection improvement in comparison with multilamellar cationic DRV liposomes, confirming the widely accepted view that the vesicle size also influences the transfection activity.
The final step prior to in vivo experiments was to assess the cytotoxic effect of our DRV preparations. To eliminate the risk of toxicity in our in vivo studies, we examined the cell toxicity of different molar ratios of cationic DRV liposomes. Our preliminary cytotoxicity data showed that none of the cationic DRV liposomes exhibited any sign of toxicity on the cells. This could be explained by the low amount of cationic lipid required by the dehydration-rehydration preparation method.

Although the optimal conditions for transfection efficiency and cytotoxicity in vitro may differ from those in vivo, and in vitro results cannot be directly related to the situation in vivo, our in vitro assays provided promising data for our immunisation experiments with a variety of DRV liposome compositions incorporating plasmid DNA. We analysed immune responses to free, complexed or encapsulated plasmid DNA expressing hepatitis B virus surface antigen (HBsAg) and established a dose response curve for this vector.

Single dose DNA vaccination is known to produce humoral responses (Davis et al, 1993a; 1993b). However, in our single dose experiments barely any anti-HBsAg IgG (IgG1, IgG2a and IgG2b subclasses) responses for naked DNA or complexed DNA were detectable even after ten weeks. This is most likely due to the fact that our doses of pRc/CMV-HBS (2 and 10μg) were much lower than those normally used for naked DNA immunisation (100μg or more) (Davis et al, 1993a). In contrast, a single dose injection of 10μg DNA encapsulated into
cationic DRV liposomes augmented the immune responses to the antigen significantly, even though the response dropped after 2 months.

Sedegah et al (1994) have reported that the dose of plasmid DNA and the number of immunisations influence the immune responses to DNA vaccines. While Gardner et al (1996) substantiated this view, they also pointed out that for DNA immunisation there is no simple guideline to follow and each plasmid must be evaluated independently. We therefore attempted to examine the influence of the immunisation regime and the total dosage by using pRc/CMV-HBS plasmid DNA in form of free, complexed with cationic liposomes, or encapsulated into DRV liposomes. Results from multiple injections with two doses (1 & 10 μg) of pRc/CMV-HBS at three weeks intervals demonstrated significant increases in specific antibody titers in the groups of mice injected with plasmid DNA encapsulated into cationic liposomes from 1μg to 10μg per immunisation. The same experiment with naked DNA showed similar results as a single dose injection.

Single injection of plasmid DNA incorporated into DRV cationic liposomes in mice induced the production of IgG₁ and very low titers of IgG₂a. Use of ELISA to detect the isotypes of anti-HBsAg antibodies demonstrated that the multiple immunisations had primed all three subclasses of IgG₁, IgG₂a, and IgG₂b. Interestingly, mice receiving multiple injections of plasmid DNA encapsulated into cationic DRV liposomes showed mixed Th1 (IFN-γ) and Th2 (IL-4) responses. From all these observations it is evident that even low doses of plasmid DNA encapsulated into cationic DRV liposomes are able to enhance both humoral and
cellular immune responses, while injecting the same amount of naked plasmid DNA results in barely detectable or much weaker immune responses.

Our experiments with multiple injections of pRc/CMV-HBS revealed dramatic differences between incorporation and complexion of DNA with DRV liposomes. While the group of mice immunised with DNA encapsulated into cationic DRV liposomes boosted all three subclasses of IgG (IgG1, IgG2a, and IgG2b) responses, sera from mice immunised with cationic liposomes mixed with plasmid DNA showed low antibody activity at all time points.

Given that cationic DRV demonstrated significantly better immunisation results than the other DRV types, the lipid compositions of the liposomes also appear to play an important role. The type of lipid in the DRV liposomal composition seems to influence the immune response, probably by affecting the interaction with the cells. The analysis of antibody subclasses provided evidence that cationic liposomal DNA acts as a potent stimulator of cells responsible for antigen-specific IgG2a production. Since this antibody isotype has been shown to be dependent on the presence of IFN-γ (Snapper & Paul, 1987; Brewer & Alexander, 1992), our data suggest that DNA incorporated into cationic DRV liposomes induces strong expansion of the Th1 subset of the T lymphocytes. According to previous studies (Yokoyama et al, 1995; Whalen et al, 1996), naked DNA stimulates the production of Th1 responses with no or low Th2 T cell response. However, we observed in all our immunisation studies that cationic DRV liposomal DNA induces both Th1 and Th2 subsets of T lymphocytes. This indicates that the higher activation of the Th1
type by cationic DRV liposomal DNA does not simultaneously inhibit the production of the Th2 associated IgG\textsubscript{1} isotype.

The final part of the thesis was focused on the ability of cationic DRV liposomal DNA to induce antibody and cytokine activity in other organs than muscle. In order to corroborate our results and confirm their reproducibility, we also wished to determine whether a DNA-mediated immune response could be elicited in a mouse strain with diverse genetic background (i.e. outbred). The results from both strains of mice demonstrated that the intramuscular, the subcutaneous, and the intravenous route of administration can be used for DNA vaccines. Although the induction of antibodies and cytokines varied depending on the route of injection, the administration of cationic DRV liposomes encapsulating DNA via the I/M, the S/C, and the I/V route primed the cell immunity system. Among the four different routes of injection only the intraperitoneal route proved to be ineffective.

In all the experiments performed \textit{in vitro} and \textit{in vivo} we observed no sign of contamination in our plasmid DNA (i.e. cell contamination, animals shivering, inflammation at the side of injection, or sudden death of animals after injection). Behaviour and appearance of animals during the time of the experiments were under constant surveillance by veterinarians and technicians at the School of Pharmacy's Animal House and they observed nothing that could have been interpreted as an indicator for contamination in the animals. However, while the plasmid DNA used in our experiments complied with all approval specifications, we cannot be certain that it was absolutely endotoxin-free. First, the level of
endotoxin was not measured in-house and we relied on the information provided by the supplier. Second, there is increasing evidence that the downstream processing of plasmid DNA production used in most laboratories and small companies may require further development and optimisation of plasmid DNA purification processes. In general, the final purification step for plasmid DNA is obtained by a single anionic exchange chromatographic (AEC) step. The copurification of gDNA, high molecular weight RNA, and endotoxins with plasmid DNA during AEC is inevitable because these molecules have similar affinities for anion-exchange matrices (Marquet, 1995). Although a final plasmid preparation with a single (AEC) step complies with approval specifications (i.e. undetectable RNA and proteins, <6 (ng gDNA) (µg plasmid)\(^{-1}\), <0.1 (endotoxin units) (µg plasmid)\(^{-1}\)) (Ferreira et al, 2000) and is sufficient for laboratory research, further desalting and buffer exchange operations, such as diafiltration or SEC, are required to obtain a final "clinical " plasmid solution. It appears that QIAGEN has now developed a new buffer system for efficient removal of lipopolysaccharides from DNA preparations, the endotoxin removal, which reduces endotoxin contamination to less than 50 E.U/mg DNA and is safe for clinical use.
VII GENERAL CONCLUSIONS

Based on the data from our *in vitro* and *in vivo* studies with cationic DRV liposomes, we believe that the level and type of immune response induced by plasmid DNA depends mainly on the following factors:

1) the protection of plasmid DNA from degradation by nuclease in the serum and at the site of injection or interstitial spaces;
2) the improved local delivery by enhanced cellular uptake and binding to a specific cell population;
3) the release of DNA into the cytoplasm and intracellular transport across the nuclear membrane.

Our immunisation studies demonstrated that the encapsulation of DNA into DRV liposomes contributes significantly to the induction of both humoral and cellular immune responses. Among all formulations examined *in vivo*, cationic DRV liposomes proved the most effective carriers. Plasmid DNA encapsulated into cationic DRV liposomes augmented antibodies 10-100 fold more than naked plasmid DNA. Serum Ig isotype patterns of immunised mice revealed that both Ag-specific IgG_1_ and Ag-specific IgG_2_a_ antibodies were induced by DNA encapsulated into cationic DRV liposomes. Given that strong humoral responses and induction of IL-4 induce switching towards IgG_1_ and the induction of IFN-γ induces switching to IgG_2_a_, it appears that both effectors arms were influenced by cationic DRV liposomes.
As we have successfully demonstrated the potential use of cationic DRV liposomes for DNA vaccination, it would be desirable to understand more about the system. Three issues in particular warrant further investigation. The first concerns the mechanism by which the liposomal formulation of pRc/CMV-HBS plasmid DNA elicits a Th1 and Th2 response. Until recently, studies on the mechanism of action of liposomes focussed on macrophages as dominant APC. However, the importance of other cell types, especially dendritic cells, has now been demonstrated and these cells have attracted considerable interest in the field of genetic vaccination. A more comprehensive understanding of the mechanisms behind the successful immune responses to cationic DRV liposomal DNA may lead to more refined delivery systems that could elicit appropriate immune responses for any given DNA.

The second area requiring further research concerns immune responses in challenge studies. In our animal immunisation, we have shown that encapsulation of pRc/CMV HBS plasmid DNA into cationic liposomes results in a dramatic increase in humoral and cellular immune responses to HBsAg. However, it would be interesting to study immune responses and the type of the immune responses obtained in animal models in which challenge studies can be performed. In the case of pRc/CMV HBS plasmid DNA, chimpanzees are the only animals that HBV can infect. A virulent challenge with the virus cannot be performed in other species. An alternative would be to utilise our formulation for the encapsulation of other envelope proteins, such as influenza HA plasmid DNA.
As a third area of further research, it may be worthwhile to enhance the efficacy of genetic vaccines by tailoring plasmids with inserts of immunostimulatory sequences or a variety of adjuvant cytokines and to examine the delivery of such constructs to immunocompetent cells with systems such as liposomes. It has been shown (Conry et al, 1996; Chow et al, 1997; Kim et al, 1998) that cytokines as well as chemokines encoded on plasmid DNA or as cDNA modulate or enhance a DNA vaccine induced immune response. The combination of a carrier system with the adjuvant effect of cytokines may significantly improve the efficacy of DNA vaccination.

The quick acceptance of genetic vaccines in experimental settings is due to the many advantages this strategy has over traditional vaccines. Indeed, DNA vaccines can circumvent many of the problems associated with recombinant protein-based vaccines, such as high costs of production, difficulties in purification, and incorrect folding of antigen. DNA also has clear advantages over recombinant viruses, which are plagued with the problems of pre-existing immunity, risk of insertion-mutagenesis, loss of attenuation, or inadvertent infection.

However, before this new technology becomes clinically available, several safety and regulatory issues need careful consideration. One of the primary concerns is that continuous expression of the antigenic protein (in particular in muscle, which is postmitotic) might induce immunological tolerance. Another safety concern is the possibility that the DNA may integrate into the host’s chromosomal DNA and
cause an insertional mutagenic event by activation of a protooncogene or by inactivation of a tumor suppressor gene. Finally, while the adjuvant effect of CpG motifs appears to be a desirable and necessary event for the induction of strong immune responses with DNA vaccines, there may be situations where a strong Th1 stimulatory effect that includes IL-12 induction is undesirable, such as in persons prone to autoimmune disease.

And last but not least, pharmaceutical aspects of DNA carrier systems require attention. Although the potential of various carrier systems for DNA delivery has been widely demonstrated in laboratories, the complexity of the preparation, technical difficulties, and the costs of raw material need to be thoroughly counterbalanced for a scale-up formulation.
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