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STRATEGIES FOR THE INDUCTION OF MUCOSAL IMMUNITY AGAINST HEPATITIS B VIRUS

By

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To my parents,

Robert Aloysius McCluskie and

Maureen Agnes Fitzgerald McCluskie

ABSTRACT

Most conventional vaccines are administered parenterally (e.g. by intramuscular (IM) or intradermal (ID) injection) and induce systemic but rarely mucosal immunity. Novel vaccination strategies capable of inducing both systemic and mucosal immune responses could greatly reduce infection and morbidity. One such strategy is DNA vaccination whereby the antigen is synthesized *in vivo* after direct introduction of its encoding sequences. In this thesis, we show that the route of administration of plasmid DNA encoding the hepatitis B surface antigen (HBsAg) influences the strength and nature of immune responses in mice and non-human primates. Mucosal immunization using plasmid DNA in saline results in no or weak immune responses. Formulation of DNA with lipid increases levels of reporter gene expression, but apparently not sufficiently to raise immune responses against expressed antigen indicating that other factors are involved. The strong immune responses induced after parenteral administration of DNA appears to be partly due to the adjuvant effect of unmethylated immunostimulatory CpG motifs present in the DNA backbone. Synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs (CpG) are potent adjuvants for induction of Th1-like systemic immune responses against parenterally-delivered proteins. Herein, the adjuvanticity of CpG ODN relative to or in combination with cholera toxin (CT), *Escherichia coli* heat-labile enterotoxin (LT), the B subunit of CT (CTB) and a non-toxic derivative of LT (LTK63) was evaluated with intranasal delivery. We also evaluate the potential of administering immunostimulatory complexes (comprised of HBsAg

complexed with antibodies against HBsAg) by a mucosal route and determine whether immune responses are modulated by using CT or CpG as adjuvants. We demonstrate that CpG ODN, CT and LT augment anti-HBs titers equally, and this is more than with CTB or LTK63. CpG ODN acts synergistically with CT and LT but not CTB or LTK63, however for all combinations, CpG induces a more Th1-like response. The mucosal immune response induced is not restricted to the site of application but is also present at distant mucosal surfaces. These studies may lead to the development of novel human vaccines that protect at the level of entry for mucosal pathogens and may thereby prevent many infectious diseases.

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2-ME	2-mercaptoethanol
Ab	antibody
ADP	adenosine diphosphate
Ag	antigen
ANOVA	analysis of variance
anti-HBs	HBsAg-specific Abs
anti-DHBs	DHBsAg-specific Abs
AP	activating protein
APC	antigen presenting cell
ATF	activating transcription factor
β-Gal	beta-galactosidase
BALT	bronchus-associated lymphoid tissue
CCAC	The Canadian Council on Animal Care
CD	complementary determinant, e.g. CD8+
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane receptor
CMCS-L	low viscosity carboxymethylcellulose sodium salt
CMI	cell-mediated immunity
CMIS	common mucosal immune system
CMV	cytomegalovirus
CO_2	carbon dioxide

CpG	cytosine-guanine dinucleotides, where p indicates the phosphate bond
CSF	colony stimulating factor
CT	cholera toxin
CTB	B subunit of CT
CTL	cytotoxic T lymphocyte
CTS106	mutant of CT bearing a proline-to-serine at position 106
DHBcAg	duck hepatitis B core antigen
DHBsAg	duck hepatitis B surface antigen
DHBV	duck hepatitis B virus
DLPE	dilauroylphosphatidylethanolamine
DMPE	dimyristoylphosphatidylethanolamine
DMRIE-C	(+)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis-(tetradecyloxy)-1-propanaminium bromide-cholesterol
DNA	deoxyribonucleic acid
DOPE	dioleylphosphatidylethanolamine
DOSPA	2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propaninium trifluoracetate
DOTMA	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride
DP$^{\Delta}$PE	dipalmitoleoylphosphatidylethanolamine
E:T	effector-to-target ratio
EDTA	ethylenediaminetetraacetic acid

ELISA	enzyme linked immunosorbent assay
FA	fumaric acid
FAE	follicle-associated epithelium
FBS	fetal bovine serum
FcγR	receptor for Fc fragment of IgG
GALT	gut-associated lymphoid tissue
GAP-DLRIE	(+)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide
GG	gene-gun
GI	gastrointestinal
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMT	geometric mean titer
GTP	guanosine triphosphate
GU	genitourinary
HA	haemagglutinin
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBsAg/Ab	HBsAg complexed with anti-HBs
HBV	hepatitis B virus
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HSK	herpetic stromal keratitis
HSV	herpes simplex virus

i.m.	intramuscular
i.n.	intranasal
i.p.	intraperitoneal
i.t.	intratracheal
ICAM	intercellular adhesion molecule
ID	intradermal
IEL	intraepithelial lymphocyte
IFκB	inhibitory factor kappa B
IFN	interferon
Ig	immunoglobulin, for example IgG
IL	interleukin, for example IL-5
IM	intramuscular
IN	intranasal
INH	intranasal inhalation
INS	intranasal instillation
IP	intraperitoneal
IPER	intraperineal
IR	intrarectal
ISCOM	immune-stimulating complex
IT	intratracheal
IV	intravenous
IVAG	intravaginal
JNK	c-Jun NH_2-terminal kinase

LFA	lymphocyte-function associated antigen
LPS	lipopolysaccharide
LT	*Escherichia coli* heat-labile enterotoxin
LTB	B subunit of LT
LTK63	mutant of LT bearing a serine-to-lysine at position 63
LTR72	mutant of LT bearing an alanine-to-arginine at position 72
M-cell	microfold cell
MALT	mucosal associated lymphoid tissue
MAPKAP	mitogen-activated protein kinase activated protein
MDP	muramyl dipeptide
Me	methyl
MHC	major histocompatability complex
mIU	milli-international unit
MPL	monophosphoryl lipid A
MRC	Medical Research Council
N/A	not applicable
NALT	nasal-associated lymphoid tissue
N/D	not determined
NFκB	nuclear factor kappa B
NK	natural killer
NMR	nuclear magnetic resonance
Oc	ocular

OD	optical density
ODN	oligodeoxynucleotides
OPD	*o*-phenylenediamine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	polyethylene
PLG	poly (DL-lactide-co-glycolide)
PP	Peyer's patches
PS	polysaccharide
RALT	rectal associated lymphoid tissue
RLU	relative light units
ROS	reactive oxygen species
RT	room temperature
s-IgA	surface IgA
S-IgA	secretory IgA
SA	sebacic acid
SC	subcutaneous
SCID	severe combined immunodeficient
SD	standard deviation
SEM	standard error of the mean
SL	sublingual
TA	tibialis anterior
TBS	tris-buffered saline

TGF	transforming growth factor
Th	T helper, e.g. Th1, Th type-1
TMTLS	tetramethyltetralaurylspermine
TMTMS	tetramethyltetramyristylspermine
TMTOS	tetramethyltetraoleoylspermine
TMTPS	tetramethyltetrapalmitylspermine
TNF	tumor necrosis factor
Tris	2-amino-2-(hydroxymethyl)-1, 3-propanediol
VW	vaginal wall
WHO	World Health Organization

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Chapter II: In the second mouse study, in which IM, ID and GG immunizations were compared, IM and ID immunizations were performed by Dr. Cynthia L. Brazolot-Millan and GG immunizations by Georg Widera. Georg Widera also shipped sera from these mice to us and live mice to the Scripps Institute. Assays on these mice were conducted by Dr. Brazolot-Millan. All other mouse experiments and assays were performed by Michael J. McCluskie.

There were two separate primate studies which were conducted at Bioqual, Rockville, MD and Primedica, Worcester, MA. Robert H. Purcell supervised all aspects of primate handling including safety etc., and also participated in the design of the primate studies. Harriet L. Robinson also helped in design of both primate studies and Joel R. Haynes helped in design of the second study. James Fuller prepared the gold particles for GG immunizations and the actual GG immunizations were carried out in both studies by Joseph C. Santoro. Robert A. Gramzinski did the ID injections in the first study, and Heather L. Davis did the ID injections in the second study and IM injections in both studies. Samples were assayed and data analysed by Michael J. McCluskie.

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Chapter I

Introduction

1.1. The need for mucosal vaccines

Two distinct compartments of the immune system have been identified: (i) the systemic, which comprises the bone marrow, spleen and lymph nodes, and (ii) the mucosal, which comprises lymphoid tissue associated with mucosal surfaces and external secretory glands (1). Each compartment is associated with both humoral (antibodies) and cell-mediated (cytotoxic T-cells, cytokines) responses, however there are differences in the nature of the immune responses induced in each compartment. Antibodies associated with the systemic compartment are predominantly of the IgG isotype, which function to neutralize pathogens in the circulatory system. In contrast, antibodies in the mucosae are primarily secretory IgA (S-IgA), which function to prevent entry of the pathogen into the body via the mucosal surface (2).

The mucosal surface area of the gastrointestinal (GI), genitourinary (GU) and respiratory tracts is more than 200 times greater than that of the skin and is the primary site of transmission of numerous diseases (3). However, most vaccines developed to date are delivered parenterally, i.e. by intramuscular (IM), intradermal (ID) or subcutaneous (SC) injection, and yet parenteral immunization, while inducing strong systemic responses, usually results in very poor mucosal immunity (1). In contrast, antigen delivered at mucosal surfaces triggers both mucosal (at local and sometimes at distant sites) and systemic responses (4, 5). Furthermore, mucosal vaccines may have a broader age range of recipients (6). Finally, mucosal vaccines are often administered by non-

invasive means (e.g., nose drops, nasal spray, inhaled nebulizer), thus they are easier and less expensive to administer, have less need for trained personnel and no risk of needle stick injury or cross contamination (for reviews see 3, 6 - 8).

1.2. The mucosal immune system

1.2.1. Overview of the common mucosal immune system

The mucosal immune system can be divided into inductive sites, where antigens are encountered, endocytosed and presented to B and T cells, and effector sites where antigen-specific B and T cells reside and perform their respective functions to protect mucosal surfaces (1, 9).

The principal inductive sites are in the: (i) GI tract, the gut-associated lymphoid tissue (GALT), which includes the Peyer's patches (PP), appendix, mesenteric lymph nodes, and small solitary lymphoid nodules; (ii) respiratory tract, the bronchus-associated lymphoid tissue (BALT) and nasal-associated lymphoid tissue (NALT) or Waldeyer's ring (i.e., palatine, lingual and nasopharyngeal tonsils), and (iii) GU tract, the less well characterized rectal-associated lymphoid tissue (RALT) of the large intestine (1, 3, 10).

The GALT (e.g., PP) are major inductive sites in most common mammalian systems, however, the degree of BALT differs considerably. In rabbits, rats and guinea pigs, the development of BALT is significant, in mice it is less, and in humans it is absent in newborns as well as healthy adults (11 - 13). In most larger mammals (e.g., humans, pigs, dogs, rabbits and horses) the major inductive sites in the respiratory tract are the palatine, lingual and pharangeal tonsils, which together form a barrier of lymphoid tissues termed

Waldeyer's ring (14). Rodents lack pharyngeal and palatine tonsils but possess bilateral strips of lymphoid tissue underlying the respiratory epithelium of the nasal cavity and the nasopharynx, collectively termed NALT (15, 16). A number of differences exist between tissues at different sites in the MALT. For example, the gut possesses more intraepithelial lymphocytes (IELs), has increased expression of costimulatory signals to T cells by epithelial cells (e.g., intercellular adhesion molecule-1, ICAM-1), and more subepithelial APCs but fewer intraepithelial APCs than the airways (17). These differences are believed to account for the preferential suppression of immune responses in the gut as compared to the airways (17).

The most highly studied mucosal lymphoid tissue is the PP of the murine small intestine, and despite functional differences between different tissues; it serves as a good model for the functioning of the mucosal associated lymphoid tissue (MALT).

The PP consists of three distinct regions: (i) a domed epithelium; (ii) follicles (B cell zones); and (iii) parafollicular regions (T cell zones) (18). The surface of the dome region is covered by a modified epithelial layer known as the follicle-associated epithelium (FAE) (19). This differs from surrounding epithelium by the absence or paucity of goblet cells and the presence of microfold (M) cells (20). M cells have small cytoplasmic vesicles, few lysosomes, and possess short microvilli on the apical membrane (21). Thin extensions extend from the M cell basolateral membrane and form an apparent pocket around lymphoreticular cells (21, 22) (see Figure 1, adapted from ref. 20).

M cells are highly specialized in the uptake and transport of intact lumenal antigens, such as proteins, bacteria, viruses and small parasites, into the underlying lymphoid

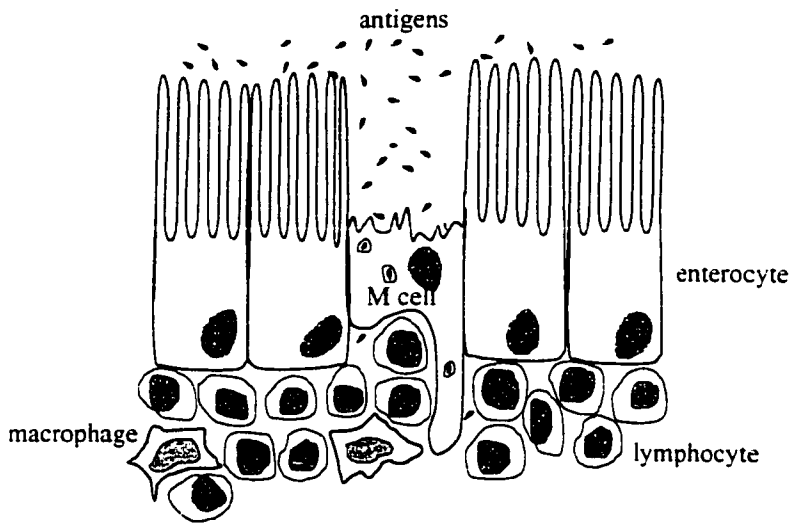

Figure 1: Schematic drawing of an M cell in the mucosal epithelium

tissue, mainly due to the invaginated membrane domain that forms the pocket (20-22). The mechanism by which M cells take up antigens remains unknown, however it may be IgA mediated, since there is selective uptake of IgA and IgA/antigen complexes by M cells, but, to date, no receptor has been identified (20). Interestingly, the FAE does not possess the secretory component necessary for secretion of IgA to the lumen (23, 24). In many species the glycocalyx of M cells is thinner and less elaborate than that of enterocytes (20), which may facilitate the diffusion of antigens to receptors lying deep in the glycocalyx or directly on the membrane surface (25).

Antigen is transported from the apical membrane to the basolateral surface and the underlying dome region where it can interact with antigen-presenting cells (APCs) such as dendritic cells, macrophages and perhaps B cells expressing major histocompatibility complex (MHC) class II molecules. In addition, in both lung and intestinal mucosae epithelial cells have the capability to present antigen. Two separate pathways for presentation of exogenous and endogenous pathways are known to exist. In general, MHC class II restricted $CD4^+$ Th cells respond to extracellular antigens, whereas MHC class I restricted $CD8^+$ CTL respond to endogenous antigens. In addition, an alternative MHC class I presentation pathway appears to exist whereby certain extracellular antigens such as inactivated or virus-like particles (e.g. hepatitis B surface antigen, HBsAg) may access the MHC class I pathway (26). Initial antigen presentation may occur within the dome region and help provide protection in this region (20-22).

Beneath the dome of the PP are distinct follicles that contain germinal centers where B cell division occurs. These germinal centers contain the majority of surface (s) IgA^+ B cells, and are considered sites where frequent B cell switches to IgA and affinity maturation occur (1). However, unlike the lymph nodes and spleen, the B cell zones do not give rise to significant numbers of plasma cells (1). Well-defined T-cell areas are adjacent. The parafollicular T cells are mature and most (>95 %) express the $\alpha\beta$ heterodimer form of T-cell receptor (1, 3). Approximately 60 % of these cells are $CD3^+CD4^+CD8^-$ T helper (Th) cells and the remainder are $CD3^+CD4^-CD8^+$ cytotoxic T-lymphocytes (CTLs) (3). In addition, the mucosal epithelium contains a large number of

intraepithelial lymphocytes (IELs) which are believed to be the primary source of effector T cells in the mucosal epithelium and play a major role in the defence of the mucosal surfaces (27). The lamina propria also contains many lymphocytes that serve as regulatory T cells for predominantly Th2-like humoral immune responses and IgA production (28). The majority of IELs are $CD8^+$ T cells possessing cytotoxic activity although subsets of IEL function as regulatory cells capable of producing Th type 1 (Th1), Th2 and Th3 cytokines (27, 29). In general, Th1 cells secrete IL-2, IFN-γ, and lymphotoxin and are important in the generation of cell-mediated immune responses; Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 and are more important in the generation of humoral immunity, regulation of cell-mediated immune responses, control of macrophage function and the stimulation of particular Ig isotypes; Th3 cells secrete IL-4, IL-10 and TGF-β and act to inhibit Th1 cell mediated responses (30). In recent years, considerable attention has focused on TGF- β which is believed to play a central role in oral tolerance, epithelial homeostasis and the promotion of class switching from $sIgM^+$ to $sIgA^+$ B cells (1, 29). Cytokines secreted by Th cells regulate the growth and differentiation of other Th cells in both positive and negative ways. For example, IL-10 secreted by Th2 cells suppresses cytokine production by Th1 cells, IFN-γ secreted by Th1 cells suppresses cytokine production by Th2 cells and TGF–β secreted by Th3 cells suppresses Th1 cytokine effects by downregulating IL-12 receptor expression (29, 31). Thus considerable cross-regulation exists between Th responses. There is no absolute dichotomy between Th1 and Th2 responses, particularly in higher animals such as humans, and established Th1 responses can convert to Th2 responses, although the reverse does not appear to occur (30).

The phenotype markers of IELs differ between mucosal sites and peripheral blood lymphocytes. In the adult mouse, 30 to 50% of IEL carry γδ receptors, compared to 3% of lamina propria or peripheral lymphocytes (28), and in the human colon epithelium up to 40% of the lymphocyte population have γδ receptors (32). T cells carrying γδ receptors are believed to play a major role in the primary defence against invading pathogens, and appear to respond in a simpler, more direct manner than the more common αβ T cells. Whereas αβ T cells can only recognize and respond to antigens which have been processed and presented by APCs in conjunction with MHC molecules, γδ T cells can respond directly to MHC proteins without added peptides and also recognize some bacterial antigens without the need for MHC presentation (32). Furthermore, γδ T cells may play a role in maintaining epithelial integrity since they respond to the MHC class I-related molecules MICA and MICB, displayed on the surface of epithelial cells when stressed by infection or inflammation (32)

Following antigenic stimulation in the inductive sites, antigen-sensitized precursor $sIgA^+$ B cells, $CD4^+$ Th cells and $CD8^+$ CTLs leave the inductive sites via the efferent lymphatics and migrate to regional lymph nodes, and into the thoracic duct to reach the bloodstream (1). These migrating cells then enter the mucosal effector sites such as the lamina propria of the respiratory, GI and GU tracts and the excretory glands (lacrimal, salivary and mammary), where IgA^+ B cells differentiate into IgA producing plasma cells under the influence of antigen, T cells and Th2-type cytokines [interleukin (IL)-5, IL-6 and IL-10] (1, 3, 33). The distribution pathway of cells from mucosal inductive to effector sites is termed the common mucosal immune system (CMIS) (see Figure 2) (9, 17, 34).

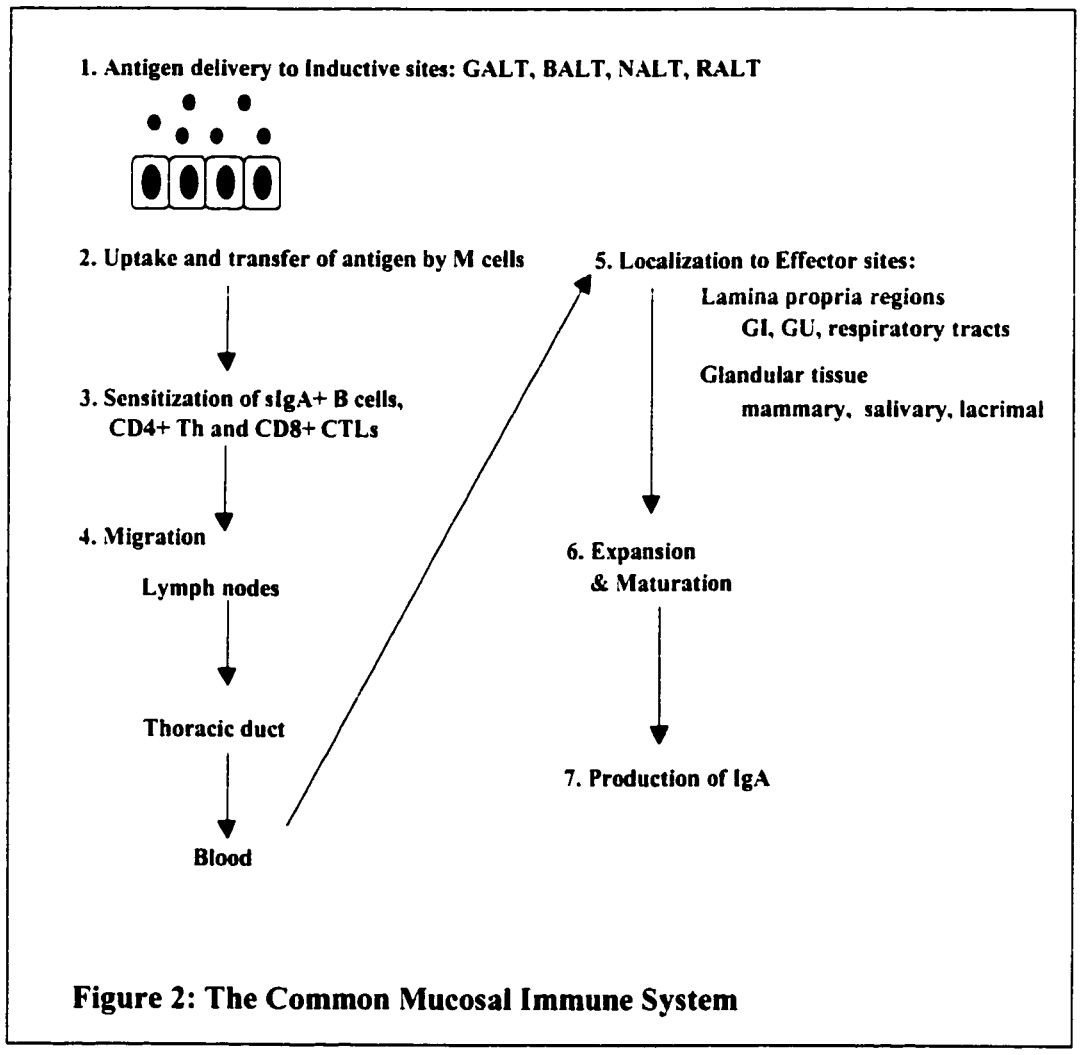

Figure 2: The Common Mucosal Immune System

1.2.2 The production of S-IgA

The hallmark of mucosal immunity is local production of S-IgA antibodies. These constitute > 80% of all antibodies in mucosa associated tissues and are induced, transported and regulated by mechanisms quite distinct from those of the systemic response. At the mucosal effector sites, IgA molecules are synthesized in plasma cells. Within the plasma cells, two IgA molecules associate with a 15 KDa polypeptide known

as J chain and the IgA dimer / J chain complex is secreted. The IgA dimer / J chain complex binds to the polymeric Ig receptor on the basolateral surface of lining epithelial cells, and the IgA dimer / J chain / polymeric Ig receptor complex thus formed is endocytosed and transported through the epithelial cell. The polymeric IgA receptor spans the epithelial surface membrane and contains, on its external side, an 80 K molecular weight glycoprotein, termed the secretory component. The secretory component remains associated with the IgA dimer / J chain complex when it is secreted at the apical surface of the epithelial cell while the remainder of the polymeric Ig receptor is retained within the cell. The secreted IgA dimer/ J chain / secretory component complex is termed S-IgA (2. 35).

1.2.3. Actions of S-IgA

S-IgA is of primary importance to host defence and acts not only to resist strict mucosal pathogens but also microorganisms causing systemic disease, many of which initially colonize mucosal surfaces. There appear to be three sites of IgA mediated mucosal defence: (i) in the lumen, where S-IgA can neutralize viruses, bacterial toxins and enzymes, and prevent viral attachment, microbial adherence and adsorption of antigen; (ii) within epithelial cells where dimeric IgA can bind to intracellular antigen; (iii) within the lamina propria where dimeric IgA can complex with antigen and the immune complex thus formed be transported to the lumen (2) (see Figure 3).

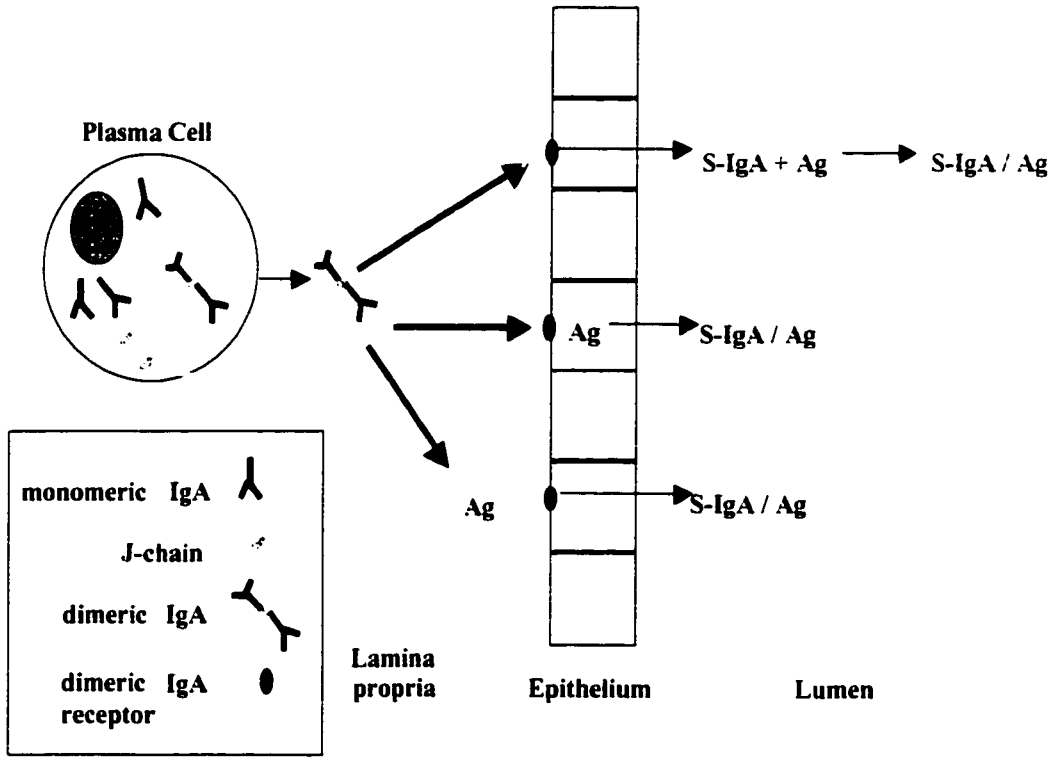

Figure 3: The actions of S-IgA

Furthermore, S-IgA synergizes with nonspecific antimicrobial substances in secretions (e.g., lactoferrin, peroxidase and lysozyme), promotes phagocytosis and antibody-dependent cellular cytotoxicity and has anti-inflammatory properties (1, 2). Finally S-IgA is resistant to proteolysis and has a high avidity for mucus and thus is well suited for the mucosal environment (1, 2).

1.3. Mucosal immunization

1.3.1. Mucosal vaccines

The mucosal surfaces are the primary site of transmission of most infectious diseases. Development of vaccines that could induce protective mucosal immunity would greatly reduce morbidity and mortality worldwide. Furthermore, mucosal immunization, in particular by oral or intranasal routes, is highly desirable for mass vaccination since it is fast and easy to administer, requires minimal trained personnel and carries no risk of needle stick injury or cross contamination.

Many mucosal vaccines in development are composed of live attenuated organisms. These are able to infect mucosal surfaces and are usually highly effective at inducing mucosal responses. Oral poliomyelitis, *Salmonella typhi* Ty21a and *Vibrio cholerae* attenuated vaccines are currently licensed for use in humans. However attenuated vaccines carry the risk of reversion to a virulent form, especially in immunocompromised individuals (e.g., neonates, HIV-infected or immunosuppressed individuals). Furthermore, not all pathogens may be attenuated.

Synthetic or recombinant antigens (peptides or polypeptides) are considered safer than traditional attenuated or inactivated whole pathogens, however they are often poorly immunogenic and require adjuvants to enhance specific immunity. For parenteral administration, aluminum precipitates (alum) may be added to the antigens to augment immune responses. Alum is currently the only adjuvant licensed for human use in North America, however it is generally considered not suitable for delivery to mucosal surfaces.

1.3.2. Mucosal adjuvants

One of the major impediments to the development of mucosal vaccines has been the lack of effective yet safe mucosal adjuvants. Cholera toxin (CT) and the structurally related *Escherichia coli* heat-labile enterotoxin (LT) are potent mucosal adjuvants commonly used in animal models (36, 37), however, toxicity prevents their use in humans. Two strategies have been proposed to circumvent the toxicity of the native toxins: (i) the use of the non-toxic B subunits that lack enzymatic activity (38, 39); or (ii) genetically detoxified mutants of CT or LT, which have little or no enzymatic activity (40).

The B subunits of CT and LT (CTB and LTB respectively) are considered non-toxic but contradictory results have been obtained with regard to their adjuvanticity. Initial reports indicated that CTB could act as a mucosal adjuvant but the purified preparations of CTB used were subsequently found to contain some holotoxin contamination (41, 42). However even with the use of recombinant CTB, where purity is not a question, the matter has not been clarified. Several investigators have found recombinant CTB either failed to stimulate mucosal responses to coadministered antigens (36, 43, 44) or was a significantly weaker adjuvant than native toxins (45, 46), while others report strong adjuvant activity of LTB or CTB (39, 47).

Another approach in the development of less toxic mucosal adjuvants has been the use of genetically detoxified mutants of CT and LT that lack enzymatic activity (40, 43, 44, 48-54). However, whether enzymatic activity is linked to adjuvanticity is also controversial. In some studies, mutants lacking enzymatic activity fail to generate

antibody responses against coadministered antigen (36), but in others they retain the adjuvant properties of the native toxin (51, 53, 55, 56). It has also recently been suggested that optimal adjuvanticity may be induced by mutants having only partial knockout of enzymatic activity, such as LTR72 or CTS106 (52, 55).

We have developed the use of immunostimulatory CpG DNA as a novel mucosal adjuvant and shown it to induce strong systemic and mucosal immune responses against coadministered antigen when delivered by the intranasal (IN) route. This is demonstrated in Chapters IV, V, VI, and VII of this thesis.

In addition, various other mucosal adjuvants and vaccine delivery systems are in development including microspheres, liposomes, proteosomes, cochleates, and immunostimulating complexes (ISCOMs) (see Table 1). These act to: (i) keep the antigen intact until it reaches the inductive sites of the mucosal immune system; (ii) increase antigen absorption; and/or (iii) skew the immune response towards a mucosal response. However, with few exceptions, antigen-delivery systems have been extensively used only in animal experimentation and often are unsuitable for human use. Thus, there is a great need to develop new approaches for mucosal immunization, capable of inducing strong mucosal and systemic immune responses without associated toxicity. In addition, many of the current vaccines are not affordable in less developed countries, where they are most needed (118).

Table 1: Common strategies for induction of mucosal immunity

Mucosal vaccine strategy	References
Antigen delivery systems	
Cochleates	57, 58
Emulsomes	59, 60
ISCOMs	61-64
Liposomes	65-69
Live bacterial vectors (e.g., *Salmonella, Escherichia coli, Bacillus calmatte-guerin, Shigella, Lactobacillus*)	10, 70-73
Live viral vectors (e.g., Vaccinia, adenovirus, Herpes Simplex)	10, 74-77
Microspheres	78-83
Nucleic acid vaccines	84-89
Polymer rings	90
Proteosomes	59, 69, 91, 92
Transgenic plants	93-95
Virosomes	96-98
Administration of adjuvants	
Bacterial toxins (e.g., CT, LT)	36, 37, 99
Toxin derivatives	38-40, 99
Lipid A derivatives (e.g., monophosphoryl lipid A, MPL)	59, 86
Muramyl Dipeptide (MDP) derivatives	100-103
Aluminium salts	104, 105
Saponins (e.g., QS21)	106, 107
Administration of cytokines/chemokines	108-115,
Administration of hormones	116, 117

1.4. Mucosal Immunization with DNA vaccines

1.4.1. DNA vaccines for mucosal immunization

One of the most exciting developments in vaccine technology in recent years has been the development of DNA vaccines, with which the antigen is synthesized *in vivo* after direct introduction of its encoding sequences. This unique method of immunization arose from the discovery that injection of pure plasmid DNA ("naked" DNA) into muscles of mice, resulted in long-term reporter gene expression in transfected muscle fibers (119). It was subsequently shown that if the plasmid encoded an antigenic protein, such as human growth hormone in a mouse, it was possible to induce immune responses against the expressed protein (120).

The induction of strong immune responses following introduction of DNA appears to be due to a combination of: (i) the sustained *in vivo* synthesis of antigen (121); (ii) antigen expression, very likely in APCs, that results in MHC class I and II presentation (122, 123); and (iii) the potent adjuvant effect of unmethylated immunostimulatory CpG motifs present in the DNA backbone (124, 125).

The potential of DNA vaccines to induce systemic immune responses even with transfection of relatively few cells, has now been demonstrated in many species including man (126-131). The vast majority of DNA vaccines have been delivered parenterally (e.g., by IM or ID injection or coated on gold particles and shot into the epidermis with a gene-gun, GG), and although these can induce potent systemic immune responses, they do not induce mucosal immunity.

1.4.2. Potential advantages of mucosal DNA immunization

Mucosal immunization with DNA vaccines if they were effective, would offer several potential advantages over the traditional parenterally delivered antigen-based vaccines:

(i) no infectious agents are used, and thus, in contrast to live attenuated vaccines, the potential for reversal to virulence does not exist;

(ii) this safety aspect may allow widespread use including immunocompromised individuals (e.g., neonates, HIV-infected or malnourished individuals);

(iii) induction of both mucosal (S-IgA) and systemic immune responses, including MHC class I restricted CTL by virtue of the *in vivo* antigen synthesis, thus protection is provided at the primary site of transmission of infectious agents and also in the submucosal tissues and bloodstream;

(iv) the generation of protective immune responses at sites distant from the immunization site;

(v) long-lasting immunity, likely due at least in part to prolonged antigen synthesis;

(vi) ease and safety of administration, without the need for highly trained personnel;

(vii) heat-stability, obviating the need for a "cold-chain" and thus highly suitable for developing countries

(viii) low cost and relative ease of manufacturing, with a single process for all DNA vaccines;

(ix) the possibility to make multivalent vaccines against several pathogens by cloning genes encoding different antigens into a single vector (for co-linear expression), or by mixing different plasmids together; and the ease of cloning allows new vaccines to be created quickly, for example in response to new or changing strains of pathogens.

1.4.3. Safety and regulatory concerns

A number of phase I human clinical trials using parenterally delivered DNA vaccines are underway or have been completed (128-131). Clinical trials using mucosal delivery of DNA vaccines have not started, however, the safety concerns will be essentially the same as for parenteral immunization. These include:

1.4.3.1. Possibility of tolerance to foreign antigen

Early studies in DNA vaccination indicated that reporter gene expression could continue for up to 2 years (119), and concern was raised that immunological tolerance might ensue. However, the luciferase reporter gene used in these studies is poorly immunogenic and subsequent studies have demonstrated that after IM injection of plasmid DNA encoding an antigen, expression persists for only a few weeks owing to immune-mediated destruction of the antigen-expressing cells (121). In addition, at mucosal surfaces there is a rapid turnover of epithelial cells. Moreover tolerance has never been demonstrated in the large number of DNA vaccination studies conducted to date.

1.4.3.2. Possibility of an Integrative Event

Another safety concern is the possibility of intergartion of DNA into the host's chromosomal DNA resulting in an insertional mutagenic event by activation of a proto-oncogene or inactivation of a tumor suppresser gene. However the incidence of such an insertional mutagenic effect is low, since i) most administered DNA is rapidly degraded in the extracellular space; ii) the plasmid vectors used are designed to remain episomal; (iii) most nonintegrated DNA would be lost during subsequent cell division (122). The incidence of an insertional mutagenic event has been estimated to be 10^3 times lower tham the rate os spontaneous mutagenisis (132).

1.4.3.3 Possibility of immune response to DNA and auto-immunity

Double stranded DNA can induce the production of anti-DNA antibodies and it was feared that this could contribute to autoimmune reactions against the host's DNA. However, plasmid DNA is of bacterial origin and the anti-DNA antibodies generated do not cross react with mammalian DNA. Indeed, even in lupus-prone animal models, DNA immunization has not resulted in autoimmune reactions. In addition, there was concern that the presentation of foreign antigen on the surface of the host's cells would lead to tissue destruction. However, viral and bacterial infection continually result in the destruction of cells and it is unlikely that the administration of antigen-expressing DNA will cause more severe effects than a natural infection.

1.4.3.4. Immunostimulatory effects of CpG motifs

The adjuvant effects of CpG motifs appear to be desirable and necessary for the induction of strong immune responses with DNA vaccines. Nevertheless, there needs to be a balance between necessary immunostimulatory effects and inflammatory responses particularly in the respiratory tract. In addition, there may be situations where a strong Th1 stimulatory response is undesirable, such as in persons prone to autoimmune disease.

1.4.4. The possibility of mucosal immunization with DNA vaccines

One of the earliest reports on DNA immunization demonstrated that protective immunity could be induced by parenteral, mucosal and GG immunizations using plasmid DNA expressing influenza virus hemagglutinin glycoproteins. Fynan *et al.* (84) immunized mice by various routes and reported excellent protection from subsequent lethal influenza challenge with IM, intravenous (IV) and GG delivery, good protection with IN, poor protection with ID or SC and no protection with intraperitoneal (IP) immunization. In the same study, chickens immunized by IV, IM or intratracheal (IT) demonstrated moderate protection after challenge, while SC, IP, intrabursal and intraorbital gave very low levels of protection. Although mucosal responses were not measured, this study demonstrated the feasibility of using mucosal routes to deliver DNA vaccines and showed that mucosal immunization, at least in this case, was superior to certain parenteral routes (e.g., ID, SC). In recent years there have been several reports of successful mucosal immunization using DNA vaccines, however, the report by Fynan *et al.*, was the only published account of mucosal DNA immunization when the work on this

thesis commenced. We have evaluated the possibility of using pure plasmid DNA or lipid-formulated DNA to induce mucosal immunity against hepatitis B and various aspects of this are found in Chapters II, III and VIII.

1.4.5. DNA Vaccine Design

DNA vaccines designed for delivery at mucosal surface are essentially identical to those used for parenteral delivery. These plasmids are similar to vectors used as reporter or therapeutic gene expression vectors, sometimes incorporating features designed to augment immune responses. The properties of DNA vaccine vectors have been previously described and include: (i) a bacterial origin of replication (ori); (ii) a prokaryotic selectable marker gene such as an antibiotic resistance gene; (iii) antigen-encoding sequences; (iv) eukaryotic transcription regulatory elements (e.g., promoter and enhancer sequences); (v) a transcription termination element; and (vi) optional elements that may be included in DNA vaccines (e.g., introns, unrelated Th epitopes to provide additional T-help, immunostimulatory CpG sequences, or additional genes expressing cytokines or co-stimulatory molecules) (127).

1.5. DNA vaccine delivery at mucosal surfaces

1.5.1. Naked DNA

DNA in an aqueous solvent ("naked DNA") is very inefficient for *in vitro* transfection of cells, a procedure which is usually carried out with transfection reagents (e.g., cationic lipids) or physical facilitation (e.g., calcium phosphate precipitation or electroporation). In contrast, pure plasmid DNA can transfect mature muscle fibers *in vivo* with a much higher

efficiency than lipid-formulated DNA (119, 133-135). Pure plasmid DNA may also be used for gene delivery by other parenteral routes including ID, SC, and IV (84, 126 136, 137), although transfection efficiency in these tissues is generally less than in muscle. Several recent studies have reported the use of naked DNA for mucosal immunization, either administered by non-invasive (non-injected) means or directly injected into mucosal tissues.

In Chapter II of this thesis, we have evaluated a plasmid DNA vaccine against hepatitis B when delivered to various mucosal surfaces by non-invasive means and direct injection.

1.5.1.1. Non-invasive delivery of naked DNA to mucosal surfaces

Early studies demonstrated that intranasal administration of DNA encoding influenza hemagglutinin glycoproteins could protect mice against lethal challenge, but the nature of immunity at mucosal surfaces was not determined (84). A number of studies have subsequently reported, with varying degrees of success, that naked DNA delivered to mucosal surfaces by non-injected means can induce antigen-specific immune responses. These include IN (85-88, 106, 138-142), oral (140), intravaginal (143, 144), and ocular (145) administrations. However, in most cases where examined, immune responses could be enhanced by the use of formulated DNA (e.g., with liposomes or microspheres) or with adjuvants (e.g., CT, MPL) as discussed below.

1.5.1.2. Direct injection of naked DNA to mucosal surfaces

Strong immune responses have been induced by direct injection of various mucosal surfaces including: (i) in the oral cavity: the tongue (89, 139), and oral mucosa (139, 140); (ii) the vaginal wall (89); and (iii) the jejunum (140). Mucosal secretions were not collected and assayed for S-IgA in these studies, however after tongue and oral mucosa injections, splenic B cells restimulated *in vitro* produced IgA (139).

1.5.1.3. Gene gun delivery to mucosal surfaces

Plasmid DNA may also be coated and dried onto very small gold particles, which are then shot under pressure (e.g., using a helium or CO_2 powered "gene-gun") into the target tissues (84, 146). This method is highly efficient even with very low doses of DNA, most likely because it can deliver the DNA directly into cells and may circumvent some of the problems associated with mucosal delivery of unformulated DNA. That being said, the gold particles can only penetrate a few mm at most, and so far other than delivery to an exposed surface (e.g., epidermis, oral cavity) use of the GG requires surgical exposure of the target tissue. The GG has been used to introduce DNA into the oral (139, 147) and vaginal mucosae (148), the PP (148, 149), tongue (150), and cornea (151). Mucosal and systemic antigen-specific antibodies have been observed after PP immunization (148, 149), which could be augmented by PP (148), dermal (149) or vaginal booster immunization (148). Local immune responses were generated following GG delivery of DNA to the vaginal mucosa, which were higher than those generated by PP immunization, and could be enhanced with boosting (148). Delivery of influenza hemagglutinin expressing DNA by GG to the tongue of pigs elicited higher levels of

influenza virus-specific systemic antibodies than epidermally immunized animals but no detectable mucosal antibodies. Moreover, subsequent to nasal influenza challenge pigs immunized by GG in the tongue had an 18-fold decrease in viral shedding relative to epidermally immunized animals (150).

1.5.2. Formulated DNA

Despite the initial early success (84), progress has been considerably slower with mucosal than parenteral delivery of DNA vaccines. Delivery of genes to the mucosal surfaces (i.e, GU, GI and respiratory tracts) may be hampered by poor plasmid uptake across the epithelium owing to biochemical (e.g., secreted enzymes), physical (e.g., mucus, cilia) or mechanical (e.g., coughing) defence mechanisms. In order to increase plasmid uptake and limit degradation, various methods of formulating DNA for mucosal delivery have been proposed. These include:

1.5.2.1. Cationic liposomes

Cationic lipids have been widely used to deliver DNA for therapeutic purposes by numerous routes including IT, IN, IV, intraarterial, intratumor, intracranial, and intraocular (152-155). Thus far, for immunization purposes, liposomes have mainly been used for DNA delivery to the respiratory tract (87, 88, 138, 156-160). However, liposomes have also been used to deliver plasmid DNA orally (140) and by IM (135, 88), IP, SC and ID (88) injections.

The liposomes form complexes with the plasmid DNA via ionic interactions and, while the exact mechanism of DNA/lipid uptake is unknown, it has been suggested that

liposomes bring about enhanced gene transfer by attaching to cell surfaces where they either fuse directly or are endocytosed with subsequent intracellular fusion with endosomal membranes. As the lipid diffuses into the membrane, lipid-DNA ionic interactions may be disrupted, thereby releasing the DNA into the cytoplasm or endosome (161). Liposomes may also improve transfection efficiency by increasing the retention time and reducing the rate of DNA degradation by extracellular nucleases (162).

Incorporation of plasmid DNA into liposomes has been shown to induce stronger and longer-lasting antigen-specific humoral (in serum and in vaginal and rectal fluids) and cellular (CTL in genital lymph nodes and spleen, T-cell proliferation) responses after IN inhalation than did uncomplexed DNA (87, 88, 138, 157, 158). Lipid composition can greatly affect level of expression (161, 163) and this may, at least in part, explain why in other studies using lipid formulated DNA delivered intranasally, no specific mucosal antibodies were detected (158, 160). Interestingly, one study has shown oral delivery of lipid-formulated DNA to induce a CTL response, albeit of a low magnitude (140).

Following lipid associated gene transfer to the lungs there is only a brief period of gene expression that peaks between 2 and 4 days with residual levels for several weeks (162-165). This may be as a result of promotor turn-off. Liposome-formulated DNA induces, in mouse lungs, elevated levels of cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α (166, 167) that are known to down-regulate viral (e.g., cytomegalovirus, CMV) (168-170) as well as some non-viral promoters (171). Increased cytokine expression in the lung appears to result, at least in part, from the presence of unmethylated CpG dinucleotide sequences within the plasmid (172, 173). Indeed,

unmethylated but not methylated CpG-containing oligonucleotides cause acute inflammation and increased levels of cytokines in mouse lungs (174). Neutralizing CpG motifs have also been identified which counter the effects of immunostimulatory CpG motifs (175). Inflammation of the lung can also be induced by the cationic and much less so by the neutral components of the liposomes used to complex the DNA (166, 171), although with some lipids no such inflammation is observed (173). Reduction of this inflammatory response leads to an enhanced transgene expression (173). It is possible that stronger immune responses could be achieved in future with a better understanding of CpG-mediated inflammation and the use of novel cationic lipids.

In this thesis we have evaluated the delivery of plasmid DNA to the respiratory tract either as 'naked' DNA or formulated with various cationic lipids (see Chapters II and III).

1.5.2.2. Microspheres

Biodegradable microspheres, such as those composed of poly (DL-lactide-co-glycolide) (PLG), have been used to encapsulate and deliver antigens, toxoids or attenuated virus by parenteral, oral or IN routes resulting in antigen-specific systemic and mucosal immune responses (78-84). Microspheres are most commonly used for oral delivery since they protect encapsulated vaccines from enzymatic degradation and acid hydrolysis in the stomach. They are readily endocytosed from the lumen of the gut by M cells in the PP and thereafter transported to the underlying APCs. Microsphere degradation time is determined by their size and composition, and thus by using different microspheres a controlled release of antigen to the immune system may be achieved (78). Oral delivery of encapsulated DNA to the PP was first achieved using DNA encoding

β-galactosidase in microspheres composed of poly anhydride copolymers of fumaric and sebacic acid [poly(FA:SA)] (176). Subsequently PLG-encapsulated plasmid DNA has been shown to induce antigen-specific systemic and mucosal antibody responses after oral administration (177, 178) and, in the case of a rotavirus DNA vaccine, to protect against subsequent live challenge (179).

1.5.2.3. Other delivery systems

Various other gene delivery systems have been used including virosomes (180, 181), dendrimers (182, 183), receptor-mediated polycationic systems (184, 185) and DNA condensing compounds (186), however, their role in mucosal DNA immunization has yet to be determined. In addition, it has been reported at a recent conference that herpes simplex virus (HSV)-1 and -2 plasmid DNA vaccines formulated with cochleates and delivered by various mucosal routes could induce immune responses and enhance protection from virus challenge (Gould-Fogerite S, *personal communication*), however these results have yet to be published.

We have attempted to use microspheres, cochleates, dendrimers and DNA-condensing compounds to augment immune responses using a DNA vaccine against hepatitis B and this is briefly discussed in Chapter VIII.

1.5.3. The use of adjuvants

Adjuvants are substances that enhance specific immune responses to coadministered antigens. Different adjuvants may influence: (i) the biological or immunological half-life of vaccine antigens; (ii) antigen delivery and presentation; and (iii) the production of

immunomodulatory cytokines that favor the development of Th1 or Th2 immune responses (187). Aluminum phosphate or hydroxide salts (alum) are currently the only adjuvant licensed for human use, and, while effective for parenteral administration of proteins and peptides, they are generally not considered suitable for delivery to mucosal surfaces nor for use with DNA vaccines.

1.5.3.1. Bacterial toxins

The bacterial toxins CT and the closely related LT have been the most commonly used mucosal adjuvants in animal models for a number of years (36, 38). To date, only native CT has been tested as a mucosal adjuvant with DNA vaccines (85, 87, 140, 159).

Co-administration of 2 μg CT with HSV-1 DNA vaccine by IN inhalation enhanced distal mucosal responses (IgA in vaginal and fecal fluids, IgA producing cells in iliac lymph nodes) and enhanced protection against viral challenge compared to DNA without adjuvant (85). In contrast, when DNA encoding HIV-1 antigens was given by IN inhalation, no increase in serum or mucosal antibody responses was seen with the addition of 10 μg CT (87). Similar findings were also reported in serum, saliva or nasal washes using an influenza virus DNA vaccine after IN delivery, however in the same study, as little as 0.1 μg CT induced significant numbers of anti-HA secreting B cells to appear in the spleen and lungs (159). This suggests that weak immune responses may be induced even in cases where no IgA is detected in mucosal fluids. The effect of CT is influenced by route of administration of DNA vaccines, in that following IN inhalation or buccal injection, CTL responses with a measles virus DNA vaccine were increased with 5 μg CT, but after oral or intrajejunal administration no increase in CTL was seen compared to

DNA alone (140). When given with a DNA vaccine by IN immunization, CT enhanced Th2 responses as indicated by a predominance of IgG1 antibodies and Th2 cytokines (IL-4, IL-5) (85). CT is generally considered a Th2-type adjuvant (188), although it has also been shown to induce mixed Th1/Th2 (189, 190) or Th1 (191) responses with protein antigens.

We have attempted to use CT and LT as mucosal adjuvants with a DNA vaccine delivered by mucosal routes and this is briefly discussed in Chapter VIII.

1.5.3.2. Other mucosal adjuvants

During recent years there has been considerable progress in the development of alternative adjuvants, often designed to induce Th1 responses that are desirable for viral clearance. Adjuvants used for mucosal immunization with DNA vaccines include: (i) MPL, an immunological adjuvant obtained from bacterial lipopolysaccharide (192), which can boost Th1-like antigen-specific immune responses after IM (86, 193) and IN administration of an HIV-1 DNA (86) vaccine, and *in vitro,* when co-encapsulated with plasmid DNA in microspheres, increases their rate of phagocytosis (194); (ii) QS21, a purified saponin isolated from the bark of the *Quillaja saponaria* Molina tree (195) which enhances systemic and mucosal responses after IN administration, and preferentially induces Th1 responses, as indicated by predominantly IgG2a antibodies and Th1-like cytokines (IL-2, IFN-γ) compared to DNA without adjuvant (106); (iii) low viscosity carboxymethylcellulose sodium salt (CMCS-L), a water soluble polymer which, when coadministered with an HIV-1 DNA vaccine by IN inhalation, enhanced antigen-specific

mucosal and systemic humoral and cell-mediated immune responses and switched responses from Th2-like to a mixed Th1/Th2 response (142).

1.5.4. Cytokine-expressing plasmids

1.5.4.1. Coadministration of cytokine-expressing plasmids

Cytokines are soluble proteins produced by a variety of cell types in response to injury, infection, or antigenic challenge that can influence growth, differentiation and function of a variety of immune cells. On the basis of the cytokines that they secrete, Th cells are divided into Th1 and Th2 subtypes. Th1 cytokines (e.g., IL-2, IFN-γ, IL-12, TNF-α) stimulate both CTL and B cells while Th2 cytokines (e.g., IL-4, IL-5, IL-6, IL-10) act predominantly on B cells. Th1 cytokines can also have direct effects on viral replication or assembly (196). Cytokines are essential for the regulation of IgA isotype switching and IgA B cell differentiation. TGF-β1 promotes heavy chain class switching by IgA^+ cells, whereas IL-2, IL-5 and IL-6 enhance secretion by IgA^- cells (197). Several cytokines have been evaluated as vaccine adjuvants, including IFN-γ, granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-12 (187). For immunization purposes with protein antigens, cytokines have been used as adjuvant in the form of recombinant proteins (109-111) or as cytokine expressing plasmids (198). Cytokine-expressing plasmids have also been shown to increase mucosal and systemic immune responses when coadministered with DNA vaccines by IN inhalation (87, 158, 199). Furthermore, depending on the cytokine expressed, Th1 or Th2 responses can be

preferentially induced (87). The use of cytokine adjuvants in humans has been limited by toxicity, as well as decreased sensitivity to repeated doses.

We have attempted unsuccessfully to co-administer DNA-expressing IL-4 or IL-6 with a DNA vaccine against hepatitis B delivered by IN inhalation and this is briefly discussed in Chapter VIII.

1.5.4.2. Therapeutic use of cytokine-expressing plasmids

There is considerable interest in the use of cytokine-expressing plasmids delivered mucosally for therapeutic purposes, particularly in the treatment of pulmonary allergic responses (200, 201) and ocular herpetic lesions (145, 202-204). Asthma is associated with Th2 responses (205, 206) and thus Th1 cytokines may help reduce allergic responses. Indeed, mucosal IL-12 or IFN-γ gene transfer inhibits pulmonary allergic responses (200, 201) and restore local antiviral immunity (201). Interestingly, inhibition of airway allergic responses is also seen by administration of synthetic immunostimulatory CpG oligonucleotides (207, 208), which preferentially induce Th1 responses (209, 210).

In contrast, herpetic stromal keratitis (HSK) is an immunopathological process which can occur after HSV reactivation and, since it is associated with $CD4^+$ Th1 T lymphocytes, may be treated with Th2 cytokines (211). Treatment with or pre-exposure to Th2 cytokines (IL-10, IL-4, or TGF-β) but not Th1 cytokines (IL-2 or INF-γ), reduced the severity of lesions associated with HSV (145, 202, 204).

1.5.5. Sites of administration

The strength and nature of immune responses in mice with DNA vaccines appear to be influenced, at least in part, by the route and method of DNA delivery. This most likely arises because of differences in the efficiency of gene transfer and the type of cell(s) transfected. For the purposes of mucosal immunization, DNA vaccines have been delivered to a wide variety of tissues by both invasive and non-invasive DNA delivery methods including: (i) into the respiratory system by IN (84-88, 106, 138-142, 156-160) or IT (84) delivery, with or without cationic lipids; (ii) into the digestive system by oral feeding of microencapsulated (177-179) or lipid-formulated DNA (140), injection into the oral mucosa (140), tongue (139) or the jejunum (140), and GG delivery into the tongue (150) or oral mucosa (139, 147), or PP of the bowel (148, 149); (iii) into the GU tract by GG delivery into the vaginal mucosae (148) or intravaginal instillation (143, 144), and (iv) by topical ocular administration (145) (see Table 2).

We have evaluated a number of routes and methods of DNA immunization and this is reported in Chapters II and III.

1.5.6. The mechanism of DNA vaccines at mucosal surfaces

There is considerable evidence that mucosal immunization with DNA vaccines primes the common mucosal immune system. A number of studies have demonstrated that mucosal responses can be generated distant from the site of immunization (85-87, 106, 138, 141, 149, 157). However, the mechanism by which immune responses are generated

Table 2: Routes of mucosal immunization with DNA vaccines

Route of immunization	Reference(s)
A. Respiratory tract	
Intranasal inhalation	84-89, 106, 138-142, 156-160, 199
Intratracheal instillation	84
B. Digestive tract	
Oral feeding	89, 140, 177-179
Injection	
tongue	89, 139
oral mucosa	140
jejunum	140
Gene-gun	
tongue	150
oral mucosa	139, 147
Peyer's Patches	148, 149
C. Genitourinary tract	
Injection (vaginal mucosa)	89
Gene-gun (vaginal mucosa)	148
Instillation	89, 143, 144
D. Ocular administration	89, 145

after mucosal immunization with DNA vaccines has not been identified. However, it is likely due to a combination of three factors: (i) efficient antigen presentation by virtue of *in vivo* synthesis, (ii) prolonged antigen synthesis and (iii) the adjuvant effect of CpG immunostimulatory motifs.

1.5.6.1. Antigen presentation with DNA vaccines

The inductive sites of the mucosal immune system contain large numbers of specialized M cells which are capable of taking up antigen and delivering it to the underlying lymphoid tissue (reviewed in ref. 20). M cells are capable of trafficking not

only soluble macromolecules but also intact bacteria, viruses, small parasites and microspheres (212, 213). It is possible that plasmid DNA, even though it is not antigenic itself, is transported in a similar fashion after mucosal immunization. Within the subepithelial lymphoid tissue, various cell types may be transfected, and these most likely include APCs (in particular dendritic cells) that can present antigen in a class I and II restricted manner along with appropriate costimulatory molecules to local $CD4^+$ and $CD8^+$ T lymphocytes. The mucosal epithelium of the GI, respiratory and reproductive tracts contains a large number of IELs with a predominance of $CD8^+$ T cells (27). In addition, the lamina propria contains many lymphocytes that serve as regulatory T cells for humoral immune responses that are predominantly Th2-like but also include IgA production (28). Local production of cytokines, in particular IL-4 and TGF-β, are thought to promote class switching from $sIgM^-$ to $sIgA^+$ B cells. Subsequently, antigen-sensitized precursor $sIgA^+$ B cells, $CD4^+$ Th cells and $CD8^+$ CTLs leave the inductive sites via the efferent lymphatics and migrate to regional lymph nodes and into the thoracic duct to reach the bloodstream. These migrating cells then enter the IgA effector cells, where terminal differentiation, synthesis and transport of S-IgA occur (1).

Alternatively, it has been suggested that DNA transfected APCs could migrate through lymph vessels and/or blood stream and disseminate to distant sites (86). In addition to DCs, other transfected cells in the epithelium may function in the initiation of an immune response. Intestinal epithelial cells produce various cytokines (e.g., IL-1, IL-6 and IL-8), express the adhesion molecules ICAM-1 and lymphocyte-function associated

antigen (LFA)-3, and functioning as non-professional APCs, may also present antigen locally to IELs (215,216).

1.5.6.2. Longevity of Antigen Synthesis

Gene expression from plasmid DNA is transient in the mucosal tissues largely due to cell turnover. DNA delivery to the lungs or nasal epithelium, results in high levels of gene expression for only a limited period (days 2 to 4 after gene transfer) (138, 162, 164, 216), although very low levels of gene expression persist for at least several weeks (138, 162, 165, 217-219). This may be due to a combination of cell turnover that is possibly enhanced by vaccine delivery, and promoter turn-off in response to the elevated levels of cytokines such as IFN-γ and TNF-α (166, 167) that are known to down-regulate viral (e.g., CMV) (168-170) as well as some non-viral promoters (171). Nevertheless, despite the relatively brief period of antigen expression, mice can develop long-lived immune responses with IN immunization of DNA vaccines (87). This is not overly surprising since a single IM injection with a DNA vaccine can induce life long immune responses in mice (220), even though antigen-expressing muscle fibers are destroyed between 10 and 20 days after gene delivery (122).

Studies after gene delivery to other mucosal tissues have been more limited. In one study, after intravaginal instillation, reporter gene expression was detected at 8 days in the ovary and stomach but not in other tissues (vagina, liver, cervix, fallopian tubes, large or small intestine, lymph nodes), at 14 days in all tissues except lymph nodes (144) and persisted for at least 21 days in some tissues (fallopian tubes and liver). In another study,

after vaginal GG delivery, expression was detected within 24 hr and persisted for at least 96 hr (148), but longer periods were not determined.

1.5.6.3. Role of CpG Immunostimulatory Sequences

DNA vaccines possess their own adjuvant activity owing to the presence of unmethylated CpG dinucleotides in particular sequence contexts (124). Such immunostimulatory DNA sequences (CpG motifs) are found frequently in bacterial, but not mammalian DNA, and may therefore be an evolutionary adaptation to augment innate immunity in response to bacterial infection. Plasmids contain many immunostimulatory CpG motifs, and indeed it has been demonstrated that DNA vaccines are dependent on the presence of such motifs to be effective (125, 209). Overall, CpG DNA induces a Th1 like pattern of cytokine production dominated by IL-12 and IFN- γ with little secretion of Th2 cytokines (209) and are thought to account for the strong CTL responses frequently seen with DNA vaccines (210). The appearance of Th1 cytokines in the bronchiolar lavage fluid following IN administration of plasmid DNA, is caused, at least in part, by the presence of CpG motifs within the plasmid (172). Thus it is possible that the CpG content of the vector may influence whether immune responses are biased towards a Th1- or Th2-type and explain, at least in part, why different plasmids induce predominantly Th1 (85, 88), Th2 (86, 88, 106, 141) or mixed Th1/Th2 (138) responses when naked DNA is delivered to the lungs. Other factors which may determine whether a Th1 or Th2 response predominates after mucosal immunization include (i) the antigen, (ii) the dose of antigen, (iii) the route and method of DNA administration, (iv) co-expression of cytokines and (vii) whether another adjuvant was used.

1.6. CpG immunostimulatory DNA

1.6.1. The adjuvant properties of CpG DNA

CpG DNA triggers most (>95%) B cells to proliferate, secrete immunoglobulin, IL-6 and IL-12, and to be protected from apoptosis (124, 209, 221). CpG DNA also directly activates monocytes, macrophages and dendritic cells to secrete IFN-α/β, IL-6, IL-12, GM-CSF, chemokines, and TNF-α (209, 222). These cytokines stimulate natural killer (NK) cells to secrete IFN-γ and have increased lytic activity (209, 221, 223-226). Overall, CpG induces a Th1-like pattern of cytokine production dominated by IL-12 and IFN-γ with little secretion of Th2 cytokines (209) (see figure 4).

No direct activation of T cells by CpG DNA has been shown, however, the induction of monocyte and NK cell cytokines secretion by CpG DNA promotes T -cell cytokine secretion and the development of a CTL response (210). While direct CpG DNA effects are T cell independent and antigen non-specific, the B cell activation by CpG DNA synergizes strongly with signals delivered through the B cell antigen receptor for both B cell proliferation and Ig secretion (125). This synergy promotes antigen-specific immune responses and accounts for the strong adjuvant effect of CpG-containing oligonucleotides given with protein vaccines including hepatitis B surface antigen (HBsAg, a virus-like particle) (227, 228), live-attenuated measles virus (229), whole killed influenza virus (230, 231), a peptide derived from tetanus toxoid (229), as well as model antigens such as ovalbumin (232-234), hen egg lysozyme (235), β-galactosidase (231) and fowl γ-globulin (236). CpG ODN has also been shown to be highly effective for enhancement of Th1-like

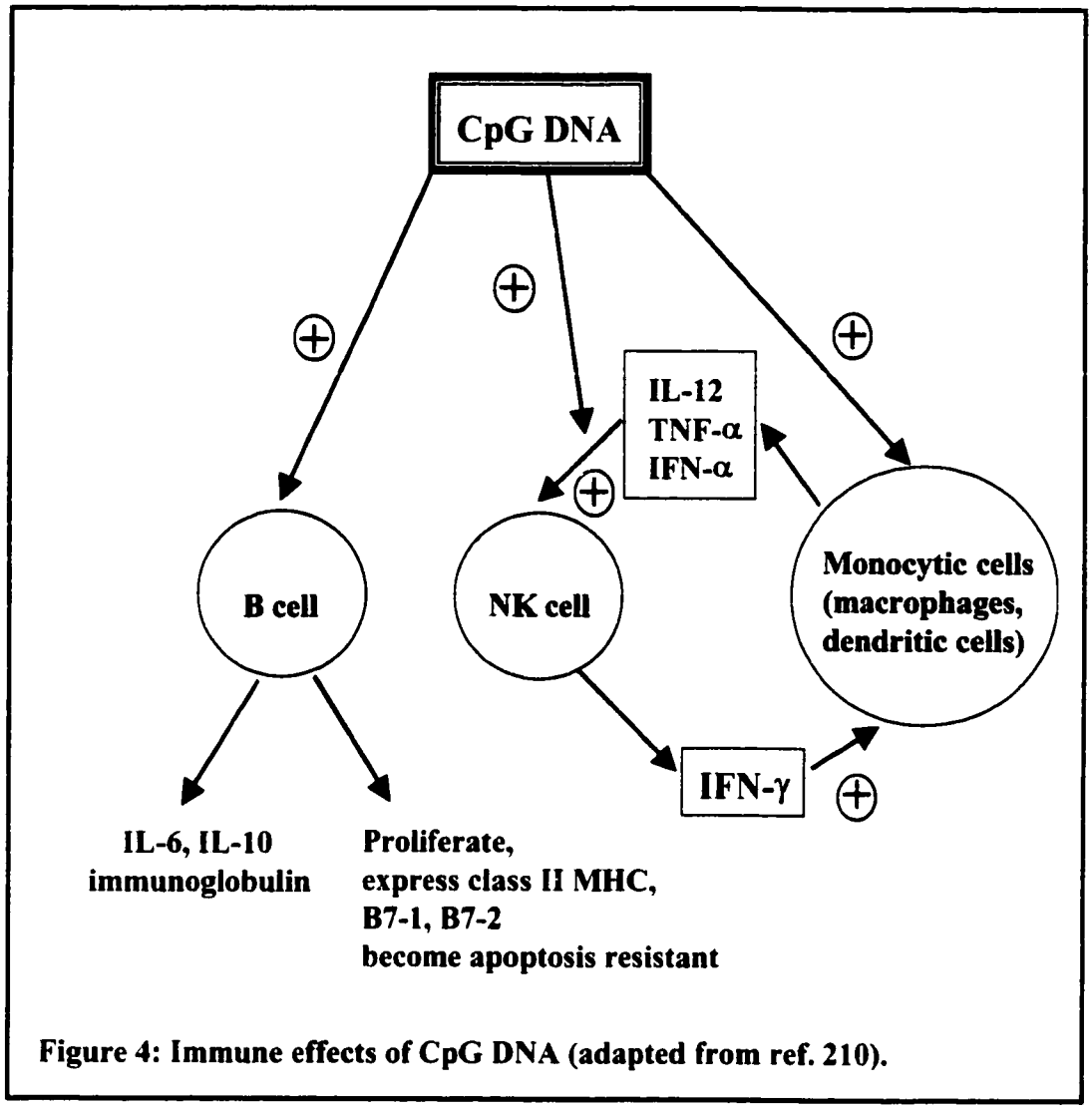

Figure 4: Immune effects of CpG DNA (adapted from ref. 210).

immune responses against a tumor antigen, the idiotype of the surface IgM of 38C13 mouse lymphoma cells, and to protect against subsequent tumor challenge (237, 238).

While CpG ODN has been shown to be a potent adjuvant for induction of systemic immune responses against a variety of antigens given parenterally (see above), prior to the work reported in this thesis (see Chapters IV, V, VI, VII), only one study had shown the use of CpG as a mucosal adjuvant. This was with inactivated virus, which when delivered

on its own to a mucosal surface, is capable of inducing immune responses (230). Subsequently, another report has confirmed that CpG DNA is a highly effective mucosal adjuvant (239).

1.6.2. The immunomodulatory mechanism of CpG DNA

The precise mechanism by which CpG DNA mediates cell activation is as yet unknown, nevertheless some important steps have been determined (see Figure 5). CpG-mediated cell activation does not appear to function through a cell surface receptor, but rather requires cell uptake and internalization of CpG DNA into an acidified intracellular compartment (124, 240). Acidification of CpG DNA within endosomes induces sequence specific generation of intracellular reactive oxygen species (ROS) (241). Endosomal maturation is essential for signaling since competition by non-CpG DNA or endosomal acidification inhibitors, such as chloroquine, quinacrine, bafilomycin and monensin, block cellular activation (241-243). Nuclear factor kappa B (NFκB) is an ubiquitous transcription factor in cells involved in immune and inflammatory reactions, and is involved in the expression of cytokines, chemokines, cell adhesion molecules, growth factors, and immunoreceptors (244, 245). Inactive NFκB is present in the cytoplasm complexed with an inhibitory protein, IκB (244, 245). The rapid generation of ROS induced by CpG DNA results in the degradation of IκB that leads to NFκB activation and the subsequent induction of leukocyte gene transcription and cytokine secretion (241).

However not all genes whose expression is induced by CpG DNA have known NFκB binding sites in their promotors and additional intracellular pathways appear to be induced in CpG activated cells (246). Activation of APCs and B cells has been shown to be

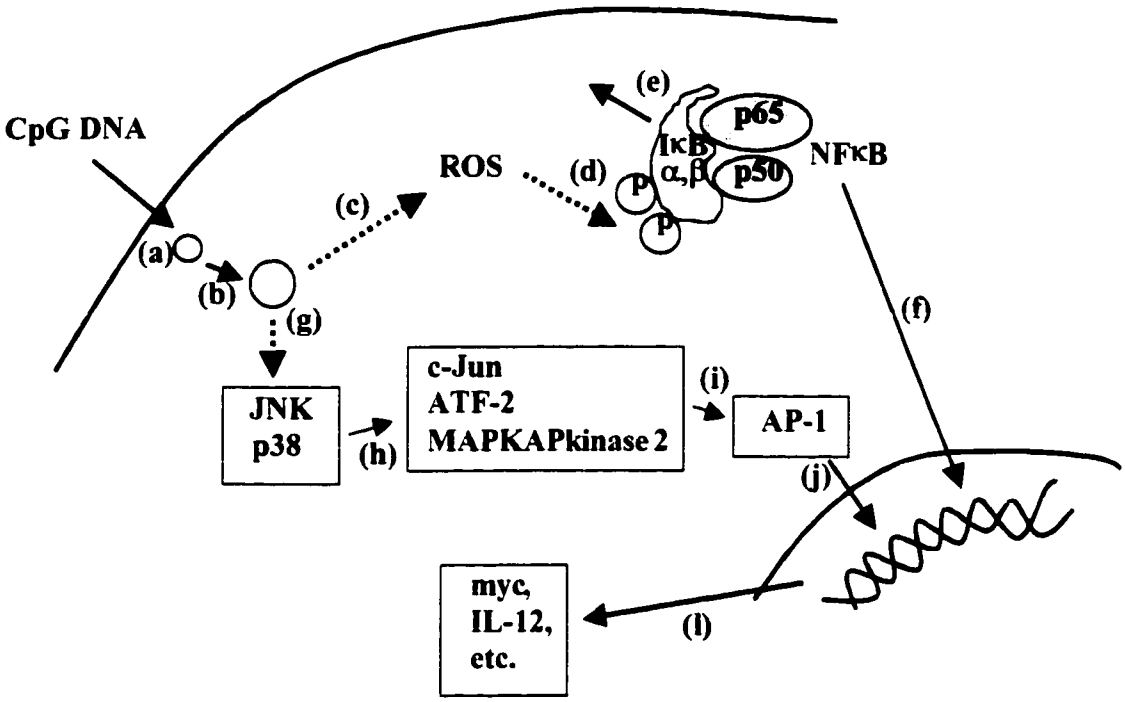

Figure 5. Schematic diagram of proposed CpG DNA-mediated signaling pathway.

The model illustrates suggested signaling pathways for CpG DNA-mediated cytokine and proto-oncogene regulation (adapted from ref. 241). (a) CpG DNA is taken up by B cells and monocytic cells via adsorptive endocytosis into an acidified compartment; (b) Endosomal acidification inhibitors (e.g., chloroquine) can block signaling pathways; (c) generation of sequence specific intracellular reactive oxygen species (ROS); (d) ROS induced phosphorylation of IκB; (e) polyubiquitination and degradation of IκB; (f) translocation of NFκB to the nucleus and upregulation of gene transcription; (g) phosphorylation of c-Jun NH_2-terminal kinase (JNK) and p38; (h) phosphorylation of c-Jun, activating transcription factor-2 (ATF-2), and mitogen-activated protein kinase activated protein (MAPKAP) kinase 2; (i) activation of activating protein-1 (AP-1); (j) translocation of AP-1 to the nucleus and upregulation of gene transcription; (k) expression of cytokines and proto-oncogenes.

mediated by CpG induced phosphorylation of mitogen-activated protein kinases (243, 246). This leads to activation of the transcription activating protein-1 (AP-1) which may participate in gene transcription in CpG activated cells (243, 246). Activation of both NFκB and AP-1 appear required for cytokine production, although other immune effects may only require one pathway (246).

1.6.3. Sequence specificity of CpG motifs

Not all DNA sequences containing CpG motifs have stimulatory effects on the immune system. Immunostimulatory CpG motifs (CpG-S) are generally preceded on the 5' side by two purines such as ApA, GpA or a purine and a T such as GpT and followed on the 3' side by 2 pyrimidines, most commonly TpT. In addition, DNA sequences containing CpG dinucleotides have been identified that can counteract the stimulatory effect of CpG-S. These neutralizing motifs (CpG-N) consist of various combinations that typically include CGCG, CCG and CGG. Interestingly, high numbers of CpG-N motifs are found in certain serotypes of adenovirus (e.g., types 2 and 5) and appear to have evolved as a defence mechanism of the virus to suppress the immunostimulatory properties of CpG-S motifs (175).

There appears to be some species specificity associated with CpG ODN. Mouse cells can be stimulated by a broad range of CpG motifs, whereas human cells have a much more restricted subset of stimulatory CpG motifs. Indeed, an excellent CpG motif for activating mouse cells (TGACGTT) does not activate human cells (A. M. Krieg *et al.*, unpublished observations). Recently CpG motifs have been identified that are highly stimulatory for human and monkey peripheral blood mononuclear cells, and different

motifs are optimal for stimulating fish, cat and dog immune responses (A. M. Krieg *et al.*, unpublished observations). The efficacy of a particular CpG ODN in a given species is influenced by several factors including: i) the surrounding base sequence; ii) number and spacing of CpG motifs; iii) the nature of the DNA backbone (phosphorothioate vs. phosphodiester).

1.7. The scope of this thesis

1.7.1. Aim and objectives of this study

The aim of this thesis is the development of immunization strategies for the induction of mucosal immunity. The hepatitis B virus was selected as model, primarily on account of the considerable experience that we have in our laboratory using HBsAg as antigenic protein or HBsAg-encoding plasmid. Although mucosal immunity does not appear to be necessary for protection against hepatitis B, the possibility to deliver vaccines by easy and non-invasive means is attractive for large-scale prophylactic vaccination.

Specific objective include:

i) to determine the effect of route and method of DNA immunization in mice and non human primates;

ii) to evaluate different DNA formulations that might facilitate gene delivery to mucosal surfaces;

iii) to evaluate the efficacy of CpG DNA as a mucosal adjuvant with a protein antigen whether used alone or in combination with other mucosal adjuvants;

iv) to evaluate the efficacy of antigen-antibody complexes (comprised of HBsAg complexed with antibodies against HBsAg) for the induction of immune responses against hepatitis B.

1.7.2. Overview of this study

In recent years there has been considerable interest in the field of DNA-based immunization. However, in March 1995, when the work described in this thesis was started, DNA-based vaccination was still a novel field. IM or ID injections were the methods of choice and only one report had studied mucosal delivery of a DNA vaccine (84). While this study offered proof of principle, only systemic but not mucosal immune responses were evaluated. We had developed a DNA-based vaccine against hepatitis B which was capable of inducing strong humoral and cellular immune responses against HBsAg with IM injection (133), however it had not been tested by other routes. At that time, the primary aim of my thesis research was to investigate the use of an HBsAg-expressing DNA vaccine for the induction of mucosal immune responses.

In recent years, a large number of routes of administration of DNA vaccines have been reported, but in spite of this, few studies have directly compared a number of routes, especially in non-human primates. In **Chapter II** of this thesis, we examined the ability of an HBsAg-encoding plasmid to induce immune responses in mice after delivery by a large number of injected and non-injected routes and compared three of the most frequently used delivery methods, namely IM and ID injection, and GG delivery to skin, for immunization of non-human primates (rhesus monkeys).

Direct gene transfer into the respiratory system is of considerable interest for either therapeutic or immunization purposes. Various reports had indicated that gene transfer could be carried out in the lung tissue using plasmid DNA either alone or formulated with cationic lipids, however there were conflicting reports as to the relative efficiencies of these two methods. In **Chapter III**, we examined direct gene transfer in the lungs of mice by administration of plasmid DNA using different techniques and formulations.

The induction of strong immune responses in animal models following introduction of DNA appears to be partly due to the adjuvant effect of unmethylated immunostimulatory CpG motifs present in the DNA backbone. Synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs (CpG) are potent adjuvants for induction of Th1-like systemic immune responses against parenterally-delivered proteins. In **Chapter IV**, we determined the efficacy of CpG as a mucosal adjuvant to HBsAg surface antigen after IN delivery. Furthermore, we compared HBsAg-specific mucosal and systemic responses using CpG as adjuvant to those obtained with CT, the most commonly used mucosal adjuvant in animal studies, and described the synergistic effect of CpG and CT. **Chapter V** further evaluated the potential of CpG ODN as a mucosal adjuvant to HBsAg and described the effect of higher doses of adjuvant and boosting on systemic (humoral and cellular) and mucosal immune responses against HBsAg. In **Chapter VI**, the adjuvanticity of CpG ODN relative to or in combination with CT, LT, the B subunit of CT (CTB) and a non-toxic derivative of LT (LTK63) was evaluated with IN delivery.

Antigen-antibody complexes have been shown to enhance immune responses against several antigens given by parenteral immunization. In **Chapter VII**, we evaluated the

potential of administering such immunostimulatory complexes (comprised of HBsAg complexed with antibodies against HBsAg) by a mucosal route and determined whether immune responses could be modulated by the use of CT or CpG as adjuvants.

The initial efforts to develop DNA vaccines for mucosal delivery were of very limited success, however we had considerable success with the antigen-antibody approach, and even more so with CpG DNA as an adjuvant to protein antigens. In an overview discussion **(Chapter VIII),** the possibility to develop safe and effective mucosal vaccines using DNA vaccines or DNA adjuvants is discussed in the context of what is known today.

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Chapter II

Route and method of delivery of DNA vaccine influence immune responses in mice and non-human primates

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Abstract

Background:

In spite of the large number of studies that have evaluated DNA-based immunization, few have directly compared the immune responses generated by different routes of immunization, particularly in non-human primates. Herein we examine the ability of a hepatitis B surface antigen (HBsAg)-encoding plasmid to induce immune responses in mice and non-human primates (rhesus monkeys: *Macaca mulatta*) after delivery by a number of routes.

Materials and Methods:

Eight different injected (intraperitoneal [IP], intradermal [ID], intravenous [IV], intramuscular [IM], intraperineal [IPER], sub-cutaneous [SC], sublingual [SL], vaginal wall [VW]), six non-injected (intranasal inhalation [INH], intranasal instillation [INS], intrarectal [IR], intravaginal [IVAG], ocular [Oc], oral feeding [oral]) routes and the gene-gun [GG] were used to deliver HBsAg-expressing plasmid DNA to BALB/c mice. Sera were assessed for HBsAg-specific antibodies (anti-HBs, IgG, IgG1, IgG2a) and CTL activity measured. Three of the most commonly used routes (IM, ID, GG) were compared in rhesus monkeys, also using HBsAg-expressing vectors. Monkeys were immunized with short (0, 4 and 8 wk) or long (0, 12 and 24 wk) intervals between boosts, and in the case of GG also with different doses, and their sera were assessed for anti-HBs.

Results:

In one study, anti-HBs were detected in plasma of mice treated by five of eight of the injected and none of the six non-injected routes. The highest levels of anti-HBs were induced by IM and IV injections, although significant titers were also obtained with SL and ID. Each of these routes also induced CTL, as did IPER and VW and one non-injected route (INH) that failed to induce antibodies. In a second study, GG (1.6 μg) was compared to ID and IM (100 μg) delivery. Significant titers were obtained by all routes after only one boost, with the highest levels detected by IM. Delivery to the skin by GG induced exclusively IgG1 antibodies (Th2-like) at 4 wk and only very low IgG2a levels at later times, ID immunized mice had predominantly IgG1 at 4 wk and this changed to mixed IgG1/IgG2a over time. Responses with IM injection (in the leg or tongue) were predominantly IgG2a (Th1-like) at all times. IV injection gave mixed IgG1/IgG2a responses. In monkeys, in the first experiment, 1 mg DNA IM or ID at 0, 4 and 8 wk gave equivalent anti-HBs titers and 0.4 μg at the same times by GG induced lower titers. In the second experiment, 1 mg DNA IM or ID, or 3.2 μg by GG, at 0, 12 and 24 wk, gave anti-HBs values in the hierarchy of GG>IM>ID. Furthermore, high titers were retained after a single immunization in mice but fell off over time in the monkeys, even after boost.

Conclusions:

Route of administration of plasmid DNA vaccines influences the strength and nature of immune responses in mice and non-human primates. However, the results in mice were not always predictive of those in monkeys and this is likely true for humans as well. Optimal dose and immunization schedule will most likely vary between species. It is not

clear whether results in non-human primates will be predictive of results in humans and additional studies are required.

Introduction

DNA-based immunization is a recently developed method of immunization that, in principle, could overcome many of the deficits of traditional antigen-based vaccines. Strong cellular and humoral responses have been demonstrated in a number of different animal models, subsequent to the *in vivo* expression of antigen from directly introduced plasmid DNA (see 1, 2), and several human clinical trials have been completed or are underway (3-6). Unfortunately, the promising results in animal models have not been realized in human trials and considerable effort is now being focused at understanding this difference and developing ways of improving the efficacy of DNA vaccines.

The induction of strong immune responses in animal models following introduction of DNA appears to be due to a combination of (i) the prolonged *in vivo* synthesis of antigen (7), (ii) in vivo antigen expression that results in major histocompatability complex (MHC) presentation (8, 9) and (iii) the adjuvant effect of unmethylated immunostimulatory CpG motifs present in the DNA backbone (10, 11). Thus, efforts to improve the efficacy of DNA vaccines include vector modifications, facilitated or targeted DNA delivery and the use of adjuvants in an attempt to increase the amount of antigen expressed, to have the antigen expressed in the most appropriate cells and/or to improve the immune responses against the expressed antigen.

The strength and nature of immune responses in mice with DNA vaccines appear to be influenced by a number of factors (see 1, 2), however these variables may not be of similar importance in larger animals including humans. As such, optimization methods developed in mice may not necessarily be applicable to humans. Factors that have

significant impact on subsequent immune responses in mice include the route and method of DNA delivery (12 -14), likely because of differences in the efficiency of gene transfer and the type of cell(s) transfected.

DNA vaccines have been delivered to a wide variety of tissues by both injected and non-injected DNA delivery methods including: (i) into the muscle by gene-gun (GG) or intramuscular (IM) injection (13, 15 - 20); (ii) into the skin by epidermal GG delivery (9, 12, 21), intradermal (ID) (12, 22, 23) or subcutaneous (SC) (12, 24) injection; (iii) into the circulatory system by intravenous (IV) injection (12, 25, 26); (iv) into the respiratory system by intranasal (IN) (12, 27 - 29) or intratracheal (12) delivery; (v) into the digestive system by oral feeding of microencapsulated (30, 31) or lipid-formulated DNA (32), injection into the oral mucosa (32), tongue (33), jejunum (32), and GG delivery into the Peyer's patches of the bowel (34), tongue (35) or oral mucosa (36); (vi) into the genitourinary tract by intravaginal GG delivery (34) or instillation (37, 38), and (vii) by topical ocular administration (39).

In spite of the large number of routes previously reported, few studies have directly compared the immune responses generated by different routes of immunization, particularly in non-human primates. We have previously described a model for DNA-based immunization against the hepatitis B virus (HBV) whereby strong systemic immunity is induced by IM or ID injection of DNA encoding HBV surface antigen (HBsAg). Herein, we examine the ability of a hepatitis B surface antigen (HBsAg)-encoding plasmid to induce immune responses in mice after delivery by a large number of routes and compare three of the most frequently used delivery methods, namely IM and

ID injection, and GG delivery to skin, for immunization of mice and non-human primates (rhesus monkeys).

Materials and Methods

Plasmid DNA:

Gene transfer studies were carried out with plasmid DNA encoding the middle (M = preS2 + S) or major (S) surface proteins of the HBV envelope. These plasmids, which were each under the control of the cytomegalovirus (CMV) immediate early promoter, with or without inclusion of the CMV intron A (A), were designated pCMV-S2.S (40), pCMV-S (16) and pCMV(A)-S (provided by PowderJect Vaccines, # WRG 7072). We have previously shown no difference in anti-HBs titers against the major (S) envelope protein after immunization with pCMV-S2S or pCMV-S (40) or with pCMV-S or pCMV(A)-S (Davis et al., unpublished results). The DNA was purified on Qiagen anion-exchange chromatography columns (Qiagen Inc., Chatsworth, CA) and resuspended in sterile saline (0.15 M NaCl, Sigma) to a final concentration of 1 to 5 mg/ml or coated onto 1 - 3 μm gold beads for GG delivery. DNA prepared in this way has only low levels of endotoxin (18). DNA in saline or coated onto gold beads was stored at -20°C or 4°C (after desiccation) respectively, until required for administration.

Animals:

Mice: All experiments were carried out using female BALB/c mice (Charles River, Montreal, QC) aged 6 to 8 wk with 3 to 12 mice per experimental or control group. Mice were shaved over the administration site for most injected routes and GG. For most injected routes as well as intravaginal instillation, mice were deeply anesthetized with Somnotol® (75 mg/kg IP; MTC Pharmaceuticals, Cambridge, ON) anesthesia. For other

non-injected routes, GG and IM injection, mice were lightly anesthetized by inhalation of Halothane® (Halocarbon Laboratories, River Edge, NJ).

Monkeys: All experiments were carried out with adult male and female rhesus monkeys (2.5 to 6.3 kg body weight) with 4 animals randomly assigned per experimental or control group. Animals were anesthetized with Ketamine HCl (10 mg/kg body weight; Phoenix Pharmaceutical, Inc., St. Joseph, Missouri) prior to immunization. Monkeys were maintained in the animal facility of Bioqual (Rockville, MD) or Primedica (formerly TSI Mason, Worcester, MA) and monitored daily by animal care specialists. No symptoms of general ill health or local adverse reactions at the sites of injection were noted.

DNA-based immunization of mice:

Each animal received a total of 100 μg pCMV-S, pCMV(A)-S or pCMV-S2.S in 0.15 M NaCl, with the exception of GG delivery where each animal received a total of 1.6 μg DNA coated onto gold particles as per our standard protocol (41). Gold particles (Degussa, Theodore, AL) used in mice and monkeys were 1 to 3 μm in diameter. DNA loading rate was 1.6 μg DNA/mg gold, 0.5 mg gold per cartridge in mice. The number and volume of administrations varied with the route of administration and had been determined in preliminary studies as optimal for that particular route. Some of the mice immunized by IM or ID injection, or GG delivery to the epidermis were boosted at different time points after priming.

Injected routes of administration and GG: GG: DNA was delivered to two separate sites in the lower abdomen using the hand-held, helium-powered PowderJect XR particle acceleration device (formerly known as Accell® gene gun). For all injected routes, DNA

was injected using an insulin syringe with a 29-gauge needle attached (Becton Dickinson, Franklin Lakes, NJ) by one of the following routes: ***intraperitoneal (IP)***: 150 µl directly into the peritoneal cavity; ***ID:*** 50 µl at each of 3 separate sites in the skin of the lower back; ***IM:*** 50 µl bilaterally into the tibialis anterior (TA) muscles; ***IV:*** 150 µl into the tail vein; ***intraperineal (IPER):*** 50 µl into the perineal region; ***SC:*** 150 µl under the skin of the lower back; ***sublingual (SL)***: 50 µl in the lower surface of the tongue; ***vaginal wall (VW):*** 50 µl into the submucosa deep to the vaginal epithelium.

Non-injected routes of administration: DNA solution was administered by one of the following routes: ***IN inhalation (INH):*** 3 x 50 µl at 15 minute intervals, applied as droplets directly over both external nares of mice; ***IN instillation (INS):*** 3 x 25 µl at 15 minute intervals, instilled directly into the nasal cavity by insertion of a gel-loading tip attached to a Gilson pipette a few mm into the nostril; ***intrarectal (IR):*** 100 µl DNA instilled via the anus using a 200 µl pipette tip, following which a small quantity of petroleum jelly was applied to prevent leakage; ***IVAG:*** 100 µl DNA slowly instilled into the mouse vaginal cavity using a 200 µl pipette tip, following which a small quantity of petroleum jelly was applied to prevent leakage (mice were anesthetized with Somnotol to prevent them from licking themselves and ingesting some of the DNA); ***ocular (Oc):*** 10 µl applied to each eyeball following which the eyelids were gently massaged; ***oral feeding (Oral):*** 100 µl administered directly into the stomach using a 1 c.c. tuberculin syringe (Becton Dickinson) attached to a 20-gauge olive tip steel feeding tube (Fine Science Tools Inc., North Vancouver, BC), which was passed through the oral cavity and into the esophagus.

DNA-based immunization of monkeys:

In one experiment (carried out at Bioqual), monkeys were immunized at 0, 4 and 8 wk with pCMV-S2.S DNA by IM or ID injection (1 mg) or GG delivery to the skin (0.4 μg). In a separate experiment (carried out at Primedica) monkeys were treated similarly except pCMV-S DNA was given at 0, 12 and 24 wk and the GG dose was increased to 3.2 μg. IM injections were performed with the Biojector® needle-free jet injection system (Bioject Inc., Portland, OR) using a barrel size previously shown to deliver almost all radio-opaque injectate into the muscle belly (data not shown) and ID injections using an insulin syringe with a 29-gauge needle attached (Becton Dickinson). We have previously shown that the Biojector is superior to IM injection for DNA-based immunization of rabbits (42) and Aotus monkeys (23). The Biojector is not suitable for IM injection of mice since the force of the liquid jet stream is too powerful for such a small animal. GG immunization in monkeys was also with the PowderJect XR system (DNA loading rates: 0.2 and 0.8 μg DNA per mg gold, in the first and second studies respectively, 0.5 mg gold per cartridge). DNA for each of these routes (4 monkeys/group) was delivered as follows: ***ID:*** 100 μl at each of 4 separate sites in the shaved skin of the lower back; ***IM:*** 250 μl at each of 4 sites by bilateral injection into the triceps and hamstring (Bioqual) or the triceps and quadriceps (Primedica); ***GG:*** 0.4 μg divided into 4 sites (Bioqual) or 3.2 μg divided into 8 sites (Primedica) over the mid-abdomen. All sites were shaved and swabbed with alcohol prior to DNA administration.

Evaluation of HBsAg-specific immune responses in mice:

Anti-HBs: Plasma was obtained by retro-orbital puncture at various time points after immunization. HBsAg-specific antibodies (anti-HBs) in plasma were detected by ELISA assay on individual samples using HBsAg-coated plates as previously described (43). End-point dilution titers for total IgG as well as IgG1 and IgG2a isotypes were defined as the highest plasma dilution that resulted in an absorbance value (OD 450) two times greater than that of non-immune plasma, with a cut-off value of 0.05. In our laboratory we have previously determined that the relationship between endpoint titers in mice and those in milli-international units (mIU), as defined by the World Health Organization (WHO), is close to 1:1 (77). A value of 10 mIU/ml is protective in humans. In addition, a panel of mouse standards was identified by comparing various mouse sera against human-derived standards defined by the WHO (Monolisa Anti-HBs Standards, Sanofi Diagnostics Pasteur, Montreal, QC) using a commercial kit (Monolisa Anti-HBs, Sanofi Diagnostics Pasteur). These mouse standards were then used to quantify anti-HBs titers in some mouse groups in mIU/ml. Anti-HBs titers were expressed as means (mIU/ml) or geometric means (end-point dilution) ± SEM of individual animal values, which were themselves the average of triplicate assays. Non-immune plasma was used as a negative control.

***HBsAg-specific cytotoxic T-lymphocyte (CTL) activity*:** Splenocytes taken at 4, 6 or 8 wk after immunization were specifically restimulated for 5 days in vitro with an irradiated HBsAg-expressing cell line (P815S) and then used in a chromium release assay with the same HBsAg-expressing target cells, or a non-expressing control cell line (P815), as

previously described (44). Effector:target (E:T) ratios of 6.25:1, 25:1, 50:1 and 100:1 were used for all assays, although only data with the 50:1 ratio is shown (Figs. 1 and 4) as relative differences were similar with all ratios tested. Data was expressed as % specific lysis = % lysis with P815S cells - % lysis P815 cells. The percent lysis was calculated as [(experimental release - spontaneous release) / (total release - spontaneous release)] x 100. Spontaneous release was determined by incubating target cells without effector cells and total release was determined by adding 100 µl of 2% Triton-X 100 to target cells. The percent specific lysis was calculated. CTL activity was expressed as group means ± SEM of individual animal values, which were themselves the average of values from triplicate assays.

Evaluation of anti-HBs responses in monkeys:

Monkeys were bled by IV puncture prior to and at various time-points after immunization and anti-HBs antibodies were detected and quantified by ELISA on individual serum samples as follows. A solid phase of plasma-derived HBsAg particles (*ay* subtype, 100 µl of 1 µg/ml per well, overnight at RT; International Enzymes, Inc., Fallbrook, CA) was used to capture anti-HBs antibodies in the serum (1 hr at 37°C), which were then detected using a non-species-specific conjugate and *o*-Phenylenediamine (OPD) as chromogen supplied as part of a commercial kit (Monolisa Anti-HBs). Anti-HBs titers were expressed in mIU/ml based on comparison with WHO defined standards (Monolisa Anti-HBs Standards).

Statistical analysis:

Data were analyzed using the GraphPAD InStat program (GraphPAD Software, San Diego). The statistical significance of the difference between values obtained from two groups was determined by Student's 2-tailed *t*-test and from three or more groups by 1-factor analysis of variance followed by Tukey's test. Anti-HBs titers were subjected to logarithmic transformation prior to statistical analysis. Differences were considered to be not significant when p was > 0.05.

Results

Effect of route of immunization on immune responses in mice:

The mouse studies were done in two separate experiments. The first compared 14 different injected or non-injected routes. The second study compared IM and ID injection and GG.

Humoral response: Anti-HBs were detected in plasma of mice immunized with 100 μg pCMV(A)-S by some of the injected, but none of the non-injected routes (Figure 1, upper panel). The IM, SL and ID routes resulted in 100% seroconversion compared to 60% with IV injection. Equivalent levels of anti-HBs were attained by 8 wk post-immunization in responding mice in the IM and IV groups ($p = 0.56$), and in the SL and ID routes ($p = 0.91$), but the former pair had significantly greater titers than the latter pair ($p < 0.05$). Both IM and SL immunization induced mixed Th1/Th2 responses, but with considerably more IgG2a than IgG1. IgG2a antibodies are considered indicative of a Th1 response, although it is not possible to directly compare levels of antibodies because of different avidities for the two antibodies. IV and ID immunization also induced a mixed IgG1/IgG2a response at early time points, and over time these shifted slightly towards Th1-like for IV and slightly towards Th2-like for ID routes (Figure 2). A humoral response was not detected with the other injected routes (IP, IPER, SC and VW) or with any of the non-injected routes (INH, INS, IR, IVAG, Oc and Oral) (Figure 1).

Figure 1:

BALB/c mice were immunized with 100 μg pCMV(A)-S in saline by various routes.

Upper panel: Each bar represents the group geometric mean titer (GMT) (± SEM) determined by end-point dilution ELISA assay for HBsAg-specific antibodies (anti-HBs, total IgG) in plasma of mice that responded (endpoint titer > 10) taken 8 wk after immunization. Number of responders per group total is indicated below graph. End-point dilution titers were determined as the highest sample dilution resulting in an absorbance value two times that of non-immune plasma, with a cut-off value of 0.05.

Lower panel: Each bar represents the mean HBsAg-specific lysis (± SEM) as a percentage of the total possible lysis (% specific lysis) at effector to target ratio (E:T) of 50:1 in splenocytes from mice that responded (% specific lysis > 10) 8 wk after immunization. Number of responders per group total is indicated above graph. Abbreviation: ND, not determined.

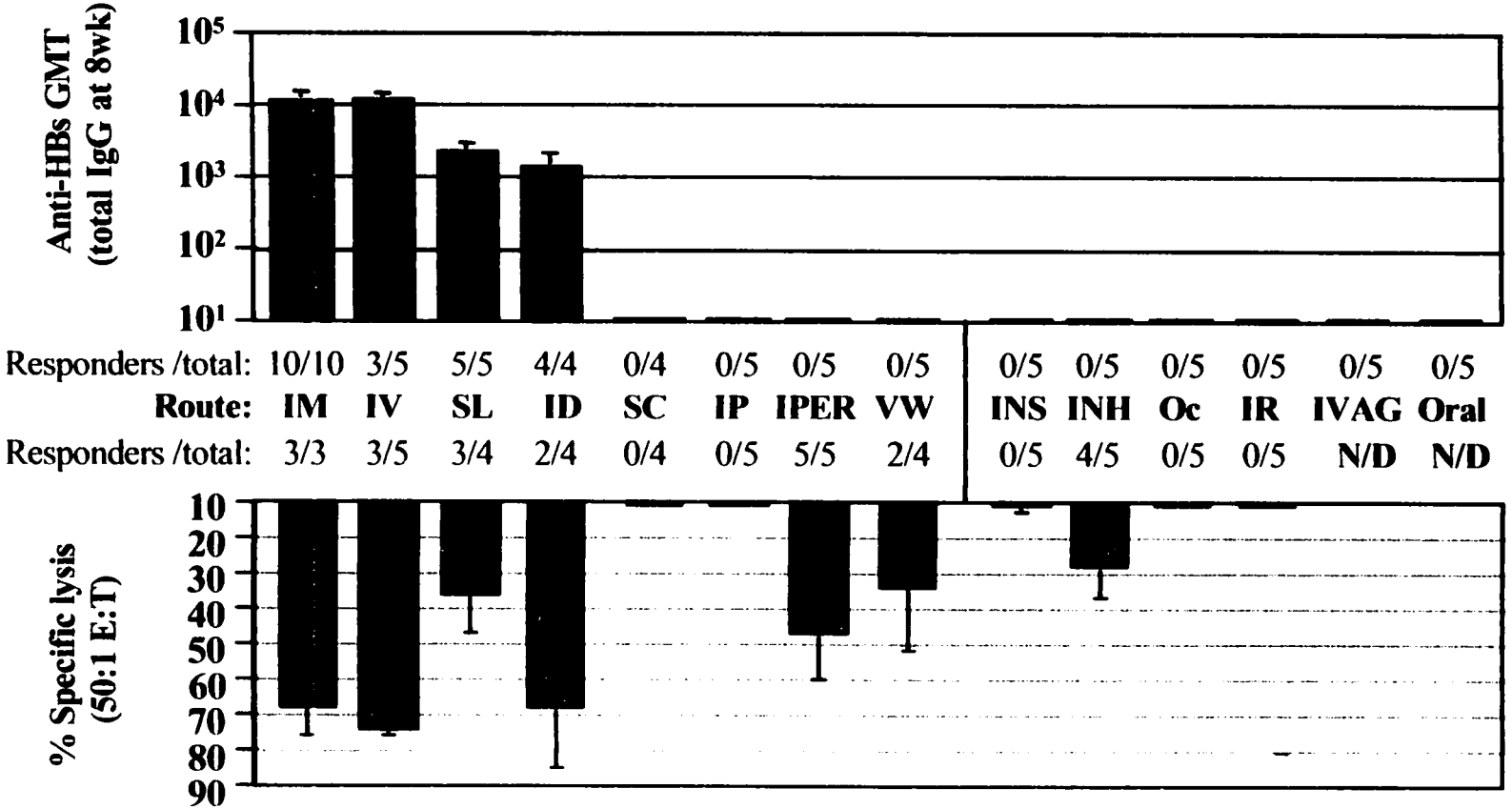
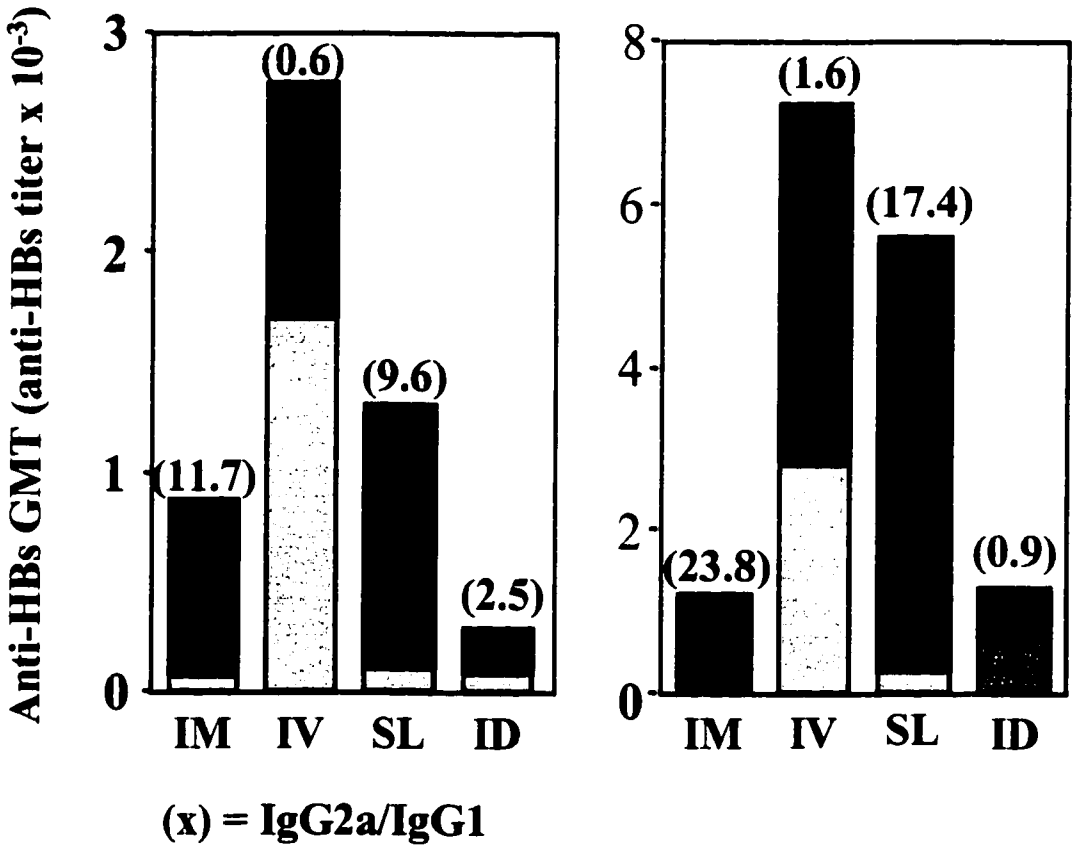

Anti-HBs GMT
(total IgG at 8wk)
10⁵
10⁴
10³
10²
10¹
Responders /total: 10/10 3/5 5/5 4/4 0/4 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5
Route: IM IV SL ID SC IP IPER VW INS INH Oc IR IVAG Oral
Responders /total: 3/3 3/5 3/4 2/4 0/4 0/5 5/5 2/4 0/5 4/5 0/5 0/5 N/D N/D
% Specific lysis
(50:1 E:T)
10
20
30
40
50
60
70
80
90

Figure 2:

BALB/c mice were immunized with 100 μg pCMV(A)-S in saline by various routes. Each bar represents the group geometric mean titer (GMT) determined by end-point dilution ELISA assay for HBsAg-specific antibodies (anti-HBs) of IgG1 (gray bars) or IgG2a (black bars) isotypes in plasma taken 4 wk (panel A) and 8 wk (panel B) after immunization. Titers were defined as the highest plasma dilution resulting in an absorbance value two times that of non-immune plasma, with a cut-off value of 0.05. Numbers above each bar indicate the IgG2a to IgG1 ratio, with a value >1 indicating a more Th-1 like response.


Anti-HBs GMT (anti-HBs titer x 10^{-3})
3
2
1
0
(0.6)
(11.7)
(9.6)
(2.5)
IM
IV
SL
ID
8
6
4
2
0
(1.6)
(17.4)
(23.8)
(0.9)
IM
IV
SL
ID
(x) = IgG2a/IgG1

Cell-mediated responses: HBsAg-specific CTL were detected following immunization by six injected routes (IM, IV, SL, ID, IPER and VW) as well as by one non-injected route (INH) (Figure 1, lower panel). The IM and IV routes resulted in significantly stronger cellular responses than INH ($p = 0.01$ and 0.006 respectively), and IV was stronger than SL ($p = 0.03$), but there were no other significant differences between routes ($p > 0.05$). Not all groups with CTL had anti-HBs (e.g. IPER, VW) and not all animals with humoral responses had cellular responses (e.g. for ID immunization, 4/4 had anti-HBs and 2/4 had CTL). Furthermore, CTL responses did not always correlate with anti-HBs isotypes. For example, there were low CTL with predominantly IgG2a (Th1-like) for SL yet strong CTL with both IgG1 and IgG2a (mixed Th1/Th2) for ID. Only with IM injection did 100% of mice both seroconvert and have CTL activity (Figure 1).

Immune responses in mice with IM, ID and GG delivery:

Humoral responses: There was 100% seroconversion (anti-HBs>10 mIU/ml) by 2 wk in mice immunized with 100 μg pCMV-S2.S by IM or ID injection (10/10). In mice immunized with more than 60-fold less (1.6 μg) DNA by GG there was 80% seroconversion at 2 wk (8/10) and 100% by 4 wk. Anti-HBs total IgG titers at 4 wk after priming were higher after IM than either ID or GG immunizations ($p = 0.0005$ and $p < 0.0001$ respectively), and ID gave higher titers than GG ($p = 0.015$). Boosting at 4 wk raised anti-HBs titers approximately ten-fold for IM and ID groups and twenty-fold for GG immunized mice ($p < 0.001$ relative to pre-boost titers for all routes).

Post-boost titers remained the highest with IM ($p < 0.05$), but there was no longer a difference between the ID and GG groups ($p = 0.59$). Animals immunized by IM and ID, but not GG, received a second boost at 8 wk. This did not increase anti-HBs titers further ($p = 0.16$ and 0.35 for IM and ID respectively compared to pre-boost levels) (Figure 3, panel A). Near-peak levels of anti-HBs were maintained until the end of the experiment in all groups (5 to 6 wk after the last boost, $p > 0.05$).

Evaluation of plasma for IgG1 and IgG2a antibody isotypes, as indicators of Th2- and Th1-like responses respectively, showed, 4 wk after priming, only IgG1 antibodies following GG, predominantly IgG1 following ID and mixed IgG1/IgG2a following IM delivery. These all shifted towards a Th1 response over time such that ID became mixed Th1/Th2 and IM became Th1-like, although they remained Th2-like for GG. Subsequent boosting did not change the ratio of antibody isotypes in any groups (Table 1). These findings indicate that, at least for the time points evaluated herein, route and method of immunization can influence the kinetics, strength and type of the humoral response after DNA-based immunization.

CTL: HBsAg-specific CTL were detected after each of IM, ID and GG immunizations. There was no difference in mean CTL activity 4 wk after IM or ID delivery of 100 μg pCMV-S ($p > 0.05$), although there was lower variability with IM than ID (Figure 4, panel A). There was no significant effect on CTL in IM or ID groups by boosting mice in an identical manner at 2 wk (not shown). CTL activity was weaker after 2 doses by GG administration than one dose by IM or ID injection ($p < 0.002$) (Figure 4, panel B). It should be noted that the dose was more than 60-fold lower with GG than the

Figure 3:

Panel A: BALB/c mice were immunized at 0, 4 and 8 wk with 100 μg pCMV-S2.S by IM (white bars) or ID injection (gray bars) or with 1.6 μg by GG delivery (black bars). Each bar represents the group mean (± SEM) of anti-HBs titer (mIU/ml) in plasma collected 4 wk after priming, or 2 wk after each boost. End-point dilution titers were determined as the highest sample dilution resulting in an absorbance value two times that of non-immune plasma, with a cut-off value of 0.05. Values in mIU/ml were determined from mouse standards identified by comparison with human-derived standards and the Monolisa Anti-HBs Detection Kit (Sanofi Diagnostics Pasteur). Abbreviation: N/D, not determined.

Panel B: Rhesus monkeys were immunized at 0, 4 and 8 wk with 1 mg pCMV-S2.S by IM (white bars) or ID injection (gray bars) or with 0.4 μg by GG delivery (black bars). Each bar represents the group mean (± SEM) of anti-HBs titer (mIU/ml) in plasma collected 4 wk after priming, or 2 wk after each boost. Values in mIU/ml were determined with human-derived standards and the Monolisa Anti-HBs Detection Kit (Sanofi Diagnostics Pasteur).

Panel C: Rhesus monkeys were immunized at 0, 12 and 24 wk with 1 mg pCMV-S by IM (white bars) or ID injection (gray bars) or with 3.2 μg by GG delivery (black bars). Each bar represents the group mean (± SEM) of anti-HBs titer (mIU/ml) in plasma collected 4 wk after priming, or 2 wk after each boost. Values in mIU/ml were determined with human-derived standards and the Monolisa Anti-HBs Detection Kit (Sanofi Diagnostics Pasteur).

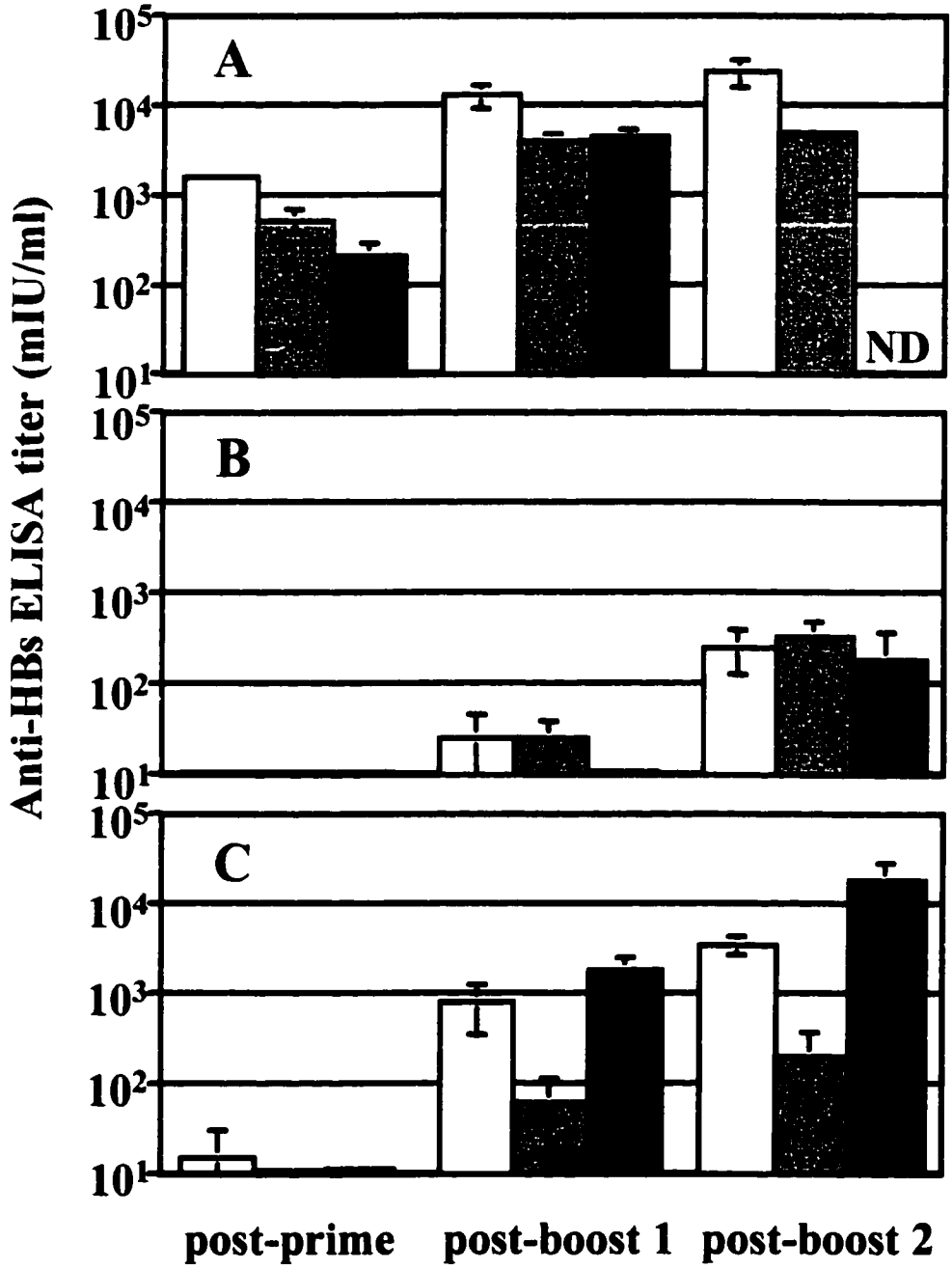

Anti-HBs ELISA titer (mIU/ml)
A
B
C
ND
10^5
10^4
10^3
10^2
10^1
post-prime
post-boost 1
post-boost 2

Table 1:

BALB/c mice were immunized by IM or ID injection with 100 μg pCMV-S2.S or by GG delivery with 1.6 μg. At 4 and 6 wk some mice were boosted in an identical manner to prime. HBsAg-specific antibodies of IgG1 or IgG2a isotypes were determined by endpoint dilution ELISA assay in plasma taken at various timepoints after immunization. Titers were defined as the highest plasma dilution resulting in an absorbance value two times that of non-immune plasma, with a cut-off value of 0.05. The IgG2a to IgG1 ratios were determined, with a value >1 indicating a predominantly Th-1 like response.

Immunization route/method	IgG2a/IgG1	IgG2a/IgG1	IgG2a/IgG1
	4 wk	***12 wk (no boost)***	***12 wk (2 boosts)***
IM	1.2	2.7	3.2
ID	0.2	0.9	1.3
	4 wk	***9 wk (no boost)***	***9 wk (1 boost)***
GG	0.01*	0.003*	0.002

* There was no detectable IgG2a with these groups, thus a value of 1 was assigned for IgG2a to determine IgG2a/IgG1.

injected routes, and we do not have single dose GG data to know whether there was a boost effect by the second dose. Nevertheless, results are comparable because all CTL assays were carried out simultaneously. There was no difference in CTL activity in mice boosted by GG at 2 or 4 wk ($p > 0.05$), nor was there a difference in mice immunized IM with 100 μg pCMV-S or pCMV-S2.S ($p > 0.05$) (Figure 4, panels A and B).

Humoral response in monkeys:

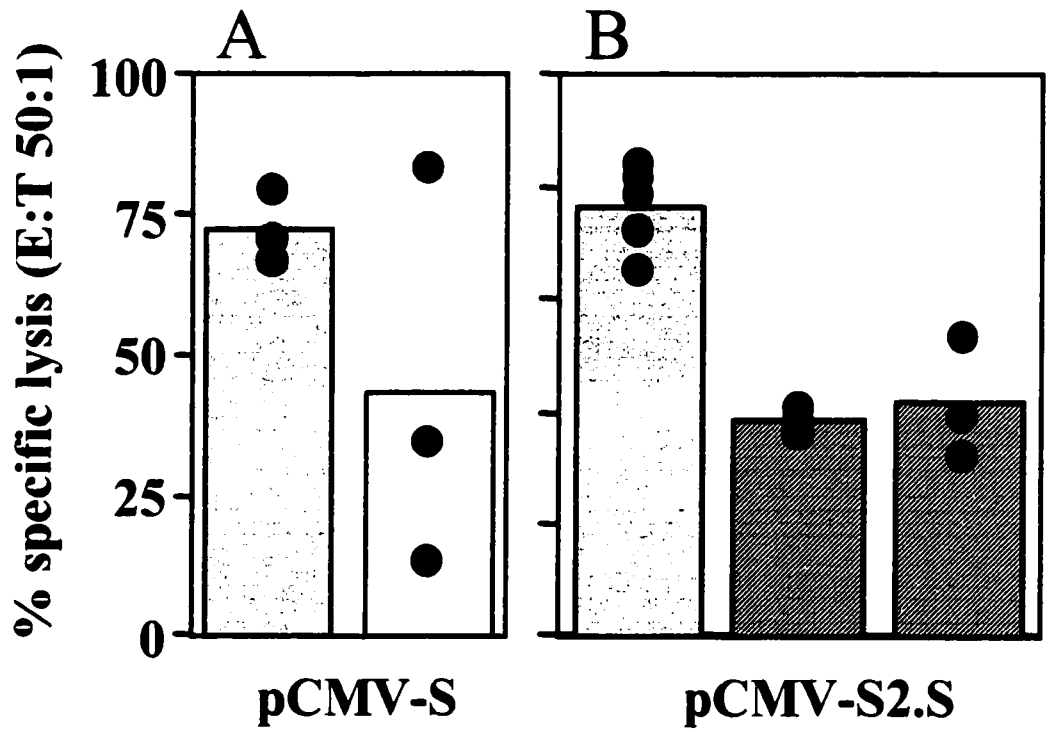
Response after priming: After administration of pCMV-S2.S or pCMV-S DNA, anti-HBs were not detected by 4 wk in ID or GG groups (0/8) and detected in only 1 of 8 (60 mIU/ml) in the IM groups. By 8 wk after priming, two monkeys in GG group (2/4) had seroconverted, as had one monkey in the ID group (1/4) by 12 wk.

Experiment one (boosting at 4 and 8 wk): Although no monkeys in this experiment had seroconverted by the time of the first boost, by two wk post-boost, low levels of anti-HBs (10 to 100 mIU/ml, $p = 0.69$) were detected in plasma of some monkeys immunized by IM (2/4) or ID (3/4), but not by GG (0/4). However by 4 wk after the first boost one of the monkeys immunized by GG had seroconverted (16 mIU/ml). By 2 wk after the second boost the number of responders increased for IM and ID groups (3/4 and 4/4 respectively), and by 4 wk post-boost for GG (2/4), with all animals attaining low to moderate levels (< 1000 mIU/ml) and no significant differences among groups ($p = 0.3$) (Figure 3, panel B).

Figure 4:

Panel A: BALB/c mice were immunized with 100 μg pCMV-S by IM (gray bars) or ID (white bars) injection. Spleens were removed 4 wk later and CTL activity was determined. Bars represent the mean HBsAg-specific lysis as a percentage of the total possible lysis (% specific lysis) at effector to target ratio (E:T) of 50:1, and black dots represent % specific lysis for individual mice.

Panel B: BALB/c mice were immunized with 100 μg pCMV-S2.S by IM (gray bars) or 1.6 μg by GG (diagonally striped bars) delivery. Mice immunized by GG were boosted in identical manner 2 wk (light gray striped bars) or 4 wk (black striped bars) later. CTL activity was determined on spleens removed 6 wk after priming for IM and ID groups, or at 2 or 4 wk after boost (which was carried out 4 wk post-prime) for GG groups. Bars represent the mean HBsAg-specific lysis as a percentage of the total possible lysis (% specific lysis) at effector to target ratio (E:T) of 50:1, and black dots represent % specific lysis for individual mice.


A
B
100
75
50
25
0
% specific lysis (E:T 50:1)
pCMV-S
pCMV-S2.S

Experiment two (boosting at 12 and 24 wk): At the time of the first boost, only a few monkeys had seroconverted (IM 1/4, ID 1/4, GG 2/4). By 2 wk after a 12 wk boost, most monkeys had seroconverted (IM 4/4, ID 2/4, GG 3/4) and anti-HBs titers had peaked at 62 ± 54, 816 ± 461 and 1827 ± 742 mIU/ml for ID, IM and GG respectively. However over the following 6 wk anti-HBs levels decreased 2 to 6 fold in all groups. A second boost given at 24 wk raised anti-HBs levels ~20 fold by 2 wk later for all groups and brought about seroconversion in one more monkey of the ID group. Anti-HBs titers decreased by 2 to 6 fold over the subsequent 6 wk (data not shown). Overall, peak anti-HBs titers attained with GG immunization were 5 and 100 fold higher than those with IM and ID delivery, respectively.

Effect of timing of boosts: It is possible to compare timing of boosts for the IM and ID groups where dose was kept constant. The anti-HBs response with IM immunized monkeys was improved by lengthening the rest period between immunizations. Anti-HBs titers attained for monkeys immunized IM were greater when boosted at 12 and 24 wk than those at 4 and 8 wk ($p = 0.008$, $p = 0.019$ for 2 wk post boost 1 and 2 respectively). In contrast, an increase in anti-HBs levels was not seen with the increased rest period for ID immunized animals ($p > 0.05$) (Figure 3, panels B and C). It is not possible to evaluate this effect with GG since the dose was also changed.

Discussion

DNA vaccines have been shown to induce strong humoral and cellular immune responses against numerous bacterial, viral and parasite antigens in animal models. This, combined with the many potential economical and practical advantages that they offer, led to intense interest in the development of DNA vaccines for use in humans. Unfortunately, early human clinical trials have not proven to be as successful as was predicted from pre-clinical studies, which were mostly carried out in mice. Considerable effort has been expended towards improving the efficacy of DNA vaccines through (i) improved efficiency of transfection with facilitated delivery methods such as liposomes (45), DNA-compacting agents (46), and gene-gun for direct intracellular delivery (47), (ii) improved vector design for high levels of expression and longer mRNA stability (1), (iii) addition of immunostimulatory CpG motifs to plasmid vectors (48), (iv) co-expression of co-stimulatory molecules or cytokines to augment immune responses (49, 50), (v) optimization of immunization schedule (51, 52), and (vi) combination with other vaccine approaches such as protein or attenuated viral vaccines (53, 54). The route of delivery of the DNA vaccine can have an impact on the efficiency of transfection as well as the types and location of cells transfected, and thus potentially on the nature of the immune response (12 - 14, 55, 56).

Many different routes have been shown to be effective for DNA delivery in mice, as has been confirmed in the present study, however few studies have compared responses obtained with different routes using the same antigen-expressing DNA, dose and immunization schedule. There have been even fewer studies to compare routes of

administration in non-human primates. We have attempted to bridge this gap by comparing the delivery of HBsAg-expressing vectors by 15 different routes in mice and three of the most common routes in rhesus monkeys.

When a large number of injected and non-injected routes were used to administer the DNA vaccine, anti-HBs were detected only in serum of mice treated by five of eight of the injected and by GG but none of the six non-injected routes. The highest levels of anti-HBs were induced by IM and IV injections although reasonable titers were also obtained with sublingual and ID injections and GG delivery of DNA-coated gold particles to the epidermis. Each of these routes also induced CTL, but notably CTL were also found with two other injected routes (perineum and vaginal wall injection) as well as one non-injected route (IN inhalation) that failed to induce antibodies. At one time it was thought that muscle was a preferred route for DNA vaccine delivery because early studies showed it to be more efficient than other tissues for the uptake and expression of plasmid DNA encoding reporter genes (57) and its post-mitotic nature allowed expression from the episomally located plasmids to continue for many months (58). However, more recent studies with antigen-encoding plasmids have shown that antigen expression does not continue indefinitely, but rather is lost by some immune-mediated mechanism around 2-3 weeks after DNA injection (7). Furthermore, even though immune responses are possible following transplantation of antigen-expressing myoblasts (59), only professional antigen-presenting cells are actually capable of priming immune responses (59 - 62). Nevertheless, based on findings that immune responses were unaffected when the injected muscle was removed within seconds of injection (63), it is uncertain what role the

relatively few antigen-expressing muscle fibers play after direct injection of DNA vaccines into muscle. Indeed it is possible that direct transfection of APC may be necessary in the case of DNA vaccines, and following IM injection, such transfected APC are found in regional lymph nodes (64). However, it is not clear whether transfection occurs only at the site of DNA administration or whether the DNA can travel to lymphoid tissues and transfect cells there. Successful delivery routes appear to deliver the DNA to regions of concentrated lymphoid tissue or organs: IV and IP to the spleen, ID to Langerhan cells, IN and sublingual or vaginal wall injections to the extensive diffuse interstitial lymphoid tissues of the naso-oropharynx and genitourinary systems respectively. Why some approaches induced CTL without antibodies is difficult to say, although this may be largely a question of efficiency since these routes tended to have the weakest CTL responses. The generally absent responses with the non-injected routes was not unexpected as the mucosal surfaces are protective barriers physiologically designed to limit uptake of bacteria, viruses and antigens, and, unless the mucosal surface has been broken or damaged, transfection efficiency using naked DNA is low. Efforts to circumvent this have included formulation of plasmid DNA with cationic lipids (24, 29, 32), the use of mucosal adjuvants (27, 28, 32), microencapsulation for oral delivery (30, 31, 65) or physical penetration of naked DNA into mucosal tissue using GG (34, 36).

A number of factors appear to influence the Th-bias of the response including: (i) the antigen (20), (ii) the dose of antigen (66), (iii) whether the antigen is secreted, cytoplasmic or membrane bound (67, 68), (iv) the route and method of DNA administration (13, 14, 62), (v) number of immunizations (14, 69, 70), (vi) the presence of

CpG motifs (10, 71), (vii) the haplotype of the mouse immunized (72), (viii) the presence of adjuvant (27, 28 32), (ix) co-expression of cytokines (73, 74), (ix) whether or not DNA is formulated (e.g. with cationic liposomes) (24, 29, 32), and (x) rest period between immunizations (50, 51). Previous studies have reported Th2-like or mixed Th1/Th2 responses with DNA delivery to the skin and more Th1-like responses with IM injection (13, 14, 19, 75, 76) and this has been corroborated in the present study. However, antibody isotype did not change as a result of booster immunizations in the present study, in contrast with findings of other studies (14, 69). These differences may be due to the nature of the encoded antigen, which can affect the antibody isotypes induced (20). While it is not clear exactly what factors determine the Th-bias of responses with DNA vaccines, comparisons between studies should take into account the time at which antibody isotypes are determined and whether it is a primary or secondary response.

Anti-HBs isotypes did not always correlate with CTL responses. For example low CTL were found with SL administration despite predominantly IgG2a antibodies (Th1-like), and strong CTL were found with ID concomitant with a Th2-like antibody response. Although IgG2a and CTL are both considered indicative of Th1 responses, we have also noted such disassociation of antibody isotype and CTL activity with mucosal administration of antigen (44) or with DNA-based immunization of neonatal mice (77). Thus precise evaluation of the Th response may also require analysis of cytokine secretion from antigen-stimulated Th cells in vitro.

The realization that results in mice often do not predict the situation in humans has also led to a large number of DNA vaccine studies in non-human primates, including

Aotus monkeys (23), rhesus monkeys (50, 78 - 80) and chimpanzees (17, 38, 81-- 84). IM injection of plasmid DNA vaccines, while highly immunogenic in mice (see 1, 2) was found to be only relatively so in chimpanzees (17, 38, 79, 83), and essentially not at all in Aotus monkeys (23). Furthermore, although early human studies have demonstrated the safety and potential of DNA vaccines, results obtained have not been as good as predicted from animal models (3-6). Collectively, these results indicate that no animal model may be ideal for prediction of efficacy in humans. The relatively greater efficacy of IM in mice than primates may be related to morphological differences. Alternatively, it may be related more to dose. The 10 to 100 μg doses of DNA vaccine typically used in a 20 g mouse would be equivalent to 35 to 350 mg in a 70 kg human, a dose range greatly in excess of what has been used to date in human clinical trials.

In summary, mice may have limited value for choosing the best route of DNA vaccine delivery for humans. While efficacy in murine models has preceeded the successful development of many human vaccines, it is probably safe to say that any vaccine that works in a human will work in a mouse, but not necessarily *vice versa*. Therefore, it is difficult to predict from mouse studies the potential of a new vaccine for humans. In fact, in those human trials that have been carried out, none of the DNA vaccines induced the strong immune responses that had been seen in mice with the same vectors. Furthermore, although non-human primate models are frequently used for development and testing of human vaccines, it is not clear how predictive they will be in the case of DNA vaccines where efficacy, by virtue of the requirement first to transfect cells and express the antigen, relies on many factors other than immunological responses to the antigen. We will not

know the answer to this until after greater experience has been achieved in non-human primates and human clinical trials.

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Chapter III

Direct gene transfer to the respiratory tract of mice with pure plasmid and lipid-formulated DNA.

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Abstract

Direct gene transfer into the respiratory system could be carried out for either therapeutic or immunization purposes. Here we demonstrate that cells in the lung can take up and express plasmid DNA encoding a luciferase reporter gene whether it is administered in naked form or formulated with cationic liposomes. Depending on the lipid used, the transfection efficiency with liposome-formulated DNA may be higher, the same as, or less than that with pure plasmid DNA. Tetramethyltetraalkylspermine analogues with alkyl groups of 16 or 18 carbons and DMRIE/cholesterol formulations proved particularly effective. Similar results for reporter gene expression in the lung were obtained whether the DNA (naked or lipid formulated) was administered by indirect, non-invasive intranasal delivery (inhaled or instilled) or by invasive, direct intra-tracheal delivery (injected or via a cannula). Reporter gene expression peaks around 4 days then falls off dramatically by 9 days. The dose-response is linear, at least up to 100 μg plasmid DNA, suggesting better transfection efficiencies might be realized if there was not a volume limitation. For a given dose of DNA, the best results are obtained when the DNA is mixed with the minimum amount of lipid that can complex it completely. These results are discussed in the context of direct gene transfer for either gene therapy or delivery of a mucosal DNA vaccine.

Introduction

The respiratory system is attractive for direct gene delivery, as it has an extensive surface area of pulmonary epithelium that is readily accessible via the bronchiolar and alveolar network. This easy and non-invasive method of gene delivery has the potential to transfect a large number of cells and allow high local concentrations of expressed gene products to be attained. Gene delivery into the respiratory system could be useful for either therapeutic or immunization purposes.

There are several potential therapeutic applications for direct gene transfer in the lung. Perhaps the most obvious is gene therapy for inborn errors of metabolism, such as delivery of the cystic fibrosis (CF) transmembrane conductance regulator gene (CFTR) to treat CF (Hyde *et al.*, 1993; Yoshimura *et al.*, 1992; Alton *et al.*, 1993; Caplen *et al.*, 1995). It might also be possible to treat allergy-induced asthma, for example, by expressing interferon-γ (IFN-γ) to shift a T helper type 2 (Th2) immune response, which is associated with IgE, to a Th1 response (Li *et al.*, 1996). Lung cancer could perhaps be treated by direct transfer of suicide genes or tumor repressor genes (Woll and Hart, 1995). Other local or systemic, acquired diseases might be managed by short-term, low-dose therapy through expression of genes encoding cytokines or co-activation factors.

Although most therapeutic strategies aim to avoid an immune response against the expressed gene product, the purposeful introduction of antigen-encoding genes can be used for either immunotherapy or prophylactic vaccination. Intranasal administration of such DNA vaccines would be simple and noninvasive and should also induce specific

mucosal immunity, which is desirable to prevent entry of pathogens via the extensive mucosal surfaces of the body.

Direct gene transfer may be carried out in many tissues by various means. The use of plasmid DNA is preferable to live viral or bacterial vectors owing to ease of production, the non-immunogenic nature of the vector itself (Krieg, 1995; Pisetsky, 1996; Klinman *et al.*, 1997) and the absence of risk of inadvertent infection. Plasmid DNA may be administered in pure or naked form, complexed with cationic lipids, microencapsulated, or coated onto gold particles introduced biolistically (Lasic and Templeton, 1996; Miller, 1992; Ledley, 1995). Delivery of genes to the respiratory system may be hampered by poor transport of molecules across the epithelium owing to biochemical (e.g., secreted enzymes), physical (e.g., mucus, cilia) or mechanical (e.g., coughing) pulmonary defense mechanisms. Previous reports have indicated that gene transfer may be carried out in the lung tissue using plasmid DNA either alone or formulated with cationic lipids. However, there are conflicting reports as to the relative efficiencies of these two methods. Here, we examine direct gene transfer in the lungs of mice by administration of plasmid DNA using different techniques and lipid formulations.

Materials and Methods

Plasmid DNA vectors

Gene transfer studies were carried out with plasmid DNA in which the immediate early promoter of the cytomegalovirus (CMV) drove expression of firefly *Photinus pyralis* luciferase (pCMV-luc) (Davis *et al.*, 1993b), β-galactosidase (pCMV-lacZ) (Davis *et al.*, 1993b) or green fluorescent protein (pEGFP-C1, Clontech Laboratories, Inc., Palo Alto, CA). The DNA was purified on Qiagen anion-exchange chromatography columns (Qiagen GmbH, Hilden, Germany) and resuspended in sterile saline (0.15 M NaCl, Sigma-Aldrich, Oakville, Canada). DNA prepared in this way has only low levels of endotoxin (Davis *et al.*, 1996b). The concentration of DNA was calculated based on absorbance of ultraviolet light (OD 260) with final concentrations usually being 5-10 mg/ml. DNA solutions were stored at -20°C until required for *in vivo* delivery. DNA was administered either as pure plasmid DNA in saline (naked DNA) or formulated with the cationic lipids described below.

Cationic and neutral lipids

Tetramethyltetraalkylspermine analogs were provided by Life Technologies, Inc. (Gaithersburg, MD) (Jessee *et al.*, unpublished observations). In brief, spermine was acylated with the desired acyl chloride (Nu-Chek-Prep, Inc., Eysian, MN), followed with lithium aluminium hydride reduction to obtain tetraalkylspermine. The tetraalklyspermine was alkylated further with methyl iodide to obtain the tetramethyltetraalkylspermine, which was then characterized by infrared and nuclear magnetic resonance spectroscopy

(NMR) and fast atomic bombardment-mass spectrometry. Four different tetramethyltetraalkylspermine derivatives were produced (Fig. 1), which were then formulated with one of DOPE, DP$^{\Delta}$PE, DMPE and DLPE (Avanti Polar Lipids, Albaster, AL) at ratios as outlined in Table 1.

Other lipid formulations tested were Lipofectin®, LipofectAMINE®, DMRIE-C and Cellfectin® as well as a mixture of GAP-DLRIE (Wheeler *et al.*, 1996) and DOPE (Life Technologies, Inc., Gaithersburg, MD) (Table 1).

Preparation of liposomes

The lipids were formulated into liposomes using the reverse evaporation method. A known amount of cationic lipid was mixed with the neutral colipid in a 2-L round-bottom flask to obtain the desired molar ratio of cationic/neutral lipid. Methylene chloride or 10% methanol in methylene chloride was added to make a 10-30 mg/ml solution. Distilled water was added to give approximately 1 or 2 mg/ml solution. The mixture was shaken 3-5 minutes, then let stand for approximately 3 minutes. The solution was subjected to rotary evaporation to remove methylene chloride and methanol. The volume was adjusted with distilled water to obtain the desired concentration (1 or 2 mg/ml). The liposome solution was flushed with nitrogen and stored at 4°C.

Figure 1

Structure of tetramethyltetraalkylspermine analogues;

Tetramethyltetralaurylspermine (TMTLS) R = $(CH_2)_{10}CH_3$.

Tetramethyltetramyristylspermine (TMTMS) R = $(CH_2)_{12}CH_3$.

Tetramethyltetrapalmitylspermine (TMTPS) R = $(CH_2)_{14}CH_3$.

Tetramethyltetraoleoylspermine (TMTOS) R = $(CH_2)_7CH{=}CH(CH_2)_7CH_3$.

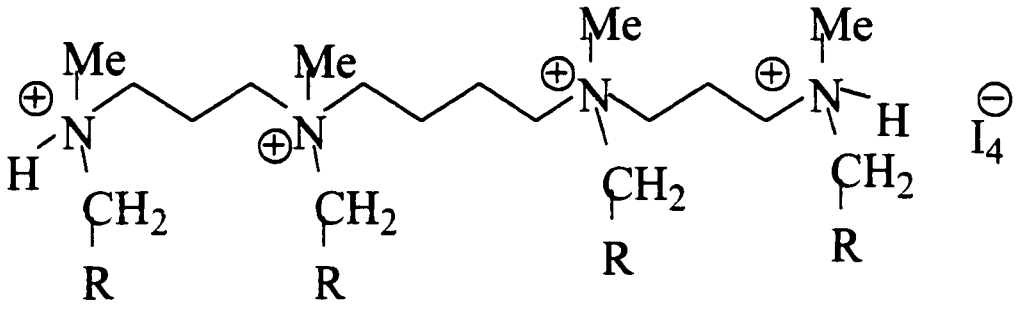
Me
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Table 1. Lipid formulations used for direct gene transfer into lungs of mice.

[a]C = cholesterol; DLPE= dilauroylphosphatidylethanolamine;

DMPE = dimyristoylphosphatidylethanolamine; DMRIE = (+)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis-(tetradecyloxy)-1-propanaminium bromide;

DOPE = dioleylphosphatidylethanolamine; DOSP = 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propaniniumtrifluoracetate; DOTMA = N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride;

DP$^{\Delta}$PE = dipalmitoleoylphosphatidylethanolamine; GAP-DLRIE = (+)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide;

TMTLS = tetramethyltetralaurylspermine; TMTMS = tetramethyltetramyristylspermine;

TMTOS = tetramethyltetraoleoylspermine; TMTPS = tetramethyltetrapalmitylspermine.

Formulation[a]	*Molar ratio*	*Other name*
Tetramethyltetraalkylspermines		
TMTLS:DOPE	1:1.5	
TMTLS:DLPE	1:1.5	
TMTLS:DP$^{\Delta}$PE	1:1.5	
TMTMS:DOPE	1:1.5	
TMTMS:DMPE	1:1.5	
TMTMS:DP$^{\Delta}$PE	1:1.5	
TMTOS:DOPE	1:1.5	
TMTOS:DP$^{\Delta}$PE	1:1.5	
TMTPS:DOPE	1:1.5	Cellfectin®
TMTPS:DP$^{\Delta}$PE	1:1.5	
Others		
DOTMA:DOPE	1:1	Lipofectin®
DOSPA:DOPE	1.5:1	LipofectAMINE®
DMRIE:C	1:1	
GAP-DLRIE: DOPE	1:1	

Preparation of plasmid DNA-liposome complexes

Lipid-formulated DNA was administered in a total volume of 150 µl containing 1-100 µg DNA and 0.2-5 times that weight of lipid. For each formulation, the required volumes of stock DNA solution, stock lipid solution, and diluent (0.15 M NaCl) were calculated.

The lipid, the diluent and the DNA were added in sequential steps, with brief vortexing after each step. The final mixture was left undisturbed (either on ice, at ambient temperature or at 37°C) for 30 minutes to allow DNA-liposome complex formation. Immediately before administration, the DNA-lipid mixture was mixed gently by tapping on the side of the microtube.

To determine whether all the DNA was complexed with the lipid, mixtures were run on a 0.5 % agarose gel and stained with ethidium bromide (0.04 µg/ml). The presence of free plasmid was detected as a band at the expected distance, whereas DNA-lipid complexes failed to enter the gel.

Determination of size and ζ potential

Particle size and ζ potential were measured with complexes formed using pCMV-luc mixed with an equal amount of the following five lipid formulations: Cellfectin, LipofectAMINE, DMRIE-C, TMTLS-DP$^{\Delta}$PE or TMTOS-DOPE. These five formulations were chosen as they represented the range of efficiencies of lung transfection obtained when complexes were administered by i.n. inhalation to mice. Additional measurements were recorded using 100 µg DNA mixed with 0.2-5 times that weight of Cellfectin to

determine the effect of different relative proportions of DNA and lipid components. Measurements were taken 30 minutes after mixing for all lipid formulations, and for Cellfectin, measurements were also taken at regular time intervals up to 4 hours.

Particle size measurements were made at a scattering angle of 90° with a Brookhaven 90Plus Particle Size Analyzer (Brookhaven Instruments Corp., Holtsville, NY). The homodyne intensity-intensity correlation function G(q,t), was obtained. The amplitude of the scattering vector

$$q = (4\pi n/\lambda)\sin(\theta/2)$$

where n is the refractive index of the medium, λ is the wavelength of the excitation light in a vacuum, θ is the scattering angle, and t is the time.

Autocorrelation functions were analyzed using the CONTIN program (Brookhaven Instruments Corp.), which employs the constrained regularization method to calculate the mean diffusion time, $<\tau>$, related to the diffusion coefficient by

$$D = \frac{\lambda^2}{16\pi^2 \sin^2(\theta/2) < \tau >}$$

From each D value, we obtained Stokes' radius, R_s, by Stokes-Einstein equation:

$$R_s = \frac{kT}{6\pi\eta D}$$

where k is Boltzmann's constant, T is the absolute temperature and η is the viscosity of the solvent.

ζ potential measurements were made with the same Brookhaven instrument as for the particle size measurements. In ζ potential measurements, the measured Doppler shift frequency, $\Delta\omega$ is given by:

$$\Delta\omega = \frac{2\pi n}{\lambda} E u \sin\theta$$

where E (volts/cm) and u [($\mu m\ s^{-1}$)/($V\ cm^{-1}$)] are the applied electric field strength and electrophoretic mobility, respectively. Therefore, u can be directly obtained from the $\Delta\omega$. Values of ζ potential were calculated by:

$$\zeta = \frac{u\eta}{\varepsilon}$$

where ε is permitivity of solution.

In vivo *gene transfer*

Animals. Experiments were carried out using female BALB/c mice (Charles River, Montreal, Quebec, Canada) or IFN-γ knockout mice (Jackson Labs, Bar Harbor, ME) aged 6-8 weeks with a total of 10-15 mice per experimental or control group. Each experiment was repeated two or three separate times (n = 5) to ensure reproducibility of results. Control groups for luciferase expression consisted of untreated mice and mice administered plasmid encoding a nonrelevant gene (i.e., β-galactosidase, β-gal). Each animal received naked or lipid-formulated DNA in a total volume of 150 μl, which was given by single or multiple administrations and which contained different doses of DNA and different relative proportions of DNA and lipid. The DNA was administered to the respiratory system of the mice via intranasal (i.n.) or intratracheal (i.t.) routes.

i.n. Instillation. Mice were lightly anesthetized with Halothane® (Halocarbon Laboratories, River Edge, NJ) and held on their backs with their head suspended inferiorly. The DNA solution was then instilled bilaterally into the nasal cavity using a gel-loading tip and a Gilson pipette. The tip was inserted a few millimeters into the nasal cavity, and each nostril was instilled three times at 15-minute intervals with 25 µl of DNA solution (for a total volume of 150 µl/mouse).

i.n. Inhalation. Droplets of DNA solution were deposited by pipette directly over both external nares of mice under either Halothane or Somnotol® (75 mg/kg i.p.) (MTC Pharmaceuticals, Cambridge, Ontarion, Canada) anesthesia. A total volume of 150 µl was given as either a single (1 x 150 µl) or triple (3 x 50 µl) administration. For the triple administration, 15-minute intervals were allowed between administrations.

i.t. Injection. Mice fully anesthetized with Somnotol had the trachea exposed through an anterior midline incision. The DNA solution (150 µl) was then injected through the anterior wall of the trachea using a 0.3-ml insulin syringe with a 29-gauge needle attached (Becton Dickenson, Franklin Lakes, NJ). The incision was sutured, and the mouse was placed in an incubator until fully recovered from the anesthetic (approximately 45 minutes).

i.t. Cannulation. Mice under Somnotol anesthesia had the trachea exposed via an anterior midline incision, and this was used to visualize the insertion of the cannula into the trachea. A 20-gauge olive tip steel feeding tube (Fine Science Tools Inc., North Vancouver, BC, Canada) attached to a 1-ml tuberculin syringe (Becton Dickenson) was

passed through the oral cavity and into the trachea. The DNA solution (150 µl) was then slowly injected directly into the lungs.

Evaluation of reporter gene expression

Mice were killed by cervical dislocation under Halothane anesthesia at various times from 6 hours to 19 days after gene transfer. For determination of luciferase reporter gene activity, the lungs and bronchial tree were dissected free and stored frozen (-20°C) until assayed using the Promega Luciferase Assay System (Madison, WI). This was carried out as described previously for muscle (Davis *et al.*, 1993b) except that the lungs were homogenized in 350 µl of 1 x lysis buffer. Measurements were taken with a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The protein content of the lungs was determined by the Bio-Rad microassay procedure using the Bio-Rad Protein Assay Reagent (Hercules, CA). Values for luciferase activity were expressed in relative light units (RLU) /sec/mg protein.

For visualization of green fluorescent protein, the lungs and bronchial tree were dissected free and thinly sectioned. The head of the mouse was severed between the upper and lower jaws, facial skin was removed and the head split sagitally to reveal the nasal passage. The lungs, bronchial tree and nasal passages were examined for fluorescence using a Zeiss Axioskop light microscope (Carl Zeiss Canada Ltd., North York, Ontario, Canada).

PCR analysis

Lungs were removed, homogenized and genomic plus plasmid DNA was isolated using QIAamp Tissue Kit (Qiagen). PCR was performed in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT) using upstream (5`-ATG GAA GAC GCC AAA AAC AT-3`) and downstream (5`-TTA CAA TTT GGA CTT TCC GC-3`) primers (Life Technologies, Inc.) designed to amplify the luciferase gene. The components of the reaction mixture were as indicated in the Basic PCR Protocol supplied with Taq DNA polymerase (Life Technologies, Inc.). After an initial denaturation at 94°C for 2 minutes, the samples were subjected to 35 cycles of the following: denaturation at 94°C for 30 seconds, annealing at 52°C for 45 seconds and extension at 72°C for 2 minutes. This was followed by a 7-minute extension at 72°C. PCR products were evaluated by 0.8% agarose gel electrophoresis stained with 0.04 μg/ml ethidium bromide. As positive control, 1 μg plasmid DNA was added to lungs of untreated mice before tissue homogenization and DNA isolation.

Assay of humoral response to expressed proteins

Plasma taken from mice at 2, 4, 8 and 12 weeks after immunization by i.n. inhalation of 100 μg pCMV-lacZ mixed with Cellfectin was evaluated for total IgG antibodies specific to β-gal as previously described (Davis *et al.*, 1997).

Experimental groups

Effect of DNA/lipid ratio. Mice were administered by i.n. inhalation with a total of 25 μg pCMV-luc DNA mixed with Cellfectin at DNA/lipid (w/w) ratios of 1:0 (pure DNA),

5:1, 3:1, 2:1, 1:1, 1:2, 1:3 and 1:5 (equivalent to charge ratios of 0, 0.05, 0.08, 0.12, 0.23, 0.46, 0.69 and 1.16 nmoles cationic lipid per nmole nucleotide respectively). Lungs were removed 72 hours later.

DNA dose. Mice received a total of 1, 10, 25, 50 or 100 μg of pCMV-luc DNA, formulated with Cellfectin at a 1:1 DNA/lipid ratio (w/w). This was given by i.n. inhalation in a total volume of 150 μl. Lungs were removed at 72 hours.

Longevity of reporter gene expression. A total of 100 μg pCMV-luc DNA formulated with Cellfectin at a 1:1 DNA/lipid (w/w) ratio was administered by i.n. inhalation to each mouse. Lungs were removed from mice killed at 6 hr, 1, 2, 3, 4, 5, 7, 9, 14 or 19 days after gene transfer and assayed for luciferase activity.

Method of DNA administration. A total of 100 μg pCMV-luc DNA formulated with Cellfectin at a 1/1 DNA:lipid (w/w) ratio was administered by each of the four methods described above (i.n. instillation, i.n. inhalation, i.t. injection, i.t. cannulation). Lungs were removed 24, 48 or 72 hours later.

Lipid composition. A total of 100 μg pCMV-luc DNA was formulated with each of the 14 different lipids (as described above) at a 1:1 DNA:lipid (w/w) ratio. These solutions were administered to mice by i.n. inhalation and lungs were removed 72 hours later.

Statistics

Data were expressed as group means ± standard errors of the means (SEM) and were analyzed using the GraphPAD InStat program (Graph PAD Software, San Diego). The

significance of the differences between groups was determined by the Mann-Whitney test for comparison of two groups or by a 1-factor analysis of variance followed by the Krustal-Wallis test for comparison of 3 or more groups. Differences were considered to be not significant with $p > 0.05$. In no case were results significantly different for the same treatment carried out on two or three separate occasions (n=5). Therefore, these data were pooled (n=10 or 15).

Results

For most of the studies, Cellfectin (TMTPS:DOPE [1:1.5 w/w]) (Table 1) was used as a model lipid as it allowed efficient transfection of the mouse lung, and i.n. inhalation was used as a model delivery method as it was easy and non-invasive.

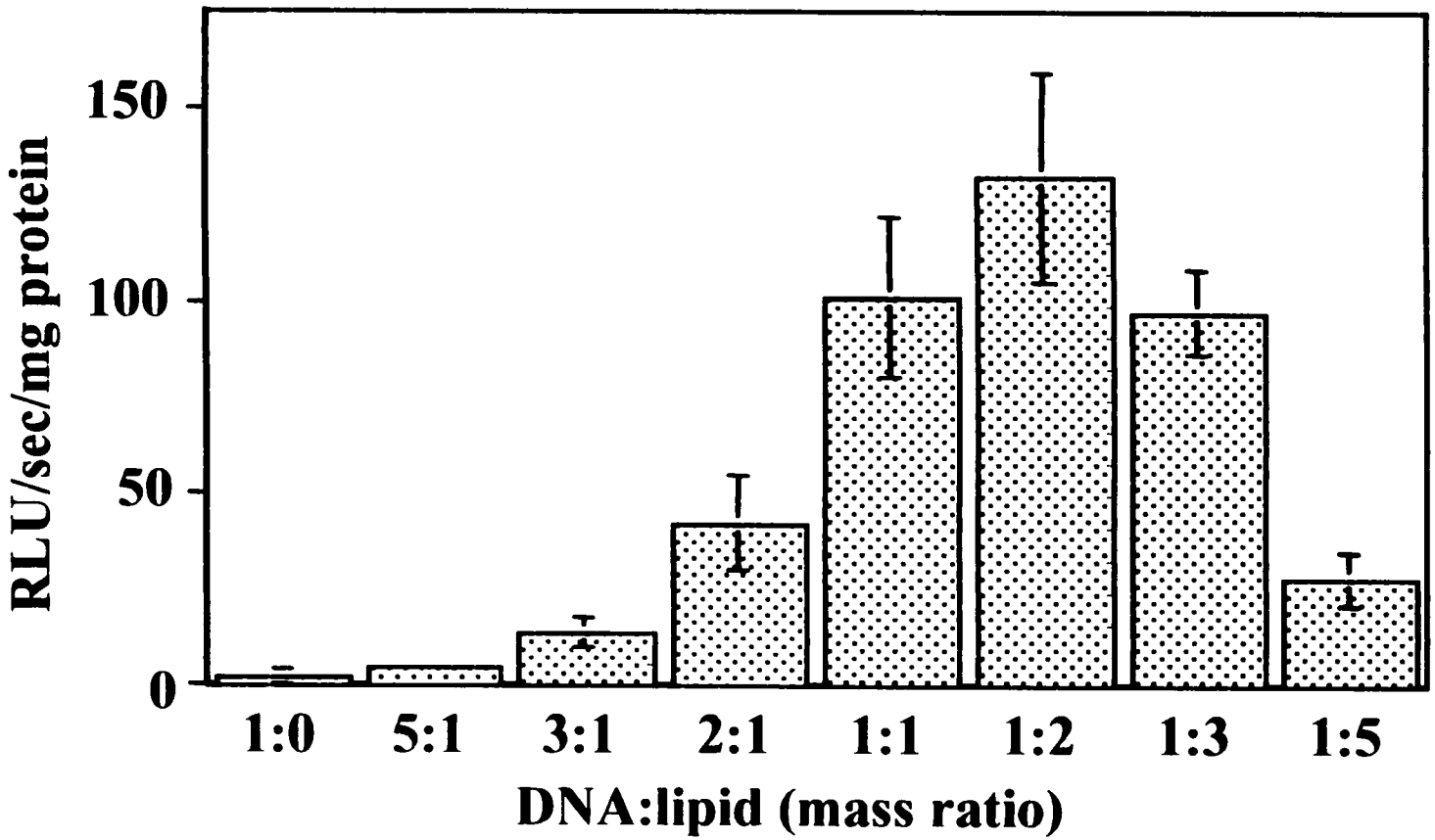
Effect of DNA/lipid ratio

The efficiency of lung tissue transfection after i.n. inhalation of DNA mixed with lipid depended on the relative amounts of DNA and lipid. For example, with Cellfectin, the highest mean luciferase expression was obtained using a DNA/lipid ratio (w/w) of 1:2, although ratios of 1:1 and 1:3 also gave good results (Fig. 2). We have shown previously by visualization of free plasmid DNA on an agarose gel (van der Woude *et al.*, 1997) that all DNA was complexed when mixed with at least twice as much (by weight) Cellfectin (McCluskie and Davis, 1997). Thus, the present results indicate that the most efficient gene transfer for a given dose of DNA was obtained when it was mixed with the minimum amount of the lipid that could completely complex it. The presence of excess uncomplexed DNA (e.g., DNA/lipid at 5:1 or 3:1, w/w) resulted in lower levels of luciferase activity, and the lowest luciferase activity was detected with pure plasmid DNA. Transfection efficiency also decreased with excess lipid (1:5), possibly owing to toxicity of the lipid.

There was no difference between expression levels in lungs removed 72 hr following i.n. inhalation of 100 μg pCMV-luc complexed with Cellfectin when complexes were formed on ice or at room temperature (mean ± SD: 466 ± 242 and 506 ± 180

Figure 2

Effect of DNA/lipid ratio on luciferase expression. DNA (25 μg pCMV-luc) was mixed with increasing amounts of Cellfectin and administered by i.n. inhalation to mice. Lungs were harvested at 72 hours and assayed for luciferase activity. Bars represent the mean luciferase activity in relative light units (RLU)/sec/mg of lung protein, and T bars indicate SEM (n = 10).


RLU/sec/mg protein
150
100
50
0
1:0
5:1
3:1
2:1
1:1
1:2
1:3
1:5
DNA:lipid (mass ratio)

RLU/sec/mg lung protein respectively, $p = 0.97$). However when the temperature was increased to 37°C, expression levels decreased significantly (220 ± 203 RLU/sec/mg protein, $p < 0.05$).

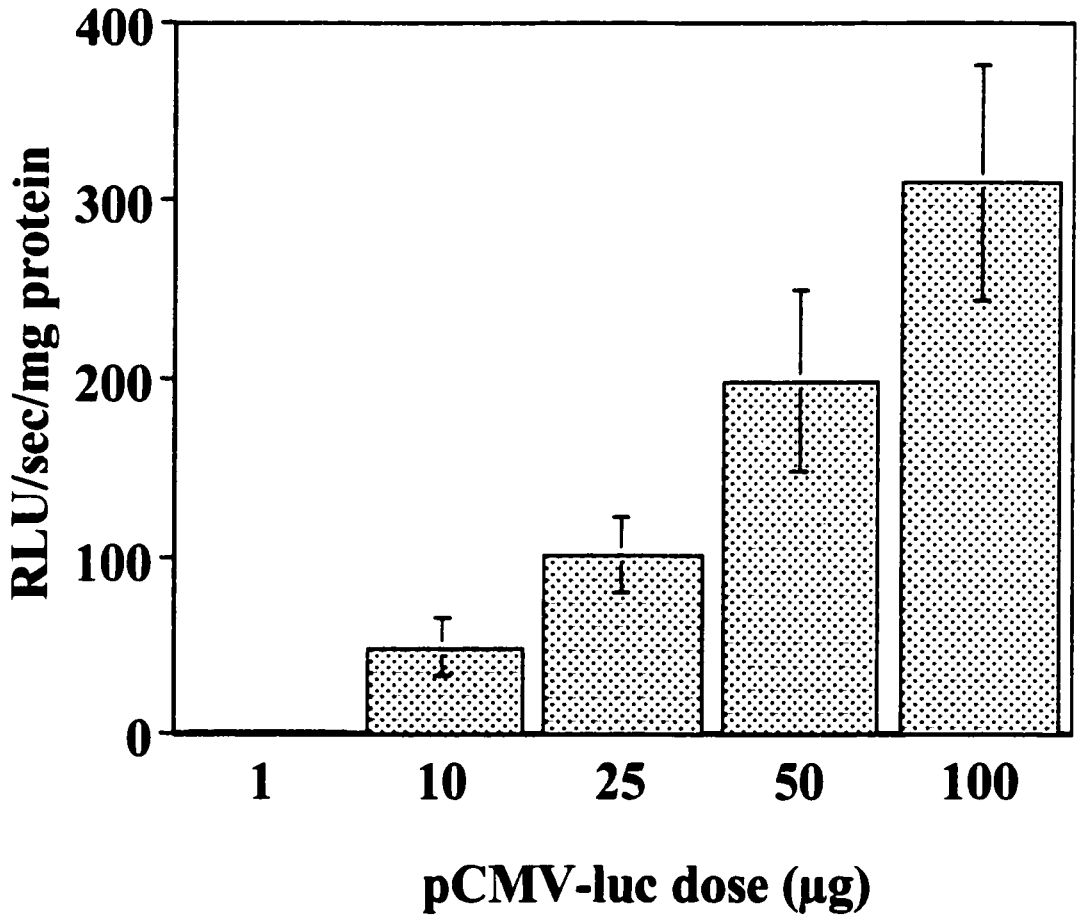
For further studies we chose a DNA/lipid ratio of 1:1, which was the lowest amount of lipid to give a relatively high transfection efficiency, to minimize any possible lipid-associated toxicity. Cationic lipids have been shown to be toxic *in vivo* in a dose-dependent manner (Scheule *et al.*, 1997).

Effect of DNA dose

The level of luciferase activity in the lung 48 hr after i.n. inhalation of pCMV-luc DNA (mixed 1:1 w/w with Cellfectin) was directly proportional to the amount of DNA from 1 to 100 μg ($r = 0.99$) (Fig. 3). The lack of plateau in reporter gene activity indicated that uptake and expression of DNA were not limited by saturation with DNA but rather by the maximum volume that could be given without causing undue stress to the animal. There was no significant difference between expression levels in lungs removed 72 hours following i.n. inhalation of 100 μg pCMV-luc complexed 1:1 w/w with Cellfectin when the complexes were given by a single (1 x 150 μl) or triple (3 x 50 μl over 30 minutes) administration schedule (mean ± SD, 595 ± 326 and 506 ± 180 RLU/sec/mg protein respectively, $p = 0.63$). On the other hand, animals were less distressed with the triple than the single administration method. A 100-μg dose of DNA given as a triple administration was used in all subsequent experiments.

Figure 3

Effect of DNA dose on luciferase expression. DNA (1-100 μg pCMV-luc) was mixed with increasing amounts of Cellfectin, at a DNA/lipid w/w ratio of 1:1 and administered by i.n. inhalation to mice. Lungs were harvested at 48 hours and assayed for luciferase activity. Bars represent the mean luciferase activity in RLU/sec/mg of lung protein and T bars indicate SEM (n = 10).


400
300
200
100
0
RLU/sec/mg protein
1
10
25
50
100
pCMV-luc dose (µg)

Longevity of reporter gene expression

Low levels of luciferase expression could be detected as early as 6 hours after i.n. inhalation of 100 μg pCMV-luc (mixed 1:1 w/w with Cellfectin). The reporter gene activity increased steadily for 4 days, but declined rapidly between days 4 and 5. Only very low levels of luciferase were detected at 9 days, although these were sustained for at least a further 10 days (Fig. 4). Because luciferase is unstable and has a short half-life, we considered the presence of high luciferase activity to indicate recent expression from plasmid DNA rather than persistence of gene product in the absence of new synthesis. The kinetics of expression were the same in IFN-γ knock-out mice as in normal (BALB/c) mice (mean ± SD, 519 ± 36 and 466 ± 242 RLU/sec/mg protein, respectively at day 3 $p > 0.99$; 92 ± 69 and 95 ± 10 at day 7, $p = 0.81$).

Method of DNA administration

Delivery of DNA with lipid to the mouse lung by either of the two non-invasive (i.e., i.n. inhalation or instillation) or one of the invasive (i.e., i.t. injection or cannulation) techniques gave similar luciferase gene expression. In comparison, i.t. injection gave somewhat lower luciferase activity (significant for two of three time points, $p < 0.05$), and this may have been because of DNA leakage from the hole created in the trachea (Fig. 5).

For delivery of pure plasmid DNA, the i.n. routes gave luciferase levels about 10-fold lower than those with lipid-associated DNA. In contrast, for the i.t. routes, similar results were obtained with lipid-associated or with pure plasmid DNA (Fig. 5). Taken together, these results indicate that the lipid may protect the DNA from degradation in the

Figure 4

Time course of reporter gene expression. DNA (100 μg pCMV-luc) mixed with Cellfectin (1:1 w/w) was administered to mice by i.n. inhalation. Lungs were harvested at various times after administration and assayed for luciferase activity. Each value represents the mean luciferase activity in RLU/sec/mg of lung protein, and T bars indicate SEM (n = 10).

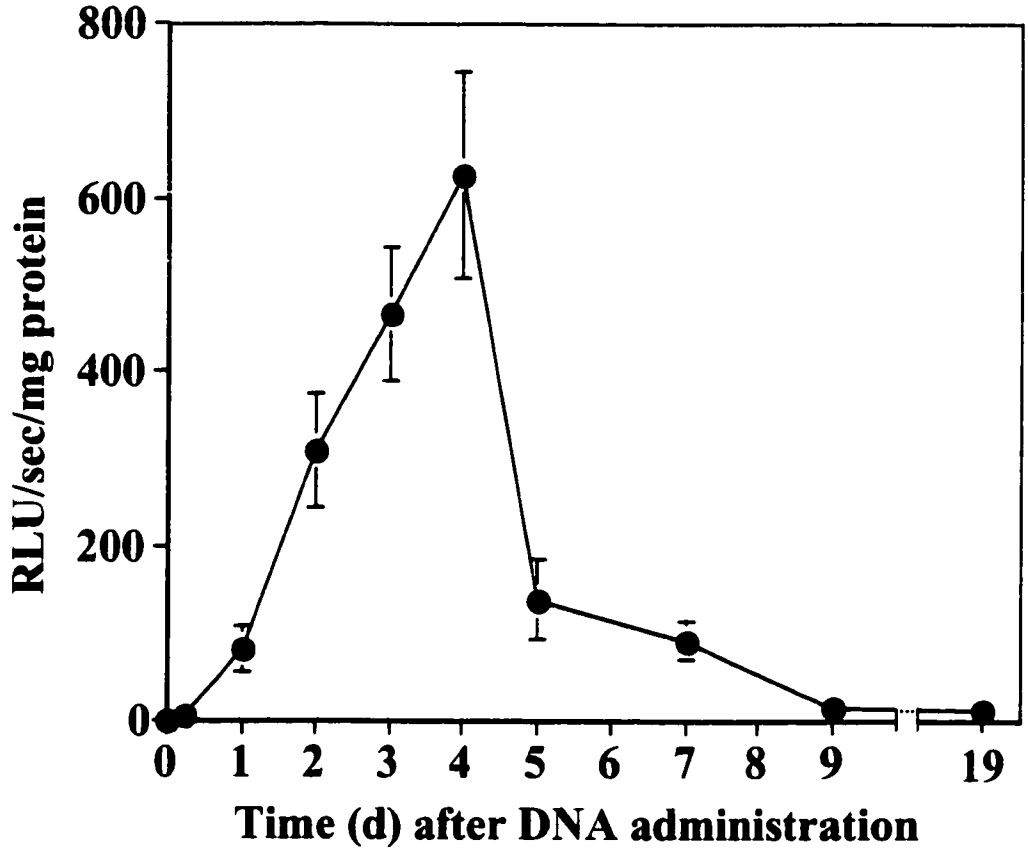
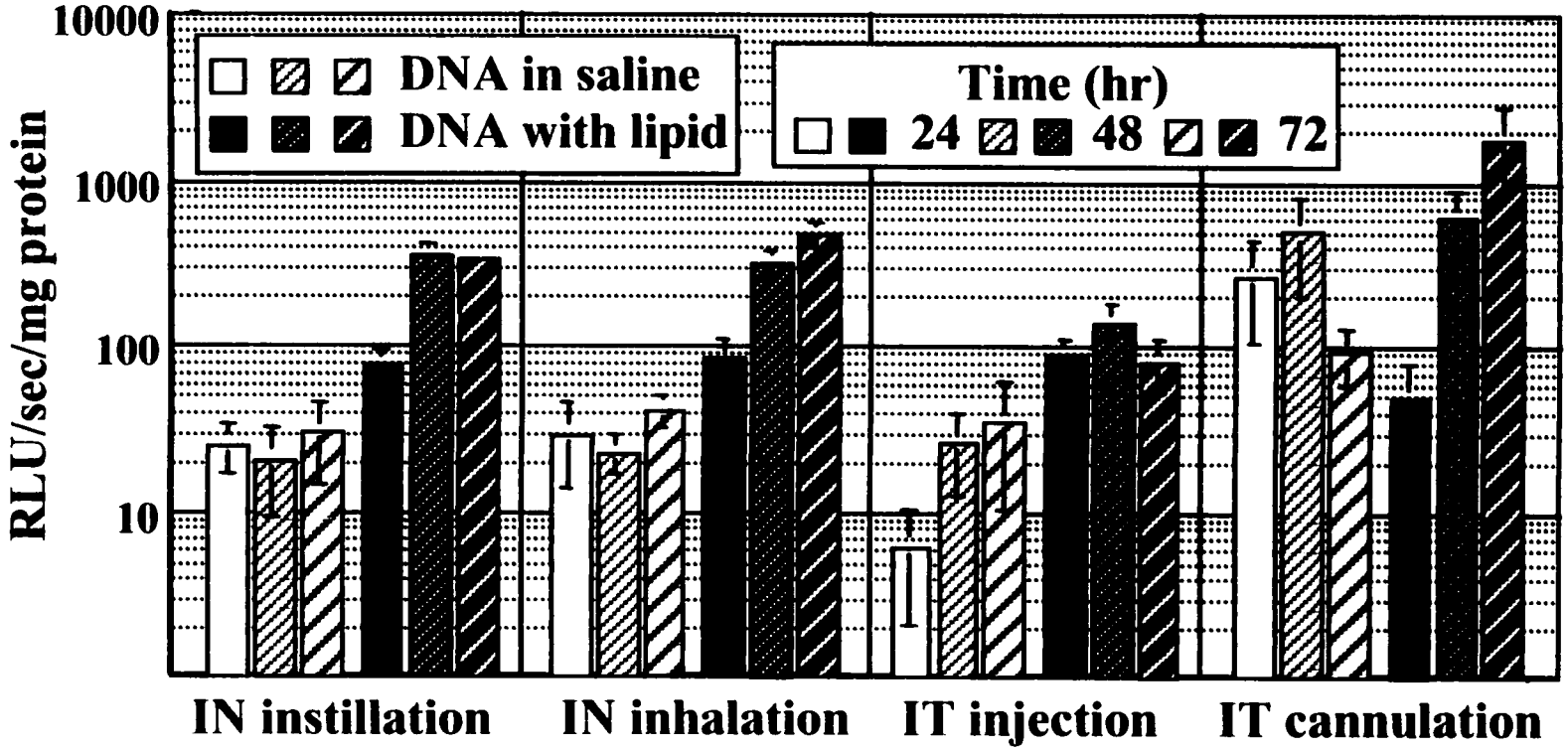

800
600
400
200
0
RLU/sec/mg protein
0 1 2 3 4 5 6 7 8 9 19
Time (d) after DNA administration

Figure 5

Routes of administration. DNA (100 μg pCMV-luc) was administered to mice either in saline or mixed with Cellfectin (1:1 w/w) by i.n. instillation, i.n. inhalation, i.t. injection, and i.t. cannulation. Lungs were harvested at 24, 48 or 72 hours and assayed for luciferase activity. Bars represent the luciferase activity in RLU/sec/mg of lung protein and T bars indicate SEM (n = 10).


10000
1000
100
10
RLU/sec/mg protein
DNA in saline
DNA with lipid
Time (hr)
24
48
72
IN instillation
IN inhalation
IT injection
IT cannulation

nasopharynx but that this is either not required or is not effective once the DNA is in the lung. Luciferase expression levels 72 hours after i.n. DNA delivery were significantly greater when the mice were anesthetized with halothane than with somnotol (mean ± SD, 595 ± 326 and 124 ± 250 RLU/sec/mg protein respectively, $p < 0.05$). This indicates that more DNA may have been inhaled under the lighter anesthetic state induced by halothane and more swallowed under the deeper anesthetic state induced by somnotol. Furthermore, administration of DNA solutions under halothane anesthesia appeared less stressful to the animals.

Of all routes, i.t. cannulation had the greatest intragroup variability (coefficient of variation > 150% compared with < 100% for i.n. methods). Although delivery by i.n. (inhalation or instillation) and i.t. cannulation gave similar results for transfection of lung tissue, there was also significant gene transfer to the nasopharyngeal epithelium with the i.n. but not the i.t. routes. When lipid-formulated plasmid DNA encoding green fluorescent protein (pEGFP-C1) was administered by i.n. inhalation or i.t. cannulation, fluorescence was detected throughout the lung in both cases, but in the upper respiratory passages only following i.n. inhalation (data not shown).

Effect of lipid composition

Different cationic lipid compositions had all been mixed with pCMV-luc (100 μg) at a DNA/lipid ratio of 1:1 (w/w), as agarose gel electrophoresis revealed them to have similar DNA-binding capacities, and delivered by i.n. inhalation (3 x 50 μl) because it was easy and non-invasive and had relatively low variability of transfection efficiencies.

The different lipid formulations (Table 1) resulted in different efficiencies of lung transfection, ranging from much better to much worse than pure plasmid DNA (Fig. 6). TMTPS or TMTOS mixed with either DOPE or DP$^{\Delta}$PE and DMRIE-C gave significantly higher luciferase activities than did DNA in saline. Although there were no significant differences in levels of expression obtained using these lipids, the intragroup variability was lower with Cellfectin, TMTOS:DOPE or DMRIE:C than with TMTPS:DP$^{\Delta}$PE or TMTOS:DP$^{\Delta}$PE. The next group of TMTMS:DP$^{\Delta}$PE, TMTLS:DP$^{\Delta}$PE and TMTLS:DLPE resulted in luciferase expression equivalent to that obtained with pure plasmid DNA. Finally, Lipofectin, LipofectAMINE, TMTMS:DOPE, TMTMS:DMPE, TMTLS:DOPE and GAP-DLRIE:DOPE all resulted in luciferase activities significantly less than that obtained with naked DNA ($p < 0.05$). The actual relative efficiencies compared to pure plasmid DNA are shown in Figure 7.

Physicochemical characteristics of DNA-lipid complexes

DNA-liposome complexes formed using five different lipid formulations had different sizes (~ 650 to > 1700 nm) and ζ potential (-36 to +32 mV) (Table 2). Maximum levels of expression in lung at 72 hr after i.n. inhalation of lipid-pCMV-luc complexes were obtained with those of 700 to 800 nm and with a ζ potential of -16 to -20 mV (Fig. 8). There is however no clear correlation between complex size and ζ potential ($r = 0.55$).

For any of the five formulations there was no difference in the size or the ζ potential at 30 min versus 4 hr, indicating a certain stability over time. All *in vivo* administrations were carried out within 30 to 60 min of complex formation.

Figure 6

Gene transfer with different cationic lipids. DNA (100 μg pCMV-luc) in saline or mixed with one of 14 different lipids at a 1:1 DNA/lipid (w/w) ratio was administered to mice by i.n. inhalation. Lungs were harvested at 72 hours and assayed for luciferase activity. Bars represent the mean luciferase activity in RLU/sec/mg of lung protein and T bars indicate SEM (n = 5).

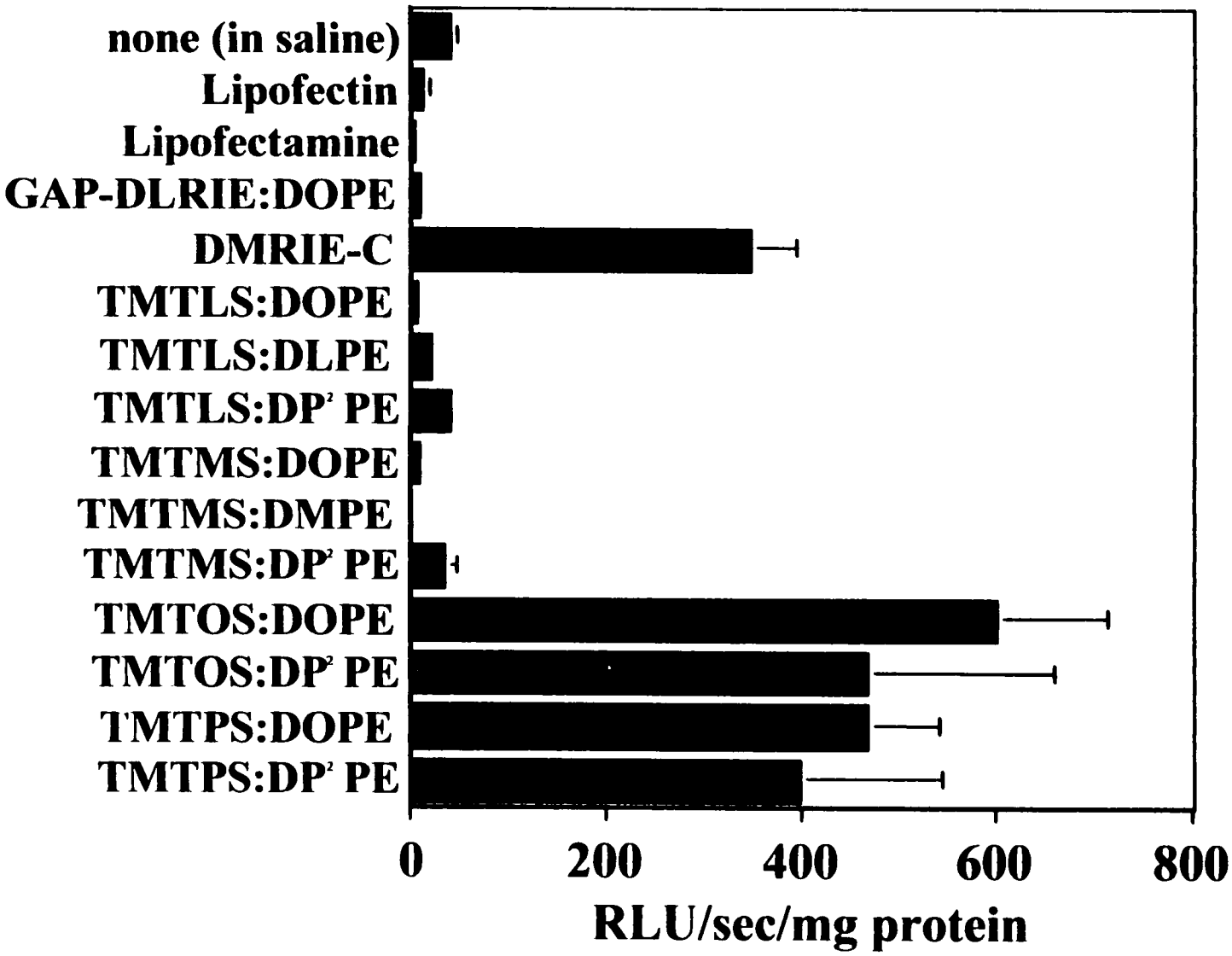
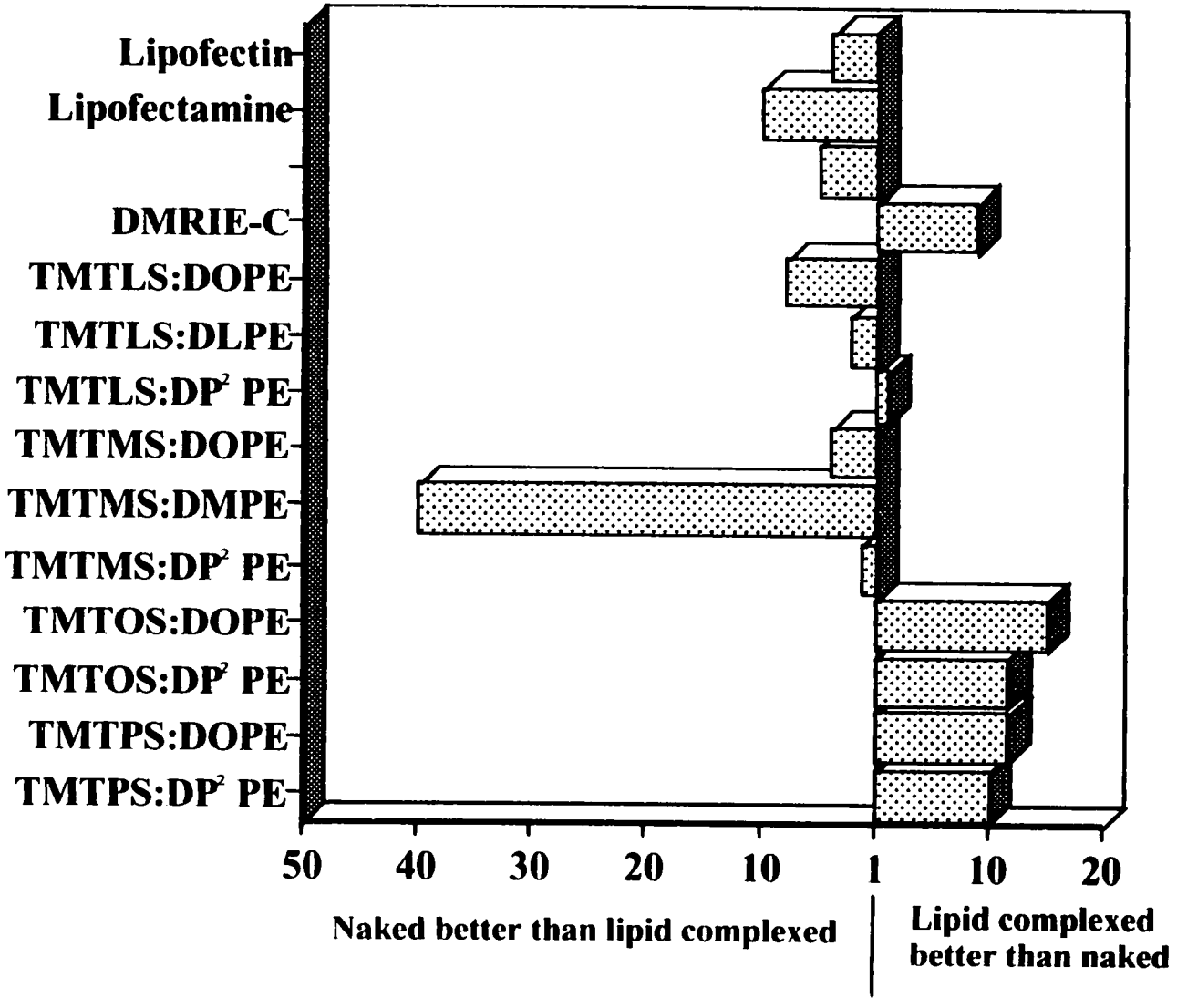

none (in saline)
Lipofectin
Lipofectamine
GAP-DLRIE:DOPE
DMRIE-C
TMTLS:DOPE
TMTLS:DLPE
TMTLS:DP² PE
TMTMS:DOPE
TMTMS:DMPE
TMTMS:DP² PE
TMTOS:DOPE
TMTOS:DP² PE
TMTPS:DOPE
TMTPS:DP² PE
0
200
400
600
800
RLU/sec/mg protein

Figure 7

Relative efficiency of different cationic lipids. DNA (100 μg pCMV-luc) in saline or mixed with one of 14 different lipids at a 1:1 DNA/lipid (w/w) ratio was administered to mice by i.n. inhalation. Lungs were harvested at 72 hours, assayed for luciferase activity to obtain RLU/sec/mg of lung protein (Fig. 6). Bars represent the efficiency of lipid-complexed DNA formulations relative to that of pure (naked) DNA in saline.



Relative efficiency of transfection with lipid complexed or naked DNA

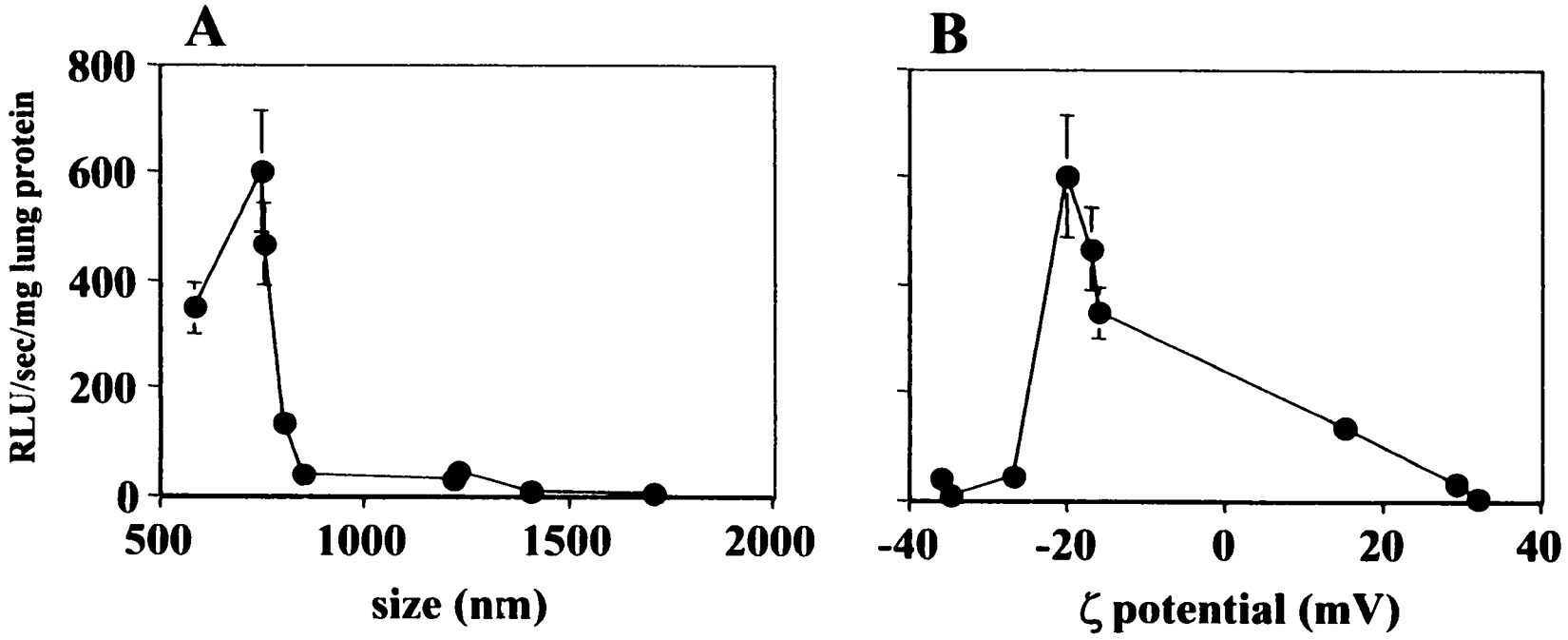
Table 2. Size and ζ potential of various formulations used for gene transfer to the respiratory tract.

[a] The w/w ratio of DNA/lipid.

Sample	*Size (nm)*	*ζ potential (mV)*
LipofectAMINE	93	60
DMRIE-C	309	42
TMTLS- $DP^{\Delta}PE$	386	30
TMTOS-DOPE	505	36
Cellfectin	365	41
pCMV-luc	128	-50
pCMV-luc: LipofectAMINE (1:1)[a]	1711	32
pCMV-luc:DMRIE-C (1:1)	658	-20
pCMV-luc:TMTLS-$DP^{\Delta}PE$ (1:1)	848	-36
pCMV-luc:TMTOS-DOPE (1:1)	751	-20
pCMV-luc:Cellfectin (1:5)	1217	29
pCMV-luc:Cellfectin (1:2)	803	15
pCMV-luc:Cellfectin (1:1)	758	-17
pCMV-luc:Cellfectin (2:1)	1232	-27
pCMV-luc:Cellfectin (5:1)	1409	-35

Figure 8

Effect of size and ζ potential. DNA (100 μg pCMV-luc) associated with different lipids at various DNA/lipid (w/w) ratios was administered to mice by i.n. inhalation. Lungs were harvested at 72 hours and assayed for luciferase activity (mean RLU/sec/mg lung protein. n = 5). Size and ζ potential of DNA/lipid complexes prepared in an identical manner were measured. (A) The effect of size on expression levels. (B) The effect of ζ potential on expression levels.


A
800
600
400
200
0
RLU/sec/mg lung protein
500
1000
1500
2000
size (nm)
B
-40
-20
0
20
40
ζ potential (mV)

Antibodies against expressed proteins

Antibodies against β-gal were detected following administration of Cellfectin-complexed pCMV-lacZ DNA, however the titers induced were much lower than those by IM injection of the same dose (100 μg) of DNA (data not shown).

Discussion

Direct gene transfer into the lung might be carried out for the purpose of gene therapy or DNA-based immunization. Depending on the application, requirements may vary for which region of lung and cell-type are transfected, as well as for the efficiency of transfection and longevity of gene expression. Many factors may influence these outcomes (Table 3).

Naked vs. lipid-formulated DNA for gene delivery to lungs

Pure plasmid (naked) DNA is very inefficient for *in vitro* transfection of cells, a procedure that is usually carried out with transfection reagents (e.g., cationic lipids, dendrimers) or physical facilitation (e.g., calcium phosphate precipitation or electroporation). In contrast, pure plasmid DNA can transfect mature muscle fibers *in vivo* with a much higher efficiency than lipid-formulated DNA (McCluskie and Davis, 1997; Davis *et al.*, 1993a; Wolff *et al.*, 1990). Pure plasmid DNA may also be used for gene delivery by intradermal, subcutaneous, i.n., and i.v. routes (Wolff *et al.*, 1992; Fynan *et al.*, 1993; Donnelly *et al.*, 1997; Raz *et al.*, 1994; Kuklin *et al.*, 1997), although transfection efficiency in these tissues is considerably less than in muscle.

Cationic lipids have been shown to be effective for direct gene transfer by numerous routes including i.t., i.n., i.v., intraarterial, intratumor, intracranial, intraocular and by topical administration to the hair follicle (Lasic and Templeton 1996; Miller, 1992; Ledley 1995). The liposomes form complexes with the plasmid DNA via ionic

Table 3. Factors to consider for gene transfer to lungs.

Outcome that may be affected by a given factor is indicated by a closed circle (●) and factors addressed in this work are indicated by an open circle (ↄ).

Factor	*Outcome which may be influenced*			Reference
	Area/cell type transfected	Transfection efficiency	Longevity of expression	
Transfection agent for cationic lipid mediated transfer				
Choice of cationic and co-lipids	●	●( )		Felgner *et al.*, 1996, , Wheeler *et al.*, 1996, Tsan *et al.*, 1995, 1997, Lee *et al.*, 1996, Eastman *et al.*, 1997,
Amounts of cationic and co-lipids		●( )		Felgner *et al.*, 1996, , Wheeler *et al.*, 1996, Tsan *et al.*, 1997,
Toxicity			●	Caplen *et al.*, 1995, Scheule *et al.*, 1997,, Eastman *et al.*, 1997, Porteous *et al.*, 1997
Interaction of lipid with surfactant	●	●	●	Fasbender *et al.*, 1997
DNA:lipid ratio		●( )		Felgner *et al.*, 1996, , Meyer *et al.*, 1995,, Wheeler *et al.*, 1996, Tsan *et al.*, 1995, Lee *et al.*, 1996, Eastman *et al.*, 1997
Suspension buffer		●		Felgner *et al.*, 1996, , Meyer *et al.*, 1995,, Eastman *et al.*, 1997
Mixing procedures		●		Felgner *et al.*, 1996,
Temperature for complex formation		●( )		Felgner *et al.*, 1996, ,
Size, ζ potential of complexes		●( )		Meyer *et al.*, 1995,, Mahato *et al.*, 1997,, Eastman *et al.*, 1997

Table 3 (continued). Factors to consider for gene transfer to lungs.

Outcome that may be affected by a given factor is indicated by a closed circle (●) and factors addressed in this work are indicated by an open circle (ↄ).

Stability of complexes		●❩		Lee *et al.*, 1996
Storage time and conditions		●❩		Lee *et al.*, 1996
Plasmid DNA:				
Choice of promoter/enhancer elements			●	Tsan *et al.*, 1995, Xiang *et al.*, 1997,, Yew *et al.*, 1997
Presence of introns			●	Yew *et al.*, 1997
Nature and number of CpG motifs			●	Schwartz *et al.*, 1997
Antigenicity of encoded protein			●❩	Lasic and Templeton, 1996, Kuklin *et al.*, 1997
Purification procedure/purity		●	●	Felgner *et al.*, 1996, , Meyer *et al.*, 1995,
Administration protocol:				
Route of administration	❩	●❩		Meyer *et al.*, 1995,, Wheeler *et al.*, 1996- Canonico *et al.*, 1994, Eastman *et al.*, 1997
Anesthetic used		❩		
Number and volume of administrations		●❩		Wheeler *et al.*, 1996
Interspecies differences	●	●	●	Fynan *et al.*, 1993,, Tomkiewicz, 1995, Hazinski, 1993

interactions and, although the exact mechanism of DNA/lipid uptake is unknown, it has been suggested that liposomes bring about enhanced gene transfer by attaching to cell surfaces, where they either fuse directly or are endocytosed with subsequent intracellular fusion with endosomal membranes. Furthermore, it has been proposed that as the lipid diffuses into the membrane, lipid/DNA ionic interactions are disrupted, thereby releasing the DNA into the cytoplasm or endosome (Felgner *at al.*, 1996).

Liposomes may also improve transfection efficiency by increasing the retention time and reducing the rate of DNA degradation by extracellular nucleases (Meyer *et al.*, 1995). In the present study, it appears that the lipid protected the DNA from degradation caused by factors encountered in the nasopharynx but not in the lung.

There have been conflicting reports as to the relative efficiencies of pure plasmid DNA and liposome-formulated DNA for direct gene transfer in the lung. Some reports state that naked DNA cannot transfect lung tissue (Hazinski *et al.*, 1991; Stribling *et al.*, 1992), whereas others say that it can with lower (Yoshimura *et al.*, 1992; Felgner *et al.*, 1996; Wheeler *et al.*, 1996) or equal efficiency to that of lipids (Meyer *et al.*, 1995; Tsan *et al.*, 1995). Many studies evaluate only lipid-formulated DNA, precluding a comparison with pure plasmid DNA. The present study is the most extensive and comprehensive to date to compare pure plasmid DNA and lipid-formulated DNA for direct gene transfer into the lung. Our findings, in agreement with others, indicate that level of expression varies greatly with different lipid formulations and thus can explain why such discrepant results have been reported in the literature. Of the 14 lipids tested in the present study, 5 were more efficient than pure plasmid DNA, 3 were about the same and 6 were less efficient.

Method of gene delivery

Numerous approaches for carrying out direct gene transfer in the lung have been reported including i.t. injection (Yoshimura *et al.*, 1992), i.n. inhalation of droplets applied to the nares (Fynan *et al.*, 1993), aspiration with the tongue pulled out (Li *et al.*, 1996), i.t. or i.n. instillation through a catheter (Hyde *et al.*, 1993; Meyer *et al.*, 1995; Wheeler *et al.*, 1996; Tsan *et al.*, 1995) and aerosol delivery (Stribling *et al.*, 1992, Canonico *et al.*, 1994b). However this is the first report to compare several different methods. We show comparable efficiencies with i.n. (inhalation or instillation) and i.t. (cannulation) methods of delivery. i.n. inhalation is our preferred method, as it is easy and quick to perform and non-invasive and offers short recovery time and the potential for repeated administrations. As well, i.n. approaches cause transfection of cells in the nasopharynx, a situation which may prove beneficial with DNA vaccines because of the large number of antigen presenting cells (APC) in the extensive lymphoid tissue of the nasal cavity and pharynx. APC have been shown to play a central role in the induction of immune responses after DNA immunization (Doe *et al.*, 1996; Corr *et al.*, 1996; Iwasaki *et al.*, 1997).

Liposome composition

Cationic liposomes are composed of a mixture of a cationic lipid for charge and a neutral co-lipid for stability and reduced cytotoxicity. The net positively charged liposome and negatively charged DNA spontaneously form complexes. We tested a series of cationic tetramethyltetraalkylspermine analogues mixed with phosphatidylethanolamine derivatives with various commercially available and experimental lipids (Table 1).

We observed the most efficient transfection with two novel lipids, TMTPS and TMTOS, which are composed of 16 and 18 carbon chain fatty acids, respectively, and also with the 14 carbon chain DMRIE. However, the novel lipids TMTLS and TMTMS, which have 12 and 14 carbon chain fatty acids, respectively, were considerably less efficient. This suggests that for tetramethyltetraalkylspermine analogs, transfection efficiency is proportional to carbon chain length and that even better results might be obtained using fatty acid chains longer than 18.

We have found GAP-DLRIE, from the 2,3-dioxy-propaniminium class of lipids, and the two commercially available lipids, Lipofectin and LipofectAMINE, to be less efficient for transfection of the respiratory epithelium than pure plasmid DNA alone. Our results with GAP-DLRIE contrast with those of Wheeler *et al.* (1996) who reported it to give better lung transfection than lipids such as DMRIE, DOTAP, DOTMA and DOSPA. Other studies with Lipofectin and LipofectAMINE have reported varied results (Yoshimura *et al.*, 1992; Li *et al.*, 1996; Meyer *et al.*, 1995; Canonico *et al.*, 1994a; Egilmez *et al.*, 1996; Brigham *et al.*, 1993).

We looked only at total gene expression in the lung, but it is possible that different lipids may preferentially transfect different regions and cell types of the respiratory system. With the lipids tested here, we detected relatively high expression in the lungs but were unable to detect any in the trachea. In contrast, another study found tracheal but not lung transfection using guanidium-cholesterol liposome preparations (Oudrhiri *et al.*, 1997), and this difference may be due to numerous factors other than the lipid used (Table 3).

Physicochemical characteristics

It is thought that the physical (i.e., size) and chemical (i.e., charge) properties of DNA/lipid complexes will influence various aspects of direct gene transfer, such as transfection efficiency and type of cell taking up the DNA. The size and ζ potential of DNA/lipid complexes have been shown to depend on (1) the amount and type of cationic and neutral co-lipids, (2) the cationic lipid/DNA ratio, (3) the final concentration of lipid and DNA, (4) the composition of the suspending substance, (5) the mixing procedures, and (6) the molecular weight, type and form of plasmid DNA (Felgner *et al.*, 1996; Mahato *et al.*, 1997). Our results indicate that both size and charge of the DNA/lipid complexes affect transfection efficiency, but owing to the lack of correlation of these two properties, and the limited number of complexes tested, it is not possible to determine their exact roles. Furthermore, because size and ζ potential of DNA/lipid complexes may vary within a given lipid formulation, it is possible that the effects seen may have been due to a minority population of a specific size.

Use of animal models to evaluate respiratory gene transfer

Animal models have been used to evaluate DNA/lipid formulations for direct gene transfer in the lung, particularly for gene therapy applications (Hyde *et al.*, 1993; Yoshimura *et al.*, 1992; Alton *et al.*, 1993). Our results indicate several factors, in addition to those affecting the physicochemical properties discussed, that can play a role in such studies.

For example, optimal results appear to be obtained when just enough lipid is used to complex all of the DNA. An excess of cationic liposome may interfere with the release of

DNA, whereas an excess of DNA would leave some DNA uncomplexed and may overneutralize the cationic charge. Although the lipids reported in this study all have similar DNA-binding capacities, this is not true for other lipids we have tested. Thus, for a given lipid, it is important to determine the optimal DNA/lipid ratio by checking for nonassociated DNA with gel electrophoresis (van der Woude *et al.*, 1997).

It is necessary as well to consider the rather brief period of peak gene expression and its rapid rate of rise and fall. Thus, assays should be timed appropriately to ensure measurement of peak expression, and groups to be compared must be evaluated after identical periods of time.

Finally, it is wise to include pure plasmid DNA as a control, as this will provide a point of reference and better comparison between different studies.

Potential of respiratory gene transfer for clinical application

DNA vaccines delivered parenterally can induce potent systemic immune responses, even with transfection of relatively few cells (Donnelly *et al.*, 1997; Ulmer *et al.*, 1996; Davis *et al.*, 1997), and even protection against subsequent mucosal challenge (Zhang *et al.*, 1997). However, specific mucosal immunity is considered more desirable (O'Hagan, 1995; McGhee *et al.*, 1992; Walker, 1994), and this might be possible with delivery of DNA vaccines to mucosal surfaces. To date, few examples of respiratory DNA vaccine delivery have been reported. However, i.n. administration of plasmid DNA expressing influenza virus haemagglutinin glycoproteins protected both mice and chickens against lethal influenza challenge (Fynan *et al.*, 1993), and both systemic and mucosal immunity have been

generated in mice following i.n. immunization with plasmid DNA expressing herpes simplex virus type 1 glycoprotein B (Kuklin *et al.*, 1997). Similarly, we detect antibodies against β-Gal following administration by i.n. inhalation of *lacZ*-encoding plasmid DNA complexed with our most effective lipid. However, these are lower than induced by i.m. delivery (Davis *et al.*, 1997) and this may be due to the lower level and shorter duration of expression and/or different types of cells transfected (Davis *et al.*, 1993a, Davis *et al.*, 1996a).

In contrast to the use of DNA for immunization, gene transfer to the respiratory system for therapeutic purposes will likely require a larger number of cells to be transfected. For example, gene therapy for CF may require transfection of nearly 100% of epithelial cells if correction of both chloride transport and sodium conductance is necessary for therapeutic effects (Johnson *et al.*, 1995) or < 10% if only the former is required (Johnson *et al.*, 1992). In either event, expression may not have to be at physiologic levels, as in CF transgenic mice, about 5% of wild type levels of CFTR mRNA can correct the pathologic condition (Dorin *et al.*, 1996). For therapeutic expression of some polypeptides, it may also be necessary to transfect a relatively large number of cells, and it is difficult to predict whether the approaches used herein will be adequate for such applications. In mice, the maximum dose of DNA was limited by the concentrations of the reagents, and thus volume, so it is possible that relatively more efficient transfection could be realized in the human with larger volumes of concentrated DNA solutions.

Another factor of obvious concern for therapeutic application is the brief period of gene expression in the lung. We show high luciferase reporter gene expression for only a 3-day period (days 2 to 4 after gene transfer), similar to previously reported findings using reporter

genes in animals (Meyer *et al.*, 1995; Hazinski *et al.*, 1991; Wheeler *et al.*, 1996) and the CFTR gene in humans (Caplen *et al.*, 1995). Although we and others (Yoshimura *et al.*, 1992; Stribling *et al.*, 1992; Wheeler *et al.*, 1996; Tsan *et al.*, 1995) show very low levels of gene expression for at least several weeks, this is unlikely to be adequate for most therapeutic effects. Nevertheless, a brief period of expression might be adequate for certain therapeutic applications, including protection of the lungs from oxidant stress by the superoxide dismutase gene (Wispe *et al.*, 1992), transfer of drug resistance genes to decrease chemotherapy toxicity (Koc *et al.*, 1996), protection of the lungs from endotoxin injury by the prostaglandin G/H synthase gene (Conary *et al.*, 1994), or activation of pro-drugs (Springer and Niculescuduvaz, 1996).

In the present study, the decrease in gene expression is unlikely to be immune mediated, as luciferase is essentially non-immunogenic in a mouse, and its expression continues for at least 60 days in muscle (Davis *et al.*, 1997). Also, gene expression in the lungs of nude (no T cells) and SCID (no B or T cells) mice is similarly short-lived (Lee *et al.*, 1996). The loss is unlikely to be due solely to normal cell turnover, which is about 4 - 5 weeks for type II alveolar epithelial cells (Bowden, 1983), although this could be accelerated by the DNA/lipid complexes. Although the turnover rate is less than 1 week for bronchial and tracheal epithelial cells (Bowden, 1983), we have found high reporter gene expression in the lung but not the trachea. We can detect by PCR plasmid DNA in lung extracts for only 6 hours after administration, as reported elsewhere (Meyer *et al.*, 1995). Luciferase is unstable and thus this likely reflects the rapid degradation of extracellular DNA rather than the loss of DNA being expressed.

Expression might decline because of promoter turn-off. Liposome-formulated DNA induces, in mouse lungs, elevated levels of cytokines such as IFN-γ and tumor necrosis factor-alpha (TNF-α) (Scheule *et al.*, 1997; Lee *et al.*, 1996), that are known to downregulate viral (e.g. CMV) (Xiang *et al.*, 1997; Harms and Splitter, 1995; Romero and Lavine, 1996) as well as some nonviral promoters (Yew *et al.*, 1997). In the present study, IFN-γ was found not to be responsible for the loss of expression, but other cytokines could have played a role. Increased cytokine expression can be induced by immunostimulatory CpG motifs within the plasmid DNA vectors (Krieg *et al.*, 1995; Klinman *et al.*, 1996; Krieg, 1996; Schwartz *et al.*, 1997) as well as by the cationic and possibly the neutral lipids (Scheule *et al.*, 1997). We have noted no long-term adverse effects with i.n. administration of up to 1 mg plasmid DNA alone, but death within 48 hrs with the addition of 0.5 to 1 mg of Cellfectin (McCluskie and Davis, unpublished observations). However, others have noted that a given dose of lipid-complexed DNA was toxic when given as a liquid by i.n. instillation but not as an aerosol (Eastman *et al.*, 1997). Nevertheless, various studies have shown liposome-formulated DNA to be safe and nontoxic in animal models and humans at doses producing physiologic responses (Caplen *et al.*, 1995; Canonico *et al.*, 1994b; Nabel *et al.*, 1993; Tsan *et al.*, 1996; Porteous *et al.*, 1997).

The results of the present study in mice are useful for establishing animal models of direct gene transfer into the respiratory system. However, extrapolation to humans should be made cautiously because of differences in the murine and human respiratory systems (Tomkiewicz *et al.*, 1995). For example, mucous-secreting surface epithelial cells that are found throughout the tracheobronchial epithelium of humans are rare in rodents beyond the

trachea, and mucous glands are found in human but not rodent airways (Hazinski, 1993). It will be desirable to carry out studies on other mammals, such as sheep, ferrets or non-human primates, where the pulmonary epithelium is more similar to that of humans.

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Chapter IV

CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice[1]

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Footnotes

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[3] Abbreviations used in this paper: CT, cholera toxin; ODN, oligodeoxynucleotide; HBsAg, hepatitis B surface Ag; i.n., intranasal; anti-HBs, HBsAg-specific Abs.

Abstract

Mucosal immunity is difficult to induce with subunit vaccines unless such vaccines are administered with a mucosal adjuvant such as cholera toxin (CT); however, CT is toxic in humans. Synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs (CpG) are potent adjuvants for the induction of Th1-like systemic immune responses against parenterally delivered proteins. Here, we show in mice that intranasal delivery of hepatitis B surface antigen, which alone has no effect, elicits good immune responses when given with CpG oligodeoxynucleotides and/or CT. Overall, CpG is superior to CT for the induction of humoral and cell-mediated systemic immunity as well as mucosal immune responses (IgA) at local (lung) and distant (feces) sites. Furthermore, CpG and CT act synergistically, giving stronger responses than those observed with 10 times more of either adjuvant alone. Ab isotypes were predominantly IgG1 (Th2-like) with CT, mixed IgG1/IgG2a (Th0) with CpG, and predominantly IgG2a (Th1-like) with CpG and CT together.

Introduction

The combined mucosal surface area is >200 times greater than that of the skin and is the primary site of transmission of the majority of infectious diseases. In general, parenteral Ag delivery induces only systemic immunity, whereas mucosal delivery can trigger both mucosal (i.e., secretory IgA Abs) immunity at local and distant sites as well as systemic responses (1, 2). Live-attenuated organisms are effective for mucosal immunization, but results have been disappointing with subunit vaccines, largely because of the lack of a safe and effective adjuvant. Cholera toxin (CT)[3] is an effective mucosal adjuvant in animal models, but it is highly toxic, especially in humans. Genetically detoxified mutants of CT or the related *Escherichia coli* heat-labile enterotoxin have been developed using site-directed mutagenesis. These mutants appear to be non-toxic, yet retain adjuvanticity in animal models (3, 4).

A new class of adjuvant is bacterial DNA or synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs. CpG DNA triggers most (>95%) B cells to proliferate; to secrete Ig, IL-6, and IL-12; and to be protected from apoptosis (5-7). In addition, CpG DNA also directly activates monocytes, macrophages, and dendritic cells to secrete IFN-αβ, IL-6, IL-12, granulocyte-macrophage CSF, chemokines and TNF- α (7, 8). These cytokines stimulate NK cells to secrete IFN-γ and have increased lytic activity (7, 9, 10). Overall, CpG induces a Th1-like pattern of cytokine production that is dominated by IL-12 and IFN-γ with little secretion of Th2 cytokines (7).

The utility of CpG as a vaccine adjuvant was suggested by 1) the strong activating effects of CpG on B cells (antigen nonspecific) (5), 2) the strong synergy between the B cell-

signaling pathways triggered through the B cell AgR and by CpG (Ag specific) (11), and 3) the induction of cytokines that could have indirect effects on B and T cells via Th pathways (7). Indeed, we have shown that for systemic immunization, CpG ODN has potent adjuvant effects on the humoral and cellular responses against recombinant hepatitis B surface Ag (HBsAg) administered by i.m. injection in mice (12). The immune responses with CpG were clearly Th1-like, with predominantly IgG2a Abs and strong CTLs.

Although CpG ODN has been shown to be a potent adjuvant for induction of systemic immune responses against a variety of other antigens (12-16), only one study has shown the use of CpG as a mucosal adjuvant. This was with inactivated virus, which is capable of inducing immune responses when delivered on its own to a mucosal surface (17). Here, we report that CpG ODN is a highly effective mucosal adjuvant for HBsAg, which by itself does not induce an immune response.

Materials and Methods

Immunization of mice against HBsAg:

Groups (n = 5-15) of female BALB/c mice (6-8 weeks, Charles River, Montreal, Canada) were immunized by intranasal (i.n.) inhalation (drops were applied to external nares under light anesthesia with Halothane (Halocarbon Laboratories, River Edge, NJ)) of 1 or 10 μg HBsAg (ad subtype, Genzyme Diagnostics, San Carlos, CA) alone or in combination with 1 or 10 μg of CT (purified from *Vibrio cholerae*; Sigma, St. Louis, MO) and/or CpG ODN (5'-TCCATGACGTTCCTGACGTT-3') or non-CpG control ODN (5'-TCCAGGACTTCTCTCAGGTT-3') in a total volume of 150 μl.

We have previously shown that i.n. inhalation is as efficient as direct intratracheal instillation for the delivery of solutions to the lungs (with minimal oral delivery); 150 μl is the optimal volume (18). Both ODN had a nuclease-resistant phosphorothionate backbone (12). Some mice were boosted in the identical manner 8 wk postprime.

Collection of samples

Plasma and fecal pellets were collected 1, 2, 4 and 8 wk postprime and 1, 2 and 4 wk postboost; lung washes were conducted at 4 wk postprime or postboost. Plasma was obtained by retro-orbital puncture. Fecal pellets were recovered from cages in which mice had been isolated without bedding for 24 hr. Fecal material (0.1 mg) was rehydrated for 30 min at RT in 1 ml of Tris-buffered saline (TBS) (0.05 M Tris-HCl and 0.15 M NaCl (pH 7.5)) with 0.1 μg sodium azide (Sigma); next, supernatants were collected after centrifugation at 6000 rpm for 15 min. Lung washes were conducted on mice immediately after anesthetic overdose by

rinsing with 1 ml TBS using polyethylene tubing (polyethylene-20, inner diameter = 0.38 mm, Becton Dickinson, Franklin Lakes, NJ); the tubing was attached to the needle of a 1 -ml insulin syringe (Becton Dickenson), inserted into the trachea via a small incision, and anchored with vascular microclamps (Fine Science Tools Inc., North Vancouver, Canada) above and below the incision. The TBS solution was slowly instilled and withdrawn three times with ~80% final recovery. Lung wash samples were centrifuged at 13,000 rpm for 7 min, and supernatants were collected. All plasma, fecal, and lung wash samples were stored at -20° C until assayed by ELISA.

Evaluation of immune responses

HBsAg-specific Abs (anti-HBs) in plasma were detected by ELISA on individual samples using HBsAg-coated plates (12). Endpoint dilution titers for total IgG as well as IgG1 and IgG2a isotypes were defined as the highest plasma dilution that resulted in an absorbance value (OD 450) of two times greater than that of non-immune plasma, with a cutoff value of 0.05. Anti-HBs titers of responding mice (titers of > 10) were expressed as means ± SEM of individual animal values, which were themselves the average of triplicate assays. Titers of HBsAg-specific IgA were detected as described for IgG, except samples were incubated on coated plates for 2 h at 37 °C and captured Abs were detected with horseradish-peroxidase-conjugated goat anti-mouse IgA (1:1000 in PBS-Tween, 10% PBS, 100 µl/well; Southern Biotechnology Associates, Birmingham, AL). IgA in lung washes and fecal extracts were expressed as endpoint dilution titers and OD 450 above background respectively. Non-immune plasma, fecal extracts, or lung wash solutions were used as negative controls.

The CTL activity of splenocytes taken 4 wk postprime or postboost was detected as previously described (12) except: 1) media used for incubating splenocytes was supplemented with 5 x 10^{-5} M 2-ME (Sigma) and 3% EL-4 supernatant as a source of IL-2, and 2) splenocytes (3 x 10^{7}) were co-cultured with 1 x 10^{6} irradiated stimulator cells. CTL activity was expressed as group means ± SEM of individual animal values, which were themselves the average of triplicate assays.

Statistical analysis

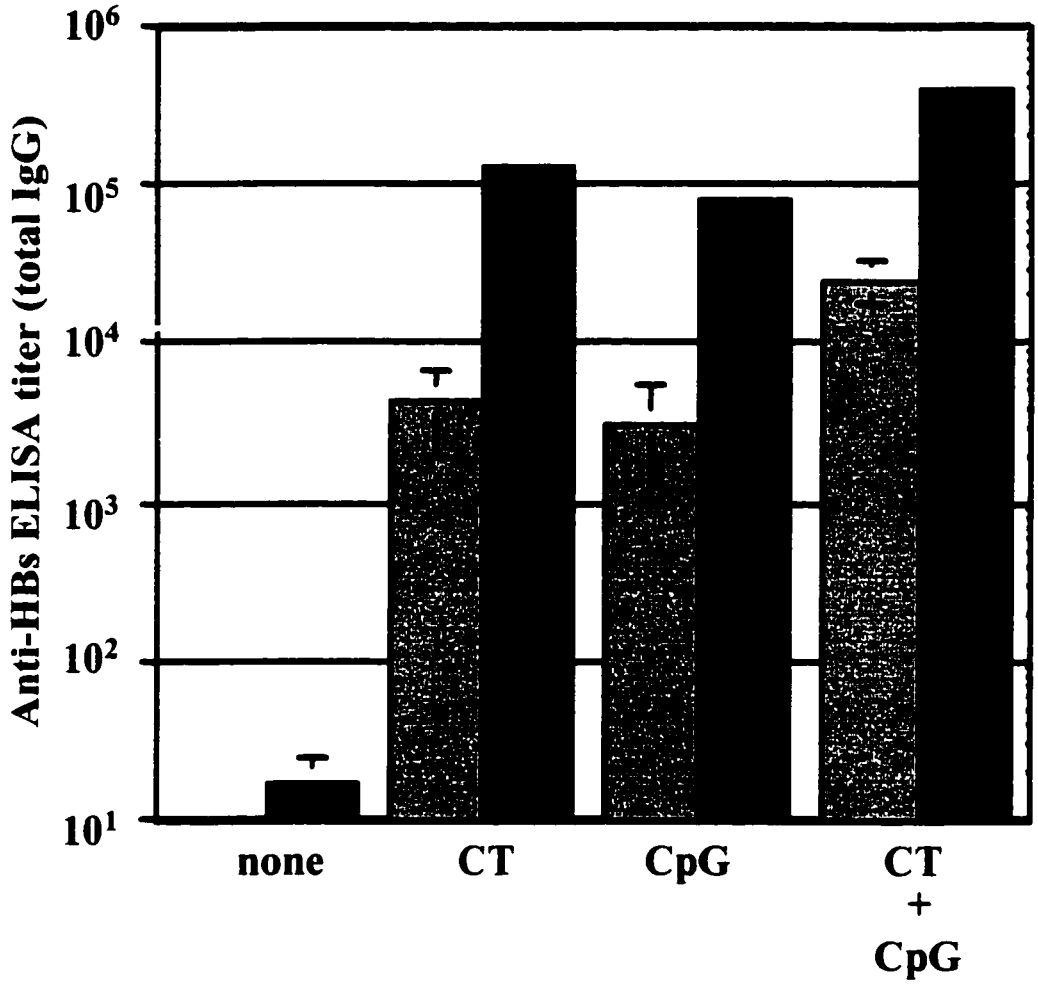
Data were analyzed using the GraphPAD InStat program (GraphPAD Software, San Diego, CA). The statistical significance of the difference between two groups was determined by the two-tailed Student's *t*-test; the difference between three or more groups was determined by one-factor ANOVA followed by Tukey's test. Differences were considered to be not significant with $p > 0.05$.

Results

i.n. inhalation of HBsAg (1 μg) alone failed to induce detectable anti-HBs IgG Abs in the plasma of any mice (0 of 15) (Fig. 1), even after boost (0 of 5) (data not shown). With the higher dose of Ag (10 μg), most mice (12 of 15) failed to seroconvert, a few (2 of 15) had extremely low titers (<40), and only one had a reasonable anti-HBs titer (~1000) (Fig. 1). In contrast, high titers of plasma anti-HBs IgG were induced in all mice when adjuvant was added to the HBsAg, and surprisingly, CpG was equal to CT when used at a 1 μg dose ($p = 0.73$ and 0.13 with 1 and 10 μg HBsAg, respectively) (Fig. 1), or a 10 μg dose ($p = 0.08$ with 10 μg HBsAg) (data not shown). CT was only superior (by sevenfold) to CpG when a high dose of adjuvant (10 μg) and a low dose of Ag (1 μg) were used ($p = 0.01$) (data not shown). Nevertheless, upon boost, titers with CpG rose nearly 500-fold, whereas those with CT were increased by <100-fold (data not shown). A synergistic effect appeared with CpG and CT together (CpG/CT), since titers of plasma IgG were 5 to 10 times higher than with either adjuvant alone (Fig. 1). Indeed, 1 μg of each adjuvant together gave a better systemic humoral response against a low dose of Ag (1 μg) than did 10 μg of CpG alone ($p = 0.01$) and was equal to that with 10 μg CT alone ($p = 0.22$) (Fig. 1). Furthermore, with a high dose of Ag (10 μg), the low dose CpG/CT combination (1 μg each) induced anti-HBs IgG titers as high as those seen with 10 times as much of either adjuvant alone (CT, $p = 0.27$; CpG, $p = 0.09$) (data not shown). Results were due to the CpG motif rather than to a non-specific effect of the ODN backbone, since mice immunized with 1 μg of HBsAg plus 10 μg of non-CpG ODNs had no (7 of 10) or very low (3 of 10) titers of anti-HBs IgG Abs.

Figure 1

BALB/c mice were immunized by i.n. inhalation of 1 μg (grey bars) or 10 μg (black bars) of HBsAg alone (none) or in combination with CT and/or CpG-containing ODNs (CpG) as adjuvants (1 μg each). Each bar represents the group mean (± SEM) of the ELISA endpoint dilution titer for anti-HBs (total IgG) in plasma taken 4 wk postimmunization. Titers were defined as the highest plasma dilution resulting in an absorbance value of two times that seen for nonimmune plasma, with a cutoff value of 0.05.


Anti-HBs ELISA titer (total IgG)
10^6
10^5
10^4
10^3
10^2
10^1
none
CT
CpG
CT + CpG

Abs were predominantly IgG1 (Th2-like) with CT and mixed IgG1/IgG2a (Th0) with CpG or CpG/CT. With boost, CT responses remained Th2; however, responses became more Th1 (IgG2a>IgG1) with CpG, and especially so with CpG/CT (Fig. 2).

Only low levels of CTLs were induced with HBsAg alone (18 ± 3% specific lysis at an E:T ratio of 25:1, mean ± SD) and there was no significant effect ($p > 0.07$) observed with the addition of 1 or 10 μg of either CT or CpG alone. In contrast, low doses of CpG/CT (1 μg each) resulted in a higher level of CTLs (60 ± 5% specific lysis), even compared with 10 times more of either adjuvant alone ($p < 0.01$).

No IgA was detected in lung washes of mice that had been given a low dose (1 μg) of HBsAg, even when 10 μg of either CpG or CT was added (data not shown). However, low IgA titers were detected with CpG/CT (1 μg each), and these titers increased 100-fold after boost (Fig. 3). In contrast, no IgA was detected in the lung washes with 1 μg doses of non-CpG ODN/CT (data not shown). With the 10 μg Ag dose, IgA was detected with the addition of CT and CpG, either alone or in combination (Fig. 3). A synergy was also noted, with lung wash IgA being significantly higher with 1 μg each of CpG/CT than with 10 μg of either adjuvant alone ($p < 0.0003$) (data not shown).

Only a few formulations induced IgA in the feces, namely 10 μg HBsAg in combination with CpG/CT (1 μg each) (OD above background = 0.08 ± 0.05 at 4 wk postprime and 0.23 ± 0.03 at 4 wk postboost) and 10 μg of HBsAg with 10 μg of CpG alone (0.39 ± 0.09 at 4 wk). Neither CT nor non-CpG ODNs (10 μg doses) induced significant IgA in the feces.

Figure 2

BALB/c mice were immunized at 0 and 8 weeks by i.n. inhalation of HBsAg alone (none) or in combination with CT and/or CpG-containing ODNs (CpG) as adjuvants. The dose of antigen and each adjuvant was 1 μg. Each bar represents the group mean of the ELISA end-point dilution titer for anti-HBs of IgG1 (gray bars) or IgG2a (black bars) isotypes in plasma taken 8 wk postprime (upper panel) or 4 wk post-boost (lower panel). Titers were defined as the highest plasma dilution resulting in an absorbance value of two times that seen for nonimmune plasma, with a cutoff value of 0.05. The numbers above each bar indicate the IgG2a to IgG1 ratio, with a value >1 indicating a more Th-1 like response.

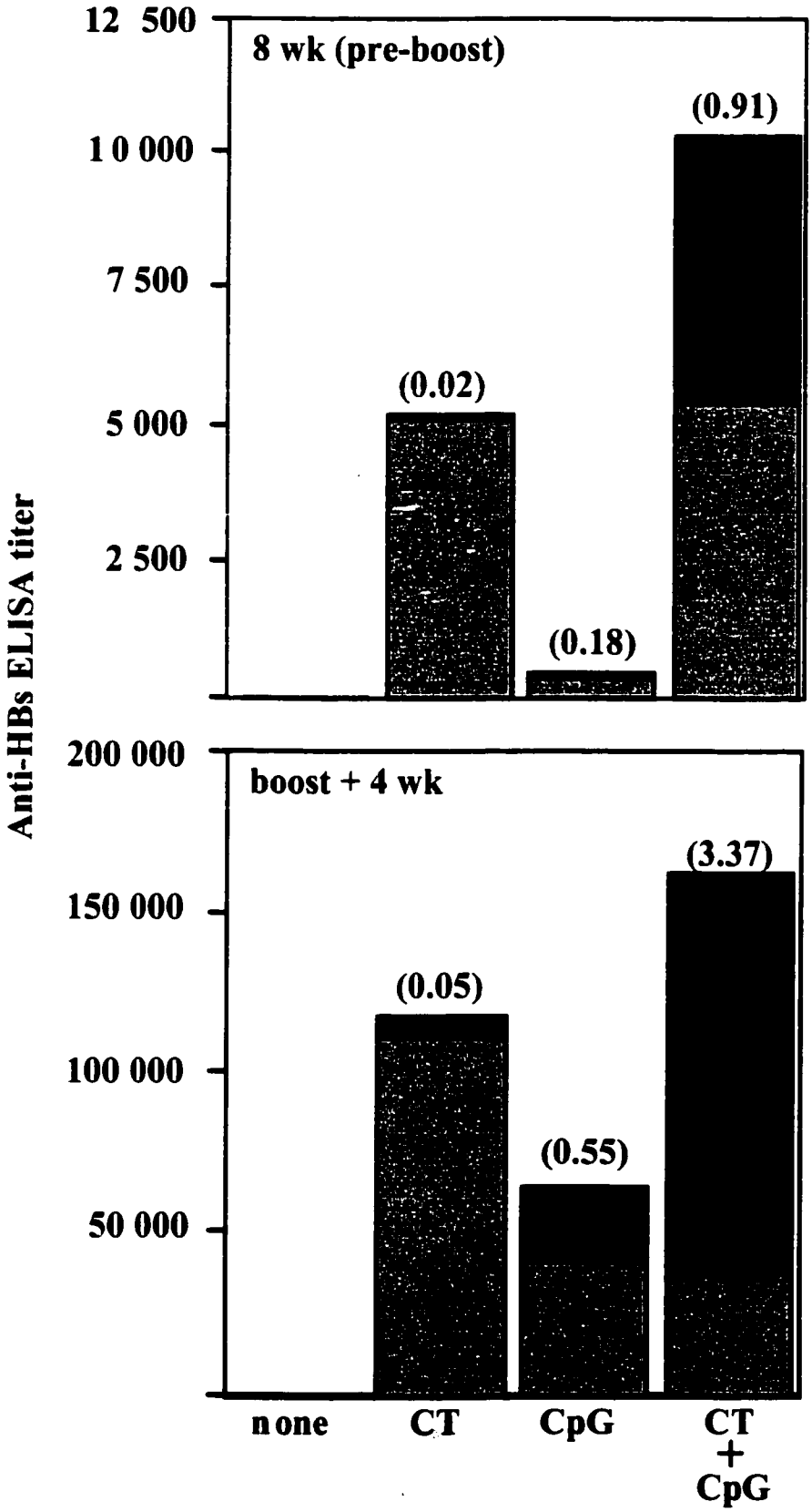
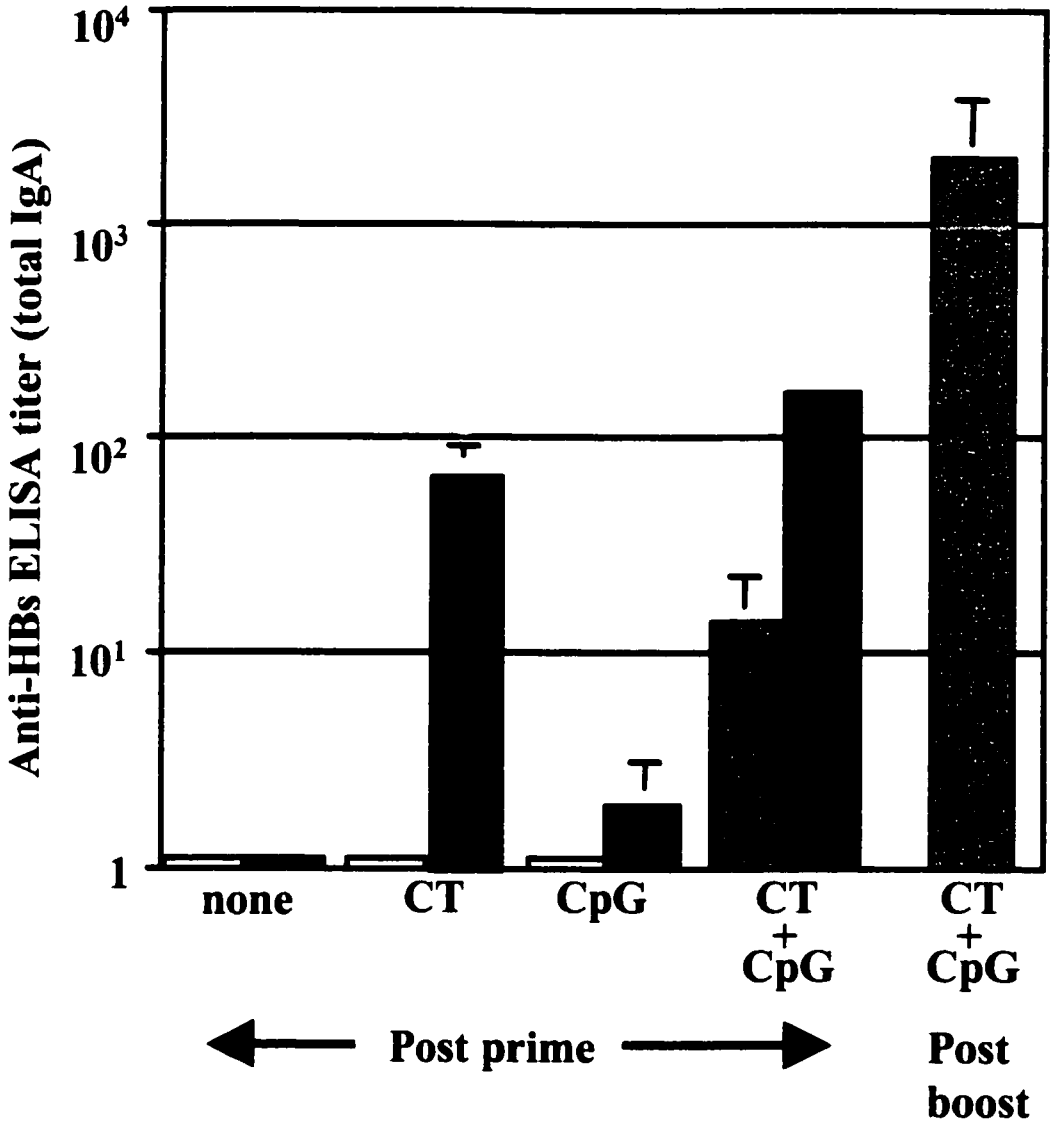

12 500
10 000
7 500
5 000
2 500
8 wk (pre-boost)
(0.91)
(0.02)
(0.18)
Anti-HBs ELISA titer
200 000
150 000
100 000
50 000
boost + 4 wk
(3.37)
(0.05)
(0.55)
none
CT
CpG
CT + CpG

Figure 3

BALB/c mice were immunized at 0 and 8 weeks by i.n. inhalation of 1 μg (grey bars) or 10 μg (black bars) of HBsAg alone or in combination with low doses (1 μg) CT and/or CpG-containing ODNs (CpG) as adjuvants. Each bar represents the group mean of the ELISA end-point dilution titer for anti-HBs in lung washes performed 4 wk postprime or postboost. Titers were defined as the highest sample dilution resulting in an absorbance value of two times that seen for nonimmune lung wash, with a cut-off value of 0.05.


Anti-HBs ELISA titer (total IgA)
10^4
10^3
10^2
10^1
1
none
CT
CpG
CT + CpG
CT + CpG
Post prime
Post boost

Discussion

Here, we show that CpG DNA can act as a potent mucosal adjuvant for both humoral and cell-mediated systemic responses as well as both local (lung) and distant (digestive tract) mucosal immunity. Surprisingly, CpG was as good as CT, and in some aspects (e.g., IgA at distant site), was even better.

The hallmark of mucosal immunity is secretory IgA Abs, which neutralize pathogens in or adjacent to mucosal epithelial cells (19). Activated IgA cell precursors can also migrate to other mucosal sites and differentiate into IgA-secreting plasma cells (20). IgA responses depend upon T-cell help, with IL-4 and TGF-β promoting isotype switching and IL-5, IL-6, and IL-10 being required for terminal differentiation and proliferation (20, 21).

CpG and CT both activate B-cells but appear to act by different mechanisms as they induce different IgG isotypes. This difference may be cytokine related, as CT induces primarily IL-4, IL-5, IL-6, and IL-10 (9, 22, 23) and CpG induces predominantly IL-6, IL-12, and IFN-γ (7, 8). Notably, both induce IL-6, which is required for development of IgA (24). Despite the predominance of IgG1 Abs with CT, its effects cannot be considered purely Th2, since CTLs are detected. Nevertheless, CpG as a mucosal adjuvant can induce IgA Abs in the context of a more pure Th1 response. This has also been reported using CpG with whole-killed influenza virus (17) and tetanus toxoid with IL-12, a Th1-like cytokine (25, 26). A Th2-like response in the lung may be undesirable because of an association with asthma (27, 28). Interestingly, CpG has recently been shown in mice to prevent allergen-induced asthmatic responses, including airway eosinophilia, Th2 cytokine induction, IgE production and bronchial hyperactivity (29).

Further support for the different mechanisms of CpG and CT is provided by their strong synergistic effects on both humoral (systemic and mucosal) and cell-mediated responses. This finding also indicates that it may be possible to use much lower doses of mucosal toxins with CpG to obtain better immune responses with less toxicity.

CpG appears to be a safe yet effective mucosal adjuvant. Even delivery of very high doses ($\leq$ 500 μg) to the lungs of mice was well tolerated; no more short-term distress was observed than with HBsAg alone, and mice displayed a rapid, complete recovery. In contrast, mice receiving high doses of CT (> 10 μg) show signs of toxicity, such as ruffling of fur and diarrhea. CT is even more toxic in humans, where a dose as low as 1 to 5 μg can cause diarrhea (30). This is the first demonstration of CpG as a mucosal adjuvant with a purified protein and of the synergistic actions of CpG and CT together. Like CT, CpG will likely be effective with other Ags such as recombinant proteins, synthetic peptides, and inactivated whole pathogens (31, 32). CpG might also be used as a mucosal adjuvant for Ags delivered to other mucosal surfaces, although the i.n. route is highly desirable for mass immunization since it is noninvasive, fast, easy, and also induces mucosal responses at distant sites (i.e., the lower digestive tract).

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Chapter V

CpG DNA as mucosal adjuvant

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Vaccine. **1999, in press.**

Abstract

We have previously found synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs to be a potent adjuvant to protein administered by intramuscular injection or intranasal inhalation to BALB/c mice. Herein we have further evaluated the potential of CpG ODN as a mucosal adjuvant to purified hepatitis B surface antigen (HBsAg) when administered alone or with cholera toxin (CT). CpG ODN and CT both augmented systemic (humoral and cellular) and mucosal immune responses against HBsAg, and these could be further enhanced with higher doses of adjuvant or boosting. Overall, antibody isotypes with CT alone were predominantly IgG1 (Th2-like) whereas they were predominantly IgG2a (Th1-like) with CpG ODN alone or in combination with CT. Results from this study indicate that stimulatory CpG ODN are promising new adjuvants for mucosal vaccination strategies, whether used alone or in combination with other mucosal adjuvants.

Introduction

The mucosal surfaces are the primary site of entry of most pathogens and thus the induction of effective mucosal immunity could greatly reduce rates of infection and morbidity. Most vaccines developed to date are delivered parenterally (e.g., by intramuscular [IM] or intradermal injection), and as such induce systemic but not mucosal immune responses. In contrast, antigen delivered at mucosal surfaces triggers both mucosal (characterized by the presence of secretory IgA antibodies at local and sometimes at distant sites) and systemic responses [1, 2].

Mucosal vaccines generally require the use of adjuvants to enhance specific immunity. Cholera toxin (CT) is an effective mucosal adjuvant in animal models, however toxicity prevents its use in humans. Genetically detoxified mutants of CT have been developed by using site-directed mutagenisis, which in animal models appear to be non-toxic yet retain adjuvanticity [3, 4]. Despite this progress, there is still a need for novel safe and effective mucosal adjuvants.

Bacterial (but not vertebrate) DNA has direct immunostimulatory effects *in vitro* [5, 6] due to the presence of unmethylated cytosine-guanine dinucleotides (CpG, where the p indicates the phosphate bond) within a particular base context (CpG motifs) [7]. Synthetic oligodeoxynucleotides (ODN) that contain one or more CpG motifs also have immunostimulatory properties [7] . It appears likely that the rapid immune activation in response to CpG DNA may have evolved as one component of the innate immune defense mechanisms that recognize structural patterns specific to microbial molecules [8].

CpG DNA can trigger B cell proliferation, polyclonal immunoglobulin (Ig), IL-6 and IL-12 secretion, and protect B cells from apoptosis [7, 9 - 11]. In addition, CpG DNA also directly activates monocytes, macrophages and dendritic cells to secrete a variety of cytokines including IFN-α/β, IL-6, IL-12 and TNF- α [11 – 14], which in turn, stimulate natural killer (NK) cells to secrete interferon-γ (IFN-γ) and have increased lytic activity [11. 13, 15 - 18]. Immunization of animals against a variety of antigens using CpG DNA as an adjuvant induces strong CTL and mainly IgG2a antibodies, indicative of a Th1 response [19 - 25]. We have found CpG ODN at low doses to be a highly effective mucosal adjuvant for HBsAg (26). Another study has used CpG ODN as a mucosal adjuvant with inactivated virus [25]. Herein we report the effect of adjuvant dose and boosting on immune responses following intranasal delivery to mice using CpG as a mucosal adjuvant.

Materials and Methods

Animals:

All experiments were carried out using female BALB/c mice aged 8-10 wk with 5-15 mice per experimental or control group.

Vaccines:

Antigen: plasma-derived HBV S protein (HBsAg, ad subtype, Genzyme Diagnostics, San Carlos, CA)

Adjuvants: CT, purified from *Vibrio cholerae*, was obtained from Sigma (St. Louis, MO).

CpG ODN 1826 of sequence TCCATGACGTTCCTGACGTT (CpG) was synthesized with a nuclease-resistant phosphorothioate backbone by Hybridon (Milford, MA). This sequence was selected since of the thousands tested, it has the strongest immunostimulatory effects on mouse immune cells *in vitro*. Furthermore, it has previously been shown to be a potent adjuvant for antigen-specific responses in mice with both parenteral and mucosal immunizations (20, 26). The ODN was resuspended in 10 mM Tris (pH 7.0), 1 mM EDTA for storage at +4°C before dilution into saline for immunization. LPS level in ODN was undetectable (< 1 ng/mg) by Limulus assay (Whittaker Bioproducts, Walkersville, MD).

Immunization of mice against HBsAg

Each animal was immunized with 1 or 10 μg HBsAg alone or in combination with 1 or 10 μg of CT and/or CpG. All vaccines were delivered in a total volume of 150 μl, which was applied as droplets directly over both external nares of mice lightly anaesthetized with

Halothane® (Halocarbon Laboratories, River Edge, NJ). Some mice were boosted in the identical manner at 8 wk after prime.

Collection of samples

Plasma: Plasma was recovered from mice at various times after immunization (1, 2, 4 and 8 wk post-prime and 1, 2 and 4 wk post-boost) by retro-orbital bleeding and stored at -20° C until assayed.

Fecal pellets: Fecal pellets were recovered from mice at various times after immunization (1, 2, 4 and 8 wk post-prime and 1, 2 and 4 wk post-boost). Mice were isolated in individual cages without bedding for a 24 hr period, following which fecal pellets were collected and weighed into 0.1 mg aliquots. One ml TBS (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5) and 0.1 µg sodium azide (Sigma) were added per 0.1 mg of fecal material. Samples were allowed to rehydrate for 30 min at RT, then were centrifuged at 6000 rpm for 15 min to remove fecal debris and supernatants were collected and stored at -20° C until assayed for IgA by ELISA.

Lung washes: Lung washes were carried out on mice 4 wk after either primary immunization or boost using a 0.33 cc Insulin syringe fitted with a 29G1/2 needle (Becton Dickenson, Franklin Lakes, NJ) that had a length of polyethylene (PE) tubing 1 cm longer than the needle attached (PE20, ID = 0.38 mm, Becton Dickinson). The mouse was killed by anesthetic overdose and the trachea was immediately exposed through an anterior midline incision made using fine-tipped surgical scissors (Fine Science Tools Inc., North Vancouver, BC). A small incision was then made in the trachea and a vascular microclamp (Fine Science Tools Inc., North Vancouver, BC) was placed above it. One ml of TBS was drawn into the syringe, the PE tubing was passed a few mm down the trachea through the incision and a

second clamp was placed just below the incision to hold the PE tubing in place in the trachea. The TBS solution was slowly instilled in the lungs then withdrawn three times (80% recovery expected). Recovered samples were centrifuge at 13,000 rpm for 7 min., and the supernatants were collected and stored at -20° C until assayed by ELISA.

Evaluation of immune responses

HBsAg-specific antibodies (anti-HBs) in the mouse plasma were detected and quantified by end-point dilution ELISA assay for individual animals as described previously [20]. End-point dilution titers for total IgG as well as IgG1 and IgG2a isotypes were defined as the highest plasma dilution that resulted in an absorbance value (OD 450) two times greater than that of non-immune plasma, with a cut-off value of 0.05. Anti-HBs titers of responding mice (endpoint titers > 10) were expressed as means ± SEM of individual animal values, which were themselves the average of triplicate assays. In our laboratory we have previously determined that the relationship between endpoint titers in mice and those in milli-international units (mIU), as defined by the World Health Organization, is close to 1:1. A value of 10 mIU/ml is protective in humans. Titers of HBsAg-specific IgA were detected as for IgG except samples were incubated on coated plates for 2 hr at 37 °C and captured antibodies were detected with HRP-conjugated goat anti-mouse IgA (1:1000 in PBS-Tween, 10% PBS, 100 µl/well; Southern Biotechnology Inc). IgA in lung washes and fecal extracts were expressed as end-point dilution titers and OD 450 x 1000 above background respectively. Non-immune plasma, fecal extracts or lung wash solutions were used as negative controls.

Cytotoxic T-lymphocyte (CTL) activity was determined using splenocytes taken from mice 4 wk post-prime or post-boost. In brief, single cell suspensions were prepared and suspended in tissue culture medium (RPMI 1640, 10 % FBS, Life Technologies, Grand Island, NY, supplemented with 5 x 10^{-5} M β-mercaptoethanol and penicillin-streptomycin solution, 1000 U/ml, 1mg/ml final concentrations respectively, Sigma, and 3% EL-4 supernatant as a source of IL-2). Splenocytes (3 x 10^7) were co-cultured for 5 days (37°C, 5% CO_2) with 1 x 10^6 syngenic HBsAg-expressing stimulator cells (P815-preS, generously provided by J. Reimann, University of Ulm, Ulm, Germany) that had been previously inactivated by irradiation (20 000 rad). Effector cells were harvested, washed, serially diluted and cultured with 5 x 10^4 ^{51}Cr-labelled HBsAg-expressing target cells (P815S) or control target cells (P815) in round bottom 96-well culture plates (37°C, 5% CO2, 4 hr). Supernatant (100 µl) was removed for radiation (gamma) counting. Spontaneous release was determined by incubating target cells without effector cells and total release by addition of 100 µl 2 N HCl to the target cells. The percent lysis was calculated as [(experimental release - spontaneous release)/(total release - spontaneous release)] x 100. The percent specific lysis was calculated as % lysis with P815S - % lysis with P815 cells. CTL activity for responding mice [% specific lysis > 10] were expressed as means ± SEM of individual animal values, which were themselves the average of triplicate assays.

Statistical analysis

Data were analyzed using the GraphPAD InStat program (Graph PAD Software, San Diego). The statistical significance of the difference between two groups was determined from the means and standard deviations by Student's 2-tailed *t*-test on raw or transformed

data ($\log_{10}$ for heteroscedastic populations) and between three or more groups by 1-factor analysis of variance (ANOVA). Differences were considered to be not significant with $p > 0.05$.

Results

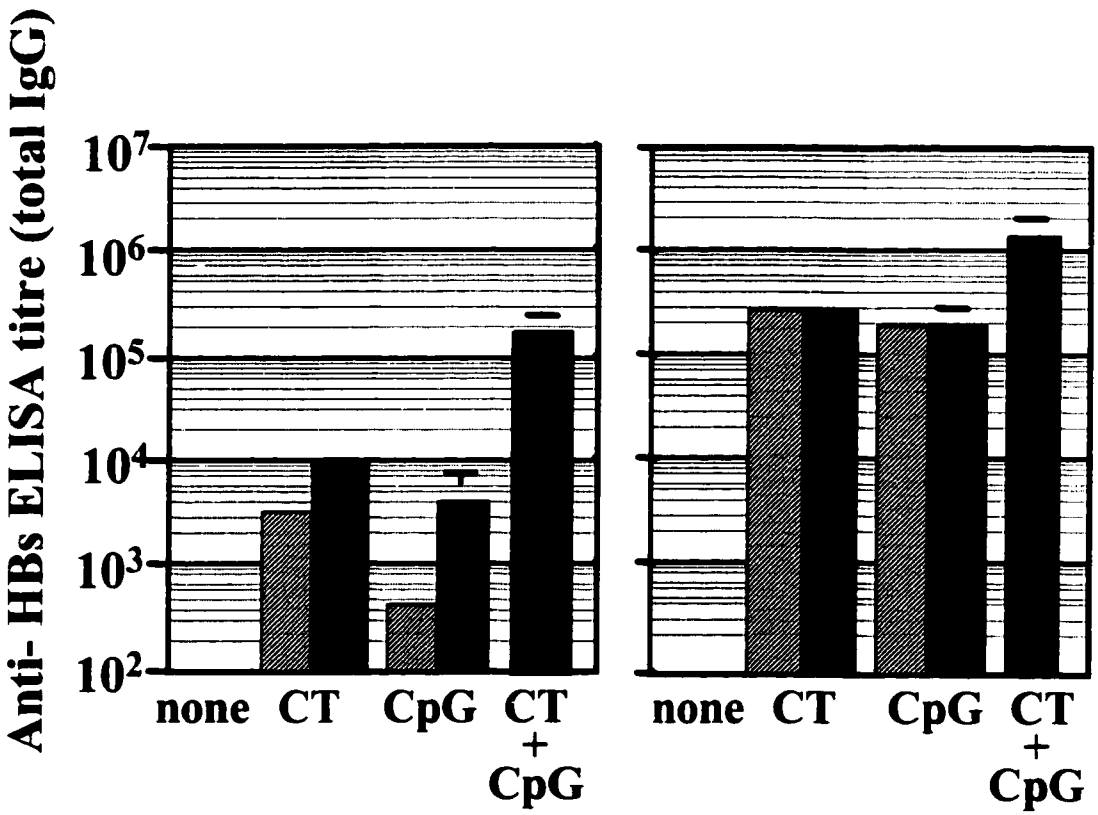
IN inhalation of HBsAg (1 μg) without adjuvant did not induce any detectable anti-HBs IgG antibodies in plasma of mice (Figure 1). In contrast, high titers of anti-HBs IgG were induced in all mice when HBsAg was administered in combination with either CpG or CT as adjuvant (Figure 1). At a high dose (10 μg), both adjuvants were equivalent for induction of plasma anti-HBs IgG ($p = 0.36$), however at a lower dose (1μg) CT was better than CpG ($p = 0.01$). CpG induced 10-fold stronger anti-HBs titres at a higher dose than at a lower dose ($p= 0.01$). In contrast, using CT as adjuvant, there was no difference between titres induced with high or low doses ($p = 0.23$). When used together, CpG and CT (low doses) appeared to have a synergistic effect since anti-HBs titres were 50- or 350-fold higher than those with low doses and 16- or 30-fold higher than those with high doses of CT or CpG respectively (Figure 1).

Boost with antigen alone still failed to induce immune responses. After boost, titres in all adjuvant groups increased a further 10-500 fold, with CT + CpG inducing remarkably high titers of 2×10^6. The magnitude of the increase was inversely proportional to the pre-boost titer (i.e., CpG > CT > CpG + CT), thus the greatest benefit from boosting was obtained with the weaker priming formulation (Figure 1).

Evaluation of plasma for IgG antibody isotypes after prime showed predominantly IgG1 antibodies (Th2-like) with low or high doses of CT. In contrast, there was a predominantly IgG2a (Th-1 like) response with CpG and equally mixed IgG1/IgG2a antibodies (Th0) with CpG + CT. Following boost, CT-induced responses remained predominantly Th2-like. In contrast, the proportion of IgG2a increased after boost with CpG indicating a shift further

Figure 1

BALB/c mice were immunized at 0 and 8 wk by IN inhalation of 1 μg of HBsAg, which was given alone (none) or with cholera toxin (CT) and/or CpG-containing oligonucleotides (CpG) as adjuvants. The dose of adjuvant was 1 μg (hatched boxes), 10 μg (grey filled boxes) or 1 μg each (black filled boxes). Each bar represents the group mean of the ELISA end-point dilution titer for HBsAg-specific antibodies (anti-HBs, total IgG) in plasma taken 8 wk after prime (left panel) or 4 wk post-boost (right panel). Vertical lines represent the SEM. Titers were defined as the highest plasma dilution resulting in an absorbance value two times that of non-immune plasma, with a cut-off value of 0.05.


Anti- HBs ELISA titre (total IgG)
10^7
10^6
10^5
10^4
10^3
10^2
none
CT
CpG
CT + CpG
none
CT
CpG
CT + CpG

towards Th1. Surprisingly, boost with CpG + CT gave now predominantly IgG2a (Th-1 like) (Table 1). These findings indicate that CpG as a mucosal adjuvant stimulates a Th1-like response even in the presence of a strong Th2-like adjuvant like CT, and that the strength of the Th1 response depends upon the dose of CpG.

A low dose of HBsAg (1μg) failed to induce any detectable CTL even after boost and there was no significant effect by the addition of 1 μg of CpG alone (Figure 2; panel A). When low doses of CT or CT + CpG were added, priming induced a weak cellular response that could be increased by boosting. After boost, CT + CpG induced stronger CTL activity than CT alone (E:T 25:1, $p = 0.02$) (Figure 2; panel B). With the high dose of HBsAg (10μg) and low dose of CT or CpG, CTL were significantly better than with HBsAg alone, (E:T 25:1, $p = 0.01$) but were not different from each other (Figure 2; panel C). Increasing the dose of adjuvant to 10 μg did not augment CTL activity (E:T 25:1, $p = 0.78$, 0.30 for CT or CpG respectively) (Figure 2; panel D). CT + CpG (1 μg each) gave stronger CTL responses than 1 or 10 μg of either CT or CpG alone ($p < 0.01$) (Figure 2; panels C and D).

With a high dose of HBsAg (10 μg), no IgA was detected in the lung washes with no adjuvant or with a low dose of CpG (1 μg). Even with the high dose of CpG (10 μg) only 1/5 mice had detectable IgA antibodies. In contrast, all mice treated with HBsAg and CT had anti-HBs IgA in their lung washes, with no difference between low or high doses of CT ($p = 0.34$). However when low doses of CpG + CT (1 μg each) were used together, levels of HBsAg-specific IgA were higher than those obtained with CT alone ($p < 0.01$) (Figure 3).

Fecal pellets were examined for IgA to determine whether any formulation had induced mucosal immunity at a distant site. No IgA was detected using HBsAg alone (either dose).

Table 1: Effect of adjuvant on HBsAg-specific antibody isotype[a]

[a] BALB/c mice were immunized at 0 and 8 weeks by IN inhalation of HBsAg alone (none) or in combination with cholera toxin (CT) and/or CpG-containing oligonucleotides (CpG) as adjuvants. HBsAg-specific antibodies of IgG1 or IgG2a isotypes were determined by endpoint dilution ELISA assay in plasma taken 8 weeks after prime or 4 weeks post-boost. Titers were defined as the highest plasma dilution resulting in an absorbance value two times that of non-immune plasma, with a cut-off value of 0.05. The IgG2a to IgG1 ratios are reported, with a value >1 indicating a predominantly Th-1 like response.

adjuvant	dose (μg)	IgG2a/IgG1 Post prime	IgG2a/IgG1 post boost
none		-	-
CT	1	0.02	0.06
	10	0.21	0.34
CpG	1	0.17	0.55
	10	3.18	6.26
CT + CpG	1 each	0.78	2.86

Figure 2

BALB/c mice were immunized by IN inhalation with HBsAg alone (open boxes), or in combination with cholera toxin (CT) (closed triangles), CpG-containing oligonucleotides (CpG, closed circles) or CpG + CT (closed boxes) as adjuvants. Some mice were boosted (the same as prime) at 8 wk. Spleens were removed 8 wk post-prime or 4 wk post-boost and CTL activity was determined. Immunization strategies were as follows: Panel A: 1 μg HBsAg, adjuvant dose 1 μg, unboosted; Panel B: 1 μg HBsAg, adjuvant dose 1 μg, boosted as prime; Panel C: 10 μg HBsAg, adjuvant dose 1 μg, unboosted; Panel D: 10 μg HBsAg, adjuvant dose 10 μg. Horizontal axes indicate effector to target ratio (E:T) and vertical axes the HBsAg specific lysis as a percentage of the total possible lysis (% specific lysis).

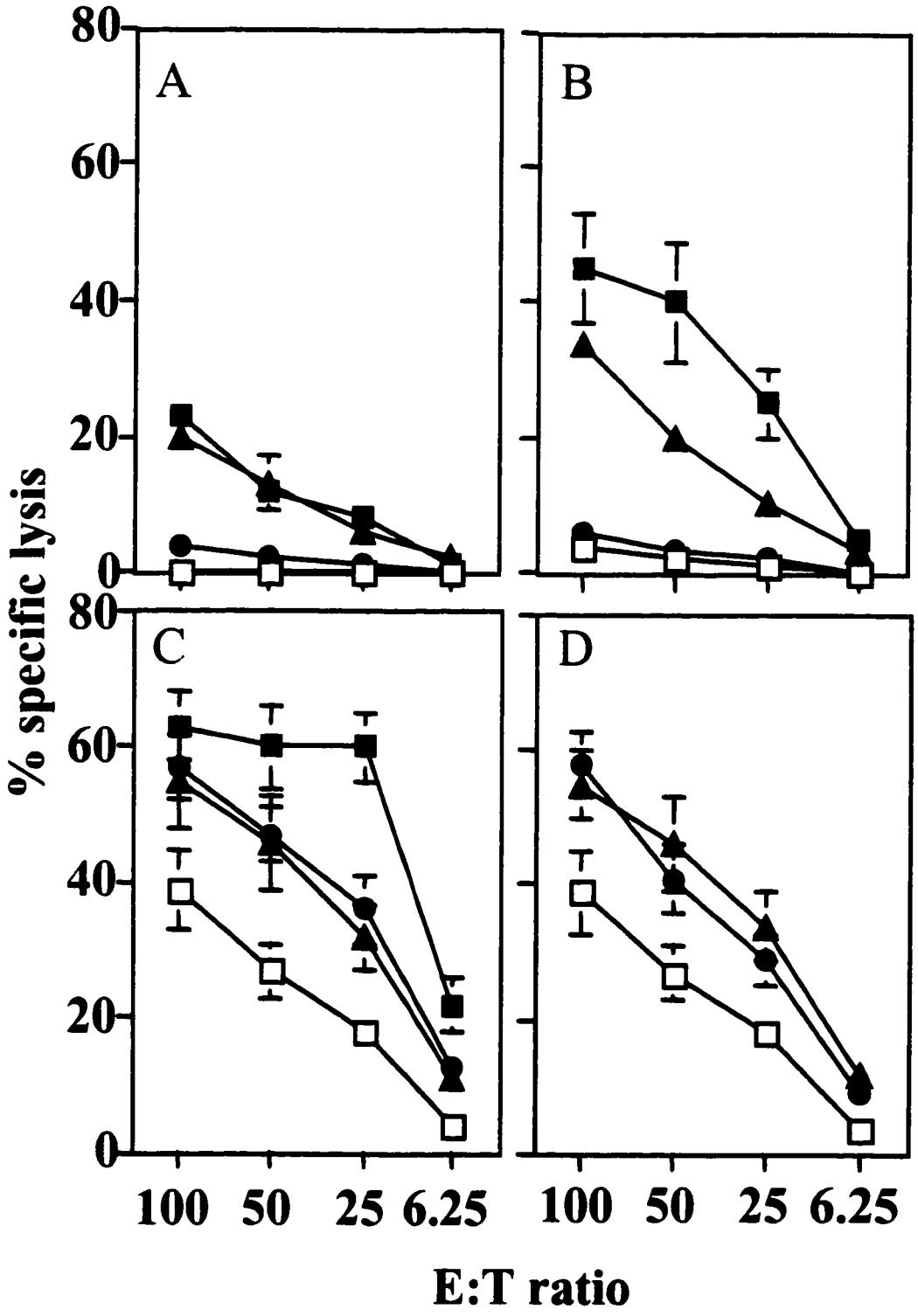
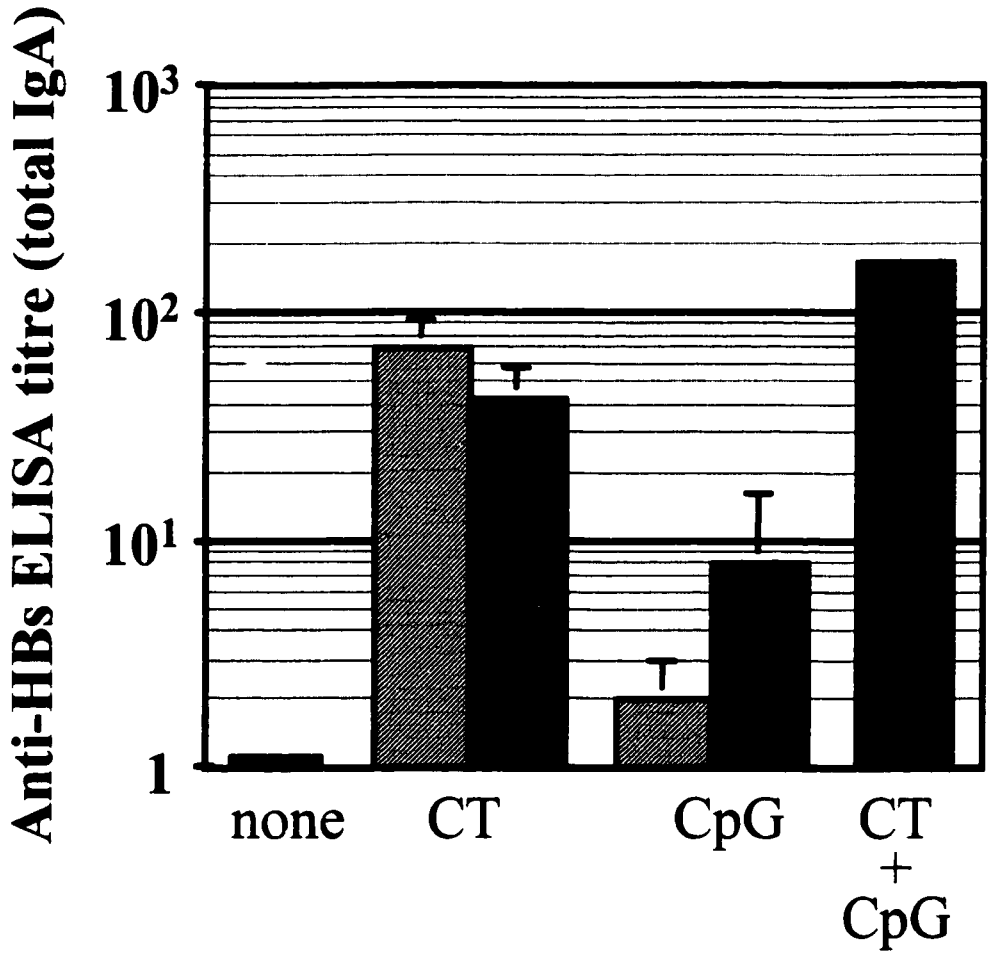

A
B
C
D
80
60
40
20
0
% specific lysis
100
50
25
6.25
E:T ratio

Figure 3

BALB/c mice were immunized by IN inhalation of 10 μg HBsAg alone or with cholera toxin (CT) and/or CpG ODN (CpG) as adjuvants. The dose of adjuvant was 1 μg (hatched boxes), 10 μg (grey filled boxes) or 1 μg each (black filled boxes). Each bar represents the group mean of the ELISA end-point dilution titer for HBsAg-specific IgA antibodies (anti-HBs) in lung washes performed 4 wk after immunization. Vertical lines represent the SEM. Titers were defined as the highest sample dilution resulting in an absorbance value two times that of non-immune lung wash, with a cut-off value of 0.05.


Anti-HBs ELISA titre (total IgA)
10³
10²
10¹
1
none
CT
CpG
CT + CpG

With a high dose of antigen and a high dose of CpG, there was significant IgA detected in the feces of all immunized mice, whereas a low dose of CpG gave a low titre in only 1/5 mice. A combination of low doses of CpG + CT induced low levels of anti-HBs IgA in 2/5 mice as did CT alone at low or high dose (Table 2).

Table 2: Effect of adjuvant on distant mucosal response[a]

[a]BALB/c mice were immunized by IN inhalation with HBsAg alone or in combination with cholera toxin (CT) and/or CpG-containing oligonucleotides (CpG) as adjuvants. Fecal pellets were collected 4 weeks after immunization and HBsAg-specific IgA antibodies were determined by ELISA assay. Mean specific OD 450 x 10^{-1} ± SEM values for responders (specific OD 450 > 2 times that of non-immune mice) are shown.

[b]Calculated with values from responding mice only.

HBsAg (μg)	CT (μg)	CpG (μg)	No. of responders	Specific OD 450 x 1000[b]
10			0/5	-
10	1		2/5	122 ± 37
10	10		2/5	95 ± 17
10		1	1/5	128
10		10	4/4	411 ± 90
10	1	1	2/5	207 ± 15

Discussion

We have previously shown that CpG ODN is a potent adjuvant for systemic (IM injection) [20] or mucosal (IN inhalation) (26) delivery of HBsAg. The effect with mucosal delivery is particularly striking since the protein alone does not induce detectable immune responses with IN delivery, whereas it gives reasonably good humoral responses with IM delivery.

Both systemic and mucosal HBsAg-specific immune responses were induced by mucosal delivery of HBsAg with CpG ODN alone or in combination with CT. Overall, the strongest immune responses were induced using a combination of CT and CpG ODN, as we had found in earlier studies in our laboratory. The synergistic interaction between CpG ODN and other adjuvants (i.e. aluminum hydroxide) has also been shown for systemic immunization of adult or neonatal mice [20, Brazolot-Millan *et al.*, unpublished data]. Using CpG ODN alone, anti-HBs IgG titres in plasma were greater with a higher dose of adjuvant and could be further increased by boosting. Significant levels of IgA antibodies were induced at a distant mucosal side in 100% of mice with CpG ODN but not with CT. This was somewhat surprising as CT is considered to be a highly effective mucosal adjuvant.

CT induces primarily IL-4, IL-5, IL-6 and IL-10 [27 - 29] and predominantly IgG1 isotype of antibodies [30], that in mice are indicative of Th2-type response. In contrast, CpG ODN induces a mixed response characterized by IL-6, IL-12 and IFN-γ but with a strong Th1 component [11 - 14]. In this study, CpG ODN gave a Th1 response (predominantly IgG2a antibodies and CTL), especially so with a high dose, after boost, or when combined with CT. The presence of IgA antibodies is classically associated with a Th2 response,

however we have detected high titres of mucosal IgA antibodies concomitant with the CpG ODN-induced Th1 response. The presence of both Th1- and Th2-like responses is probably associated with the induction of both IL-12 (Th1) and IL-6 (Th2) cytokines by CpG ODN [11, 13, 14]. In particular, IL-6 has a critical role in the generation of IgA responses [31]. High levels of IgA have also been reported by co-administration of tetanus toxoid with IL-12, a Th1-like cytokine [32, 33]. Thus, with mucosal immunization IgG antibody isotypes are not necessarily predictive of IgA responses as had been previously thought. Neither do mucosally-induced IgG isotypes predict cellular responses. CTL are normally associated with Th1 responses and high levels of IgG2a, however here we show co-existence of strong CTL with predominantly IgG1 antibodies in mice immunized with HBsAg and CT.

This study shows CpG ODN to be a potent mucosal adjuvant to induce immune responses against a protein antigen administered by intranasal inhalation. Delivery of vaccines to the respiratory system is particularly desirable since it is non-invasive (avoids needles), quick and easy to carry out, and thus can be used for rapid immunization of large numbers of people. In addition, CpG ODN might also be useful as a mucosal adjuvant for antigens delivered to other mucosal surfaces such as the genitourinary tract (e.g., intravaginal instillation) or the digestive tract (e.g., oral delivery of microencapsulated antigen). The experiments here were carried out with a particulate protein but it should also be possible to use CpG ODN to induce mucosal immune responses against other proteins, peptides and whole (killed or attenuated) pathogens.

The studies presented herein were all conducted on mice. Since results of vaccination studies in mice often do not predict the situation in humans, extrapolation to humans should

be made cautiously. Furthermore, with respect to mucosal delivery, there are differences in the murine and human respiratory systems [34]. For example, mucous-secreting surface epithelial cells which are found throughout the tracheobronchial epithelium of humans are rare in rodents beyond the trachea, and mucous glands are found in human but not rodent airways [35]. In addition, most larger mammals, including humans, possess palatine, lingual and pharangeal tonsils, which act as the major inductive sites for mucosal immune responses in the respiratory tract [36]. In contrast, rodents lack pharyngeal and palatine tonsils and instead possess bilateral strips of lymphoid tissue underlying the respiratory epithelium of the nasal cavity and the nasopharynx [37, 38]. It will be desirable to carry out studies using CpG DNA as a mucosal adjuvant on other mammals such as sheep, ferrets or non-human primates, where the pulmonary epithelium is more similar to that of humans.

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Chapter VI

Use of CpG DNA as a mucosal adjuvant alone or in combination with bacterial toxins or their derivatives.

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Abstract:

Cholera toxin (CT) and related *Escherichia coli* heat labile enterotoxin (LT) are effective mucosal adjuvants, but too toxic for humans. The B subunit of CT (CTB) and non-toxic derivatives of LT (e.g. LTK63) may reduce toxicity and retain adjuvanticity. Synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs are potent adjuvants systemically and mucosally in mice, and have synergistic action with other adjuvants, such as alum and CT. Herein we evaluate CpG ODN with intranasal (IN) delivery of purified hepatitis B surface antigen (HBsAg), relative to and in combination with CT, LT, CTB or LTK63. CpG ODN, CT and LT augmented anti-HBs titers equally, and this was more than with CTB or LTK63. CpG ODN acted synergistically with CT and LT but not CTB or LTK63, however for all combinations, CpG induced a more Th1-like response. These studies indicate that CpG ODN may be useful for allowing effective immunization through IN delivery of vaccines alone or with mucosal adjuvants of reduced toxicity, such as those that may be suitable for use in humans.

Introduction:

The vast majority of infectious diseases are transmitted at the mucosal surfaces of the gastrointestinal, genitourinary and respiratory tracts. In order to prevent infection, an effective local immune response is required. However, most vaccines developed to date are delivered parenterally (for example by IM or intradermal injection), and as such induce systemic but little or no mucosal immunity. Thus there is a great need to develop effective mucosal vaccines (1, 2).

One of the major impediments to the development of mucosal vaccines has been the lack of effective yet safe mucosal adjuvants. Cholera toxin (CT) and the structurally related *Escherichia coli* heat-labile enterotoxin (LT) are potent mucosal adjuvants commonly used in animal models (3, 4), however, toxicity prevents their use in humans. Two strategies have been proposed to circumvent the toxicity of the native toxins: (i) the use of the non-toxic B subunits that lack enzymatic activity (5, 6); or (ii) genetically detoxified mutants of CT or LT, which have little or no enzymatic activity. Such compounds, which have greatly reduced toxicity, retain some adjuvanticity in animal models (7-16). Nevertheless, the efficacy of these new adjuvants has yet to be proven in humans, and in consideration of the extreme sensitivity of humans to the toxicity of CT and LT, there is a clear need for alternative safe and effective mucosal adjuvants.

A new class of adjuvant is DNA, most often in the form of synthetic oligodeoxynucleotides (ODN), containing immunostimulatory CpG motifs. Systemic immunization of animals against a variety of antigens indicate that addition of CpG DNA induces strong CTL and predominantly IgG2a antibodies, indicative of a Th1 response (17-

21). Recently, we and others have shown CpG ODN to be a potent adjuvant for antigens delivered by IN inhalation (22-24). In an earlier study we showed CpG DNA to be as potent as CT and that there were strong synergistic responses when the two adjuvants were used together (22). While CT is too toxic for use in humans, these results suggested that CpG DNA might be combined with nontoxic derivatives of CT or the closely related LT to obtain better immune responses. Herein, we have examined this possibility using intranasal delivery of hepatitis B surface antigen (HBsAg) to mice as a model system. CpG DNA was used alone or in combination with the native toxins (CT, LT), or the forms of them that lack the toxic enzymatic activity, namely the B subunit of CT (CTB) and a genetically detoxified LT mutant (LTK63). Results indicate that when used alone, CpG ODN is greatly superior to the non-toxic adjuvants, however the synergistic response seen with CpG DNA and CT or LT is largely lost with CTB and LTK63.

Materials and Methods

Immunization of mice against HBsAg:

Groups (n=5-10) of female BALB/c mice (8-10 weeks, Charles River, Montreal, QC) were immunized by IN administration of 1 μg HBsAg (plasma-derived HBV S protein, ad subtype, Genzyme Diagnostics, San Carlos, CA), alone or combined with 1 or 10 μg of CT (purified from *Vibrio cholerae*, Sigma, St. Louis, MO), LT (purified from *Escherichia coli*, Sigma), CTB (purified from *Vibrio cholerae*, Sigma), LTK63 (mutant of LT bearing an Ser → Lys at position 63, generously provided by Dr. Rino Rappuoli, IRIS, Chiron S.p.A., Italy) and/or CpG ODN (5'-TCCATGACGTTCCTGACGTT-3') or non-CpG control ODN (5'-TCCAGGACTTCTCTCAGGTT-3') (Hybridon Speciality Products, Milford, MA). The antigen and adjuvant(s) were made up to a total volume of 150 μl with 0.15 M NaCl, and were administered by IN inhalation, as previously described (22). Both ODN had a nuclease-resistant phosphorothionate backbone (19).

Collection of samples and evaluation of immune responses:

Plasma and fecal pellets were collected 1, 2, and 4 wk post-immunization, and lung washes were carried out 4 wk post-immunization, as previously described (22). All samples were stored at -20° C until assayed by ELISA.

Anti-HBs specific antibodies in the individual samples were detected and quantified by end-point dilution ELISA assay as described previously for IgG, IgG1, IgG2a (19) and IgA (22) isotypes. End-point dilution titers for IgG isotypes in plasma and IgA in lung washes were defined as the highest plasma dilution that resulted in an absorbance value (OD 450)

two times greater than that of non-immune plasma or lung wash, with a cut-off value of 0.05. Anti-HBs titers for a group of animals were expressed as geometric mean titers ± the standard error of the mean (GMT ± SEM) of individual animal values, which were themselves the average of triplicate assays. IgA in fecal extracts were expressed as OD 450 x 10^3 above background (non-immune fecal extract).

Statistical analysis

Data were analyzed using the GraphPAD InStat program (GraphPAD Software, San Diego). The statistical significance of the difference between group means was calculated with non-transformed data for % specific lysis or OD 450 or with transformed data ($\log_{10}$) for ELISA titers by Student's 2-tailed *t*-test for two groups or by 1-factor analysis of variance (ANOVA) followed by Tukey's test for three or more groups. Differences were considered to be not significant with $p > 0.05$.

Results

Systemic immune responses

IN delivery of HBsAg (1 μg) without adjuvant did not induce detectable anti-HBs IgG antibodies in the plasma of any mice (0/15). In contrast, high titers of anti-HBs IgG were induced in all mice when HBsAg was administered in combination with CpG, CT or LT as adjuvant (Figure 1). At a low dose (1 μg) LT, CT and CpG gave equivalent anti-HBs IgG titers ($p = 0.22$). At a high dose (10 μg) CT and LT gave higher titers than CpG, however 5/10 mice receiving this dose of LT died within 10 days. No detectable anti-HBs IgG was detected with a low dose (1 μg) of CTB or LTK63, however a high dose (10 μg) of CTB gave low anti-HBs IgG endpoint ELISA titers and a high dose (10 μg) of LTK63 gave as good levels of anti-HBs IgG as a high dose (10 μg) of CpG ($p = 0.97$) (Figure 1). Thus, at low doses, CpG DNA as mucosal adjuvant can induce equivalent systemic immune responses to native toxins (CT, LT) and better responses than their nontoxic derivatives (CTB, LTK63).

When used together, CpG and either LT or CT (1 μg each) appeared to have a synergistic effect since anti-HBs titers were 5 to 10 times higher than with any one of the three adjuvants alone (Figure 1). Indeed, CpG plus LT (1 μg each) gave a better response than 10 μg of CpG or LT alone ($p = 0.007$, 0.015 respectively) and the response with CpG plus CT (1 μg each) was equal to that with 10 μg CT alone ($p = 0.65$). In contrast, there was no synergistic effect with LTK63 plus CpG (1 μg each) for anti-HBs IgG titers, which were equivalent to those with 1 μg CpG alone ($p = 0.40$). Surprisingly, CTB plus CpG (1 μg each) gave lower anti-

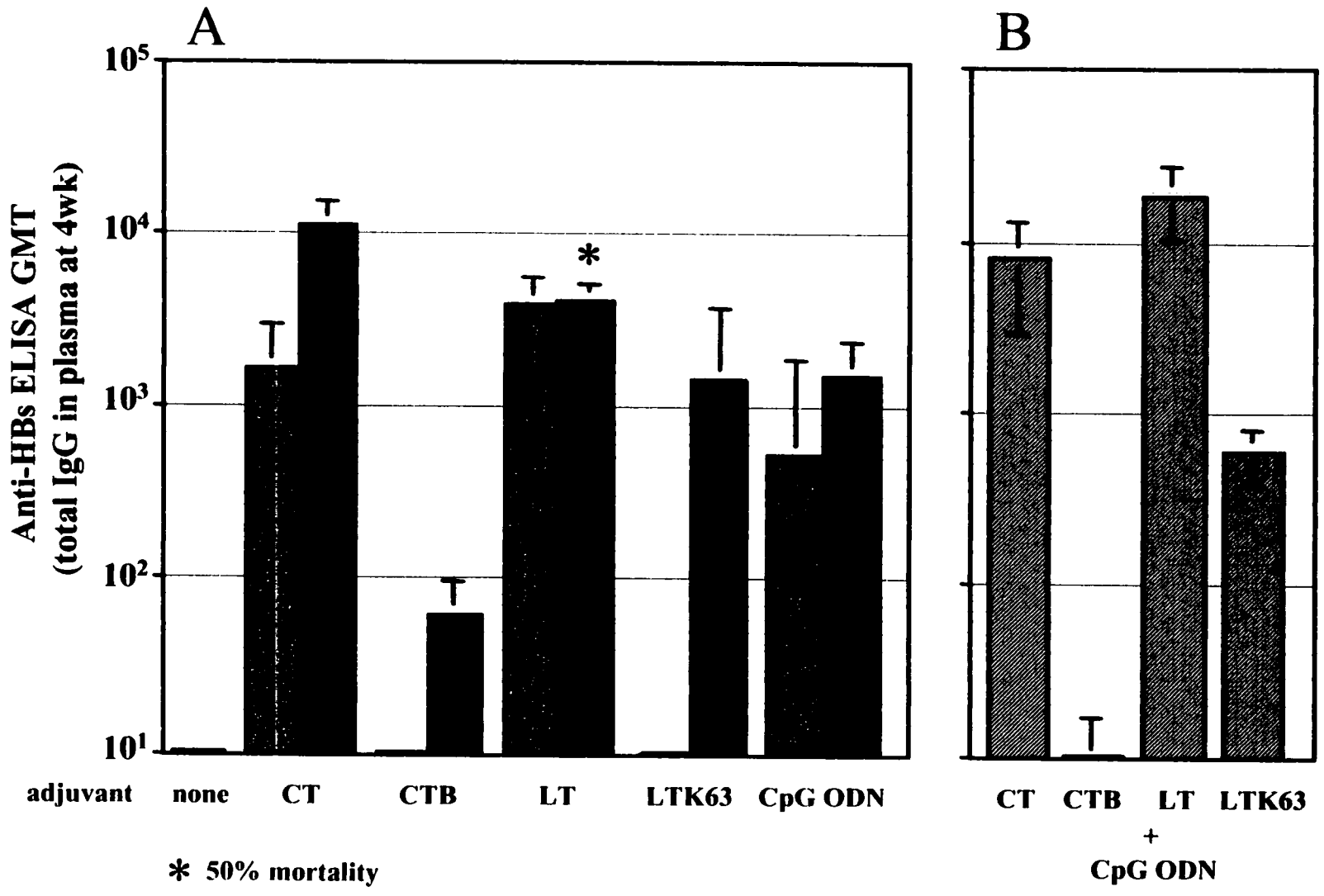
Figure 1

HBsAg-specific antibody responses after IN immunization with HBsAg alone or with mucosal adjuvants.

Panel A: BALB/c mice were immunized by IN inhalation of 1 μg HBsAg alone (none) or with 1 μg (grey boxes) or 10 μg (black boxes) of CT, CTB, LT, LTK63 or CpG ODN as adjuvants.

Panel B: BALB/c mice were immunized by IN inhalation of 1 μg HBsAg with CpG ODN and either CT, CTB, LT or LTK63 (1 μg each) as adjuvants.

Each bar represents the group geometric mean titer (GMT) (± SEM) of the ELISA end-point dilution titer for HBsAg-specific antibodies (anti-HBs, total IgG) in plasma taken 4 weeks after immunization. Titers were defined as the highest plasma dilution resulting in an absorbance value two times that of non-immune plasma, with a cut-off value of 0.05.


A
B
Anti-HBs ELISA GMT
(total IgG in plasma at 4wk)
10^5
10^4
10^3
10^2
10^1
*
adjuvant
none
CT
CTB
LT
LTK63
CpG ODN
CT
CTB
LT
LTK63
+
CpG ODN
* 50% mortality

HBs titers than 1 μg CpG alone (p = 0.007) (Figure 1). Adjuvant effects with CpG ODN were due to the CpG motif rather than a non-specific effect of the ODN backbone since mice immunized with 1 μg of HBsAg plus 10 μg of non-CpG ODN had no (7/10) or very low (3/10) titers of anti-HBs IgG antibodies (data not shown). Therefore, a synergistic effect of CpG DNA on levels of HBsAg-specific IgG occurs with native toxins (CT, LT) but not with their less toxic derivatives (CTB, LTK63).

Antibody isotypes for the induced responses are shown in Table 1. Antibodies were predominantly IgG1 (Th2-like) with CT, CTB and LT and mixed IgG1/IgG2a (Th1/Th2) with LTK63. At a low dose (1 μg) responses with CpG were mixed IgG1/IgG2a (Th1/Th2), but at a higher dose (10 μg) were more Th1 (IgG2a >> IgG1). Responses were mixed Th1/Th2 with CT/CpG or CTB/CpG and more Th1 with LT/CpG. At a low dose (1 μg each) LTK63/CpG responses were Th1/Th2, but at a higher dose (10 μg each) were more Th1. Coadministration of CpG with other adjuvants shifted responses towards a more Th1-like response as indicated by a greater proportion of IgG2a antibodies. Thus, even with those adjuvants (CTB and LTK63) with which CpG DNA did not appear to act synergistically to augment levels of HBsAg-specific antibodies, the use of CpG was beneficial since it promoted a preferred Th1-like responses.

Mucosal immune responses

When adjuvants were used alone, only mice receiving LT or LTK63 had detectable IgA in lung washes. However when CpG ODN was included with CT or LT a greater number of animals responded or titers were higher than with comparable doses alone, suggesting a

Table 1. Effect of adjuvant on HBsAg-specific antibody isotypes

*BALB/c mice were immunized by IN inhalation of 1 μg of HBsAg without adjuvant (none) or in combination with CT, CTB, LT, LTK63, and/or CpG ODN (1 or 10 μg) as adjuvants.

‡Values represent the group geometric mean (GMT) of the ELISA end-point dilution titer for HBsAg-specific IgG1 or IgG2a antibodies in plasma taken 4 wk after immunization. Titers were defined as the highest plasma dilution resulting in an absorbance value two times that of non-immune plasma, with a cut-off value of 0.05.

§The IgG2a to IgG1 ratios (IgG2a:IgG1) are reported, with a value >1 indicating a predominantly Th-1 like response.

¶ †: All mice immunized with these adjuvant combinations died within 96 hours.

** N/A: Not applicable since no antibody responses detected.

Adjuvant*	dose (μg)	Anti-HBs response		
		IgG2a‡	IgG1‡	IgG2a:IgG1§
none	-	0	0	N/A**
CT	1	36	1632	0.02
CT	10	406	3849	0.1
CTB	1	0	0	N/A
CTB	10	6	59	0.1
LT	1	226	6457	0.04
LT	10	895	2024	0.44
LTK63	1	0	0	N/A
LTK63	10	231	455	0.5
CpG ODN	1	146	403	0.4
CpG ODN	10	549	41	13.4
control ODN	1	0	0	N/A
control ODN	10	0	0	N/A
CT + CpG ODN	1 each	3376	2374	1.4
CTB + CpG ODN	1 each	0	0	N/A
LT + CpG ODN	1 each	6268	1438	4.4
LTK63 + CpG ODN	1 each	185	272	0.7
CT + control ODN	1 each	402	5087	0.08
CT + CpG ODN	10 each	†¶	†	†
CTB + CpG ODN	10 each	227	208	1.1
LT + CpG ODN	10 each	†	†	†
LTK63 + CpG ODN	10 each	3170	413	7.7

synergistic effect. CpG alone did not induce IgA. Neither did CTB, alone or combined with CpG (Table 2).

Only a few adjuvants on their own (LT and CpG) induced IgA in the feces, and then only in some animals. No significant IgA was detected with CT, CTB, LTK63 or non-CpG ODN. CpG and LT together resulted in IgA in the feces of a greater proportion of animals than either adjuvant alone suggesting an additive or synergistic effect. No such effects were evident with other combinations (Table 2).

Table 2. Effect of adjuvant on HBsAg-specific IgA responses

*BALB/c mice were immunized by IN inhalation of 1 μg of HBsAg without adjuvant (none) or in combination with CT, CTB, LT, LTK63, and/or CpG ODN (1 or 10 μg) as adjuvants. All groups contained 5 mice unless otherwise indicated.

‡Values represent the geometric mean titers ± the standard error of the mean (GMT ± SEM) of the ELISA end-point dilution titer for HBsAg-specific IgA antibodies in lung wash or fecal solutions taken 4 wk after immunization.

§IgA titers in lung washes were defined as the highest dilution that resulted in an absorbance value (OD 450) two times greater than that of non-immune lung wash, with a cut-off value of 0.05.

¶IgA titers in fecal extracts were expressed as OD 450 x 10^3 above background (non-immune fecal extract). Seroconversion was defined as an endpoint titer for total IgG > 100.

**†: All mice immunized with these adjuvant combinations died within 96 hours.

Adjuvant*	dose (μg)	Anti-HBs response‡			
		lung		fecal	
		IgA§	no. of responders	IgA¶	no. of responders
none	-	0	0	0	0
CT	1	0	0	0	0
CT	10	0	0	0	0
CTB	1	0	0	0	0
CTB	10	0	0	0	0
LT	1	160 ± 68	5	100, 200	2
LT	10	17 ± 5	3/3 (2 dead)	200 ± 50	3/3 (2 dead)
LTK63	1	0	0	0	0
LTK63	10	26 ± 6	4	0	0
CpG ODN	1	0	0	100	1
CpG ODN	10	0	0	0	0
control ODN	1	0	0	0	0
control ODN	10	0	0	0	0
CT + CpG ODN	1 each	17, 49	2	120	1
CTB + CpG ODN	1 each	0	0	0	0
LT + CpG ODN	1 each	232 ± 34	5	150 ± 20	4
LTK63 + CpG ODN	1 each	14	1	0	0
CT + control ODN	1 each	0	0	0	0
CT + CpG ODN	10 each	†**	†	†	†
CTB + CpG ODN	10 each	17	1	0	0
LT + CpG ODN[s]	10 each	†	†	†	†
LTK63 + CpG ODN	10 each	28 ± 46	3/4	130	1/4

Discussion:

Mucosal immunization is desirable for mass vaccination since it is fast and easy to administer, requires minimal training of personnel and carries no risk of needle stick injury or cross contamination. CT and LT have been widely used as adjuvants for mucosal immunization of animals (3, 4) but are too toxic for use in humans. We have previously shown CpG to be an effective non-toxic mucosal adjuvant, similar to CT for induction of systemic humoral responses and with synergistic effects when used together with CT (22). Furthermore, CpG gave a more Th1-like response than CT; this is desirable because of its association with enhanced levels of neutralizing IgG2a antibodies and CTL activity. Here we show similar findings with LT, which is not surprising considering the structural and functional similarities between CT and LT (3, 4). The amino acid sequences for CT and LT have 80% homology and both comprise two subunits. The monomeric A subunit has enzymatic activity and the pentameric ring–shaped B subunit binds GM1 gangliosides at the surface of eukaryotic cells and enables insertion of the A subunit into the cytosol, where it ADP-ribosylates a GTP-binding regulatory protein associated with adenylate cyclase (4).

In the present study, LT was found to be better than CT or CpG for induction of mucosal immunity, however, it is noteworthy that LT was more toxic than CT. Mice receiving a 10 μg dose of CT showed some signs of toxicity with ruffled fur and weight loss, but half the animals receiving an equivalent dose of LT died. No evidence of toxicity was detected with CpG, even at very high doses (up to 500 μg) (22). The synergistic effect of CpG ODN with the native toxins allowed better immune responses at lower and less toxic doses.

Nevertheless, while a mouse can tolerate doses of up to 10 μg of native toxin, an equivalent dose will cause severe diarrhea in humans (25, 26), and it is unlikely that wild-type bacterial toxins will ever be used in humans.

However, there have been variously successful attempts to develop modifications of CT and LT that have reduced or no toxicity but retain some adjuvant activity. The B subunits of CT and LT are non-toxic but contradictory results have been obtained with regard to their adjuvanticity. Several investigators have found recombinant CTB either failed to stimulate mucosal responses against admixed antigens (3, 14, 15) or was a significantly weaker adjuvant than native toxins (27, 28), while others report strong adjuvant activity of LTB or CTB (6, 29). Here we have found that while CTB has some adjuvant activity, it is considerably weaker than the native form and, since a purified form of CTB was used, it is not possible to rule out residual holotoxin contamination (30, 31).

Another approach in the development of less toxic mucosal adjuvants has been the use of genetically detoxified mutants of CT and LT that lack enzymatic activity (7-16). However, whether adjuvanticity is linked to enzymatic activity remains controversial. In some studies, mutants lacking enzymatic activity fail to generate antibody responses against coadministered antigen (3), but in others they retain the adjuvant properties of the native toxin (10, 13, 32, 33). It has also recently been suggested that optimal adjuvanticity may be induced by mutants having only partial knockout of enzymatic activity, e.g. LTR72 or CTS106 (12, 33). The detoxified mutant used in this study (LTK63) lacks ADP-ribosylating activity (7, 8, 10), and was found to be a moderately good mucosal adjuvant, but only when used at a high dose.

Here we show CpG ODN to induce much stronger humoral responses than either subunit (CTB) or detoxified forms (LTK63). While CpG ODN showed strong synergy with LT and CT for antibody titers, it was somewhat surprising that similar results were not seen with CTB or LTK63. Indeed CpG ODN plus CTB induced weaker humoral responses than CpG ODN alone. It is possible that for synergy with CpG ODN, at least some enzymatic activity may be required. If so co-administration of CpG ODN with partially detoxified mutants (e.g., LTR72) may be a more highly effective combination of mucosal adjuvants that may nevertheless be suitable for use in humans. Despite the lack of synergy with the detoxified adjuvants, CpG ODN shifted their responses towards a more Th1-biased response similar to with the holotoxins, as indicated by a greater proportion of IgG2a relative to IgG1 antibodies. A 10 μg dose of CpG alone gave the strongest Th1-type response of all adjuvants or combination of adjuvants. Th1 responses may be preferential in the lungs since allergic asthma is generally associated with Th2-like responses (35, 35). Interestingly, CpG has recently been shown in mice to prevent allergen-induced asthmatic responses, including airway eosinophilia, Th2 cytokine induction, IgE production and bronchial hyperactivity and redirect the immune response towards a more Th1-like profile (36, 37).

Thus, CpG ODN is a promising new mucosal adjuvant that gives systemic humoral responses as strong as those with CT or LT and better than those with nontoxic CTB or LTK63 derivatives. Furthermore, CpG DNA induces a more Th1-biased response whether used alone or in combination with other mucosal adjuvants. These findings may allow the development of novel vaccines to protect against mucosal pathogens as well as easy delivery of vaccines against a wide range of infectious diseases.

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Chapter VII

Immunization against hepatitis B virus by mucosal administration of antigen-antibody complexes

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Abstract

Antigen-antibody complexes have been shown to enhance immune responses against several antigens given by parenteral immunization. Herein, we have evaluated the potential of administering such immunostimulatory complexes by a mucosal route. Hepatitis B surface antigen (HBsAg) complexed with antibodies against HBsAg (anti-HBs) (HBsAg/Ab) was administered to BALB/c mice by intranasal inhalation. HBsAg by itself did not induce immune responses, whereas with HBsAg/Ab complexes, both systemic and mucosal immune responses were observed and these could be modulated by adjuvants. With HBsAg/Ab (1 or 10 μg), anti-HBs antibodies induced were predominantly of the IgG1 isotype (Th2-like). In contrast, anti-HBs induced by HBsAg/Ab plus cholera toxin (CT) or oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs (CpG) (1 μg each) were predominantly IgG2a (Th1-like). Results from this study indicate that HBsAg/Ab complexes can induce strong humoral immune responses when delivered by a noninvasive route, whether used alone or in combination with other mucosal adjuvants.

Introduction

Hepatitis B virus (HBV) remains a serious worldwide problem and more than 2 billion people have evidence of past or current HBV infection. Approximately 350 million people are chronically infected with HBV today and about 25% of them will ultimately die as a result of hepatocellular carcinoma or cirrhosis [28]. In 1992, the World Health Assembly announced the target of introducing an HBV vaccine into national immunization programs in all countries by 1997. The first vaccines against HBV comprised plasma-derived hepatitis B surface antigen (HBsAg), however, they have been largely replaced by yeast-derived recombinant HBsAg. Antibodies against HBsAg (anti-HBs) alone are sufficient to confer protection against HBV infection, with a titer $\geq$ 10 milli-International units/ml (mIU/ml) being widely accepted as the protective level [4]. Despite the availability of an effective HBV vaccine, widespread application of this vaccine has been restricted on account of the high cost and multiple immunization schedule required (typically 0, 1 and 6 or 0, 1 and 12 months). The current HBsAg-containing vaccines, which contain aluminum hydroxide (alum) as adjuvant, are given by intramuscular (IM) injection. Mass vaccination programs would be facilitated if HBV vaccines could be given by a non-invasive route that would not require highly trained personnel or pose the risk of needle-stick injury or cross-contamination. In addition, administration of a vaccine to a mucosal surface can induce both systemic and mucosal (i.e., secretory IgA) immune responses, whereas parenteral administration will usually induce only systemic immunity.

Although HBV can be transmitted via a mucosal surface, specific mucosal immunity does not appear to be necessary for protection against it. Nevertheless, for many other

diseases, a mucosal response is desirable to prevent initial infection through the mucosal surfaces of the genitourinary, gastrointestinal or respiratory tracts. Immunization at one mucosal site can give protection at distant sites [7, 8], making the easy-to-administer intranasal (i.n.) route desirable even for sexually transmitted diseases.

Several different vaccines have been shown to be effective when given intranasally (e.g., nose drops or nasal spray) [1, 14, 15, 18]. However, these usually contain live attenuated pathogens and little success has been obtained with mucosal delivery of subunit vaccines, which usually require adjuvants even for parenteral delivery. We have shown previously that HBsAg alone does not induce immune responses when given to mice by i.n. inhalation [17]. While alum is not a suitable adjuvant for i.n. administration, there are effective mucosal adjuvants such as cholera toxin (CT) or the closely related heat-labile enterotoxin (LT). These are useful for animal models but are too toxic for human use [12], although subunit or genetically detoxified versions of these toxins have been developed for use in humans [21]. Recently, we have shown synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs to be potent adjuvants for HBsAg delivered by i.n. inhalation [17].

An alternate immunization strategy that does not necessarily require adjuvants is administration of antigen-antibody immune complexes. Such complexes have been found to induce immune responses against a number of antigens including those from HBV [24], simian immunodeficiency virus [11], infectious bursal disease [9] and Newcastle disease virus [20]. In each of those studies, the complexes were administered by parenteral routes. Herein, we evaluate i.n. administration of anti-HBs complexed with HBsAg (HBsAg/Ab),

with and without mucosal adjuvants, for immunization against HBV and report for the first time induction of mucosal responses with immunostimulatory complexes.

Materials and Methods

Animals: All experiments were carried out using female BALB/c mice (Charles River, Montreal, QC) aged 8-10 wk with 5-10 mice per experimental or control group.

HBV vaccines:

Antigen: The HBsAg vaccine comprised plasma-derived HBV S protein (ad subtype, Genzyme Diagnostics, San Carlos, CA).

Antigen-antibody complexes: The HBsAg/Ab complexes were made from plasma-derived HBsAg (ad subtype, Shanghai Institute of Biological Products) and mouse anti-HBs with ELISA titer 1:25,600 as previously described [24]. In brief, appropriate amounts of HBsAg and anti-HBs were mixed, kept at 37°C for 1 hour, then overnight at 4°C. The solution was spun at 10,000 rpm for 30 minutes, and then the supernatant was removed and assayed for uncomplexed HBsAg or anti-HBs. The mixture was also checked by electron microscopy to ensure that complexes had formed. The final product of the complex was prepared with excess antigen as we have previously found this to give superior results (unpublished results).

Adjuvants: CT purified from *Vibrio cholerae* was obtained from Sigma (St. Louis, MO). CpG ODN 1826 of sequence TCCATGACGTTCCTGACGTT was synthesized with a nuclease-resistant phosphorothioate backbone by Oligos Etc. (Wilsonville, OR). The ODN was resuspended in 10 mM Tris (pH 7.0), 1 mM EDTA for storage at 4°C before dilution in saline for immunization. LPS level in ODN was undetectable (< 1 ng/mg) by Limulus assay (Whittaker Bioproducts, Walkersville, MD).

Immunization of mice against HBsAg: Each animal was immunized with 1, 10 or 20 μg of HBsAg/Ab without adjuvant or in combination with 1 μg of CT and/or CpG ODN. Control mice were immunized with 1 or 10 μg HBsAg without adjuvant. All vaccines were delivered in a total volume of 150 μl, which was applied as droplets directly over both external nares of mice lightly anaesthetized with Halothane® (Halocarbon Laboratories, River Edge, NJ). Some mice were boosted in the identical manner at 8 wk after prime. Other mice that were immunized with 20 μg HBsAg/Ab were "challenged" by intramuscular (i.m.) injection of 1 μg HBsAg without adjuvant after their anti-HBs IgG titers were seen to fall to low levels (32 wk post-prime).

Collection of samples: Plasma and fecal pellets were collected 1, 2, 4 and 8 wk post-prime and 1, 2 and 4 wk post-boost, and lung washes were carried out on some animals 4 wk post-boost, as previously described [17]. All Samples were stored at -20° C until assayed by ELISA.

Evaluation of immune responses: Anti-HBs antibodies in the mouse plasma were detected and quantified by end-point dilution ELISA assay for individual animals as described previously for total IgG, IgG1, IgG2a [5] and IgA [17] isotypes. End-point dilution titers for IgG isotypes in plasma and IgA in lung washes were defined as the highest plasma dilution that resulted in an absorbance value (OD_{450}) two times greater than that of non-immune plasma or lung wash, with a cut-off value of 0.05. Anti-HBs titers for a group of animals were expressed as geometric mean titers ± the standard error of the mean (GMT ± SEM) of individual animal values, which were themselves the average of triplicate assays.

IgA in fecal extracts was expressed as OD_{450} x 10^3 above background (non-immune fecal extract). Seroconversion was defined as an endpoint titer for total IgG > 100.

Cytotoxic T-lymphocyte (CTL) activity of splenocytes taken from mice 4 wk post-prime or post-boost was determined using a chromium release assay with HBsAg-expressing target cells, as previously described [17]. CTL activity (% specific lysis at a given effector-to-target ratio) was expressed as group means ± SEM of individual animal values, which were themselves the average of triplicate assays.

Statistical analysis: Data were analyzed using the GraphPAD InStat program (Graph PAD Software, San Diego). The statistical significance of the difference between groups was determined on means (% specific lysis or OD_{450}) or transformed raw data (log_{10} for ELISA titers) by Student's 2-tailed t-test for two groups or by 1-factor analysis of variance (ANOVA) followed by Tukey's test for three or more groups. Differences were considered to be not significant with $p > 0.05$.

Results

Intranasal delivery of HBsAg (1 μg) without adjuvant failed to induce anti-HBs IgG antibodies in the plasma of any mice (0/15), even after boost (0/5). With the higher dose of antigen (10 μg), there was only 7% seroconversion rate after prime (1/15) but 80% seroconversion after boost (4/5), although only half of these (2/4) had titers > 200.

In contrast, all mice immunized with either 1 or 10 μg HBsAg/Ab seroconverted after a single immunization, with anti-HBs IgG titers being about 100-fold higher with the 10 μg (GMT~10^4) than the 1 μg (GMT~10^2) dose of complexes. Boosting with the same formulation at 8 wk increased titers by 19- and 30-fold with low and high doses respectively (not shown).

The addition of 1μg CT to 1 μg HBsAg/Ab enhanced anti-HBs IgG titers by 10-fold ($p \leq 0.01$). In contrast, addition of CpG ODN did not augment the systemic humoral response with HBsAg/Ab (post-prime $p = 0.23$, post-boost $p = 0.53$). Upon boost, anti-HBs IgG geometric mean titers were increased for all groups (HBsAg/Ab alone × 19, HBsAg/Ab + CT × 33, HBsAg/Ab + CpG ODN × 3) (Figure 1).

Antibodies induced by HBsAg/Ab without adjuvant were predominantly of the IgG1 isotype (Th2-like) and the IgG2a:IgG1 ratio was not affected by boosting. In contrast, anti-HBs induced by HBsAg/Ab (1 μg) plus CT or CpG (1 μg each) were predominantly IgG2a (Th1-like) and the IgG2a:IgG1 ratio increased further after boost, especially with CpG (Table 1).

Figure 1

BALB/c mice were immunized at 0 and 8 weeks by i.n. inhalation with 1 μg HBsAg/Ab complex without adjuvant (none) or with cholera toxin (CT) or CpG-containing oligonucleotides (CpG) (1 μg each). Each bar represents the group geometric mean titers (GMT) ± the standard error of the mean of the ELISA end-point dilution titer for HBsAg-specific IgG antibodies (anti-HBs) in plasma taken 8 weeks post-prime (gray bars) or 4 weeks post-boost (black bars). Titers were defined as the highest sample dilution resulting in an absorbance value two times that of non-immune plasma, with a cut-off value of 0.05. The statistical significance of the difference between groups was determined on means of transformed raw data ($\log_{10}$ for ELISA titers) by Student's 2-tailed *t*-test for two groups (1 or 10 μg dose) or by 1-factor analysis of variance (ANOVA) followed by Tukey's test for three or more groups (different adjuvants). Differences were considered to be not significant with $p > 0.05$. Titers obtained post-boost were greater than those post-prime with the same formulation ($p < 0.03$), and those with HBsAg/Ab complex + CT were greater than those with HBsAg/Ab without adjuvant ($p \leq 0.01$).

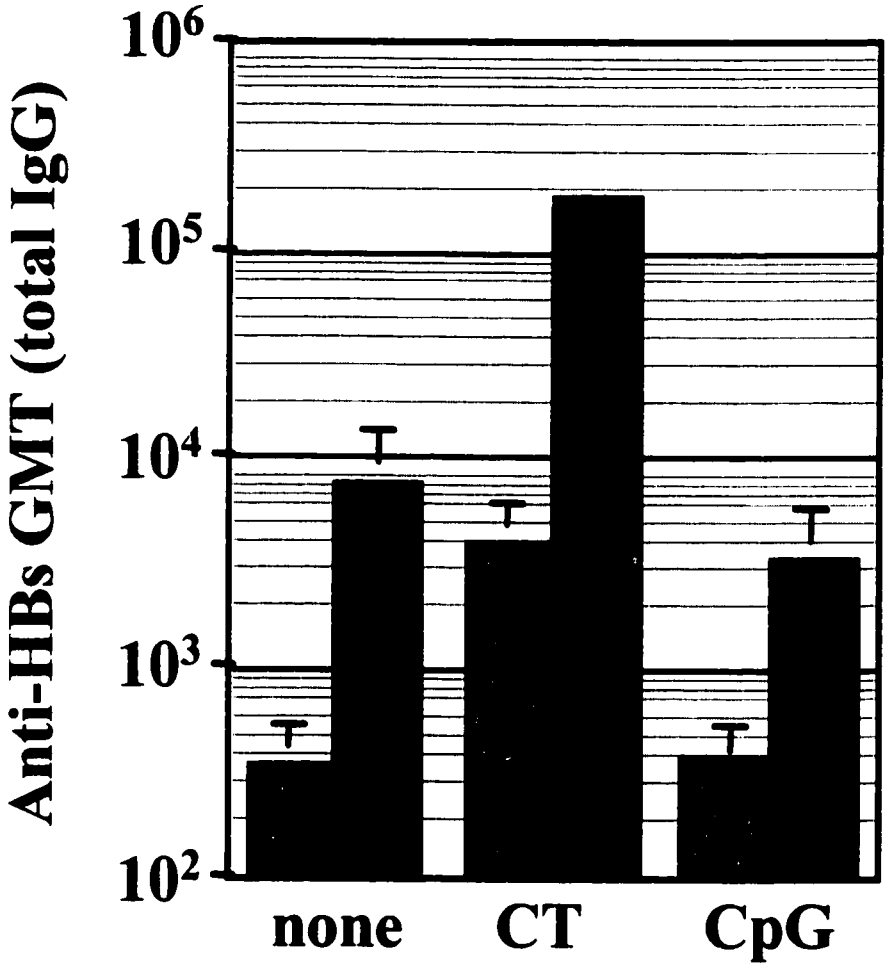
Anti-HBs GMT (total IgG)
10^6
10^5
10^4
10^3
10^2
none
CT
CpG

Table 1. Effect of adjuvant on HBsAg-specific antibody isotype

BALB/c mice were immunized at 0 and 8 weeks by i.n. inhalation of 1 or 10 μg of HBsAg/Ab complexes without adjvuant (none) or in combination with cholera toxin (CT, 1 μg) or CpG-containing oligonucleotides (CpG, 1 μg) as adjuvants. Values represent the group geometric mean (±SEMs) of the ELISA end-point dilution titer for HBsAg-specific IgG1 or IgG2a antibodies in plasma taken 8 weeks post- prime or 4 weeks post-boost. Titers were defined as the highest plasma dilution resulting in an absorbance value two times that of non-immune plasma, with a cut-off value of 0.05. The IgG2a to IgG1 ratios (IgG2a:IgG1) are reported , with a value >1 indicating a predominantly Th-1 like response.

Dose HBsAg/Ab (μg)		Anti-HBs response					
		Post-prime			Post-boost		
	Adjuvant	IgG2a	IgG1	IgG2a:IgG1	IgG2a	IgG1	IgG2a:IgG1
1	none	6	88	0.1	185	1 315	0.1
10	none	541	2 087	0.3	48 166	145 602	0.3
1	CT	1 535	110	14	78 270	3 892	20
1	CpG	51	5	10	1 148	19	60

HBsAg-specific CTL were not detected in mice immunized with HBsAg/Ab alone. The addition of CT or CpG adjuvants also failed to induce CTL despite the concomitant strong IgG2a responses (Th1-like). In contrast, positive control mice injected intravenously (i.v.) with 100 μg of plasmid DNA encoding HBsAg ($pCMV_{[A]}$-S, gift of Georg Widera, PowderJect Inc., Middleton, WI) gave a % specific lysis value of 71 ± 5 at E:T 100:1 (a typical value for that approach) when assayed on the same day.

Secretory IgA was not present in lung washes of mice primed with 1 or 10 μg HBsAg/Ab; however, after boost, HBsAg-specific IgA was detected in lung washes of all mice receiving the higher dose of complexes (GMT ± SEM, 1191 ± 449).

IgA was not detected in the feces of any animals after prime with any of the immunization formulations, or even after boost with HBsAg/Ab (1 μg) without adjuvant or with CpG. Even in groups where fecal IgA was found after boost, only a small proportion of animals responded, namely 33% (1/3) with 10 μg of HBsAg/Ab (OD_{450} above background x 10^3 = 200) and 20% (1/5) with 1 μg HBsAg/Ab + CT (OD_{450} above background x 10^3 = 130).

Anti-HBs IgG titers induced in mice immunized i.n. with HBsAg/Ab (20 μg) declined to low levels (~150) by 32 weeks. When subsequently "challenged" by i.m. injection of HBsAg without adjuvant (1 μg) plasma IgG titers rose quickly to high levels (10^4 by 1 week), indicating a strong memory response. In contrast, unprimed control mice receiving 1 μg HBsAg by i.m. injection had 0% seroconversion at 1 wk and only low titers (~10^2) by 2 weeks.

Discussion

Antigen-antibody complexes have been shown to induce significantly better immune responses than antigen alone in a number of different animal models [9, 11, 20, 24]; however in all of these studies the complexes were given by parenteral routes. The present study demonstrates that HBsAg/Ab complexes can also induce strong immune responses when given by a mucosal route. This is particularly remarkable since mucosal delivery of HBsAg alone essentially does not induce immune responses unless given in high doses and boosted.

The greater efficacy of the antigen/antibody complexes over antigen alone appears to be due, at least in part, to facilitated antigen uptake by antigen presenting cells (APC). It has been shown that uptake of HBsAg/Ab by murine APC following parenteral administration of the complexes is mediated by Fc receptors on the cell surface [2, 3]. APC have receptors for the Fc fragment of IgGs (FcγRs) but not for the F(ab')2 or Fab fragments of IgG and IgM [2, 3]. It has also been shown that the interaction between leukocyte Fc receptors and immune complexes enhances diverse functions such as phagocytosis, cell cytotoxicity, production and secretion of inflammatory mediators, and modulation of the immune response [22].

Recently, we have shown that peritoneal macrophages and dendritic cells from mice previously immunized by intraperitoneal (i.p.) injection of HBsAg/Ab, have better uptake of antigen, as well as enhanced T cell proliferation and secretion of Th1 cytokines (IFN-γ, IL-2) than those from mice injected with HBsAg alone [30]. This appears to depend largely on receptor-mediated uptake rather than non-specific effects associated with the large size of the complexes, since immune responses in mice are greatly enhanced when complexes are made with mouse-derived anti-HBs but only slightly improved when made with human anti-HBs

[24]. In addition, removal of the Fc fragment prior to making the HBsAg/Ab complexes essentially abrogates the effects (Y.-M.W., unpublished data).

CpG ODN has been shown to promote primarily Th1-like responses characterized by, among other effects, the *in vitro* induction of interleukin-12 (IL-12) and interferon-γ (IFN-γ), although there is also significant production of IL-6, a Th2 cytokine [10, 16]. On the other hand, CT is generally considered to be a Th2-promoting adjuvant inducing primarily IL-4, IL-5 and IL-6 [19, 23, 29]. When used *in vivo* as a vaccine adjuvant with HBsAg, CpG ODN gives Th1-biased responses characterized by predominantly IgG2a antibodies and good CTL responses whether the formulation is administered by i.m. injection [5] or i.n. inhalation [17]. Intranasal administration of HBsAg with CT gives a Th2-like antibody response (IgG1>IgG2a), although CTL responses were found suggesting that Th1 cytokines were present, even if they did not dominate the humoral response [17]. Indeed, CT has been shown in other studies to promote antigen priming of both Th1 and Th2 types of $CD4^+$ T precursor cells [13] and to induce Th1-like responses against inhaled allergens [27]. In the present study using i.n. delivery of HBsAg/Ab complexes, both CpG and CT adjuvants induced Th1-like antibody responses, but no CTL, suggesting a selective stimulation of humoral responses. Thus it appears that the Th bias of immune response depends on factors in addition to the adjuvant, and this may include efficiency of antigen uptake or altered presentation. Furthermore, our results show that antibody isotype cannot reliably be used to predict CTL activity.

The results of the present study indicate that it may be possible to immunize against infectious diseases through i.n. delivery of antigen-antibody complexes, and even to induce

mucosal immunity. The complexes could be administered alone or with a mucosal adjuvant such as one of the detoxified forms of CT or LT. With respect to hepatitis B, mucosal immunity does not appear to be necessary, but nevertheless the possibility to deliver HBsAg/Ab complexes by an easy and non-invasive means is attractive for large-scale prophylactic vaccination. Although this could offer many practical, safety and economical advantages, the use of human anti-HBs serum may pose manufacturing or acceptance problems. Alternatively, it might be possible to make humanized antisera in mice, or even recombinant antibodies, although cocktails might be required to include different HLA types.

Activation or deactivation of effector cells via FcγRs also has possible applications in the treatment of cancer, infectious diseases and autoimmune disease, and various clinical trials have been initiated using this approach [6]. For example, HBsAg/Ab immune complexes have been safely used in 14 human HBV chronic carriers, where, after three i.m. injections, nine subjects became negative for HBV DNA and six for HBeAg [25]. Likewise, in ducks chronically infected with duck hepatitis B virus (DHBV), DHBsAg complexed with anti-DHBs bound to a solid matrix could clear viraemia and antigenaemia, whereas DHBsAg or DHBcAg on their own did not [26]. Whether similar results could be obtained after i.n. delivery needs to be evaluated.

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Chapter VIII

General Discussion

The vast majority of infectious diseases are transmitted through the mucosal surfaces. These surfaces are protected by the mucosal immune system, with S-IgA as a major defence factor. However, conventional vaccination strategies (i.e., parenteral immunization), while inducing systemic immunity, rarely induce mucosal immunity. Therefore, novel vaccination strategies capable of inducing mucosal immunity are required. In this regard, we set out to investigate in a murine model the potential of (i) mucosal DNA immunization, (ii) CpG DNA as a mucosal adjuvant, and (iii) antigen-antibody complexes to induce mucosal immunity.

8.1. The effect of route and method of delivery of DNA vaccine

In recent years a large number of studies have evaluated DNA-based immunization. However, in spite of this, few have directly compared the immune responses generated by different routes of immunization, particularly in non-human primates. In **Chapter II** we show that the route of administration of plasmid DNA vaccines influences the strength and nature of immune responses in mice and non-human primates. When a large number of injected and non-injected routes were used to administer the HBsAg-encoding DNA vaccine in saline, anti-HBs were detected in plasma of mice treated by five of eight of the injected but none of the six non-injected routes. The highest levels of anti-HBs were induced by IM and IV injections, although significant titers were also obtained with SL and ID. In addition, each of these routes also induced CTL, as did IPER and VW and one non-injected route (INH) that failed to induce antibodies. There was a preferential induction of IgG1 (Th2-like) in ID immunized mice which changed to mixed IgG1/IgG2a over time. Responses with IM

injection (in the leg or tongue) were predominantly IgG2a (Th1-like). IV injection gave mixed IgG1/IgG2a responses.

The lack of strong immune responses following mucosal delivery was not totally unexpected due to the protective nature of the mucosal surfaces. However, this study confirmed results of other groups that non-invasive administration of plasmid DNA could induce antigen-specific immune responses, albeit at a low level (1 - 14).

In efforts to improve immune responses after mucosal delivery, we formulated DNA with various delivery systems including cationic liposomes (15, 16), cochleates (17, 18), dendrimers (19, 20), microspheres (21), and DNA condensing compounds (22). Mice were immunized by a number of mucosal routes and preliminary results indicated that direct gene transfer to the lung using lipid-formulated DNA was the most promising of these approaches (results not shown), and thus it was decided to further investigate this approach.

8.2. Direct gene transfer to the respiratory tract of mice with pure plasmid and lipid-formulated DNA.

Direct gene transfer in the lungs of mice by administration of plasmid DNA was further examined in **Chapter III** using different techniques and lipid formulations. We demonstrated that cells in the lung could take up and express DNA encoding a luciferase reporter gene whether it was administered in "naked" form or formulated with cationic liposomes. However in the latter case, levels of expression varied depending on the lipid used. Of the cationic lipid formulations tested, tetramethyltetraalkylspermine analogues with alkyl groups of 16 or 18 carbons and DMRIE:cholesterol formulations proved most effective. We showed

that equivalent levels of reporter gene expression in the lung were obtained whether the DNA (naked or lipid formulated) was administered by indirect, non-invasive IN delivery (inhaled or instilled) or by invasive, direct IT delivery (injected or via a cannula). Reporter gene expression peaked around 4 days then fell off dramatically by 9 days. This was most likely as a result of cell turnover and promotor turnoff due, at least in part, to the presence of unmethlyated CpG dinucleotide sequences within the plasmid (23) The dose-response after gene transfer to the lung with lipid-formulated DNA was linear, at least up to 100 μg plasmid DNA, suggesting better transfection efficiencies might be realized if there was not a volume limitation. For a given dose of DNA, the best results were obtained when the DNA was mixed with the minimum amount of lipid that could complex it completely.

In spite of the reasonably good expression obtained after IN administration of plasmids encoding reporter genes, when they encoded antigens either no or very low immune responses were detected whether the DNA was used alone or formulated with liposomes (McCluskie and Davis, unpublished observations). Other groups have also reported poor immune responses using lipid-formulated DNA in the lung (24, 25). In attempts to improve immune responses, we tested various adjuvants including CT, LT or CpG as well as coadministration of DNA encoding cytokines IL-4 or IL-6, and costimulatory molecules B7-1 or B7-2. However, with no approach were strong immune responses induced (results not shown).

It is possible that in some of our experiments levels of HBsAg-specific IgG or IgA may have been too low for detection by ELISA but that anti-HBs secreting cells could have been detected in the spleen and lungs. This was the case in one study using CT as adjuvant, in

which no significant antibody response was detected in serum, saliva or nasal washes using an influenza virus DNA vaccine, but significant numbers of haemagglutinin-specific antibody (anti-HA) secreting B cells did appear in the spleen and lungs (24).

Thus, our efforts to develop DNA vaccines for mucosal delivery had very limited success. This was somewhat disappointing as several of the strategies we tested have subsequently been shown to enhance immune responses to antigen expressed by mucosally delivered plasmid DNA. These include: (i) delivery of lipid formulated DNA (4 - 7, 26); (ii) co-expression of cytokines (4, 26, 27); (iii) the use of adjuvants such as CT (1, 9); and (iv) encapsulation of plasmid DNA in microspheres (28, 29). Nevertheless. these successes generally have not induced the strong immune responses seen after IM injection of plasmid DNA.

The differences in relative efficiencies of different mucosal DNA vaccines are likely due to a combination of factors including: (i) the antigen expressed; (ii) whether the antigen is secreted, cytoplasmic or membrane bound; (iii) the efficiency of expression; (iv) the dose of DNA; (v) the nature of the delivery system (e.g. lipid composition, size of microspheres); (vi) the route and method of delivery; (vii) the number of administrations: (viii) the number and type of CpG motifs present in the vector backbone; (ix) the use of other adjuvants and/or coadministration of cytokine expressing DNA.

8.3. Future direction for mucosal DNA immunization

For mucosal DNA immunization to be a viable vaccination strategy in humans it will be necessary to improve DNA vaccines so that they will be more efficacious with smaller doses

and fewer immunizations. The increased efficacy could result from: (i) optimization of the method or site of immunization that could bring about more efficient transfection of APCs; (ii) coadministration of plasmids expressing cytokines or costimulatory molecules (4, 26, 31); (iii) development of novel non-toxic mucosal adjuvants (32); (iv) development of novel strategies to target either the DNA or the encoded protein to APCs (33); (v) optimization of immunostimulatory and/or neutralizing CpG content (34 - 36); and (vi) combination with other vaccine approaches such as protein or attenuated viral vaccines (37 - 39).

Several phase I human clinical trials using parenterally delivered DNA vaccines are underway or have been completed (40 - 43). The safety and regulatory issues that have been considered include: (i) possibility of tolerance to foreign antigen; (ii) possibility of an integrative event; (iii) possibility of immune response to DNA and auto-immunity; and (iv) immunostimulatory effects of CpG motifs (see ref. 44). Clinical trials using mucosal delivery of DNA vaccines have not started, however, the safety concerns will be essentially the same as for parenteral immunization. In addition, IN DNA immunization may present the possibility of genetic material reaching the intracranial cavity and possibly the brain through the cribriform plate.

8.4. CpG DNA as a mucosal adjuvant

The induction of strong immune responses in animal models following introduction of DNA is due, at least in part, to the adjuvant effect of unmethylated immunostimulatory CpG motifs present in the DNA backbone. Studies in our laboratory and in others had shown that synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs (CpG) were potent adjuvants for induction of Th1-like systemic immune responses against

parenterally-delivered proteins (45 - 49). In **Chapter IV, V and VI** we demonstrated that CpG DNA was an effective non-toxic mucosal adjuvant to HBsAg after IN delivery. In **Chapter IV**, we showed CpG ODN was able to induce antibodies and CTL against HBsAg, which by itself did not induce any detectable responses, even with high doses and boosting (50). Indeed, this study showed CpG ODN to be as effective as CT, and to have a strong synergy with CT when the two adjuvants were coadministered. One previous study had demonstrated that systemic (IgG) and mucosal (S-IgA) humoral responses against whole killed influenza virus could be significantly enhanced by the addition of CpG ODN (51), however, the experiments in **Chapter IV** were the first reported use of CpG DNA as a mucosal adjuvant with a protein antigen (50). Subsequently, another group has reported similar mucosal adjuvant properties of CpG DNA with the model antigen β-galactosidase (52).

In **Chapter V**, we further evaluated the role of CpG DNA as a mucosal adjuvant and showed that using CpG ODN alone, anti-HBs IgG titers in plasma were greater with a higher dose of adjuvant and could be further increased by boosting (53). Better mucosal immune responses were obtained with CpG than with CT, which was somewhat surprising as CT is considered to be a highly effective mucosal adjuvant. In **Chapter VI**, the systemic and mucosal immunogenicity following IN delivery to mice of CpG ODN was determined when used in combination with native toxins (CT, LT), the less toxic B subunit of CT (CTB) and the genetically detoxified LT mutant LTK63. We demonstrated that CpG ODN, CT and LT augmented anti-HBs titers equally, and this was more than with CTB or LTK63. CpG ODN

acted synergistically with CT and LT but not CTB or LTK63, however for all combinations, CpG induced a more Th1-like response (54).

The most commonly used mucosal adjuvants in animal studies are CT and LT but they are too toxic for use in humans. Therefore novel mucosal adjuvants to replace CT and LT are extremely important and it is of considerable interest that we have shown CpG ODN to be non-toxic even at high doses. Furthermore, the findings in **Chapters IV, V and VI** indicate that CpG as a mucosal adjuvant stimulates a Th1-like response even in the presence of a strong Th2-like adjuvant like CT and LT, and that the strength of the Th1 response depends upon the dose of CpG. Strong Th1 responses are associated with cell-mediated immunity, including CTL, and are essential for protection against many diseases, and therefore desirable for most vaccines. Although not essential for protection against HBV, CTL may be important for avoiding or overcoming the chronic carrier state. Most current adjuvants whether designed for parenteral (e.g. alum) or mucosal (e.g. bacterial toxins) delivery induce Th2 responses. It is believed that the growing incidence of chronic fatigue syndrome and allergen hypersensitivity in developed countries may be due to the high number of Th2-inducing immunizations given in childhood together with the modern lifestyle, i.e. reduced exposure to Th1-inducing stimuli (e.g. bacteria) and over exposure to Th2-inducing stimuli (e.g. allergens, pollutants) (55). It has been speculated that the large number of veterans suffering from Gulf War syndrome may be due, at least in part, to the large number of Th2-inducing vaccinations (e.g. plague, anthrax, typhoid, tetanus, cholera) given prior to deployment of troops to the war zone (55 - 57). The shift in immune responses towards Th2 may have been increased in United Kingdom troops by the use of pertussis vaccine as an adjuvant, a potent

inducer of Th2 responses (55, 57, 58). In contrast, French soldiers who were vaccinated according to a different protocol have not reported Gulf War syndrome, although their exposure to chemical warfare agents may also have varied (55). A Th2-like response in the lung may be particularly undesirable because of an association with asthma (59, 60). Thus, our findings that IN delivery of CpG DNA can overcome the Th2 bias of adjuvants like CT and LT are particularly interesting. Indeed, it has recently been shown that CpG ODN can prevent and treat allergen-induced asthmatic responses in mice (61, 62).

8.5. Future directions for CpG DNA as a mucosal adjuvant

To date, the greatest experience using CpG ODN as adjuvant (mucosal or systemic) has been with protein antigens, including live-attenuated measles virus (63), whole killed influenza virus (46, 51), HBsAg (45, 50, 64) as well as model antigens such as ovalbumin (47, 48, 65), hen egg lysozyme (49), β-galactosidase (46, 52) and fowl γ-globulin (66). It is possible that CpG ODN may also be an effective adjuvant with polysaccharide (PS) antigens. This would be highly beneficial since it is particularly difficult to develop PS-based vaccines since immune responses against them are largely T-cell independent. As such, PS vaccines induce no cell-mediated immunity (CMI), antibody responses are short lived and there is no memory response. Furthermore, they are poorly immunogenic in infants, the elderly and the immunocompromised. At the time of writing this thesis, there were no published reports using CpG ODN as a mucosal adjuvant with PSs and only one report in which CpG was administered parenterally with PS (67). This study found that CpG ODN actually reduced the level of antibodies against a high molecular weight PS in mice (67). However, a very high dose of CpG ODN (500 μg) was used, greatly in excess of the optimal dose for mice (~10

μg), which may have influenced the results. In contrast, other groups have more recently found modest to good increases in antibodies against PS antigens, including antibody isotypes recognized as being T-dependent (e.g., IgG2a), with parenteral administration of CpG ODN and pneumococcal PS (C.A. Siegrist, personal communication), meningitis type C PS (J. Westerink, personal communication) and TNP-Ficoll (T. Waldschmidt, personal communication), although these results have yet to be published. It will be of particular interest to determine the role of CpG ODN as a mucosal adjuvant with different types of antigens (protein and/or polysaccharide) from mucosal pathogens, such as HIV, *Helicobacter pylori*, *Neisseria meningitidis*, *Haemophilus influenza*, and *Streptococcus pneumoniae*.

The strong immune responses that we have generated using CpG as mucosal adjuvant with HBsAg are most likely due to the strong synergy between the direct activation of B cells by CpG DNA and signals delivered through the B cell receptor for both B cell proliferation and Ig secretion (68). In addition, antigen-specific humoral responses are likely enhanced by the induction of cytokines that could have indirect effects on B cells via T help pathways. The synergistic effect of mixing CpG DNA with bacterial toxins is most likely due to the different mechanisms of CpG and bacterial toxins. Although both adjuvants activate B cells, they appear to act by different mechanisms as they induce different IgG isotypes. This difference is likely cytokine related, as CT induces primarily IL-4, IL-5, IL-6 and IL-10 (69, 70) and CpG induces primarily Il-6, IL-12, and IFN-γ (34, 71). Both adjuvants indice IL-6, which is required for IgA production (72). In this study, we have not measured cytokine secretion and it would be of interest to do so for IN delivery of HBsAg plus CpG alone or in combination with different mucosal adjuvants. In addition, it would be useful to investigate

the pharmokinetics of CpG after mucosal delivery and to determine any differences between cell types involved in CpG uptake compared to parenteral delivery, and whether this is altered by the coadministration of other mucosal adjuvants.

The role of CpG as a mucosal adjuvant has thus far only been evaluated for IN delivery of vaccines. It is possible that CpG ODN may also be effective by other mucosal routes (e.g., oral, intravaginal and intrarectal) and this will have to be determined. In addition, in the studies presented herein, we have used relatively large volumes (150 μl) for immunization, and while smaller volumes (30 μl) have been used with a model antigen (β-galactosidase), multiple administrations were necessary (52). Thus, it will be of interest to determine whether CpG DNA can be used with a low volume and a single administration, although this may require increased doses of antigen and/or adjuvant.

We have demonstrated a strong synergy of CpG DNA with the native toxins CT and LT, however similar results were not seen with CTB or LTK63. Thus, it is possible that for synergy with CpG ODN, at least some enzymatic activity of the mucosal adjuvant may be required. A number of partially detoxified mutants have been developed (e.g., LTR72 or CTS106) (32, 73), and these may provide a more effective combination with CpG DNA that would be suitable for use in humans.

Phase I clinical trials using CpG DNA as a parenterally delivered adjuvant are planned for this spring and if found to be safe, a mucosal vaccine trial may follow in 2000. Prior to this, on account of the differences between the murine and human respiratory tracts, it would be desirable to carry out studies using CpG DNA as a mucosal adjuvant on other mammals

such as sheep, ferrets or non-human primates, where the pulmonary epithelium is more similar to that of humans.

8.6. Immunization against hepatitis B virus by mucosal administration of antigen-antibody complexes

An alternate immunization strategy that may not necessarily require adjuvants is administration of antigen-antibody immune complexes. Parenteral administration of such complexes has been shown to induce significantly better immune responses than antigen alone in a number of different animal models (74-78). In **Chapter VII,** we evaluated the potential of administering such immunostimulatory complexes by a mucosal route. Results from this study indicated for the first time that immunostimulatory complexes could induce strong humoral immune responses when delivered by a non-invasive route and that these could be modulated by adjuvants (79). Administration of immunostimulatory complexes resulted in a Th2 biased immune response as indicated by a predominance of IgG1 antibodies which was significantly stronger than that induced by uncomplexed antigen, most likely due to increased FcγR-mediated antigen uptake. Surprisingly, both CpG and CT when administered with HBsAg/Ab complexes induced Th1-like antibody responses, but no CTL, suggesting a selective stimulation of humoral responses. This suggested that the Th bias of immune response may depend not only on the adjuvant, but also other factors such as efficiency of antigen uptake or altered presentation. Furthermore, this study shows that antibody isotype are not always predictive of CTL activity.

8.7. Conclusions

In conclusion, this thesis presents studies on the development of novel strategies for immunization at mucosal surfaces. Mucosal immunization using plasmid DNA in saline is shown to result in no or weak immune responses. Formulation of DNA with lipid increases levels of reporter gene expression, but apparently not sufficiently to raise immune responses against expressed antigen indicating that other factors are involved. In contrast, however we were able to induce good immune responses with the antigen-antibody approach, and even more so with CpG DNA as an adjuvant to protein antigens. When CpG ODN is used as a mucosal adjuvant, strong immune responses are generated and a synergy is seen with other mucosal adjuvants. This mucosal immune response is not restricted to the site of application but is also present at distant mucosal surfaces. The lack of toxicity and strong immunomodulatory effect make CpG an ideal candidate for mucosal vaccines for humans. Mucosal delivery, particularly by oral or IN routes, would be simple, fast and non-invasive and would therefore be highly suitable for mass immunization. The possibility of inducing mucosal (both locally and at distant sites) and systemic (humoral and cellular) responses is of particular interest in the prevention of mucosally transmitted viral pathogens such as HIV, HSV and influenza. These studies may lead to the development of novel human vaccines that protect at the level of entry for mucosal pathogens and may thereby prevent many infectious diseases.

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