EDIBLE VACCINES: IMMUNOLOGICAL RESPONSE TO HEPATITIS B VIRUS NUCLEOCAPSID DERIVED FROM TRANSGENIC PLANTS

BY

Darryl Lee Falzarano

A Thesis submitted to
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In Partial Fulfillment of the Requirements for the Degree of

Master of Science

Department of Medical Microbiology and Infectious Diseases
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FACULTY OF GRADUATE STUDIES

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LIST OF ABBREVIATIONS

α	alpha
AP	alkaline phosphatase
APC	allophycocyanin
β	beta
bHBc153	Baculovirus-derived HBc153
Bluo-gal	5-bromo-3-indolylβ-D-galactopyranoside
BSA	bovine serum albumin
°C	degrees Celsius
CD4 /CD8	cluster of differentiation 4 / 8
CO_2	carbon dioxide
conA	concanavilanA
dH_2O	distilled water
DTT	dithiothreitol
DNA	deoxyribonucleic acid
ECL	electrogenerated chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylene diaminetetracetic acid
eHBc153	*E. coli*-derived HBc153
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	gram
GIT	gastrointestinal tract
GM	genetically modified
H_2SO_4	sulfuric acid
HBc153	recombinant protein: N-terminal 153 amino acids of HBcAg
HBcAg	Hepatitis B core antigen
HBV	Hepatitis B virus
HEPES	hydroxyethylpiperazine ethanesulfonic acid
HRP	horseradish peroxidase
IFN-γ	interferon-γ
Ig	immunoglobulin
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-2 / IL-4	interleukin-2 / 4
kDa	kilo Daltons
KLH	neo-antigen-keyhole limpet hemocyanin
LB	Luria-Bertani
LT-B	heat-labile enterotoxin
M	molar
mA	milliamp
MALDI-TOF	matrix assisted laser desorption ionization – time of flight
µg	microgram (10^{-6} g)

min	minute(s)
ml	milliliter (10^{-3} L)
µl	microliter (10^{-6} L)
mM	millimolar
MOI	multiplicity of infection
MS	mass spectrometry
MV-H	measles virus hemagglutinin
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometer
NML	National Microbiology Laboratory
NP-40	Nonidet P-40
OD	optical density
OPD	o-phenylene diamine
PerCP	peridinin chlorophyll protein
PAGE	polyacrylamide gel electrophoresis
PAHO	Pan American Health Organization
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with 0.05% Tween20
PE	phycoerythrin
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonyl fluoride
RBC	red blood cell
rpm	rotations per minute
SDS	sodium dodecyl sulfate
sIgA	secretory IgA
TBS	tris-buffered saline
TBS-T	tris-buffered saline with 0.05% Tween20
TFA	trifluroacetic acid
tHBc153	GM-tobacco-derived HBc153
USD	United States dollar
v/v	volume per volume
VLP	virus-like particle
w/v	weight per volume

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The Hepatitis B virus (HBV) nucleocapsid self-assembles into virus-like particles and can be exploited for the presentation of various foreign B- and T-cell epitopes for HBV and non-HBV vaccine design. A construct containing a codon-optimized truncated HBV nucleocapsid gene (HBc153) was expressed in tobacco, carrots, and rice. Recombinant HBc153 was also expressed in *E. coli* and insect cells via the Bac-to-Bac Baculovirus expression system to serve as a control. The immunogenicity of HBc153 from transgenic plants was assessed in BALB/c mice using different routes of immunization, including feeding and subcutaneous immunization. The *E. coli* recombinant HBc153 was also delivered by oral gavage and intranasally. In order to characterize the immune response the serum total IgG, as well as the prevalence of the various IgG subtypes, was monitored. Fecal samples were also collected to determine the extent of IgA production. At the termination of the experiments the cytokine secretion profile for HBc153-stimulated splenocytes was determined for the following cytokines: IFN-γ, IL-2, IL-4, IL-4 and IL-10. The production of predominantly IFN-γ and IL-2, in subcutaneously and intranasally immunized mice, indicates a T_{h1}-like cellular response. The total serum results indicated that the route of immunization is a critical factor in the extent of the immune response, with subcutaneous being more efficient (response per quantity of antigen) than intranasal immunization and more efficient than the oral gavage and feeding route. The IgG isotype profile (predominantly IgG_3 and IgG_{2b}) elicited by

mucosal immunization, both intranasally and by oral gavage, indicates that IgA is produced and IgA was detected in fecal extracts from mucosally immunized mice. These results demonstrate that HBc153 antigen can illicit an immune response when delivered by a oral, intranasal and subcutaneous immunization; however, the GM-plants created for feeding trials did not contain a sufficient quantity of antigen in order to induce an immune response when fed to animals.

1.0 Introduction

Despite an effective vaccine, hepatitis B virus (HBV) remains a major cause of morbidity and mortality worldwide. More than 2 billion people have been infected with HBV and over 350 million remain chronic carriers. HBV is the prototype of the family Hepadnaviridae. It is the smallest autonomously replicating DNA virus known with a genome composed of approximately 3200 nucleotides in a partially double-stranded relaxed circular orientation (Vanlandschoot et al., 2003). As is true with many infectious diseases, the cost of producing and administering vaccines to achieve the elimination of disease is prohibitively expensive for developing countries. Therefore, to simplify production and delivery of vaccine preparations, genetically modified (GM) food plants are proposed as novel method to induce immunization as edible vaccines.

This method of vaccination is particularly attractive for developing countries as it would be safe, cost-effective, requires low technology input and abolishes the need for a cold chain, needles and syringes. It would also eliminate the possibility of vaccine contamination with animal pathogens as it is generated in plants. Furthermore, antigens delivered via GM-plants are protected by the rigid plant cell wall thus allowing relatively safe passage through the stomach (Rigano, 2001). A number of immunologically relevant proteins have been expressed in plants including Norwalk virus capsid (Mason et al., 1996), rabies glycoprotein (McGarvey et al., 1995), HIV-1 p24 (Zhang et al., 2002), heat-labile enterotoxin (LT-B) from *E. coli* (Haq et al., 1995), human cytomegalovirus immunodominant glycoprotein B complex (Tackaberry et al., 1999) and HBV surface antigen (Mason et al., 1992). Many viruses and bacteria establish infections at mucosal surfaces; therefore, a vaccine strategy that involves immunization at these surfaces is of

great interest. To investigate the possibility of using plants to deliver antigens a truncated form of HBV nucleocapsid or core protein (HBcAg), designated HBc153, was expressed in GM-plants, including carrots, rice and tobacco. Similar to HBcAg, HBc153 is capable of spontaneous self-assembly into virus-like particles (VLP). There are a number of unique immunological features of HBcAg that make it an attractive vaccine component: when injected in saline as little as 6ng can elicit antibody production (Milch et al., 1997a), HBcAg preferentially primes T_{h1} cells (Milch et al., 1997b) and it is an effective carrier of heterologous epitopes (Pumpens & Grenns, 2001). While the use of non-replicating antigens poses a difficulty in oral immunization due to the low pH and proteolytic enzymes of the digestive tract, particulate antigens are resistant to damage from low pH. In this project the immunogenicity of the *E. coli*- and transgenic plant-derived HBc153 protein was assessed using different routes of immunization.

1.1 Vaccines

While major achievements have been made worldwide in the control of infectious diseases they continue to cause a significant burden. In 2000, communicable diseases accounted for 26% of all deaths worldwide (Bloom & Lambert, 2001). Despite this figure, vaccination has been and continues to be one of the most important health interventions in history (Rappuoli et al., 2002) and represents the most cost-effective and successful approach to prevent infectious diseases (Giudice, 2003). Smallpox has been eradicated, paralytic poliomyelitis is virtually non-existent in developed countries and measles is well on its way to being eliminated. Since the advent of routine childhood vaccination in the United States there has been a 95-100% decline in morbidity and mortality of numerous childhood infections, including diphtheria, pertussis, tetanus,

Haemophilus influenzae B, measles, mumps and rubella (Ogra et al., 2001). While this achievement has not been without considerable financial burden, the cost represents a very small fraction of the per capita income. Furthermore, with cost-benefit ratios ranging from 1:2 for pertussis to 1:10 for poliomyelitis and measles, it continues to be more cost effective to vaccinate than to treat disease (Ogra et al., 2001).

Currently there are 25 vaccines available for human use, almost all of which are delivered parenterally. Most of these vaccines contain either attenuated or inactivated forms of the original pathogen (Jegerlehner et al., 2002). Live, attenuated vaccines work well and generally induce a good immune response as they mimic a real infection. In the case of the oral polio vaccine (OPV) and the oral *Salmonella* Typhi vaccine they also mimic the natural route of infection and presumably also induce a mucosal immune response. While attenuated and inactivated vaccines are considered safe, there exists the possibility that they could cause disease. Although unlikely it is possible that the vaccine organism may not be inactivated due to a technical issue during manufacture. There also is an ever present risk of attenuated vaccines reverting to a virulent phenotype *in vivo* and regaining the ability to cause the subsequent disease. Live vaccines can also cause illness in people who are immunosuppressed due to a genetic disorder, various medications and infection with HIV. In some circumstances people may be unaware of the incompetence of their immune system and become severely ill after receiving an attenuated vaccine.

In what appears to be a contradiction, successful vaccination programs can lead to decreased coverage in a population. When a population perceives that there is a decreased risk of contracting a vaccine preventable disease, due to a decrease in disease incidence, the acceptance of vaccine-induced side effects decreases. As an example, the small risk

of developing acute infection following immunization with OPV has led to a switch to the less efficacious inactivated polio virus vaccines (IPV) in the United States. In an effort to ensure safety, modern vaccine research has moved to investigate recombinant subunit vaccines. In addition to the safety benefits of subunit vaccines they have another potential benefit. Chronic infectious diseases such as HIV, Hepatitis C virus, tuberculosis and parasitic infections seem refractory to attenuation. This may be due in part to how these organisms evolved under selective pressure so that they have variable antigenic epitopes or epitopes that do not induce a strong immune response. The advent of peptide vaccines had promised to overcome this limitation. However, soluble proteins and peptides by themselves tend to be weak immunogens in the absence of inflammatory stimuli and have not been particularly useful as vaccines. The most obvious solution to this problem was to administer soluble protein and/or peptide concomitantly with an adjuvant. Unfortunately, the most effective adjuvants, such as cholera toxin (CT) and lymphotoxin (LT), are too toxic for human use. In animal models both CT and LT are routinely used with little negative effect; however, when delivered orally in humans even a small amount results in moderate to severe diarrhea. To circumvent the poor immunogenicity of soluble proteins a new delivery mechanism using virus-like particles has been proposed. These particles form repeating motifs that tend to be highly immunogenic. Some of these VLPs, such as HBcAg, can tolerate the insertion of antigenic epitopes from other proteins. This can lead to the creation of chimeric particles that contain repeating structures which may enhance the immunogenicity of the inserted epitope (Berzofsky et al., 2001).

The success of immunization is dependent upon reducing the susceptible population to levels below which the disease can remain endemic. Despite the capacity, herd immunity remains unachievable for numerous diseases in a large proportion of the world's population due to economic and logistical difficulties (Webster et al., 2002). Maintaining a cold-chain, the need for syringes and trained staff to administer vaccines leads to increased cost. In the United States vaccine rates are dropping due to cost (Rappuoli et al., 2002). In addition the majority, if not all of the currently available vaccines are manufactured using methods that are not easily scalable. This can be a problem even in the developed world where there tends to be sporadic and/or continuing vaccine shortages. The most recent examples include the lack of availability of the smallpox vaccine in the United States (Rappuoli et al., 2002) and the recent shortage of influenza vaccine across North America.

There is also a paucity of effort in developing vaccines that would exclusively serve the developing world. In order to reduce morbidity and mortality worldwide there needs to be more research into infectious diseases that affect underdeveloped countries. Almost all of the vaccines that are available today have been developed to prevent diseases that plague the developed world. There are multiple factors responsible for this situation not the least of which is profit. Even inexpensive vaccines prove difficult for developing countries to afford and with small profit margins it is unattractive for companies to investigate vaccines that are exclusive to diseases of the developing countries. Currently there are no mechanisms for developing vaccines that are needed only by developing countries and in developed countries these are not a priority. Localized vaccine production using inexpensive technology would allow more countries

access to a wider range of vaccines. The production of vaccines in plants may be new method to solve this problem (Masignani et al., 2003).

The only vaccines delivered via a mucosal route are oral vaccines for polio (OPV), typhoid, cholera, adenovirus and rotavirus as well as a nasal influenza vaccine. All of these vaccines make use of live attenuated pathogens and while they are generally considered safe they present a higher risk than non-replicating and subunit vaccines. Furthermore, the typhoid and cholera vaccines are only available for restricted use and the adenovirus vaccine is only approved for military use. Recently, the rotavirus vaccine has been temporarily withdrawn due to rare but serious complications (intestinal intussusception) and an inactivated virosomal-subunit intranasal influenza vaccine containing *E. coli* heat-labile toxin as an adjuvant was recently removed after a strong association between vaccine use and Bell's palsy was noted in Switzerland (Mutsch et al., 2004). Clearly there is a need for safe vaccines that can be easily delivered. Today's challenge is to develop vaccines that induce immunity at the mucosal level and to be able to produce the vaccine at the lowest cost.

1.2 Recombinant Protein Production in Plants

While conventional vaccines are considered safe there are a number of potential risks that they can pose. There is a small possibility that attenuated strains of bacteria and viruses could mutate and revert to a pathogenic state. Attenuated pathogens may also pose a slight risk for people who may be unaware that they are immunocompromised at the time of vaccination. A number of subunit vaccines and live virus vaccines are produced in mammalian cell lines allowing for the possibility of contamination with other agents. While there are mechanisms in place to prevent this it has occurred in the past,

when a number of polio vaccine lots were contaminated with simian virus 40 (SV40) in the 1950's. Furthermore, the presence of fetal bovine serum (FBS) in mammalian cell culture systems carries an unknown risk of transmitting prion diseases in vaccines produced in this culture system. Vaccines produced in plants can overcome these safety issues and therefore will provide an additional level of assurance. Only a single protein from the pathogen would be produced in the plant and as the pathogens' genome would not be present in the plant the product of this system cannot and would not be infectious. Furthermore, as they are produced in plants, the possibility of animal viruses contaminating the vaccine has been eliminated. Some subunit vaccines, such as the recombinant Hepatitis B vaccine produced in yeast, are very effective and have excellent safety profiles. However, in many countries the purchasing and delivery costs of this vaccine are prohibitively expensive. Oral vaccination coupled to plant-based production may provide a cost-effective route to overcoming this barrier. Plants have been shown to successfully produce properly processed antigens such as Hepatitis B surface antigen (Smith et al., 2003).

1.2.1 Plants as Producers of Recombinant Proteins for Vaccination

The development of genetic transformation technology has allowed the expression of foreign genes in an increasing number of plant species. Initial work was performed in plants that were neither suitable (i.e. tobacco) or nor palatable (i.e. potatoes) when eaten raw. The use of plants to produce immunogens is a relatively new idea first proposed in the early 1990's by Arntzen (1992) and Kowprowski (1998). Subsequently many different immunogens have been produced in plant-based systems. Edible vaccines are currently being pursued for measles, cholera, foot and mouth disease and hepatitis B

and C (Webster et al., 2002). Despite some differences in post-translational modifications, most immunogens maintain their neutralizing epitopes, when produced in plants. For example, the hemagglutinin protein of measles virus (MV-H) is able to stimulate a neutralizing antibody response (Marquet-Boulin et al., 2003).

Previous studies have demonstrated that the oral delivery of plants expressing vaccine antigens were capable of producing a detectable immune response. Plants were used to produce the heat-labile nontoxic subunit of *E. coli* enterotoxin (Lauterslager et al., 2001). Humans fed these GM-potatoes produced neutralizing responses against *E. coli* heat-labile toxin. GM-potatoes producing the capsid protein of Norwalk virus were also successful in eliciting a humoral immune response (Tacket et al., 2003; Tacket et al., 2000). GM-tobacco expressing MV-H was delivered to mice by oral gavage using cholera toxin B as an adjuvant and induced low levels of specific antibodies. While the acidic pH of the stomach and digestive degradation may interfere with the generation of antibodies it is possible that there will be little effect on T cell immunity (Muller et al., 2003). Plants could also provide an inexpensive antigen source that could be further purified for parenteral inoculation. HBsAg was produced in tobacco plants and demonstrated to be immunogenic when administered parenterally (Mason et al., 1992).

1.2.2 Generation of Transgenic Plants

There are multiple methods for the generation of transgenic plants; however, the most common method utilizes the bacterium *Agrobacterim tumefaciens* to transfer the gene of interest into the plant genome in a process known as transformation. *A. tumefaciens* is the causative agent of crown gall disease and can infect a wide range of dicotyledonous (broad-leaved) plants. Infection results in the bacteria transferring part of

its DNA to the plant, which then integrates into the plant genome. This transfer does not involve the bacterial genome, instead in occurs via a large plasmid, termed the T_i (tumor-inducing) plasmid. It is possible to clone foreign genes into the T_i plasmid, which are then subsequently introduced into the plant during the bacterial infection. In this case, the gene of interest that was cloned into a T_i plasmid was a truncated form of the HBV nucleocapsid that codes for the 153 N-terminal amino acids. The T_i plasmid carrying the truncated nucleocapsid gene was then delivered via *A. tumefaciens* to the different plants. Cells that contain the gene are selected for with a selectable marker and single positive transformants are stimulated to differentiate and regenerated into a transgenic plant. This method can lead to the generation of stable transgenic plants (Alli et al., 2002). The final product of this process is a GM-plant (carrots, rice and tobacco) that contains HBc153 protein. While tobacco is the model plant protein expression system its toxic compounds make it unsuitable for vaccine delivery. Potatoes are the most frequently used GM-plant thus far; however, lettuce, corn, spinach, tomatoes, bananas, rice and a number of cereal crops are currently being pursued as potential production plants. The plant is then fed to an animal and the animal produces an immune response to the recombinant protein.

1.2.3 Advantages Conferred by Plant Delivery

The production and delivery of vaccines via plants has numerous advantages over traditional methods of vaccine manufacturing. In addition to the generation of new vaccines oral delivery may be used to replace existing parenterally delivered vaccines. Traditional vaccines are generally not heat-stable and thus require constant refrigeration to maintain their effectiveness. This poses considerable logistical problems in less developed areas of the world especially those with warm climates. Proteins produced in

plants are heat-stable for an extended period of time, whether they are stored in the unprocessed plant form or in a dehydrated state (Biemelt et al., 2003; Rigano et al., 2003; Webster et al., 2002). Delivery of vaccines in plants would abolish the need to maintain a cold-chain, one of the key factors that make vaccination in developing countries more difficult and costly. If the plant material was administered as an oral vaccine the hazards, such as bloodborne infections acquired by improperly sterilized needles and/or syringes, and the expense of injections could be overcome (Biemelt et al., 2003). Oral immunization also has several additional benefits over parenteral delivery including increase in compliance, greater acceptability by the recipient (i.e. no sore arms, fear of needles) and enhanced immune responses at mucosal sites, including the production of IgA, while still stimulating humoral immunity (Guerrero et al., 2001; Kong et al., 2000).

As many viruses and bacteria establish infections at mucosal surfaces oral vaccination is a desirable route of immunization. One of the problems facing oral delivery of recombinant proteins is that they are degraded in the gastrointestinal tract (GIT) prior to being recognized by the immune system. Immunogens contained within plant cells are protected by the rigid cell wall of the plant from the acidic pH of the stomach as the majority of plant cell breakdown occurs in the intestine as a result of digestive and bacterial enzymes (Kong et al., 2000; Rigano et al., 2003). Even with the additional protection provided by the cell wall of the plant non-particulate, soluble proteins may require adjuvants for effective oral vaccination as foreign proteins are generally tolerated when delivered orally. This major obstacle of oral immunization may be circumvented by the creation of particulate vaccine delivery systems (Estes et al.,

2001). The repeating epitopes present on particles are recognized in the same manner as a whole virus and thus tend to induce stronger immune responses than soluble antigens.

Producing vaccines in plants would also provide an additional level of safety over vaccines produced in mammalian culture systems. Plant derived vaccines would not be subject to the possibility of contamination with animal viruses as these viruses do not grow in plants (Biemelt et al., 2003; Rigano et al., 2003; Webster et al., 2002). If a plant derived immunogen was administered orally this would effectively eliminate all risk of disease transmission via vaccines as the vaccine itself is non-infectious, there is no possibility of contaminating viruses being present and the use of needles, which when reused can transmit blood-borne diseases, is avoided. This would be an important factor in areas where acceptance of vaccines is low. It is possible that plant viruses could be present in such a vaccine; however, viruses are very specific as to what classification of organisms they can infect and their presence would not be a safety concern. Plants could also be engineered to contain multiple antigens (Rigano et al., 2003) reducing the number of vaccinations required while reducing the production costs. While the effect of glycosylation and other post-translational modifications of the immunogen would need to be determined on a protein by protein basis the post-translational modifications in plants are similar to those observed in mammalian tissues (de Peyer et al., 1999).

Compared to the fermentation facilities used to manufacture traditional vaccines, plant-derived vaccines are expected to provide considerably lower production costs. To date no subunit vaccine has ever been licensed for oral delivery in part due to a lack of cost-effective production. For example, production of the current Hepatitis B vaccine, a subunit vaccine containing Hepatitis B surface antigen that is expressed in yeast, purified

and chemically refolded to yield virus-like particles that are delivered parenterally, is technology intensive and comparatively expensive for developing countries. While it has an excellent safety and efficacy record the HBV vaccine was initially limited to use in industrialized countries due to its cost. Since its release its price has decreased considerably; however at the current Pan American Health Organization (PAHO) negotiated price of $0.90 USD per immunization it still costs more than the daily wage of more than 1 billion people. While the cost of plant vaccines remains uncertain it is expected that it would be substantially lower than vaccines produced via fermentation. The use of plants as the production source could be advantageous to developing countries as plants can be produced at low cost, while the higher technological costs to develop the vaccine-producing plant would occur at the outset of the project and could be covered by developed nations (Guerrero et al., 2001; Webster et al., 2002). Locally grown plants also allow sustainability of supply and independence from foreign supply. Developing countries should be able to support this sort of vaccine production which will enhance their long-term public health and provide for local business development. In addition, plants can easily be produced in large quantities. If some processing, such as dehydration and/or encapsulation was required, these processes could be performed using existing technology from the food processing industry (de Peyer et al., 1999). Furthermore, plants are essentially infinitely scalable thus allowing annual fluctuations in demand to be met within a growing season (Biemelt et al., 2003).

1.3 VLPs as Carrier Molecules for Vaccines

A strong antibody response is frequently sufficient to provide protection from a number of infectious agents. The B cell response is typically responsible for protection in this manner. VLPs are known to induce potent B cell responses even in the absence of adjuvants (Jegerlehner et al., 2002). The repetitive nature of the surface of whole viruses and VLPs allow them to cross-link B cell receptors thus creating a strong activation signal. The advantage of using VLPs for immunization is that while they are highly immunogenic, similar to a whole virus, they are a single recombinant protein and are not capable of replication. Also, these proteins are capable of spontaneous self-assembly into VLPs and can often tolerate inserts into the immunodominant region – generally the spikes on the surface of the particle. This creates the potential for epitopes from weakly immunogenic, soluble proteins to be presented on the surface of VLPs. This may result in weak immunogens gaining the ability to induce a strong B cell response due to the repetitive structure of the VLP. Furthermore, VLPs are relatively stable at low pH thus increasing the possibility of successful passage through the stomach (Li et al., 2001).

1.3.1 Hepatitis B Virus Nucleocapsid Protein

Wild-type human hepatitis B virus nucleocapsid (or core) antigen (HBcAg) is a 183-185 amino acid protein that has a mass of 21 kDa. During HBV infection it is produced in the cytoplasm of infected cells. HBcAg is primarily composed of hydrophilic and charged amino acids. It is not glycosylated and is not modified by the addition of lipids. The core protein readily forms compact dimers which are stabilized by a homologous disulfide bridge at Cys61 (Kazaks et al., 2003; Vanlandschoot et al., 2003). The dimers associate and self-assemble into icosahedral structures, also termed virus-like

particles (VLPs), that are heterogeneous in size. The larger particles are 28-30nm in diameter and consist of 240 copies of HBcAg arranged in a T=4 symmetry while the smaller particles are approximately 25nm in diameter and possess 180 copies of HBcAg in a T=3 symmetry. The predominant fraction (>95%) of the VLPs exist in a T=4 configuration when produced in *E. coli* and baculovirus expression systems (Newman et al., 2003). However, it is also possible to find both smaller and larger particles in both recombinant expression systems and from natural human infections. The core protein has been observed to spontaneously assemble into VLPs when produced in bacterial, yeast, mammalian, vaccinia virus and baculovirus expression systems (Brown et al., 1991). The structural characteristics of the core protein are quite different from other small viral capsids in that it is largely α-helical (65%) with low β-sheet jelly-roll content (Bottcher et al., 1997; Vanlandschoot et al., 2003). Dimer clustering of subunits is responsible for the spikes on the surface of the capsid which consist of radial bundles of 4 long α-helices.

Only the first 140 N-terminal amino acids are required for the protein to self-assemble into VLPs (Neuman et al., 2003). The arginine-rich carboxy-terminus binds RNA and DNA (Hatton et al., 1992) but is dispensable for assembly, thus it was not included in our construct. We chose to create a construct that contained the first 153 N-terminal amino acids producing a protein, designated HBc153, with a predicted mass of 17.4 kDa. Compared to proteins that exist in non-particulate forms VLPs tend to be very stable. HBcAg is no exception, the particles remain assembled after 15 minutes exposure to 37, 50 and 75°C, however they do dissociate at 100°C. Experiments have demonstrated that the C-terminal 34 amino acids are not involved in temperature stability (Newman et al., 2003) thus HBc153 should behave similarly. In addition to being heat resistant the

particles are stable at pH 4, 7 and 10 for 30 minutes at 37°C but will dissociate at pH 2 and 14. They are also stable in ionic detergents such as SDS to 0.1% but dissociate at 0.5% (Newman et al., 2003). It is reported that the anterior murine stomach has a pH of 4.5 while the posterior has a pH of 3.1 (Kararli, T.T., 1995) thus it is likely that particles exposed to the pH of the stomach while passing through will remain assembled. It is vital that non-replicating antigens delivered orally be able to tolerate conditions in the gut in order to induce an immune response. HBc153 seems to be well suited for this task.

The HBcAg is extremely immunogenic in all of the vertebrate hosts tested (Milich, 1997a). Only 6 ng of HBcAg in saline is sufficient to induce a detectable antibody response when injected into mice. The repetitive, highly ordered protein spikes on the core shell surface, as seen in Figure 1, are thought to be responsible for the high immunogenicity of this antigen (Vanlandschoot et al., 2003). The spikes also seem to be arranged for optimal cross-linking of surface Ig-receptor of B cells, an important B cell activation signal (Lazdina et al., 2003). This is thought to be the main factor contributing to HBcAg's unique ability to bind a high frequency of naïve human and murine B cells. After binding, the B cells process the antigen and present it to T helper cells. The major B cell epitope, which is conformationally dependent, is located at amino acids 74-83 (Ferrari et al., 1991; Kazaks et al., 2003) and this maps to the tip of the spikes (Bottcher et al., 1997). Despite the high affinity for B cells, amelioration of B cell binding ability has only a marginal effect on the overall B cell immunogenicity which suggests T helper cell involvement. (Lazdina et al., 2003). HBcAg functions as both a T cell-dependent and T cell-independent antigen in mice and humans (Cao et al., 2001; Ferrari et al., 1991;

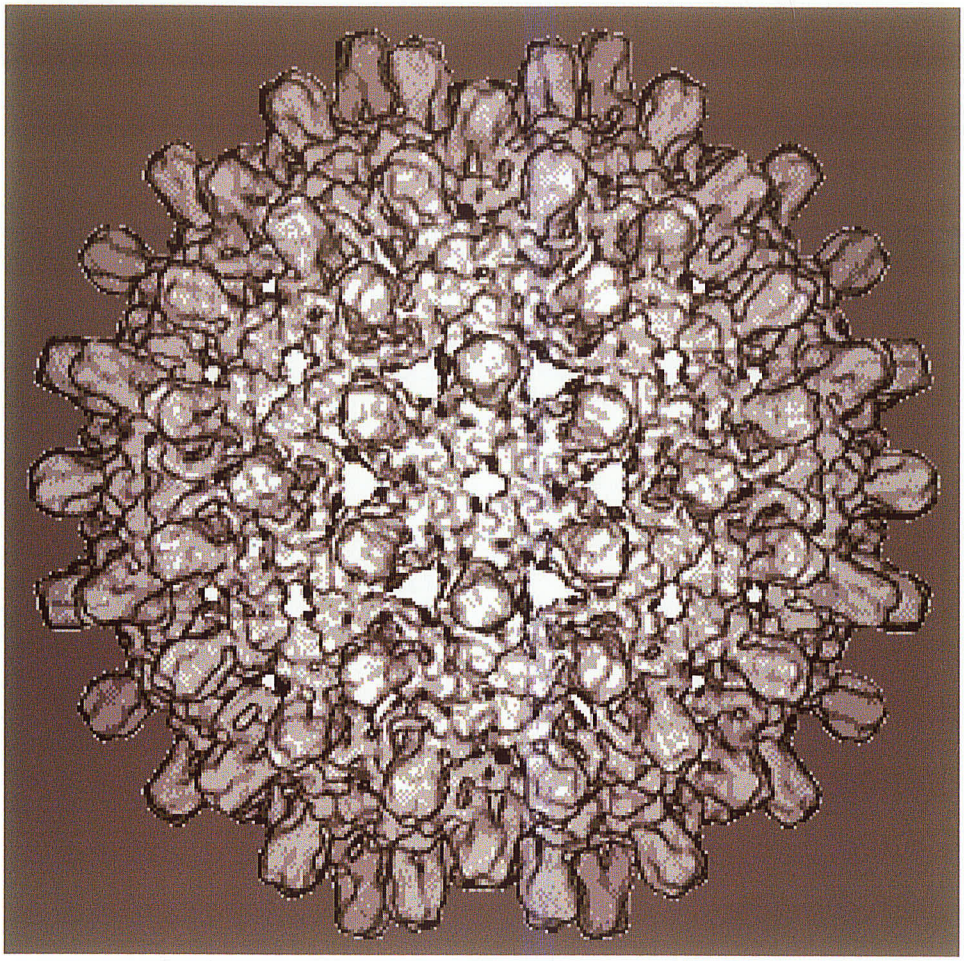

Figure 1. A three-dimensional map of the hepatitis B virus nucleocapsid protein shell demonstrating the spike portion of the protein. The core protein is seen arranged in a T=4 configuration as viewed down a 2-fold axis (from Bottcher *et al.*, 1997, with permission).

Milich & McLachlan, 1986). In athymic mice IgM and IgG antibody responses are still induced. This property requires the protein to be assembled into a capsid as denatured HBcAg is far less immunogenic while retaining its T cell activity. HBcAg also has numerous CD4+ T cell epitopes (Ferrari et al., 1991, Vanlandschoot et al., 2003).

While HBcAg is not a vaccine candidate as the humoral immune response to it does not seem to be involved in either protection from or clearance of the virus, it is of interest as a carrier molecule for epitopes of other pathogens (Lazdina et al., 2001; LoMan et al., 1995). It is possible to produce chimaeric virus particles (CVPs) that express foreign peptides on their surfaces and could have considerable vaccine potential. CVPs from VP2 of canine parvovirus were used to protect mink from experimental infection with mink enteritis virus (LoMan et al., 1995). CVPs may also allow the difficulties with peptide vaccines to be overcome. While peptide vaccines present a safe method of inducing an immune response by themselves they have proved to have low immunogenicity. Fusion or coupling peptides to polymeric particular structures have been the most successful method to presenting peptides to the immune system. Carrier molecules have been developed utilizing the nucleocapsid and surface antigen of Hepatitis B virus, yeast Ty protein and rotavirus VP6 (Brown et al., 1991). For HBcAg the c/e1 loop region was determined to be the most suitable insertion site for foreign epitopes (Ulrich et al., 1998; Pumpens et al. 2001) as it has tolerated insertions of up to 240 amino acids and still assembled correctly (Koletzki et al, 1999; Kratz et al., 1999). A number of different epitopes have been successfully inserted into the spike region of HBcAg and it has been demonstrated that they are antigenic; however the use of HBcAg in a mucosal vaccine has not been thoroughly investigated.

Despite the inability of HBcAg to induce a neutralizing antibody response to HBV infection, this antigen was considered useful for vaccine research. Investigating the properties of the native HBcAg would potentially provide insight on the future success of using the particles to carry foreign epitopes. If oral delivery of the highly immunogenic HBcAg did not yield a productive immune response then it would seem unlikely that creating chimeric particles for oral delivery would be successful. In previous studies the N-terminal 114 and 213 amino acids from the nucleocapsid protein (N) of Puumala Hantavirus (PUUV) were inserted with a stop-codon bearing linker into a C-terminal truncated HBc sequence and the presence of assembled particles was observed. These chimeric particles were capable of inducing both anti-HBc and anti PUUV-N IgG_1 antibody responses in BALB/c mice (Kazaks et al. 2002), indicative of a T_{h2}-like response. However, other particulate antigens have been observed to induce T_{h1}-like responses. As HBcAg can act as a T cell independent antigen foreign B cell epitopes inserted into the immunodominant loop may induce a T cell-independent B cell response.

1.4 Vaccination at Mucosal Surfaces

There are multiple reasons for pursuing the development of vaccines that are administered via a mucosal route. Currently, almost all vaccines are delivered parenterally via intramuscular, subcutaneous or intradermal injection. This induces systemic but little or no local mucosal immunity creating a situation where effective immune responses are not occurring at the site of infection. The majority of pathogens initiate infection at or depart from a mucosal surface (McCluskie et al., 2000; Yuki & Kiyono, 2003). This is not surprising as the average adult human has a mucosal surface area of approximately 400 m^2 (Corthesy, 2003) giving pathogens easy access to a large

area which is continually exposed to the environment. The list of pathogens that infect humans by entering via a mucosal surface is extensive. In the gastrointestinal tract (oral route) these pathogens would include *Helicobacter pylori*, *Vibrio cholerae*, enterotoxigenic *E. coli*, *Shigella* spp., *Clostridium difficile*, rotavirus, Hepatitis A virus, poliovirus, and the calici viruses. Pathogens that infect via the respiratory tract include M*ycoplasma pneumoniae*, measles, mumps, rubella, influenza virus, respiratory syncytial virus and SARS-CoV. The urogenital tract can be the entry site for *Chlamydia*, *Neisseria gonorrhoeae*, *E. coli*, herpes simplex virus, human papillomavirus, HIV and Hepatitis B (Guerrero et al., 2001; McCluskie et al., 2000). Effective vaccines for these agents would induce immunity that would prevent the infectious agent from attaching and colonizing at the mucosal epithelium (non-invasive bacteria) or from penetrating and replicating in the mucosa (virus and invasive bacteria) and/or block the binding or actions of microbial toxins. The main protective effector function for the majority of these cases would be secretory IgA (sIgA) and the associated mucosal immunological memory. In some cases a mucosal CD8+ cytotoxic T lymphocytes, CD4+ helper T cells as well as natural killer cells may also be involved.

Vaccines that are applied locally to the mucosa are more likely to be effective at inducing both systemic and local immunity of both T cells and antibody responses. In the case of inhalational anthrax, only mucosal immunization was effective at stimulating both mucosal and systemic immunity (Boyaka et al., 2003). In order to stop the infection at the site of entry a mucosal immune response is essential. Also, mucosal immunity does not appear to wane as a person gets older as systemic immunity does. The mucosal immune system is an interconnected network of lymphocytes and accessory cells that are located

throughout the mucosa of the oral, nasal, bronchial, gastrointestinal and genitourinary tracts. It provides both specific and non-specific defense against pathogens invading the body through this route. For pathogens that infect through a mucosal route, the mucosal immune system is the body's first line of defense. In the gut it is also involved in the mechanism of tolerance induction to food and other ingested antigens. Inductive sites include the lymphoid follicles in the nasal and rectal mucosa in addition to the Peyer's patches in the small intestine. The major effector molecule secreted by the lymphocytes of the mucosa is sIgA. sIgA directly blocks pathogen entry by enhancing antibody-dependant cellular cytotoxicity, by neutralizing bacterial toxins and by inhibiting viral uptake and initial replication. The major cellular effectors are CD4+ T helper cells, (both T_{h1} and T_{h2}) that produce cytokines that enhance antibody and cellular immune responses; and CD8+ CTLs which kill cells infected with pathogens; and intraepithelial leukocytes which also mediate cytotoxicity. The intestinal mucosa also contains CD4+ suppressor cells that are involved in regulation of immune responses and tolerance.

The value of mucosal IgA has been demonstrated in passive transfer experiments where it has provided a high level of protection against influenza virus, rotavirus, RSV, poliovirus, *Vibrio cholera*, *Salmonella enterica* serovar Typhi and *Helicobacter felis* when the pathogen is delivered by a mucosal route (Corthesy, 2003). While natural infection of the mucosal surface frequently results in mucosal and serum antibody production, in addition to cell-mediated immunity, not all pathogens can be sufficiently attenuated for vaccine delivery. Unfortunately, it is difficult to achieve consistent immunization with non-replicating antigens because of the rapid elimination of the antigen in the feces and due to inactivation by mucosal enzymes and bacterial flora. Live

oral vaccines have their own problems and a new method of vaccination dependant on non-replicating antigens must be sought. Mucosal vaccines may be more effective in preventing systemic illness as well as mucosal infections during subsequent challenge with wild-type pathogens.

1.5 The Mucosal Immune System

Most mucosal surfaces contain organized follicles and scattered antigen-reactive lymphoid elements including B cells, T lymphocytes and their subsets and plasma cells. All of these cells are involved in the induction and maintenance of the immune response thus constituting a multi-factor defense against foreign pathogens. Many anatomically distant immunological compartments are thought to form an intercommunicating network as induction of immunity at one site can confer protective immune responses at distant sites (McCluskie et al., 2000). This intercommunicating network is known as the common mucosal immune system (CMIS). Within the CMIS there are areas of organization that are the inductive sites. In the gastrointestinal tract the gut-associated lymphoid tissues (GALT) includes the Peyer's patches, appendix, mesenteric lymph nodes and small solitary lymphoid nodules. In the respiratory tract the bronchus-associated lymphoid tissue (BALT) and the nasal-associated lymphoid tissue (NALT) or Waldeyer's ring (palatine, lingual and nasopharyngeal tonsils) make up the effector sites. In addition there are other effector sites involved in the mucosal response including the ocular tissue, upper airway, salivary glands, tonsils, middle ear cavity, genital tract, and the products of lactation (Ogra et al., 2001). However, the organized lymphoid follicles of the GALT and BALT are considered the principle inductive sites of the mucosal immune response, hence the effort to develop vaccines that work at these sites. After

initial exposure to antigen at one of the inductive sites, lymphocytes leave the area and home to mucosal effector sites via recognition of addressins on the epithelium (Corthesy, 2003). The ability of the lymphocytes to migrate to the mucosal (lamina propria in the gastrointestinal, respiratory and reproductive tracts) and the exocrine tissues (mammary, salivary and lacrimal glands) makes the common mucosal immune system possible.

The inductive surfaces of the GALT and BALT consist of an epithelial surface which contains M cells that overlie the organized lymphoid follicles. In addition to M cells, the mucosal epithelium contains mucin-producing glandular cells, lymphocytes, plasma cells, dendritic cells and macrophages. Dendritic cells are present in both the organized lymphoid tissue and in the mucosal epithelium. They are strongly associated with the potentiation of immune responses and promote the development of active immunity. They are potent antigen-presenting cells and are critical for initiating immune responses, graft rejection, autoimmune disease and the generation of T-cell dependant B cell responses (Ogra et al., 2001). M cells are critical in the transport of luminal antigens as well as the entry of pathogens such as poliovirus, reovirus and *Salmonellae*. Rats, mice and hamsters have organized lymphoid tissue at the entrance to the nasopharyngeal duct. It is morphologically and functionally similar to the GALT and the BALT; however, the NALT appears to have better developed lymphoid follicles with marked lymphocyte infiltration. These follicular areas are organized into B cell and T cell areas of approximately the same size. There is also a large dendritic cell presence. The lymphoid follicles are covered with ciliated epithelium made up mostly of M cells. NALT M cells are similar to those of the Peyer's patches and BALT and participate in the same immunological functions, such as antigen uptake and the mucosal response to specific

antigens. While rodents have a well-developed NALT, humans have multiple structures that make up what is known as Waldeyer's ring as the important induction site for nasal vaccines (Yuki & Kiyono, 2003). It is unknown if the tissues of Waldeyer's ring function in the same manner as the NALT of rodents. Unfortunately, the only animal models that have comparable nasopharyngeal lymphoid tissue to humans are higher primates and pigs, thus making the study of this immunological compartment difficult (Mestecky, 2004).

The CMIS is unique in that it can provide both positive and negative signals for the induction of the immune response in both the mucosal and systemic immune systems following mucosal antigen exposure (Corthesy, 2003). It seems that the response to mucosally delivered antigens can take a number of different paths including the generation of: 1) a mucosal immune response with sIgA antibody production, 2) a systemic immune response (or prime) with production of serum IgG, IgM and specific cellular immunity and 3) development of mucosal tolerance with systemic hyporesponsiveness with or without any change in the antigen specific-IgA mucosal response (Ogra et al., 2001). Factors, such as IFN-γ, IL-12 and cholera toxin (CT), that favor T_{h1}-like responses generally inhibit tolerance while T_{h2}-like response factors such as IL-4 and IL-10 and the T_{h3} factor TGF-β enhance the development and persistence of mucosal tolerance (Ogra et al., 2001).

Even though the different mucosal surfaces are networked through the CMIS there appears to be significant compartmentalization. Immunization at the GALT and BALT is associated with the development of IgA antibody response and the subsequent distribution of plasma cells to the lamina propria of the respiratory, intestinal and genital

mucosa and in the nasopharynx and mammary glands. After initial immunization at the BALT or GALT re-exposure to the antigen at a distant site leads to an enhanced immune response. It appears that nasal immunization is more effective than oral immunization in inducing an immune response in the genital tract. It also appears that both nasal and oral immunizations are effective at inducing an antibody response in the milk and mammary glands. Studies have shown that oral priming can benefit parenteral immunizations and that parenteral priming can be followed by an oral boost. The ability to induce a balanced systemic and secretory response following immunization is determined by numerous interacting factors including: the nature of the antigen, route of administration, nature of the mucosal microenvironment, immunological vehicles employed in delivery and the effectors of by-stander immunologic and antigen-related events occurring concomitantly in the mucosal environment (Ogra et al., 2001).

1.5.1 Mucosal IgA Production and Function

Adult humans produce more IgA per day (approximately 40mg of IgA per kg of body weight) than all the other antibody isotypes combined, a majority of which is secreted into the gastrointestinal tract (Ogra et al., 2001). It is not surprising that this makes IgA the predominant antibody at mucosal surfaces and the primary antibody involved in mucosal defense (Corthesy, 2003). IgA is produced locally by IgA-committed plasma cells and can exist in a number of different forms primarily dependent upon its location in the body. Both monomeric and dimeric IgA exist in the serum. Monomeric IgA is analogous to IgG and IgE but dimeric IgA has additional carboxy-terminal tailpieces that interact with the J-chain to form covalently-linked dimeric IgA as well as larger polymeric IgA (pIgA) (Herr et al., 2003; Ogra et al., 2001). The mucosa

has high concentrations of dimeric IgA while the IgA in the serum tends to be monomeric. Dimeric and polymeric IgA is bound by the polymeric immunoglobulin receptor (pIgR) on the basolateral surface of the mucosal epithelium, transcytosed through the epithelia layer and released on the apical surface into mucosal secretions as a complex of dimeric IgA and the cleaved pIgR ectodomain or secretory component (Corthesy, 2003, Herr et al., 2003). The complex of dimeric IgA with bound secretory component is known as sIgA. The resulting sIgA then participates in host defense at the mucosal surface. IgG also contributes to this defense, reaching mucosal sites via passive diffusion from the bloodstream and occasionally by local synthesis.

The multi-valency of sIgA allows it to participate in immune exclusion by complexing with the pathogen and limiting its luminal uptake. It has been shown to neutralize viruses and can interfere with viral replication, assembly and/or release (Corthesy, 2003; Ruggeri et al., 1998). IgA-mediated effector responses are composed of phagocytosis, antibody-dependant cell-mediated cytotoxicity, respiratory burst and cytokine release which are all mediated through the $F_c\alpha RI$ (CD89), an IgA-specific receptor that is expressed on monocytes, eosinophils, neutrophils and macrophages. sIgA's primary function seems to be immune exclusion – the steric hindrance of adherence to mucosal epithelia (Herr et al., 2003).

The development of a sIgA response is linked to M cell-mediated antigen uptake. Following antigen exposure and uptake by M cells there is a variable level of T cell, dendritic cell and B cell activation. Typically T cell activation and cytokine release leads to B cell activation and isotype switching. Both T_{h1}- and T_{h2}-like responses appear to enhance the production of sIgA. While T_{h2}-like cytokines (IL-4, IL-5, IL-6, IL-9, IL-10

and IL-13) are thought to be involved in the upregulation of antibody production, T_{h1}-like cytokine production (IL-2 and IFN-γ) upregulates sIgA production in studies involving intracellular pathogens (Ogra et al., 2001). IFN-γ promotes transport of IgA across the epithelial barrier by upregulating pIgR expression. B cell isotype switching to pIgA-producing plasma cells appears to occur in the mucosal inductive sites. Maturation to IgA-secreting plasma cells requires TGF-β, IL-10 for isotype switching while IL-4, IL-5 and IL-6 allow maturation and final differentiation into IgA-producing plasma cells (Corthesy, 2003). Despite the large amount of IgA produced by the cells of the mucosal immune system the majority of the effector cells are CD4+ and CD8+ T lymphocytes, comprising approximately 80% of the mucosal lymphoid population (Ogra et al., 2001).

1.5.2 Uptake of Particulate Antigens

M cells in the Peyer's patches selectively take up particulate antigens, such as virus-like particles, and transport them from the intestine to underlying follicles. Activation of the mucosal immune response leads to the production of sIgA and serum IgG. Transport of particulate antigens in the nasal cavity occurs in a similar fashion via M cells (Davis, 2001). It is important that the antigen is presented to the M cells in the particulate state. While this does not pose a problem for intranasal immunization it is a potential problem for oral delivery as the antigen must survive passage through the stomach intact. The plant cell wall may provide some protection to the antigen during passage through the stomach. One of the major limitations to using plants is the low yields of antigen which may not be sufficient for immunization. Specialized M cells of the lymphoid follicle-associated epithelium conduct vesicular transport of antigens from the mucosal surface into organized lymphoid tissues where the immune response is

induced. Viral pathogens such as poliovirus, reovirus and possibly HIV can adhere to the apical membranes of M cells (Neutra, M. 2001). While the epithelium of the intestinal tract is sealed by tight junctions that prevent paracellular transport of macromolecules this barrier is not impenetrable. Instead this barrier is a mediator of molecular traffic between the lumen and the mucosal tissues. Immune surveillance requires the transport of foreign microorganisms and other antigens across this barrier and while it can be exploited for infections it also allows the induction of potentially protective immune responses. M cells will endocytose any molecule, particle or microorganism that adheres to its surface. It is hypothesized that VLPs are targeted to GALT via M cells of the Peyer's patches and pinocytosed from the gut lumen or mediated by a specific cellular receptor to cross the mucosal epithelia (Li et al., 2001). Then they are transported to the underlying lymphoid cells where they activate a mucosal response. IgA is probably the only method of preventing a virus from crossing the epithelial barrier and may be necessary for a potential HIV vaccine to prevent the virus from entering the body (Neutra, 2001).

1.5.3 Tolerance

Instead of inducing an immune response the oral delivery of antigen can also lead to the induction of peripheral tolerance. Oral tolerance is usually directed against food and autoantigens while strong immunogens generally elicit a mucosal immune response. Exposures to the antigens in food and in commensal bacteria tend to be frequent and repetitive. This appears to be one of the factors that allows the generation of tolerance. As vaccine antigens delivered orally would only be ingested a limited number of times there would not be a constant input of antigen which could lead to tolerance. Some proteins

tend to induce T cell anergy, while other generate a strong immune response; however, it is not clear what intrinsic properties are necessary to make an antigen induce a strong immune response. A mucosal immunization may give rise to a strong local IgA response while suppressing or tolerizing the systemic cellular response (Guerrero et al., 2001). The cytokines involved in local IgA response and in oral tolerance are the same, TGF-β and IL-4; however, induction of mucosal immunity as evidenced by the appearance of sIgA can occur concomitant to systemic cell-mediated tolerance. Moreover, peripheral tolerance generally involves only T cells and not the humoral response. If this is the case then the generation of T cell tolerance may be irrelevant to successful immunization (Mestecky, 2004). This would probably be an acceptable situation for intercellular pathogens while possibly posing a risk for intracellular pathogens.

1.5.4 Nasal Delivery

While direct consumption of unprocessed plant material would be the easiest method of delivering antigen mucosally it is also possible to process the plant and then administer the antigen by a nasal route. The aerosol route of immunization follows the mode of entry for numerous pathogens (measles, influenza, diphtheria, pertussis and tuberculosis) and leads to the generation of immunity at the point of entry as well as the possibility of a systemic response. Nasal vaccination is possible because the nasal passages are highly vascularized, with lots of microvilli covering the nasal epithelium, thus creating a large surface area (Davis, 2001). In addition, this route of immunization can induce both mucosal and systemic responses as well as stimulating responses at distant sites due to the CMIS. The nasal delivery route is also easily accessible, easy to administer to large groups and it does not requires needles and syringes. Nasal

immunization results in the production of mucosal IgA and systemic IgG responses which protect the entire respiratory tract from infection (Jones et al., 2001). Nasal vaccination would allow for mass and rapid vaccination, as it is easy to administer in developing countries or disaster areas (McCluskie et al., 2000; Roth et al., 2003). The delivery of small volume of antigen (20µl) restricts it to the nasal cavity (NALT) as would occur with nasal drops or nasal spray.

1.6 Current Status of Mucosal Vaccines

Despite all these attractive features there have been few successful mucosally administered vaccines. At present there are only a handful of mucosally delivered vaccines, almost all of which use living organisms, including the oral polio vaccine, live-attenuated cholera vaccines, live-attenuated typhoid vaccine, an oral BCG vaccine and the oral adenovirus virus vaccine. The only mucosally delivered non-live vaccine is the oral killed whole-cell B subunit cholera vaccine. Recently two relatively new mucosal vaccines, the oral live-attenuated rotavirus vaccine and the nasal influenza vaccine, which includes enterotoxin as an adjuvant, were withdrawn due to serious safety concerns. The immune response to mucosal vaccines can be enhanced by using adjuvants such as CT or LT but these are not suitable for use in humans as they are too toxic. Various modifications of the toxins have been made to render them less toxic but decreased toxicity leads to decreased adjuvanticity (McCluskie et al., 2000). There needs to be improvement in the efficiency of delivery of mucosal antigens and development of safe, effective mucosal adjuvants (Holmgren et al., 2003). Virus-like particles, which are self-assembling non-replicating viral core structures are produced recombinantly *in vitro*. VLPs are cheap and easy to make but more importantly tend to be highly immunogenic.

Some success has been achieved using VLPs from papillomavirus, Norwalk virus and hepatitis E virus (Guerrero et al., 2001). VLPs while of interest themselves can also be used as carriers for foreign antigens that are expressed on the surface of the VLP.

One of the problems with mucosal immunization is that monitoring the response is difficult and relies on antibody levels in external secretions such as tears or saliva (Roth et al., 2003). Another difficulty is that the most effective routes to induce mucosal and systemic immunity are the least desirable - intranasal and intrarectal immunization (Mitchell & Galun, 2003). However, the major concern of oral immunization is the development of tolerance to the antigen. The gastro-intestinal tract was not developed to make significant immunological reactions to food entering this organ. Also, despite the use of powerful adjuvants and microparticle carrier systems non-replicating antigens delivered orally tend to induce insufficient levels and duration of immunity (Czerkinsky et al., 1999). Oral administration of non-replicating antigens can elicit a detectable immune response, but they are often low. When oral immunization is preceded by systemic immunization a substantial boost can be observed but IgA production is only observed after the oral boost. This demonstrates that multi-route immunization schemes may be useful.

1.7 Hypothesis

A C-terminal truncated form of Hepatitis B nucleocapsid protein (HBc153) can be expressed in GM-plants which will induce both a systemic and local immune response when fed to small mammals.

1.8 Objectives

1.) To determine the expression level of the C-terminal truncated form of Hepatitis B nucleocapsid protein when expressed in GM-tobacco, -carrots, and -rice plants.

2.) To assess the immunogenicity of HBV nucleocapsid protein expressed in plants.

3.) To assess the systemic and local immune response to mammals (mice and rabbits) fed GM-carrots and –rice.

4.) To assess the effectiveness of immunization delivered through parenteral, oral and nasal routes.

5.) To determine the cellular response (T_{h1} vs T_{h2}) to both parenteral and mucosal immunization.

2. Materials and Methods

2.1 HBc153 Production and Purification from *E. coli*

The sequence coding for the first 153 N-terminal amino acids of Hepatitis B nucleocapsid was cloned into pkk233-2 (Clonetech) and designated pkk-HBc153, transformed and expressed in *E. coli* strain JM109 and cultured in Terrific broth with 100µg/ml ampicillin at 37°C for 36 hours. The culture was centrifuged at 2000*g* for 30 min. at 4°C, the supernatant discarded and the pellet washed in a small volume of Terrific broth. This was then centrifuged at 2000*g* for 10 min. at 4°C and the supernatant removed. The pellet was resuspended in lysis buffer (25mM HEPES, 100mM KCl, 20% glucose, 2mM EDTA, 0.2mM PMSF). Lysozyme was added to 1mg/ml, cell clumps were broken up by passaging through a syringe and consecutively smaller needles (18, 20, 22 Gauge), NP-40 was added to a final concentration of 0.1%. The suspension was then sonicated for 30 seconds and then placed on ice for 1 minute for a total of 5 times. The extract was clarified by centrifugation at 2000*g* for 30min at 4°C, the supernatant was collected and centrifuged at 15000*g* for 10min. at 4°C. The supernatant was collected and loaded onto a discontinuous sucrose gradient (20 to 60 % w/w sucrose in dialysis buffer (20mM Tris-HCl, 150mM NaCL, 1mM EDTA, 0.05% NP-40, pH7.4) and centrifuged at 30000 rpm in a SW40 rotor (Beckman) for 15 hours. Fractions (1ml) were collected with an Auto-DensiFlow (Labconco) and analyzed by SDS-PAGE and ELISA. Appropriate fractions were pooled and dialyzed against dialysis buffer overnight at 4°C. The dialyzed fraction was centrifuged at 54000rpm in a SW60 (Beckman) rotor for 5½ hours, the supernatant removed, pellet resuspended in dialysis buffer. The resuspended protein was dialyzed against Buffer A (20mM Tris-HCl, 10mM NaCl, pH8.5), filtered

through a .45um filter (Millipore) and applied to a Q-Sepharose fast flow (Amersham Biosciences) column on an AKTA FPLC (Amersham Biosciences) using Buffer A. HBc153 was eluted on a linear gradient, 0-100% Buffer B (20mM Tris-HCl, 1M NaCl, pH 8.5). Peak fractions were analyzed by SDS-PAGE and/or ELISA, dialyzed against 20mM Tris-HCl, 150mM NaCl and passed through a Sephadex S-200 gel filtration column. Peak fractions were analyzed as before. The purified HBc153 was quantitated with the Bio-Rad Protein Assay (Bio-Rad) and run on a SDS-PAGE gel and silver stained to determine purity. The purified particles were negatively stained and visualized by electron microscopy to confirm the protein was assembled into a virus-like particle.

2.2 HBc153 Production and Purification from Tobacco

Antigen from tobacco leaves was prepared from fresh leaves collected from live GM-HBc153 tobacco plants maintained at the NML, snap-frozen in liquid nitrogen, and ground in a pre-chilled stainless steel blender until only a fine powdery material remained. This was removed from the vessel to an Erlinmyer flask and resuspended in Buffer Z (PBS, 2mM EDTA, 0.2% Triton-X100, 50mM L-ascorbic acid, 2mM PMSF, pH 7.2) overnight at 4°C with constant stirring. Alternatively, crude GM-HBc153 tobacco plant extracts were prepared by Zaman Ali at the University of Ottawa (Ottawa, Ontario) and shipped on dry ice. In either situation the crude extracts were centrifuged at 15000g for 40 minutes, the supernatant removed and centrifuged at 200 000g in a 50.2Ti rotor (Beckman) for 2 hours. The pellet was resuspended in dialysis buffer and sonicated. This was then centrifuged at 15000g for 30 minutes and the supernatant removed. MAB842 ELISA and western blot were used to follow the presence of HBc153 in the fractions during purification. Purified GM-HBc153 was quantitated with Western blot.

2.3 Quantification of Recombinant HBc153 Protein from Transgenic Plants

To determine the effects of the various enhancer elements Western blots were quantitated to determine the amount of HBc153 expressed by the various constructs. The relative band intensities were estimated using Western blots performed at the NML of the various plant extracts using a known amount of eHBc153 as a standard. Images were sent to Zaman Ali at the University of Ottawa for densitometry analysis using the Kodak Image Station 440 CF from (Mandel Scientific Company). Standard curves were generated and the unknown concentrations of the plant recombinant protein were approximated as previously described by Curtiss and Cardineau (1997).

2.4 Construction of Recombinant Baculovirus Vector Expressing HBc153 Protein

pFastBac1 (Invitrogen) and pkk-HBc153 frozen stock cultures were streaked onto LB plates containing 100μg/ml ampicillin (LB-Amp) and incubated overnight at 37°C. Single colonies were inoculated into 5ml LB broth with 100 μg/ml ampicillin and incubated at 37°C for 8 hours. A 1/500 dilution of the 8 hour culture was inoculated into 200ml of LB broth with 100μg/ml ampicillin and incubated overnight at 37°C. The cultures were maxi-prepped using the Endofree Maxi Kit (Qiagen) as directed. Plasmid pkk-HBc153 containing the sequence coding for HBc153 was digested with *Nco* I, Klenow (both from New England Biolabs) filled and cleaned up with PCR Reaction Clean Up Kit (Qiagen) as directed. The cleaned product was digested with *Hin*d III (New England Biolabs) and the restriction enzyme was heat inactivated. This product was ligated into the *Hin*d III site of pFastBac1 with T4 DNA ligase (New England Biolabs) in 1x final concentration ligation buffer, water and 1μl of ligase overnight at 16°C. The ligation reaction was cleaned up with a PCR Reaction Clean Up Kit (Qiagen) and transformed into

TOP10 cells (Invitrogen) via electroporation. The cells were allowed to recover for 45 minutes at 37°C and then plated onto LB-Amp plates and incubated overnight at 37°C. 8 colonies were inoculated to 5ml of LB-Amp broth and incubated overnight at 37°C. The cultures were mini-prepped with QiaQuick Mini-Prep Kit (Qiagen), DNA concentration adjusted to 150µg/ml and submitted to the DNA Core Facility at the NML for sequencing with primers GM0006F (5'-TATTCCGGATTATTCATACC-3') and GM0007R (5'-AGTAAGACAAGAGATGTG-3').

The sequence was confirmed and pFB1-HBC153 was transformed into DH10Bac (Invitrogen) cells by electroporation and plated onto triple selection plates (LB agar plates containing 50µg/ml kanamycin, 7µg/ml gentamicin, 10µg/ml tetracycline, 100µg/ml Bluo-gal and 40µg/ml IPTG). The plates were incubated for 48 hours at 37°C and white colonies were picked and restreaked to triple selection plates and incubated overnight at 37°C. Single white colonies were inoculated into 5ml LB broth with 50µg/ml kanamycin, 7µg/ml gentamicin and 10µg/ml tetracycline and incubated overnight at 37°C. The culture was mini-prepped as before and PCR screened with the following primer sets M13 Forward (-40) (5'-GTTTTCCCAGTCACGAC-3') and M13 Reverse (5'-CAGGAAACAGCTATGAC-3') and M13 Forward and HBV109r using standard PCR conditions. The bacmid containing the gene for HBc153 was maxi-prepped using the Endofree Maxi Kit (Qiagen) as previous.

The insect ovary cell line *Spodogtera frugiperda*-21 (Invitrogen) was propagated in complete supplemented Grace's media (supplemented Grace's media with 5% FBS and penicillin/streptomycin and gentamicin). Cells were maintained in spinner flasks at a concentration of $1x10^6$ cells/ml and incubated at 27°C. Cell viability was monitored using

trypan blue (10% v/v) exclusion assay. The live and dead (blue in color) cells were counted to determine the viability.

The baculovirus recombinant (Bac-HBc153) containing the HBc153 gene was obtained by the Cellfectin transfection method (Invitrogen). To obtain HBc153 VLPs, $5x10^8$ Sf21 cells in a spinner flask were infected at an MOI of 5 PFU per cell with Bac-HBc153. HBc153 VLPs were harvested from the cells at 72 hours post-infection, subjected to 3 freeze-thaw cycles and resuspended in extraction buffer (50mM Tris (pH7.8), 150mM NaCl, 0.1mM PMSF with one Complete Mini Protease Inhibitor Cocktail tablet (Roche) and subjected to 10 stokes in a Dounce homogenizer and then sonicated. Cellular debris was pelleted and the supernatant removed and loaded onto a discontinuous sucrose gradient as previously described.

2.5 Animals

Approximately 6-week-old BALB/c mice (Charles River) were used for all experiments. To determine if plant-derived and *E. coli*-derived HBc153 had similar antigenicity, 4 mice per group were subcutaneously injected with either plant-derived or *E. coli*-derived HBc153 with Quill-A (Cedarlane). Mice administered antigen via feeding were fasted for approximately 16 hours prior to antigen administration and their normal diet was resumed when all the administered material was consumed or after 24 hours. Three groups of mice were fed either HBc153-containing carrot, tobacco seed or rice. To ensure that each mouse received a similar quantity of antigen, the HBc153-containing carrots were sliced horizontally and then divided into the required number of sections for the group. HBc153-containing tobacco seeds were partially crushed with a mortar and pestle and pre-weighed in individual tubes. Liquified Transgel was added to the tube and

the seed powder distributed equally throughout the gel. The gel was allowed to solidify and was then fed to the mice. HBc153-rice grains were shelled, weighed and given raw to the mice to eat. The remainder of the groups were immunized with purified *E. coli*-derived HBc153 by oral gavage, intranasally or subcutaneously. Serum was collected at weekly or bi-weekly intervals and analyzed by ELISA for total IgG. Positive samples were then isotyped to determine the relative quantity of IgG_1, IgG_{2a}, IgG_{2b} and IgG_3. Spleens were harvested and splenocytes subjected to ^{3}H-thymidine proliferation assay and ELISPOT.

2.6 ELISA

2.6.1 HBcAg-capture

Flat-bottomed 96-well MaxiSorp microplates (Nunc) were coated with 50µl of MAB842 (Chemicon) at 1µg/ml in PBS and incubated overnight at 4°C. Plates were blocked with blocking buffer (5% skim milk, 1% BSA, 0.05% Tween20 in PBS) for 1.5 hours at room temperature. Subsequently, the plates were incubated with sucrose or FPLC fractions in PBS for 1 hour at 37°C then washed three times with PBS containing 0.05% Tween20 (PBS-T). Plates were then incubated at 37°C for 1 hour with anti-HBcAg (human) conjugated to HRP (Abbott). Plates were washed 3 times with PBS-T and incubated with o-phenylene diamine (OPD) (Abbott) at room temperature for 30 minutes. The reaction was stopped with 1N H_2SO_4. Plates were read at 492 nm on a microplate reader (Molecular Devices).

2.6.2 Total IgG

Flat-bottomed 96-well MaxiSorp microplates (Nunc) were coated with 50µl of purified eHBc153 VLPs at 1µg/ml in PBS and incubated overnight at 4°C. Plates were blocked as above. Subsequently, the plates were incubated with serum diluted in blocking

buffer for 1.5 hours at 37°C then washed three times with PBS containing 0.05% Tween20 (PBS-T). Plates were then incubated at 37°C for 1.5 hours with sheep anti-mouse IgG conjugated to alkaline phosphatase (Jackson) diluted 1:10000 in PBS. Plates were washed 5 times with PBS-T and incubated with p-nitrophenol phosphate (Sigma) at room temperature for 30 minutes. The reaction was stopped with 3M NaOH. Plates were read at 405 nm on a microplate reader (Molecular Devices). The geometric mean titer (GMT) was calculated using the reciprocal of the highest serum dilution that had an absorbance value ≥ twice the control.

2.6.3 IgG isotyping

Flat-bottomed 96-well MaxiSorp microplates (Nunc, USA) were coated with 50µl of purified eHBc153 VLPs at 1µg/ml (for unknowns) or purified anti-mouse F_c (reference standard) in 0.1 M sodium carbonate buffer and incubated at 37°C for 1 hour. Plates were washed twice with wash buffer (10mM Tris, 0.1M NaCl, 0.05% Tween20). Then plates were blocked with postcoat (50mM Tris, 1% BSA, 0.15M NaCl) for 30 minutes at 37°C and washed twice with wash buffer. Subsequently, the plates were incubated with serum (unknowns) or IgG1, IgG2a, IgG2b, IgG3 isotype reference standards (Bethyl Laboratories, Montgomery, TX) diluted in postcoat with 0.05% Tween20 (postcoat-T) for 1 hours at 37°C then washed two times with wash buffer. Plates were then incubated at 37°C for 1 hour with the appropriate goat anti-mouse IgG isotype conjugated to HRP (Bethyl Labs) diluted 1:20000 in PBS. Plates were washed three times with wash buffer and incubated with OPD (Abbott) at room temperature for 30 minutes. The reaction was stopped with 1N H_2SO_4. Plates were read at 492 nm on a microplate reader (Molecular Devices).

2.6.4 Fecal IgA

Fecal samples were collected from individual mice at certain time points and stored at -80°C until processed as per deVos and Dick (1991). To extract IgA 0.1g of fecal pellets were weighed in a 1.5ml microfuge tube. 1ml of PBS, 2mM PMSF (10 volumes, w/v) was added to the weighed pellet and incubated at room temperature for 15 minutes. The tube was vortexed and incubated on ice for 15 minutes. The tube was then vortexed until all the material was resuspended. It was then centrifuged at 13000 rpm for 10 min at 4°C and the supernatant removed and stored at 4°C. Extracts were then tested in HBcAg-specific ELISA

2.7 ELISPOT

96 well Multiscreen IP (Millipore) filter plates were coated with 50µl of anti-IFN-γ, -IL4 or –IL5 in PBS or anti-IL-2, -IL6 or IL-10 at 10µg/ml in 0.1M carbonate buffer. Plates were incubated overnight at 4°C. Spleens were harvested and placed in RPMI-10 (RPMI-1640 supplemented with 10% heat inactivated fetal calf serum, penicillin/streptomycin). Red blood cells were lysed with RBC lysis buffer (eBioscience) and resuspended at $1x10^6$ cells/ml in RPMI-10 or MACS buffer (PBS, 2mM EDTA; 0.5% BSA; pH 7.2) as required. Portions of the total cell population were depleted of CD8+ cells, CD4+ cells and B cells respectively using MACS. Populations were analyzed on a FACSCalibur (Beckton-Dickson) flow cytometer to determine the efficiency of depletion. Multiscreen plates were washed with PBS and blocked with 200µl/well of RPMI-10 for 2 hours at room temperature. Cells were seeded in triplicate at $2x10^5$ cells per well. 0.1µg of purified HBc153 was added as required. Plates were incubated at 37°C in 5% CO_2 for 48 hours. Wells were washed five times with PBS-T and

once with distilled water. Biotinylated anti-IFN-γ or anti-IL-10 (1μg/ml) detection antibody was added to the appropriate wells in PBS with 0.5% FCS and incubated at room temperature for 1.5 hours. Plates were washed and strepdavidin-HRP (Mabtech at 1:100 in PBS-0.5%FCS or avidin-HRP (eBioscience at 1/1000 in assay diluent) was added and incubated at room temperature for 1 hour. Plates were washed and AEC (3-Amino-9-ethylcarbazole) (Sigma) substrate was added. Plates were developed at room temperature for 20-60 minutes, washed extensively with water and air dryed. Spots were subsequently counted with an ELISPOT reader (AID).

2.8 Proliferation Assay

Murine spleen cells were washed with complete RPMI and resuspended at 10^6 cells/ml. 10^5 cells were added to a 96 well round bottom plate along with 100μl of the appropriate antigen (10μg/ml conA, 1μg/ml HBc153 (*E. coli*- and tobacco-derived) or complete RPMI as a control). Plates were incubated for 5 days at 37°C in 5% CO_2 and then pulsed with 50μl per well of a 1:50 dilution of ^{3}H-Thymidine (1μCi/well final). The plate was further incubated for 18 hours at 37°C in 5% CO_2. Plates were harvested with a 96 well Filtermate Harvester (Packard Bioscience) harvester and washed extensively. The filter plate was removed and left to dry at room temperature in the dark for at least 1 hour. The bottom of the plate was sealed and 25μl of scintillation media was added to each filter well. The top of the plate was sealed and then the plate was placed in a TopCount NXT Microplate Scintillation Counter (Packard Bioscience).

2.9 Depletion of Selected Lymphocyte Populations

Splenocytes were washed with RPMI-1640, spun down and resuspended in 90μl of MACS buffer (PBS with 0.5% BSA; pH 7.2) per 10^7 total cells. 10μl of MACS anti-

CD4 (L3T4) or anti-CD45R (L220) microbeads (Miltenyi Biotec) per 10^7 total cells were added, mixed and incubated at 6-12°C for 15 minutes. The labeling reaction was stopped by adding 20x the labeling volume of MACS buffer, the cells were centrifuged at 300xg for 10 minutes, the supernatant removed and the cell pellet resuspended in 500µl of buffer per 10^8 total cells. MACS LD columns (Miltenyi Biotec) were washed with degassed MACS buffer and the cell suspension was loaded through a cell single mesh in 500µl of MACS buffer per 10^8 total cells onto the depletion column. The cell suspension was allowed to run through the matrix and the effluent was collected as the depleted fraction. The column was washed twice more (1ml) to ensure that all unbound cells were washed through. Cells were centrifuged at 300xg and resuspend in complete RPMI.

2.10 Flow Cytometry

In order to determine the efficiency of depletion, untouched and depleted cell populations were stained for with the following markers: CD3-PerCP, CD4-PE, CD8-APC and CD19-FITC (BD Biosciences). The appropriate isotype controls were also performed. Cells (10^6) were resuspended in 100µl of PBS with 2% FBS. F_c receptors were blocked with 2µl of block solution (BD Biosciences) diluted 1:1 with PBS and incubated for 15 minutes at room temperature. 2µl of each antibody conjugated dye was added and incubated for 15 minutes at 4°C. 400µl of PBS with 2% FBS was added to FACS tubes and then the labeled cells were added to the appropriate tube. Cells were then processed on a FACSCalibur (Beckton-Dickson) flow cytometer and analyzed with CellQuest to determine the efficiency of depletion.

2.11 Protein Electrophoresis

Purified HBc153 from *E.coli*, insect cells or tobacco plants was mixed with 5 µl of 6X loading buffer (Maniatis, 2003), heated at 100°C for 5 minutes and loaded onto a pre-cast 15% Tris-HCl Criterion (Bio-Rad) acrylamide gel. Following electrophoresis gels were either stained with Bio-Safe Coomassie Blue (Bio-Rad) as per the manufacturers instructions, silver stained or transferred to nitrocellulose for a western blot.

2.11.1 Western Blot

Gels were removed and placed in Towbin buffer for 10 minutes. The transfer case was assembled and the proteins transferred at 250 mA for 60 minutes onto nitrocellulose (Bio-Rad). The nitrocellulose was removed and washed three times in TBS for 5 minutes. The membrane was then blocked overnight at 4°C or for 1 hour at room temperature in blocking buffer (5% skim milk, 1% BSA, 0.05% Tween20 in TBS). The membrane was incubated for 2 hours at room temperature with the primary antibody, which was polyclonal human serum from a patient with a chronic HBV infection diluted 1/4000 in blocking buffer. The membrane was then washed in TBS-T and incubated with an anti-human Ig-HRP conjugate (Amersham) at 1/4000 in blocking buffer for 2 hours at room temperature. The membrane was washed, ECL substrate (Amersham) was added as per the manufacturer's instructions and then the membrane was exposed to Hyperfilm (Amersham) and developed with an automated X-ray film developer.

2.11.2 Silver Stain

Following electrophoresis the gel was silver stained using the Plus One silver stain kit (Amersham) using a modified protocol. The gel was fixed in 40% ethanol, 10%

acetic acid for 1 hour, with a solution change after 30 min. Then the gel was sensitized for at least 30 minutes in 30% ethanol, 0.2% sodium thiosulphate (w/v), 0.8M sodium acetate. The gel was washed in dH_2O three times for 5 min. The gel was stained for 20 minutes in 0.25% silver nitrate (w/v) and washed twice in dH_2O for 1 minute. The gel was developed in 0.2M sodium carbonate, 0.0074% formaldehyde (w/v) until the desired darkness was reached. The developer was stopped with 0.4M EDTA for 10 minutes. The gel was then washed in dH_2O twice for 10 minutes and the bands of interest excised with a clean scalpel. The gel slices were stored in 1% acetic acid at 4°C until further processed.

2.12 Mass Spectrometry

Gel slice destaining was performed as described previously (Sumner et al. 2002). Briefly, gel slices were washed twice with water (Sigma) followed by two washes in 25mM ammonium bicarbonate (Sigma). The silver stain was removed with 1% hydrogen peroxide (Fisher) in 25mM ammonium bicarbonate and then washed with water. The pH of the gel plug was then lowered with 1% formic acid (Sigma). The gel slices were then cut into approximately 1mm pieces and covered with acetonitrile (Sigma):100mM ammonium bicarbonate (1:1, v/v). The liquid was removed and the gel pieces were covered with acetonitrile. All of the preceding steps were incubated at room temperature for 5 minutes. The liquid was removed and the gel pieces were dried in a SpeedVac vacuum centrifuge for 5 minutes without heat. The gel was rehydrated with 100mM ammonium bicarbonate containing 10mM dithiothreitol (DTT) (Sigma) to reduce sulphide bonds and incubated at 56°C for 1 hour. The tube was allowed to cool to room temperature, the liquid removed and 2-3 gel volumes of 100mM ammonium bicarbonate

containing 55mM iodoactamide (Sigma) was added to block reformation of disulphide bonds. The reaction was incubated in the dark for 45 minutes at room temperature. The liquid was discarded and the gel washed with 100mM ammonium bicarbonate. The gel pieces were sequentially dehydrated as before then rehydrated in digestion buffer (50mM ammonium bicarbonate with 5mM calcium chloride and 5ng/µl sequencing grade trypsin (Sigma) or glucosidase-C (Roche) on ice for 45 minutes. The liquid was removed, replaced with digestion buffer without enzyme and the gel pieces were incubated at 37°C (trypsin) or 30°C (glucosidase-C) overnight. Following digestion the tube was spun briefly and the liquid transferred to a new tube. Digested peptides were sequentially extracted in 2-3 gel volumes 0.01% trifluroacetic acid (TFA) (Sigma) followed by 0.01% TFA: acetonitrile (1:1, v/v) and placed in a sonicating water bath for 10 min. The liquid was removed to the new tube after each step. The extraction was repeated for a total of three times. The gel was further extracted with 2-3 gel volumes acetonitrile followed by extraction with 5% formic acid in 50% acetonitrile. This was repeated for a total of two times. 10mM DTT was added to the pooled extracted peptides to a final concentration of 1mM. The pooled extracts were then dried with a SpeedVac without heat and stored at -20°C. Prior to MALDI-TOF MS analysis the sample was reconstituted in 10µl of 0.1% TFA. A ZipTip (Millipore) was used to clean-up the sample which was eluted in 2µl of 50% acetonitrile in water. An equal volume of 0.2% TFA was then added to the sample. 1µl of the sample was loaded onto a gold plate and mixed with 1µl of matrix (cinnamic acid), allowed to dry and then placed in the MALDI-TOF MS. Analysis was performed using MoverZ and Profound (Genomic Solutions).

3. Results

3.1 Production and Purification of HBc153 from *E. coli* and Baculovirus Expression Systems

HBc153 was expressed in *E. coli* JM109 cells transformed with the pkkHBc153 plasmid and in initial studies, partially purified on a discontinuous sucrose gradient. The *E. coli*-derived (eHBc153) VLPs were characterized by SDS-PAGE, Western blot and HBcAg-capture ELISA. As the product from the discontinuous sucrose gradient contained many other proteins an effort was made to further purify eHBc153 by column chromatography. It was determined that eHBc153 would bind to the ion exchange matrix, Sepharose Q-FF, at a pH 8.5 in 20mM Tris-HCl, 10mM NaCl. It was eluted from the column on a linear gradient going to 20mM Tris-HCl , 1M NaCl. (Solution B). All of the peak fractions were collected and analyzed in HBcAg-capture ELISA to determine which fractions contained HBc153. The flow through (X1) always contained some eHBc153 that would not bind the column under these conditions; however, the majority of the eHBc153 was contained in the peak eluting at approximately 40% Solution B (Figure 2a) and collected in fractions B1-B4. The other fractions did not contain detectable amounts of eHBc153. To further purify the protein it was loaded on to an S-200 gel filtration column where it was eluted as a single peak in the void volume. The concentration of eHBc153 was determined by a modified Bradford Protein Assay (BioRad) and corroborated by Coomassie stained SDS-PAGE using known quantities of lysozyme as a reference standard. To determine its purity, 1µg of HBc153 was run on an SDS-PAGE gel that was silver stained and only a single band was observed (Figure 2b). This

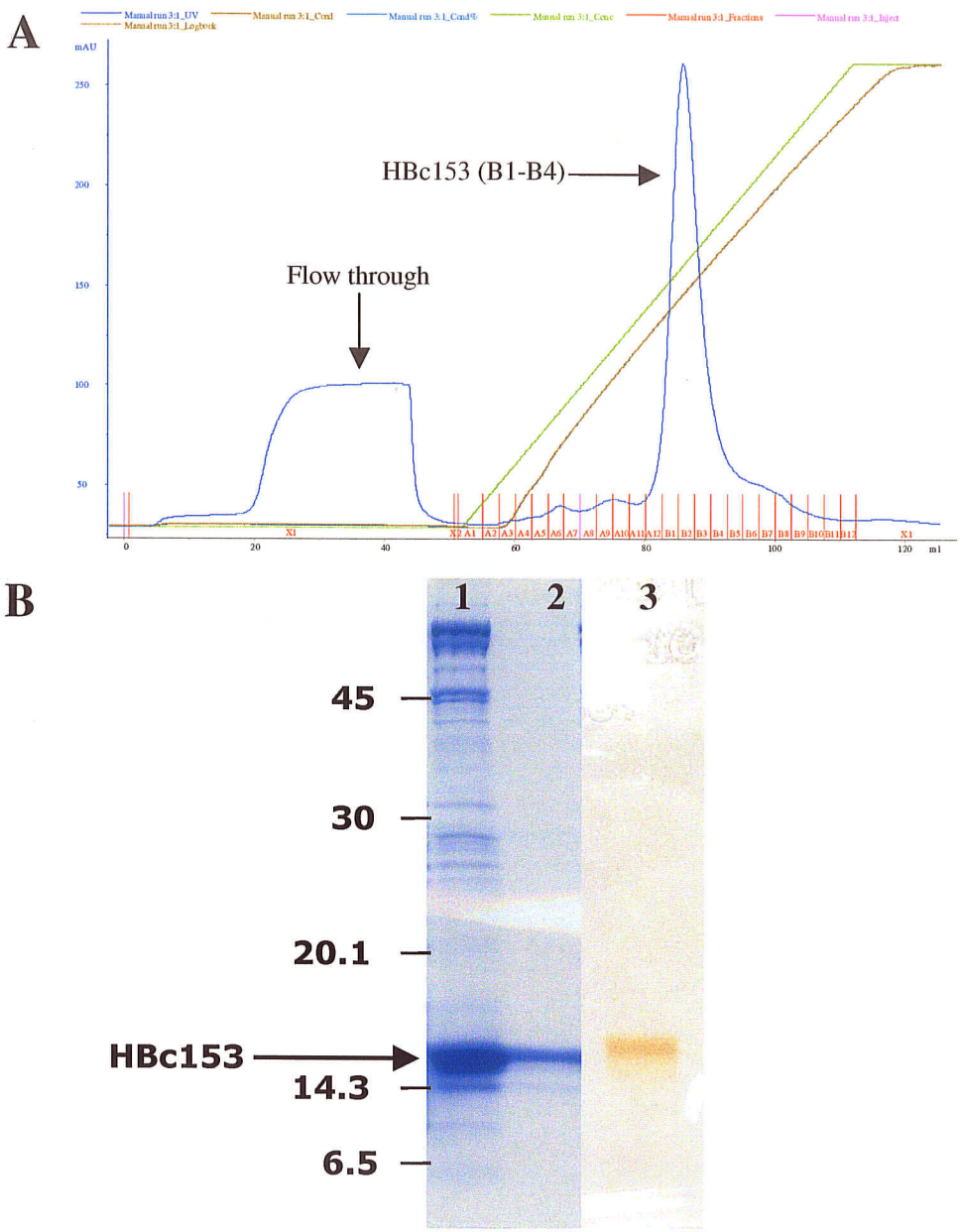

Figure 2. Purification of recombinant HBc153. A representative AKTA-FPLC run using a Q-FF ion exchange column (A). Fractions X1 and B1-B4 contain HBc153 as determined by MAB842 ELISA, the HBc153 content in the remainder of the fractions was negligible. HBc153 preparations were run on an SDS-PAGE gel (B) and stained with Coomassie after sucrose density-gradient purification (lane 1) and ion exchange/gel filtration (lane 2). 1µg of ion exchange/gel filtration purified HBc153 was also run on an SDS-PAGE gel and silver stained (lane 3) revealing a single band, indicating purity of greater than 90%.

indicates that the purity of eHBc153 was greater than 90% based on the detection limit of the silver stain kit.

To eliminate the possibility of cellular activation by contaminants such as lipopolysacharide (LPS) in the *E. coli* expression system and lectins and/or toxins in the tobacco expression system, HBc153 was also produced in the Bac-to-Bac Baculovirus expression system. Recombinant virus was propagated in *Sf*-9 cells, as described below, and optimal HBc153 accumulation was attained at 72 hours post-infection. Cells were harvested and extracted baculovirus-derived HBc153 (bHBc153) was purified following the same procedures as above and was analyzed by Western blot and antigen capture ELISA.

3.2 Expression of HBc153 in Tobacco, Rice and Carrots

Development of transgenic plants expressing HBc153 was carried out by Zaman Ali at the University of Ottawa using the four different constructs described in Figure 3. Transformants were selected for kanamycin resistance and were confirmed by Southern and Northern blot analysis by Zaman Ali at the University of Ottawa. Crude extracts were sent to our lab at the NML for verification of protein expression by antigen capture ELISA (Figure 4, 6A, 6C), Western blot (Figure 5, 6B) and for visualizing particles by electron microscopy (Figure 7). Based on our results transformants with the highest levels of expression were maintained by Zaman Ali at the University of Ottawa. Initially, transgenic tobacco plants (H1) were generated expressing the wild-type coding sequence of HBc153 under the control of the double Cauliflower Mosaic Virus 35S (d35S) promoter sequence which was followed by an Alfalfa Mosaic Virus- (AMV) RNA4 translational enhancer sequence (Figure 3). While HBc153 from H1 plants was detected

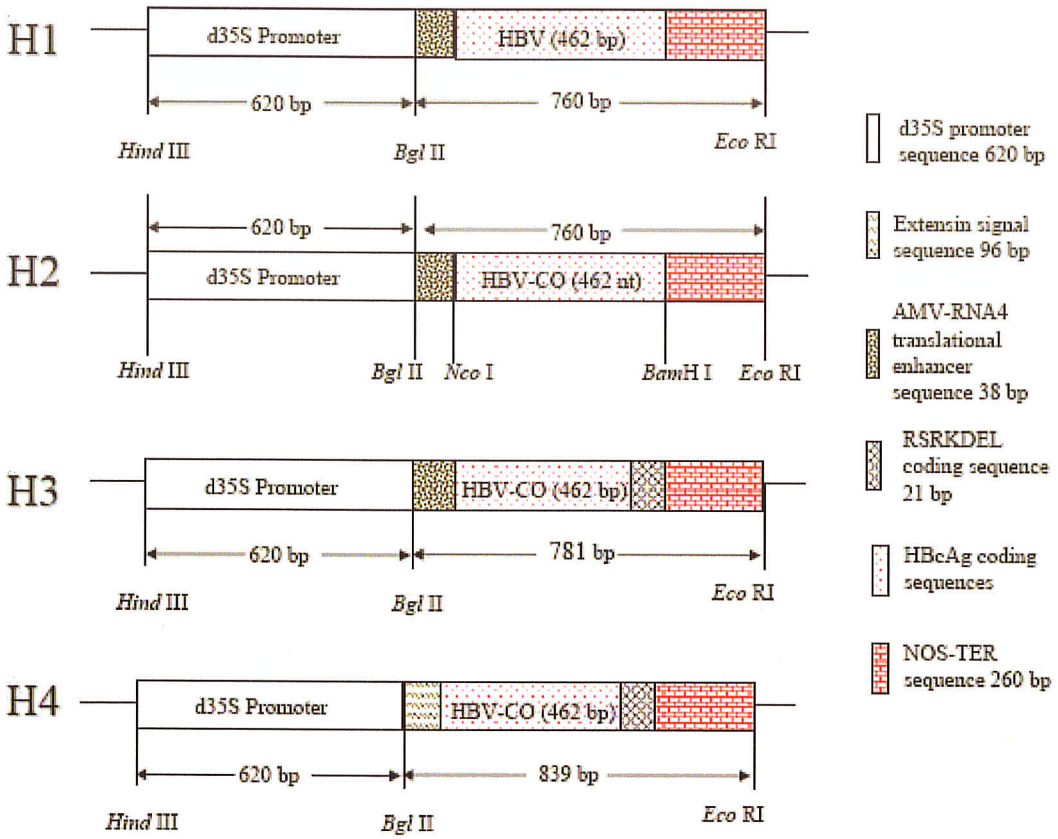

Figure 3. Schematic of the 4 different HBc153 constructs used in the GM-plants. These expression constructs were based on the wild-type HBcAg gene (H1) and the synthetic HBcAg gene that was condon optimized for rice, carrots and tobacco (H2-H4). Each complete expression construct was subcloned into independent plant transformation vectors (pRD400) using the restriction endonucleases *Hind* III and *Eco*R I. The pRD400 backbone vectors with independent inserts H1-H4 were used to transform *A. tumefaciens* strain LBA 4404 for subsequent transformation of tobacco leaf sections. Figures are not drawn to scale.

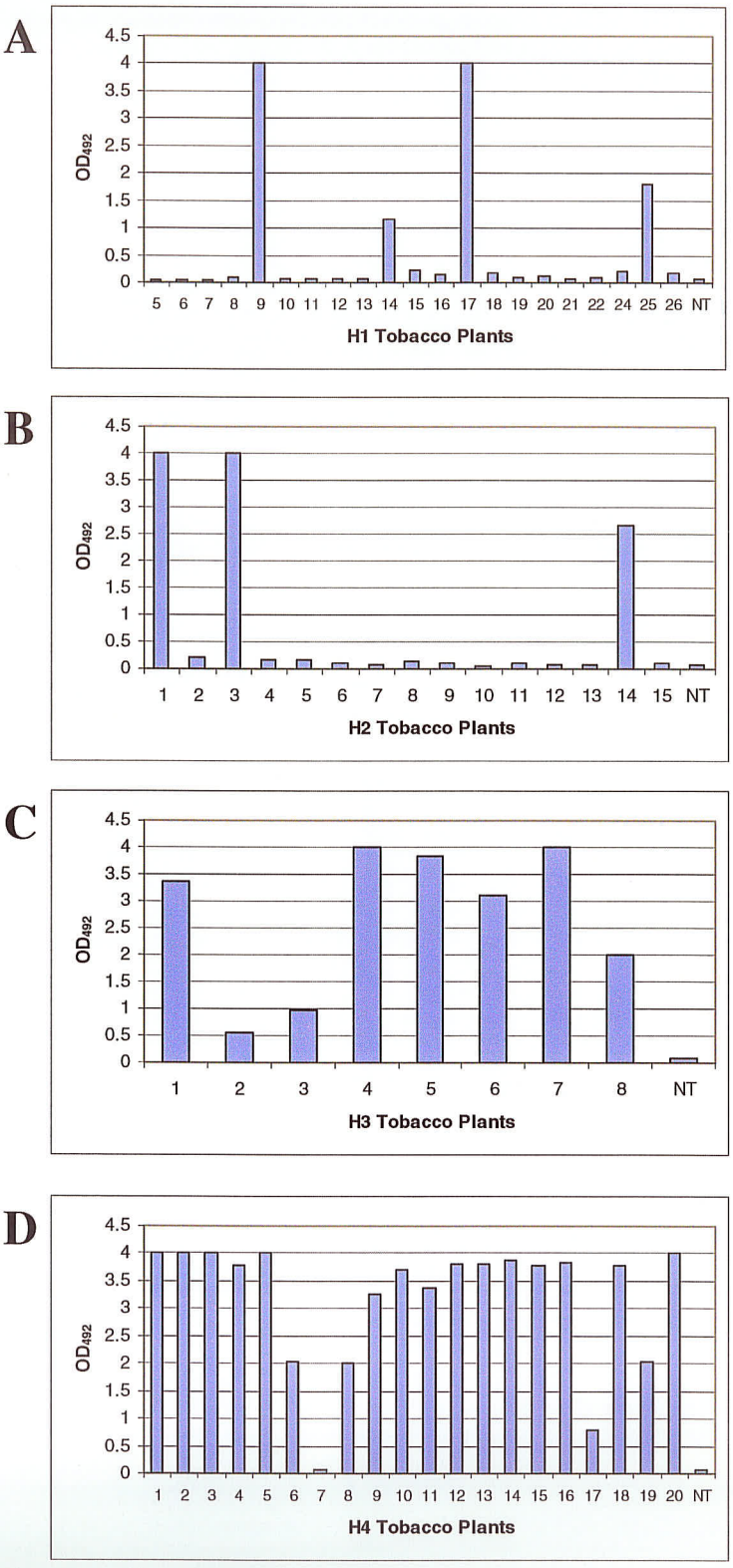

Figure 4. HBc153-capture ELISA values from extracts of the 4 different constructs expressed in GM-tobacco. HBc153 was extracted from GM-tobacco leaves of H1 (A), H2 (B), H3 (C) and H4 (D) plants and analyzed by antigen capture ELISA. It was observed that the H4 plants consistently produced plants that had comparatively high levels of expression of HBc153.

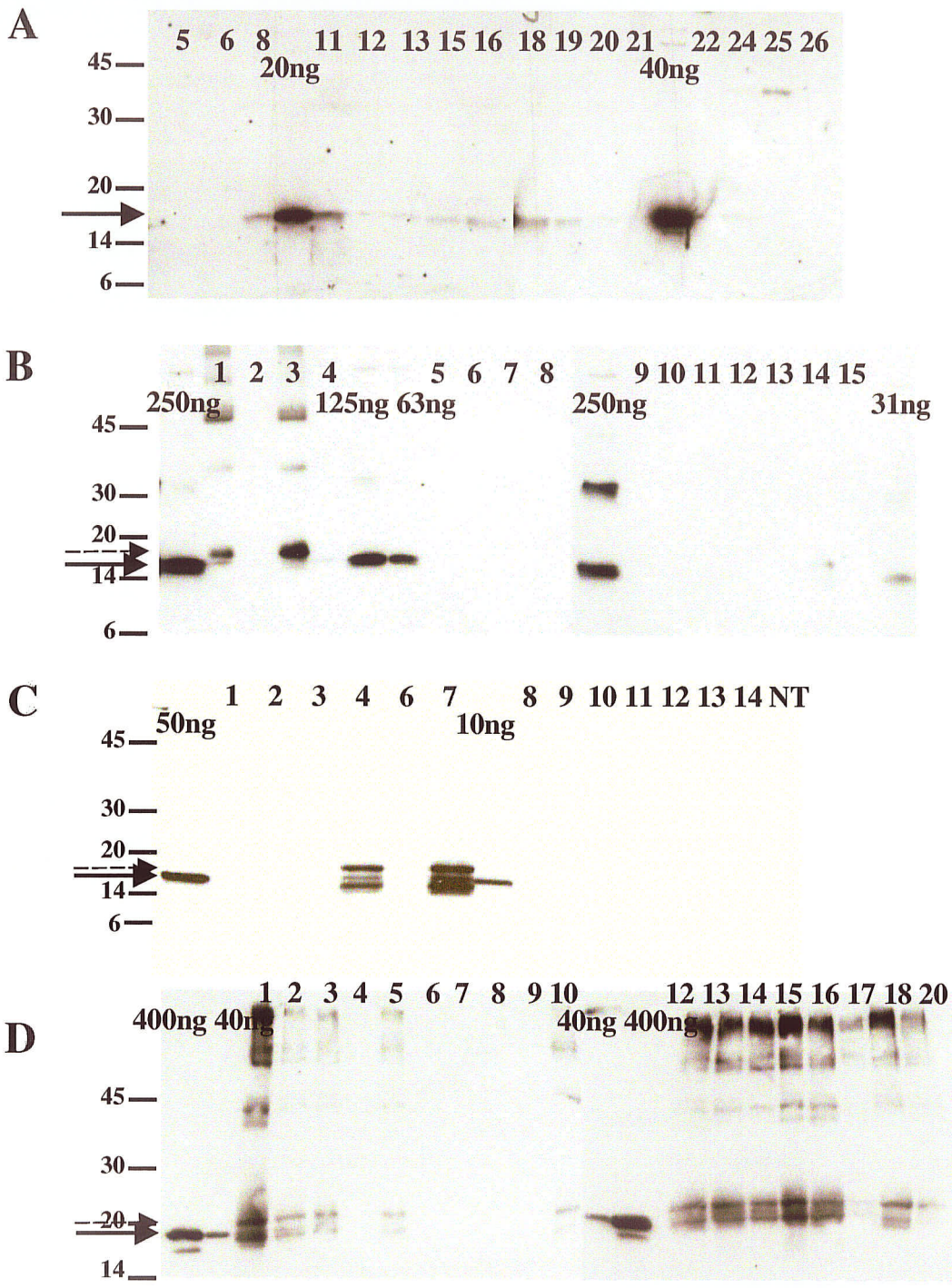

Figure 5. Western blots of extracts from the 4 different constructs expressed in GM-tobacco. HBc153 was extracted from GM-tobacco leaves of H1 (A), H2 (B), H3 (C) and H4 (D) plants. The extracts were subjected to reducing SDS-PAGE and Western blot analysis with a human polyclonal serum. Purified *E. coli*-derived HBc153 (solid arrow) that had been quantitated by protein assay was used as a reference standard as indicated. The higher MW HBc153 product observed in the H2-H4 constructs is also indicated (dashed arrow).

in both Western blots and ELISA (Figure 4a and 5a) the level of HBc153 expression was quite low. In an effort to enhance the amount of protein produced in the plants a codon-optimized version of HBc153 was constructed and transformed in tobacco plants (H2). The level of HBc153 expression in the H2 plants was somewhat improved (Figure 4b and 5b) compared to the H1 plants. To target HBc153 to the endoplasmic reticulum a RSRKDEL (0.903kDa) coding sequence was added to C-terminal end of the codon-optimized HBc153 gene (H3). This did not yield a significant improvement in the level of expression. The final construct (H4) contained an extensin signal sequence in place of the AMV-RNA4 enhancer sequence in addition to the RSRKDEL sequence. This construct provided the highest HBc153 expression levels and the largest percentage of successful plants (Figure 4d and 5d). All constructs were terminated with a nopaline synthetase terminator and polyadenylation sequence (NOS-TER). To estimate the amount of HBc153 produced by the various plant constructs densitometric analyses of Western blots was conducted by Zaman Ali of the University of Ottawa using a protocol developed by Curtiss and Cardineau (1997) for quantification of recombinant plant proteins. Briefly, the highest yield observed from the leaf was from H4 plants, with 9.8% of the fresh weight being HBc153. The best H3, H2 and H1 plant contained 3.8, 3.4 and 0.63% HBc153 respectively.

While tobacco plants are useful as a model system for studying protein production in plants they are not edible due to the high level of toxins they produce. Therefore, GM-carrots and -rice expressing HBc153 were produced for animal feeding experiments. Expression of HBc153 in the rice seed was confirmed by ELISA and Western blot and actually achieved quite high (2.12% of fresh weight) with the H1 construct. Expression studies with the rice plants were discontinued due to the loss of the plants following

exposure to cold temperatures. Carrots are certainly palatable raw and represented the most desirable plant to eat in our study. However, expression of HBc153 was not as high as in the other plants (Figure 6). The carrot roots of H1 plants contain only 0.45% fresh weight of HBc153. Carrots containing the H4 construct were also generated and contained 1.73% HBc153 of fresh weight. As the highest amount of HBc153 was produced in the H4 carrots they were used in the feeding trials.

3.3 Visualization of Virus-Like Particles Produced in Various Expression Systems

Electron microscopy of the partially purified HBc153 VLPs from the various expression systems was performed to ensure that the particles were correctly formed. The *E. coli*-, GM-tobacco-, GM-rice- and GM-carrot-derived HBc153 electron micrographs all showed particles that were morphologically similar (Figure 7) to those described previously (Newman et al., 2003). Consistent with ELISA and western blot analysis, the number of particles observed in the eHBc153 samples was much greater than that of any of the plant-derived samples.

3.4 Mass Spectrometry Analysis of HBc153

Western blots of HBc153 produced in baculovirus, GM-tobacco and –carrots exhibited a band identical in mass to that of the eHBc153 but an additional band with a higher (approximately 2 kDA) molecular weight was also observed (Figure 4c, 4d, 6b). This higher MW band was not observed in the H1 tobacco construct which may indicate that it is the result of the codon-optimization but this does not explain the observed difference. To determine if this higher molecular weight band was due to any post-translational modifications the extracts from the *E.* coli, tobacco and baculovirus

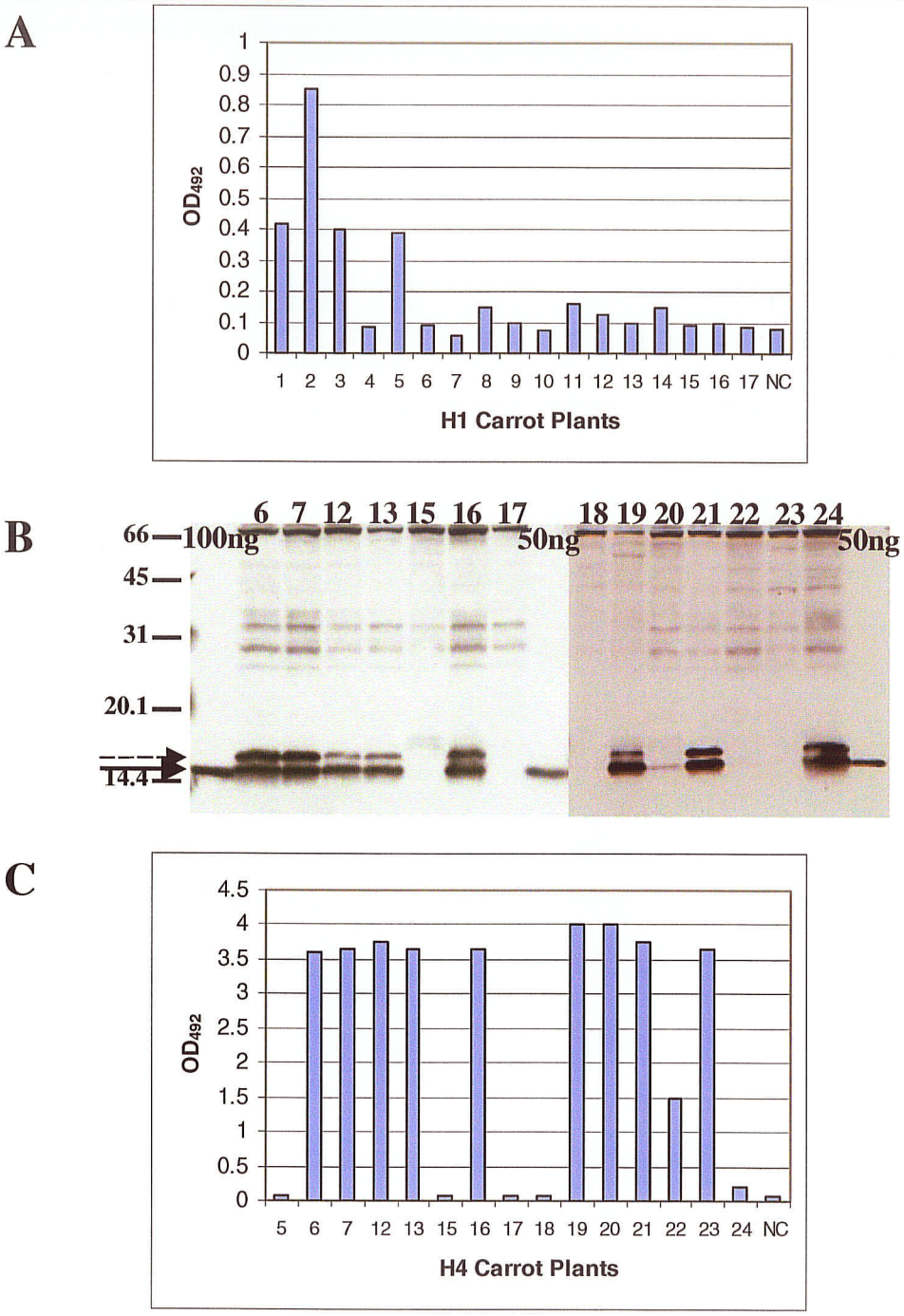

Figure 6. HBc153-capture ELISA values and Western blots of extracts from GM-carrots . HBc153 was extracted from GM-carrots and subjected to reducing SDS-PAGE followed by Western blot with a polyclonal human serum as well as antigen capture ELISA. H1 carrots did not have high enough expression of HBc153 to be detected by Western blot however HBc153 was detectable by ELISA (A). The expression of HBc153 in the H4 carrots was much higher than the H1 carrot as observed by the detectable levels in Western blot (B) and ELISA (C). Purified *E. coli*-derived HBc153 (arrow) that had been quantitated by protein assay was used as a reference standard as indicated. The higher MW HBc153 product observed in the H2-H4 constructs is also indicated (dashed arrow).

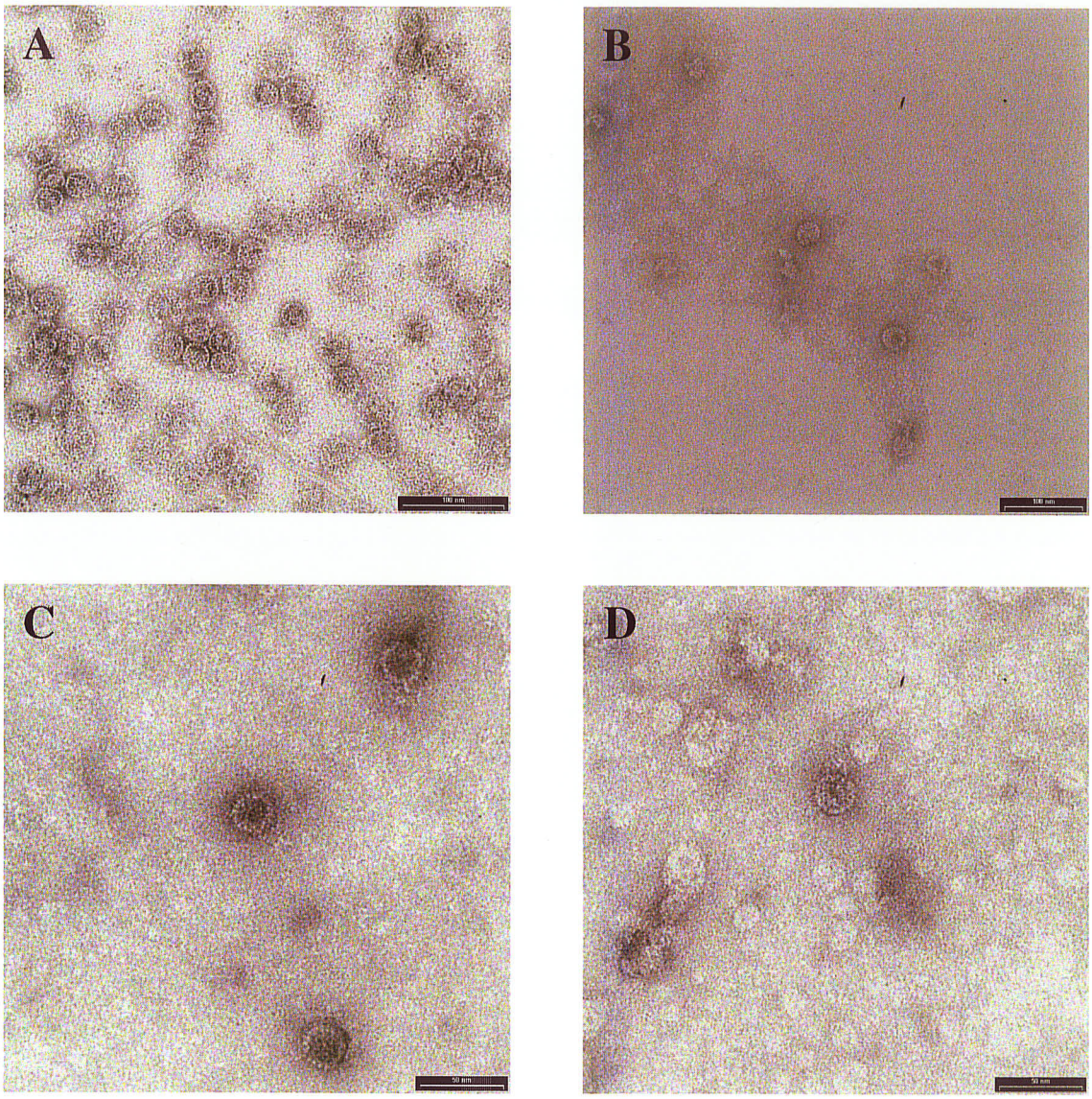

Figure 7. Electron micrographs of HBc153 VLPs from *E. coli*, GM-tobacco, GM-rice and GM-carrot. Negative stained electron micrographs of purified HBc153 VLPs produced in *E. coli* (A), GM-tobacco (B), GM-rice (C) and GM-carrot (D). Magnification bar equals 100nm in A & B and 50nm in C & D.

production systems were run on an SDS-PAGE gel, silver stained and then the bands were subsequently extracted. MALDI-TOF MS analysis was carried out at the Time of Flight Laboratory at the Department of Physics and Astronomy at the University of Manitoba (Winnipeg, Canada). Trypsin digests provided 53% and 43% sequence coverage for the lower and higher MW bands respectively (Figure 8). However, no predicted N- or O-glycosylation peak shifts were identified from the higher MW band (Figure 8b) despite the absence of the 1578.8 and 3219.6 peaks in the higher MW spectra. In an attempt to determine if the protein was properly terminated N-glycosidase digests were also performed. However, after multiple attempts no relevant data was obtained.

3.5 Immunogenicity of the Tobacco-Derived HBc153

3.5.1 Humoral Response

To ensure that plant- and *E. coli*-derived HBc153 had a similar immunogenicity, two groups of female BALB/c mice were injected subcutaneously with 3μg of sucrose gradient purified tobacco-derived (tHBc153) or *E. coli*-derived HBc153 (eHBc153), containing 100ng of Quill-A as an adjuvant. The mice were monitored for serum antibody responses by total IgG ELISA (Figure 9). The geometric mean titer (GMT) and the standard error (SE) were calculated for each group with non-responders included in the calculation. Baseline samples were taken from all mice prior to the first immunization and determined to be negative for HBc153-specific IgG. An untreated group was monitored concurrently with the other groups and did not show an increase in HBc153-specific IgG titer throughout the experiment. A positive result was considered an absorbance value twice the average untreated mice value. As seen in Figure 9 serum was assayed in an ELISA and had a reciprocal geometric mean titer (GMT) ± standard error

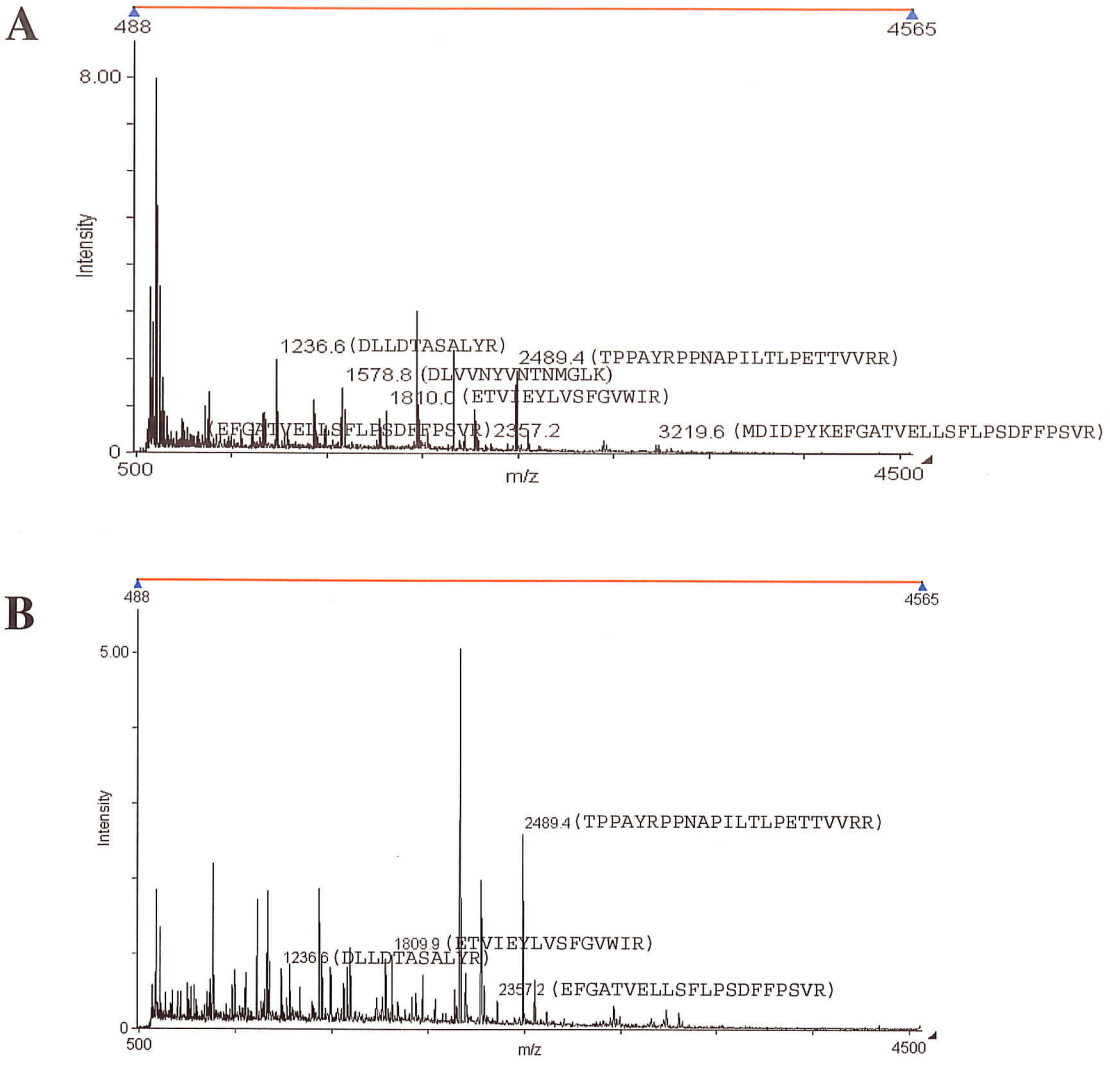

Figure 8. Mass spectrometry spectra for the expected and higher MW products observed in the GM-tobacco extracts. Representative spectra from MALDI-TOF analysis of trypsin digests of SDS-PAGE gel slices containing the expected MW band (A) and the higher MW (B) band from GM-tobacco derived HBc153.

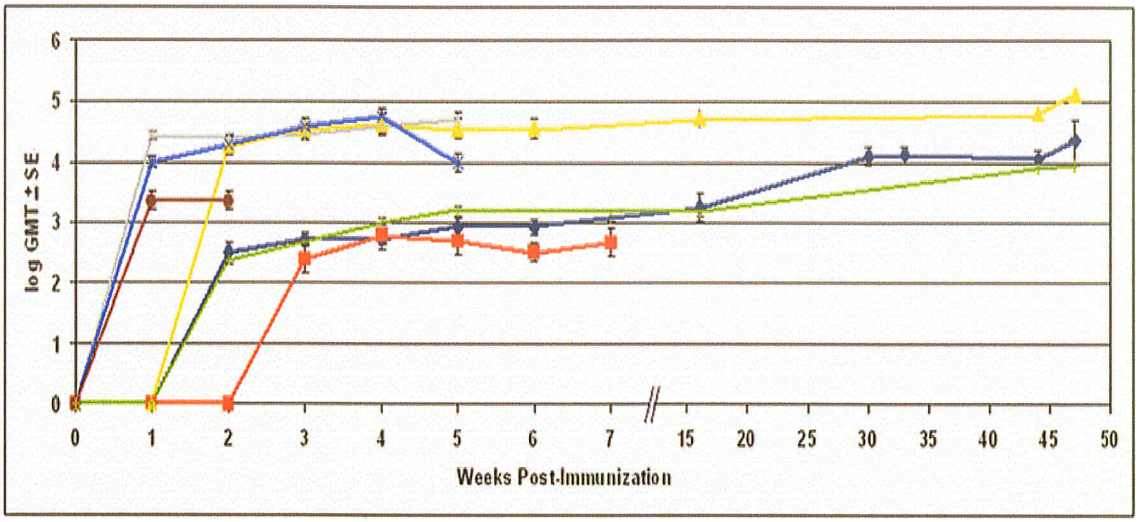

Figure 9. Mean geometric titer for total serum IgG from all mice groups. The reciprocal log geometric mean titer ± the standard error for all total IgG positive groups of mice. Mice were immunized as described and serum collected at multiple time points and analyzed by HBc153-specifc ELISA. Titer is expressed as a reciprocal of the highest serum dilution required to yield an OD_{405} value twice the control.

of the mean (SEM) of 26910±4000 and 10060±1890 for tobacco- and *E. coli*-derived HBc153-immunized mice respectively on day 7. All mice in both groups had a positive antibody titer. Both groups were boosted 21 days after the first immunization and reached a maximum reciprocal GMT of 47570±15000 (tobacco) and 56570±15000 (*E. coli*) by day 42 and 35 respectively. The similarity in magnitude and timing of the serum IgG response demonstrates that the HBc153 expressed in tobacco has an equivalent immunogenicity to HBc153 produced in *E. coli*.

3.5.2 Cellular Response

Splenocytes from mice immunized with either eHBc153 or tHBc153 were obtained and stimulated with eHBc153 and tHBc153 in ELISPOT assays to determine what cytokines these cells were producing. An increase in the number of IL-2 and IFN-γ secreting cells was only observed when the cells were stimulated with antigen from the same source as immunization. To ensure that stimulation was specific to the antigen, cells from untreated mice were also stimulated. The eHBc153 immunized mice had 134 IFN-γ secreting cells per 100000 cells while the tHBc153 immunized mice had 69 cells (Figure 10a). This is a substantial increase in IFN-γ secreting cells as the non-immunized mice showed no non-specific activation (approximately 1 cell per 100 000). For all assays, cells from each population were stimulated with the mitogen concanavilan A (conA) to ensure that the cells used were inducible. All the groups showed an equal response to conA of approximately 200 IFN-γ secreting cells per 100 000 cells. A comparable level of induction of IL-2 secreting cells was also observed with eHBc153 stimulated cells having 172 and tHBc153 having 111 IL-2 secreting cells per 100 000 cells respectively (Figure 10b). Induction of proliferation was also noted for cells from eHBc153

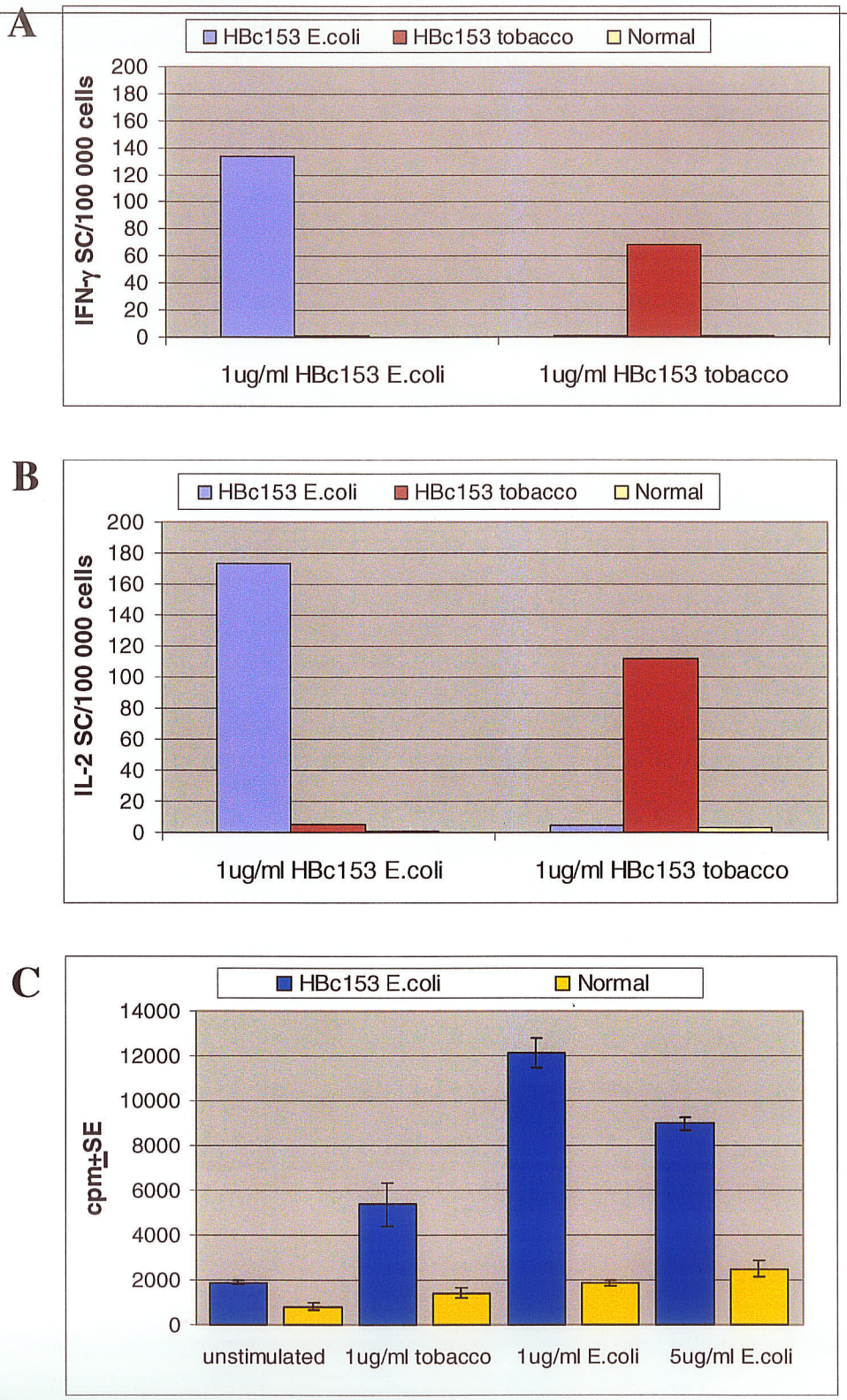

Figure 10. ELISPOT and cellular proliferation data from mice immunized with *E. coli*- and tobacco-derived HBc153. The number of cytokine producing lymphocytes from BALB/c mice immunized with 3ug of E. coli- or GM-tobacco-derived HBc153 with Quil-A. The stimulation of cells appeared to be specific to the source of the immunizing antigen.

immunized mice. For eHBc153 stimulated cells a 5-fold increase in counts per minute was noted and an almost 2-fold increase was noted when the cells were stimulated with tHBc153 (Figure 10c). Cells from the tHBc153 immunized mice were not stimulated by eHBc153, tHBc153 or conA and thus no reliable data is available for them. Cells were also screened for IL-4 and IL-10 production but no cells producing these cytokines were observed. It was presumed that the majority of the cytokines were being produced by CD4+ T helper cells. In order to ensure that this was correct CD4+ cells were removed from the population with MACS and then stimulated as with the whole population. Depletion of CD4+ cells was confirmed by flow cytometry (Figure 11). There was a drastic drop in the number of IL-2 and IFN-γ-secreting cells in the cell populations that lacked CD4+ T helper cells thus indicating their role in cytokine productions. As previous information had indicated that B cells may play an important role as antigen presenting cells for HBcAg the B cells were depleted; however, the depletion was not very effective with only a 25% reduction in B cells. However, an increase in the number of IL-2 and IFN-γ secreting cells was observed as CD4+ cells now comprised a larger proportion of the cells.

3.6 Lack of Humoral Immune Response by Orally Administered Plants

In an effort to demonstrate that GM-plants expressing HBc153 could induce immunization 10 female BALB/c mice were each fed 4g of GM-carrot containing 70μg HBc153 on day 0, 2.5g of GM-carrot containing 60μg of HBc153 on day 14 and 5g of GM-carrot containing 50μg HBc153 on day 35. Prior to carrot feeding the mice were fasted overnight; however, some mice did not consume all of the carrot that was given to them. Serum was collected to monitor the antibody response by ELISA. None of the mice

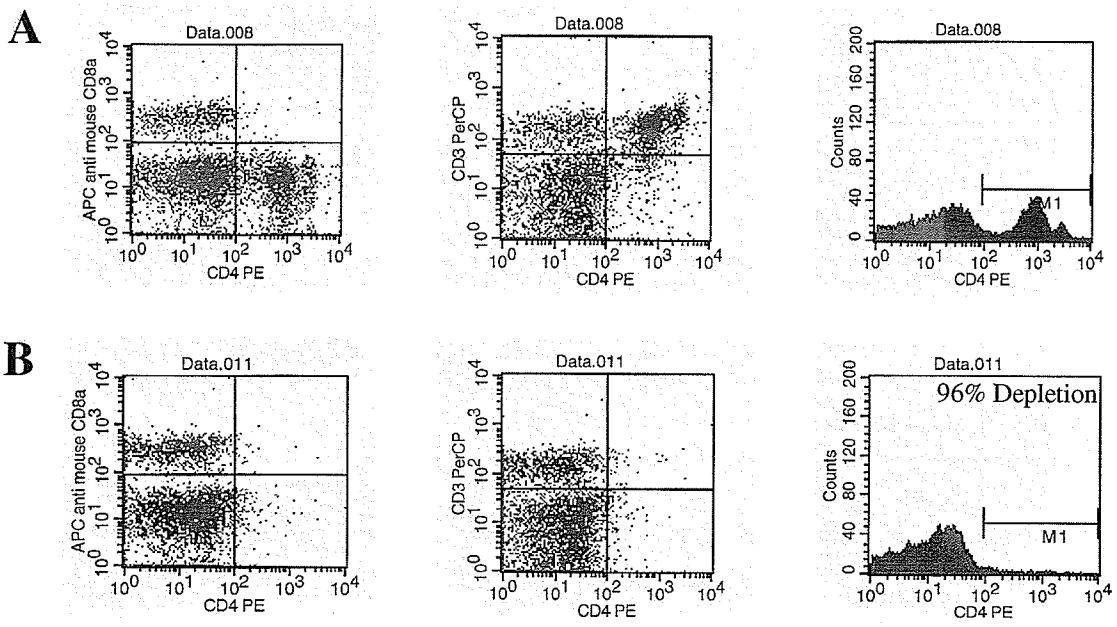

Figure 11. FACS analysis of splenocytes following CD4+ T cell depletion. Representative murine splenocytes before (A) and after (B) depletion with an anti-CD4 (L3T4) antibody using the MACS system. Following depletion, cells were stained with anti-CD3-PerCP, anti-CD4-PE and anti-CD8-APC then analyzed on a FACSCalibur flow cytometer. After depletion only 4% of the CD4+ cell population remained.

exhibited a positive IgG response by ELISA up to day 56 when the experiment was terminated. A majority of the mice ate most of the carrot given to them over the 8 hour period where there was no other food in their cage; however, the carrot was not always readily eaten. This creates a problem where all of the antigen is not administered at once as well in some cases not all of the carrot was consumed, therefore the antigen dose is less than planned. Separate groups of mice were also fed GM-rice and -tobacco seed containing HBc153. Rice was shelled and approximately 7 grains were fed to each mouse. Tobacco seed was partially crushed with a mortar and pestle and incorporated in Trans-gel. Both groups exhibited a titer of 1/50, which is the minimum titer that could be separated from the controls, after the third feeding but no further increase in titer was observed and at the time no transgenic plant material was available to attempt a further boost.

3.7 Induction of Systemic Immune Response from Orally Administered HBc153

To evaluate the amount of antigen required to induce an immune response via oral delivery we administered purified eHBc153 without adjuvant by oral gavage to female BALB/c mice and monitored serum antibody responses by ELISA (Figure 9). Mice gavaged with 150μg of *E .coli*-derived HBc153 on days 0, 14 and 35 had a reciprocal IgG GMT of 340±144 by day 14 with an initial peak GMT of 845±250 on day 49. Their GMT continued to increase as it was 1790±470 on day 112. The mice were boosted on day 204 and scheduled to be euthanized 7 days later but it was later decided to wait until baculovirus-derived HBc153 (bHBc153) was prepared for cellular stimulation studies. The boost however increased the GMT to 13450±5650. Again 1 week prior to euthanization the mice were boosted and developed a GMT 7 days later (day 328) of

22630±14890. Mice gavaged with 100μg of *E. coli*-derived HBc153 had a reciprocal IgG GMT of 250±179 by day 28 (3 out of 4 mice) with a peak GMT of 635±170 on day 49. By day 49 all mice had a positive GMT (range 200-800). Mice gavaged with 50μg of HBc153 did not produce a detectable serum response to HBc153. To summarize, HBc153 VLPs are capable of inducing a systemic immune response after two immunizations with 100μg and or after one immunization with 150μg but not after up to three immunizations with 50μg.

3.8 Induction of Systemic Immune Response from Intranasally Administered HBc153

To evaluate the effectiveness of the nasal route of delivery we administered purified eHBc153 without adjuvant by depositing 15μl of antigen, containing 45μg of eHBc153, into each nostril of inbred female BALB/c mice and monitored serum antibody responses by ELISA (Figure 9). The geometric mean titer (GMT) and the standard error (SE) were calculated for each group. Mice immunized on days 0, 14 and 35 with 90μg of *E .coli*-derived HBc153 intranasally had a reciprocal IgG GMT of 18720±6070 by day 14 with an initial peak GMT of 42110±13000 on day 35. Their GMT continued to increase as it was 52640±22170 on day 112. The boost one week prior to euthanization increased the GMT to 134540±20000.

3.9 Poor Induction of Humoral Immune Response in Rabbits Orally Administered Transgenic Carrots

Two rabbits were fed 50, 35 and 38g of GM-carrots each on days 0, 14 and 35 that contained 400, 550 and 300μg of HBc153 respectively. The carrots had to be rolled in alfalfa to coax the rabbits in to eating them but were consumed within 1 hour of

presentation. At day 42 one of the rabbits had an IgG titer of 1/100 that was specific for tobacco-derived HBc153 only. This titer persisted until week 25 when the rabbit was euthanized. No HBc153-specific IgA was detected in fecal extracts.

3.10 Induction of Mucosal Immune Response from Orally and Intranasally Administered HBc153

To evaluate the ability of orally and intranasally delivered HBc153 VLPs to induce the production of mucosal antibodies fecal specimens were extracted to test for the presence of HBc153- specific IgA by ELISA. Pre-immunization fecal samples were obtained and a control group that was not manipulated was used for baseline comparison. A 2-fold increase in OD value over the control was considered a positive result. Oral delivery of 50 and 100μg was not sufficient to observe an increase in OD; however, 150μg delivered orally was sufficient to observe an increase in OD at week 5 post-immunization (Figure 12). The amount of IgA did not appear to increase immediately after the third immunization as no increase was observed at 8 weeks. The peak amount of IgA was detected at 24 weeks with an OD value approximately 8 times the control value. HBc153 delivered intranasally induced an IgA response at week 5. There was an increased amount of HBc153-specific IgA in the orally immunized mice as compared to the intranasally immunized mice. The initial peak IgA occurred at week 8 but a further increase was noted at week 33 following a boost on day 204 (week 30). This indicates that eHBc153 VLPs are immunogenic when delivered orally and intranasally in a sufficient amount that they are capable of inducing an intestinal IgA in the absence of a mucosal adjuvant; however, a relatively large dose of protein is required in induce a detectable immune response.

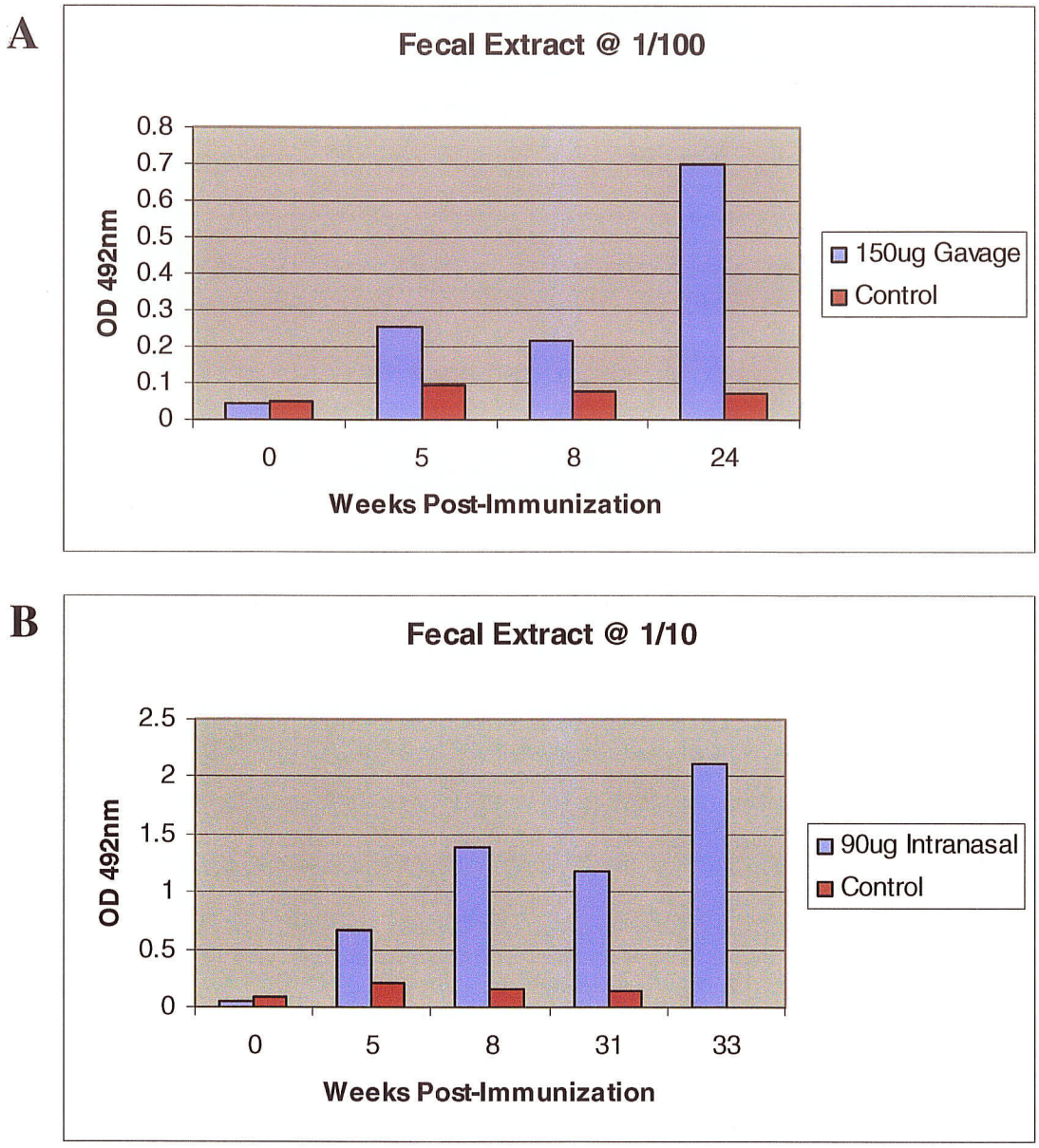

Figure 12. HBc153-specific IgA from fecal samples of mucosally immunized mice. To determine if there was an increase in HBc153-specific IgA, feces from mice immunized either intranasally or by gavage was extracted and subjected to HBc153-specific ELISA. An increase in HBc153-specific IgA was noted over the control mice at a 1/100 dilution for the mice immunized by gavage and at a 1/10 dilution by mice immunized intranasally,

3.11 Isotyping the Serum IgG Response of Parenterally and Mucosally Immunized Mice

In order to determine the nature of the immune response serum IgG isotypes were also monitored (Figure 13). Mice immunized with HBc153 by a mucosal route showed primarily IgG_{2b} (500±235 ng/ml) at the outset of the immune response (day 14 – 150µg gavage, 90µg intranasal; day 35 – 100µg gavage), however an increase in the IgG_1 isotype was noted by day 35 in the 150µg gavage group (530±250ng/ml). Until day 136 the 150µg gavage group continued to increase production of IgG_1 and IgG_{2b}. After the boost the amount of all isotypes increased substantially with the predominant isotype being IgG_{2a} (14600±6300 ng/ml) at day 252 with nearly equal levels of IgG_1 and IgG_{2b} (7200±1100 and 8300±1500 respectively). The IgG_3 level also increased (3800±1430ng/ml). After the final boost 7 days prior to day 352 IgG_3 became the predominant isotype (24000±12990) with nearly equal levels of IgG_1 (9830±2560) and IgG_{2b} (11500±4680). The IgG_{2a} level had decreased at this time (5780±1870). The 100µg gavage group showed an increase in IgG_{2b} (1315±780), the only isotype produced, at day 49. After a boost there was increase in IgG_1 (1800±1750), and IgG_{2a} (1480±630) such that they were similar to IgG_{2b} (1520±535). There was also a small increase in IgG1 (500±350). Intranasal mice showed an increase in IgG_{2a} (10750±1750) and IgG_3 (56375±23295) and a decrease in IgG_{2b} (30000±5400) on day 49. Post-boost IgG_{2a} (127500±22500) was the primary isotype with decreased IgG2b (7960±5300). Substantial levels of IgG_1 (66250±1320) and IgG_3 (51200±20500) were also present.

Mice immunized subcutaneously with eHBc153 initially produced IgG_{2b} (41125±13500 and 25500±10500) with substantially lower levels of IgG_1 (11525±3780

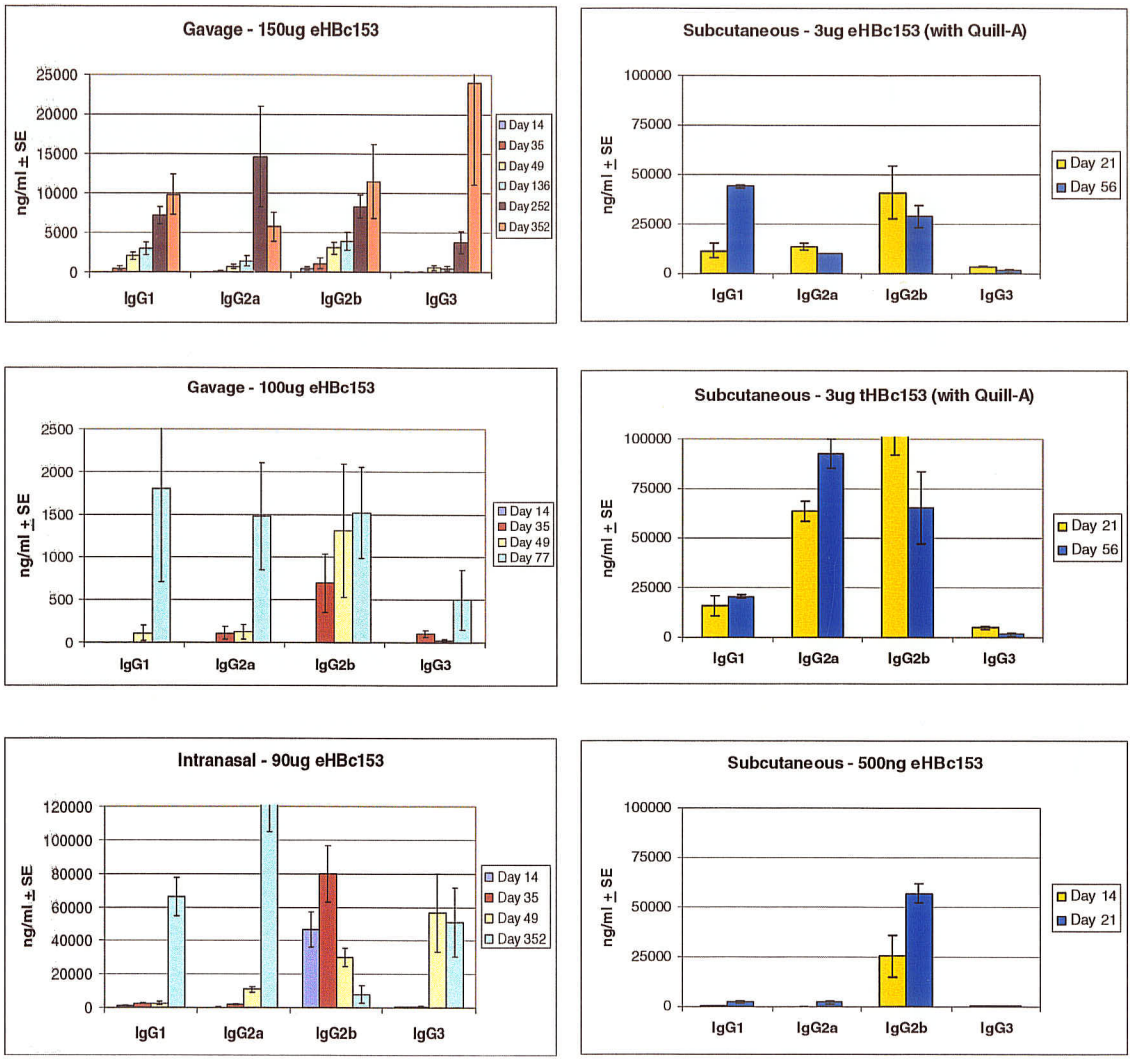

Figure 13. Serum IgG isotype profile from mice immunized by different routes. The anti-HBc153 IgG isotype profile from the serum of mice immunized with HBc153 by the route and source indicated was determined by isotype specific ELISA using commercially available purified IgG isotypes as standards. The value for each isotype was expressed in ng/ml ± the standard error of the mean.

and 320±170) and IgG2a (13500±1810 and 155±55). Mice immunized with the GM-tobacco-derived HBc153 also produced more IgG_{2b} than the other isotypes; however, they also produced substantially higher levels of IgG_{2a} but comparable levels of IgG_1. After boosting mice immunized with eHBc153 produced similar levels of IgG_{2b} and IgG_{2a} but showed an increase in IgG_1. Mice immunized with tHBc153 showed an increase in IgG2 to where it became the dominant isotype and a decrease in IgG_{2b}. Mice immunized with 500ng without adjuvant showed only an IgG_{2b} response. In all three groups the amount of IgG_3 produced was negligible.

3.12 Cellular Response to Mucosally Delivered HBc153

The T cell memory response to eHBc153 delivered orally was assayed for cytokine production. To account for any non-specific activation cells from control mice were stimulated with the same antigens as the immunized mice which was then subtracted from the immunized mice. To ensure cell viability all cells from all groups were stimulated with conA and induction of IFN-γ and IL-2 secreting cells was observed. While a low level induction of IFN-γ was observed in mice immunized by the nasal route and subcutaneously (2.5- and 2-fold increase respectively), (Figure 14a) when the standard error is included there appears to be no stimulation, furthermore the non-responders were omitted. As observed in the subcutaneously immunized mice, stimulation appeared to be dependant on the source of the antigen. These mice were immunized with eHBc153 and the strongest induction was with eHBc153. The bHBc153 was not able to induce stimulation with any of the cells. The tHBc153 induced a small increase in the IFN-γ from intranasally and subcutaneously immunized mice. A 3-fold increase in the number of IL-2 secreting cells was noted for the mice immunized

intranasally (Figure 14b); this value was just beyond the standard error range. A 2.5-fold increase was noted for mice immunized with 150μg of eHBc153 by gavage; however, the SEM with the controls was overlapping. Production of IL-4 and IL-10 was not observed.

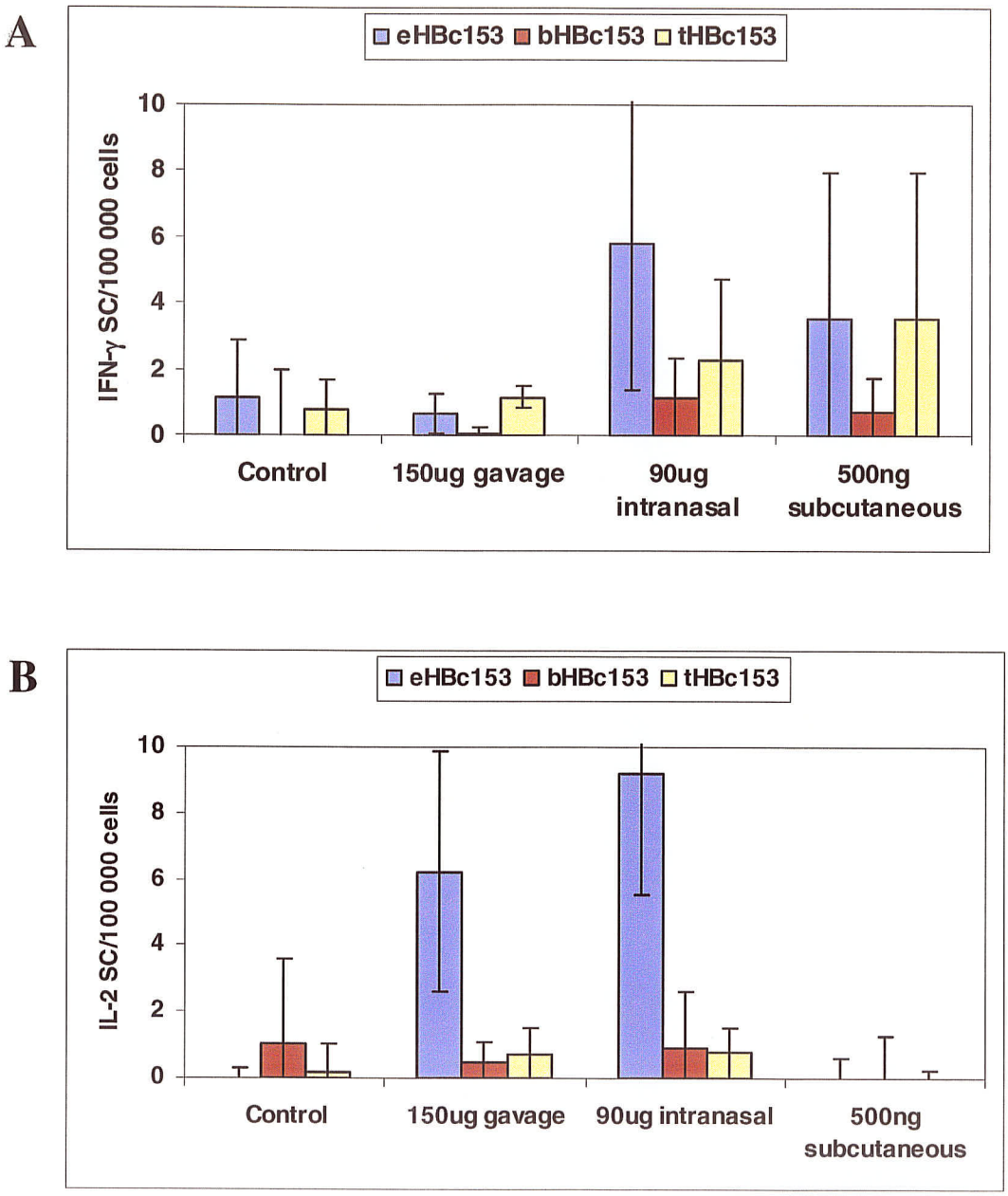

Figure 14. ELISPOT data from mice immunized via a mucosal route with *E. coli*-derived HBc153. The number of IFN-γ (A) and IL-2 (B) secreting cells per 100 000 cells harvested from murine spleen following immunization as described previously for the groups indicated on the X-axis. The legend indicates the source of the antigen used to re-stimulate the cells (eHBc153: *E. coli*-derived HBc153, bHBc153: Baculovirus-derived HBc153 and tHBc153: tobacco-derrived HBc153).

4. Discussion

4.1 Overview

While immunizations represent one of the most effective health interventions in history there are still numerous infectious diseases that lack vaccines. In fact, infectious disease currently account for approximately 26% of all deaths worldwide and 45% of all deaths in low-income countries (Bloom & Lambert, 2001). In addition, there is also a widespread inability to deliver immunizations to populations in developing countries thus creating a need to develop additional vaccines and new methods of delivery. Despite the experience that vaccination significantly reduces mortality from infectious disease there are 30 million children born each year that are not immunized to today's standard (Arntzen et al., 2005). The WHO and other organizations have encouraged the development of new technologies that will increase global immunization compliance and decrease the cost of delivery. Ideally new vaccines will be heat-stable and not require the infrastructure associated with parenterally delivered vaccines; namely a cold-chain, syringes, needles and highly trained personnel. A novel method involving the production of vaccines in GM-plants has been proposed (Mason *et al.* 1992) as a way to produce a cheap, transferable and safe source of vaccines. The number of potential vaccine antigens that have been expressed in plants continues to grow with more than 45 relevant antigens now expressed (as of 2003). These include antigens from anthrax, classical swine fever virus, diphtheria, enterotoxigenic *E. coli*, hepatitis B virus, hepatitis E virus, HIV-1, human cytomegalovirus, measles virus, Newcastle disease virus, Norwalk virus, rabies virus, respiratory syncytial virus, SARS-CoV, and smallpox virus. Oral delivery of plant-based vaccines is particularly attractive for developing countries as it would be safe, cost-

effective, requires low technology input, abolishes the need for a cold chain and needles and syringes. The potential of vaccine contamination with animal pathogens is avoided when the antigen is generated in plants. Many viruses and bacteria establish infections at mucosal surfaces therefore a vaccine strategy that involves immunization at these surfaces is of great interest.

Previous studies have indicated some success at eliciting an immune response from animals fed GM-plants expressing a foreign protein. In an effort to create an improved method of vaccination genetically modified plants; including carrots, rice and tobacco; were created that produced a truncated form of HBcAg containing the N-terminal 153 amino acids. Similar to HBcAg, HBc153 is capable of spontaneous self-assembly into virus-like particles (VLP). There are a number of properties of HBcAg that make it an attractive vaccine component. The ability of HBc153 to form VLPs results in repeating epitopes that appear to be very effective at stimulating an immune response, which probably results in the observation that nanogram quantities are sufficient to induce an immune response in mice. Also, HBcAg tends to induce a T_{h1}-like response and it has been shown to be an effective carrier of heterologous antigens. While the use of non-replicating antigens poses a difficulty in oral immunization due to the low pH and proteolytic enzymes of the digestive tract, particulate antigens are resistant to damage from low pH. In this project we characterized HBV nucleocapsid produced in plants and its immunogenicity using different routes of immunization.

4.2 Production of VLPs

Expression of potentially useful quantities of foreign proteins in a number of plant species is possible with the construction of appropriate enhancing elements and codon-

optimization. Generally, codon-optimization of non-plant sources results in increased expression of the recombinant protein (Cheng et al., 1998; Hovarth et al., 2000). In the case of HBc153 produced in tobacco, codon-optimization alone (H1 vs. H2 constructs) provided the largest increase (5.4x) in HBc153 expression (Figure 4, 5). Retention of HBc153 in the ER (H3 construct), thus avoiding trafficking to the cytosol where degradation may take place, resulted in a negligible increase in HBc153 expression demonstrating that protection from cytosolic degradation may not be necessary. In our work the optimal elements were contained in the H4 construct, which was codon-optimized and contained an extensin signal sequence that directed HBc153 to the cell wall via ER (De Loose et al., 1991). Signal sequences allow for precise targeting of proteins into specific storage locations and have been demonstrated to increase protein expression. The result was that the H4 construct showed a 3x increase in the expression of HBc153 versus the H2 construct. Our results confirm that for the expression of foreign proteins in plant systems the effort to optimize the codon usage to the species of production is well worthwhile. Despite the presence of a higher molecular weight band in the HBc153 purified from plants there appeared to be no difference in the immunogenicity and reactivity of the antibodies produced. The production of antigens in systems other than mammalian may result in different glycosylation patterns; however, the importance of these differences must be determined on a per antigen basis. In the case of hemagglutinin from influenza virus it appears that it is irrelevant if the antigen is produced in a baculovirus or a mammalian system (Jones et al., 2003).

In the GM-carrot system the H4 construct also showed an improvement over the wild-type sequence of HBc153 (3.8x increase) (Figure 6). However, despite using the

best construct for expression, in that they are the most palatable, the highest observed percentage of fresh weight of HBc153 was 1.73% in the carrots. This is much lower than the tobacco plants and resulted in suboptimal quantities of antigen being delivered to the animals in the feeding trial. Further modification of the promoter and enhancers may provide a higher level of protein expression; however, it may also be possible that carrots may not be the optimal plant for the delivery of edible vaccines. Carrots seem a suitable production vehicle to deliver an oral vaccine as they are regularly consumed uncooked, have a long shelf-life and are highly transportable. At the outset of the project there was no information on using carrots to deliver foreign proteins despite the attractiveness of this plant. Since then, in only one publication (Marquet-Blouin et al., 2003) carrots were used as a production system for vaccine preparation (measles hemagglutinin); however, they only investigated using partially purified product delivered intraperitoneally and found that it was immunogenic.

One of the benefits of using plants as the production system is the relatively lower cost. A brief look at a couple of eukaryotic expression systems reveal that at least in the case of producing recombinant IgA plants have the lowest production cost (Figure 15). Assuming that purification costs and loss during purification were similar for all of the systems the main cost was associated with production. Transgenic plants (either from green biomass or from seed are substantially lower cost than mammalian cell culture with transgenic goats being somewhere in between. Unfortunately, the production cost for yeast expression systems were not included as this system is used to produce HBsAg for the HBV vaccines. Presumably, both yeast and Baculovirus expression systems would

Figure 15. Cost per gram for purified Immunoglobulin A produced in different expression systems (see Daniell *et al.*, 2001, Figure 1, p.221). The costs for protein production in mammalian cell culture (red diamonds), transgenic goats (green circles), seed production (blue squares) and green biomass (green triangles) are based primarily on the production costs and assume similar costs and loss during purification.

result in a lower production cost than mammalian cell culture systems but it is unknown if they would be more cost effective than plants.

4.3 Humoral Immune Response

Our results demonstrate that tobacco-derived HBc153 administered subcutaneously to BALB/c mice has a similar immunogenicity to that of the eHBc153 (Figure 9). Both groups of mice showed a rapid increase in anti-HBc153 titer (by day 7) that was similar in magnitude and reached a comparable peak titer on a similar timeline. Furthermore, the antibodies produced were equally reactive to either antigen source. It was important to demonstrate that the HBc153 antigen from different sources has similar immunological properties as it allowed comparisons to be made between different delivery mechanisms.

When initial feeding trials, using GM-carrots, rice seed and tobacco seed did not yield a detectable immune response it was decided to immunize mice by oral gavage with purified eHBc153 to determine the quantity of antigen that was required orally to induce an immune response. Two doses of 100μg or one dose of 150μg of eHBc153 were required to induce a detectable anti-HBc153 IgG response (Figure 9). Furthermore, after 3 doses the titer was still not particularly high. Three doses of 50μg was not sufficient to induce an immune response. The mice immunized with the 150μg dose were maintained for an extended period of time to study the effect of a later boost. Boosting approximately six months after the initial immunizations caused a significant increase in titer. This indicates that a better immunization schedule would include a booster immunization after the initial regime; however, further studies would be necessary to optimize a schedule.

Mice fed 70, 60 and 50μg of HBc153 delivered in GM-carrots did not exhibit a detectable anti-HBc153 IgG immune response. Unfortunately, the quantity of antigen they received fell below that of the lowest value used (100μg) that elicited an immune response from eHBc153 gavaged mice. Therefore, the amount of antigen delivered was less than optimal. This was due to the level of expression of HBc153 in the carrots and the fact that mice would not eat more than 5g of carrots at a time. A number of other complicating factors were also present in the feeding trial. In some instances all of the carrot was not eaten over the 8 hour period where that was the only food available. Also, some mice did not eat all of the carrot at once. The implication of these observations is that some animals did not consume the amount of antigen that we desired them to in addition to spreading the ingestion of antigen over an 8 hour period in contrast to receiving all of it at once as in the gavage. Presumably this would not be problematic in humans as compliance could be conveyed verbally. In addition, there was some evidence that the carrots were not totally digested, as evidenced in the mouse feces, thus it is unlikely all of the antigen in the carrots would have been released. A further attempt to boost these animals at a later date was not possible as no more GM-carrots were available.

In initial feeding experiments using GM-carrots, -rice seed and -tobacco seed the amount of HBc153 delivered was not accurately calculated; however, it was less than 50μg per dose. The mice in these groups did not produce a detectable immune response to the antigens, which in retrospect is not surprising as the amount of antigen delivered was far below the required amount. Mice that were immunized intranasally with 90μg of eHBc153 showed a rapid and large increase in anti-HBc1533 titer by day 14. Others have

shown that intranasal delivery is more effective than oral administration for the induction of immune responses to low doses of antigen (Estes et al., 2000). While nasal administration of antigen can cause antigen to enter the lung or the digestive tract the small volumes used in our experiments (15 µl) should have ensured retention of particles in the nasal passages (Davis, 2001). If the vaccine was to target pathogens that primarily enter through the respiratory tract it is important to note that despite the CMIS nasal delivery is superior to oral delivery for stimulation of IgA production in the upper respiratory tract (Rudin et al., 1999). In a previous study, mice that were immunized against influenza virus and then challenged were clearly better protected when immunized through a nasal route (100% survival, <8% weight loss) versus intramuscular injection (80% survival, 15% weight loss) (Jones et al., 2003). While both groups had identical serum IgG titer only the mucosally immunized mice had anti-influenza IgA indicating that this may provide extra protection by minimizing infection at the point of entry.

The difficulties in inducing an immune response from antigen have been experienced by other groups as well. Mice fed potatoes expressing HBsAg (average of 42µg per dose plus 10µg CT as an adjuvant, weekly for three weeks) developed a weak antibody response after the second dose. Shortly after the last dose the titer returned to baseline. However, mice that were boosted with an additional dose exhibited a stronger secondary response. While these results were encouraging, CT cannot be used in human vaccines and feedings without CT did not induce a substantial prime-boost as seen with CT, indicating that a strong adjuvant was necessary. Furthermore, they were only able to

deliver a limited amount of antigen as the mice would only eat approximately 5g of potato.

In order to try to compare the efficiency, as determined by the serum IgG titer, for each route of vaccination the titer per quantity of antigen was calculated and indicates that gavage is 3000x less effective than SC with Quill A, 2000x less effective than SC and 85x less effective than intranasal immunization. It was expected that SC immunization would be considerably more efficient than the immunizations delivered to mucosal surfaces. The efficiency was in the order expected with subcutaneous > intranasal > oral as previous results had observed a 100-fold difference between SC and oral delivery (LoMan et al., 1995). The difference between oral and intranasal immunization is similar to results seen with Norwalk virus capsid particles (Guerrero et al., 2001). The consequence of the poor efficiency of oral delivery is that if an edible vaccine were to be used in humans or other large animals the amount of plant that would have to be consumed could be quite substantial.

Other groups have recently demonstrated the utility of using a prime/boost strategy utilizing different delivery routes for the prime and the boost. Following subcutaneous, intranasal or intraperitoneal but not oral priming an oral boost could enhance the serum IgG and fecal IgA levels to ovalbumin (Lauterslager et al., 2003). The prime/boost regimen could have enhanced the oral immunization with eHBc153 and may have allowed the GM-carrots to be useful as a booster following a subcutaneous or intranasal prime. Unfortunately, additional GM-carrots were not available after the completion of the other feeding trials; however, it would have been beneficial to attempt a prime-boost trial with eHBc153. A prime/boost strategy, while still having the

increased costs associated with the initial prime, would still result in a decreased overall cost as the boost would presumably be much less expensive to deliver.

4.4 Isotyping

IgG_{2a} is most often induced by viral infections or antigens, which is not surprising as IgG_{2a} and IgG_{2b} are the most effective at complement activation and antibody-dependent cell-mediated cytotoxicity (Ball et al., 1998). Isotyping of the serum antibody response revealed some differences in the predominant isotype dependant on the route of immunization and quantity of antigen. All groups with a positive serum IgG titer initially produced IgG_{2b} in the highest quantity (Figure 13). This is in accordance with previous studies that indicated that immunization with HBcAg results in production of $IgG_{2a/2b}$ over IgG_1 with little IgG_3 (Milch et al., 1997; Pumpens et al., 2002). This profile is also consistent with a predominantly T_{h1}-like response (Nicholas et al., 2003). However, at later time points the gavaged mice showed an increase in IgG_{2a} and IgG_1 which is more indicative of a mixed T_{h1}/T_{h2} response. Furthermore, the mice gavaged with 150µg of eHBc153 showed a large increase in IgG_3 following a boost one year after the initial immunization. The tHBc153 SC immunized mice show a marked $IgG_{2a/2b}$ predominance; however, the eHBc153 immunized mice show an increase in IgG_1 that exceeded the IgG_{2b} levels. The serum IgG isotyping indicates that the different routes of immunization appear to produce, at least initially, a T_{h1}-like immune response. The later development of a mixed response demonstrates the complexity of the immune response but is encouraging as it appears that a potentially beneficial immune response is ongoing.

4.5 Cellular Immune Response

The observed prevalent isotype is indicative of an initial T_{h1}-like response. This is in agreement with previously published data that indicated that HBcAg primed T cells generated T_{h1}-like responses with production of IL-2 and IFN-γ and low level production of IL-4 (Milich et al., 1997 b). The production of IL-2 and IFN-γ by stimulated cells from SC immunized mice confirms what the isotyping indicated – HBc153 is inducing a T_{h1}-like response (Figure 10). IL-2 is a T cell growth and survival factor mainly produced by $CD4^+$ T cells that induces the proliferation of T lymphocytes in response to antigenic stimulation, including cytotoxic and regulatory T cells (Lin & Warren, 2003). T_h $CD4^+$ cells secrete IL-2, IFN-γ, and lymphotoxin-α (LT-α). IFN-γ, IL-12 and IL-18 promote a T_{h1} phenotype. T_h cells activate macrophages resulting in delayed-type hypersensitivity responses and lysis of intracellular parasites. (Okada *et al.*, 2003). The proliferation observed with ^{3}H-thymidine also supports positive T cell response is occurring (Figure 10). An unexpected observation in the cellular response assays was that stimulation was dependent on the source of the antigen. For example, cells from animals immunized with eHBc153 were only stimulated by eHBc153. This was unexpected as the antibody response to the antigens from different sources was cross-reactive. While the presence of LPS in the eHBc153 and lectins in the tHBc153 are possible these antigens did not non-specifically stimulate cells from control mice. Ultimately, the explanation for the cellular response being specific to the antigen source is unknown.

The absence of T_{h2}-like cytokine secreting cells (IL-4 and IL-10) supports that a T_{h1}-like response is the predominant response occurring; however, detection of IL-4 is difficult. T_{h2} $CD4^+$ cells secrete IL-4, IL-5, IL-6, and IL-10 the induction of, which was

not observed. IL-4 induces a T_{h2} phenotype except in the presence of TGF-β, which causes a T_{h1} phenotype. T_{h2} cells control humoral responses including the production of IgE and associated eosinophilia. Helper cell subsets directly negatively regulate the activity of the other subset. IFN-γ inhibits the proliferation of T_{h2} cells while IL-4 inhibits cytokine production by T_{h1} cells. Regulation also occurs at the effector cell level where IFN has an inhibitory effect on IL-4 induced B-cell activation and IL-4 has an inhibitory effector on IL-2 induced T- and B-lymphocyte proliferation (Okada et al., 2003).

The very minor to total lack of inducation of IFN-γ and IL-2 secreting cells in the mice immunized by an intranasal or gavage route may indicate that a these routes may not induce a cellular response (Figure 13). It is possible that the amount of antigen delivered was not sufficient; moreover, it is also possible that systemic T cell toleranace was induced. This is difficult to determine; however, these mice demonstrated strong serum IgG levels indicating that at least humoral systemic tolerance was not generated.

4.6 Mucosal Immune Response

The only orally immunized group of mice to demonstrate a significant increase in the amount of HBc153-specific IgA was the group that was administered the 150μg of antigen (Figure 12). The increase was slightly delayed as it was first detected 5 weeks after the first immunization; however, an increase was observed following boosting. Therefore, oral delivery of antigen in sufficient quantity is capable of inducing an increase in antigen-specific IgA. The ability to generate a secretory IgA response is important especially for pathogens that enter through the mucosal surfaces. The group of mice that were immunized intranasally produced less antigen-specific-IgA as compared to the orally immunized group. The time course to development of this response was

similar as the increase was first detected at the same time point as the orally immunized mice (week 5); however, the peak amount after the initial immunization occurred at week 8. Also, the increase in IgA was not substantial after a booster. The time to detectable mucosal IgA response was similar to Li et al. (2001) who observed the intestinal IgA response to HEV VLPs peaked around week 8. This observation of HBc153-specific IgA in fecal extracts from mice that were immunized intranasally provides a clear demonstration of how an immunization at one location of the mucosal immune system can result in an effect at a distant site. It is also suggestive that nasal vaccinations may provide protection against pathogens that enter from non-respiratory mucosal surfaces.

4.7 Tolerance

One of the most frequent concerns raised about edible vaccines is the possibility of inducing tolerance to the delivered antigen. However, in what seems rather paradoxical it is possible to both stimulate mucosal immunity and induce systemic tolerance concomitantly. This has been observed by the presence of secretory antibodies and by diminished T-cell responses following oral antigen administration (Mestecky et al. 2005). However, it appears that particulate antigens, such as HBc153, when delivered orally do not induce tolerance and when delivered at a sufficiently high dose can induce a secretory antibody response. This is in contrast to non-particulate soluble antigens and antigens that are delivered daily which tend to induce oral tolerance that is mediated by cellular or humoral suppressor factors. In our studies we did not observe the induction of systemic humoral tolerance. Mice that were immunized with eHBc153 by oral gavage showed high initial serum IgG titers, which subsequently increased following an oral booster at weeks 30 and 45, indicating that they had a systemic humoral response. The absence of T

cell activation in the orally immunized mice could be an indication of systemic T cell tolerance; however, it is also possible that a detectable cellular response was not induced. Previous studies by another group using neo-antigen-keyhole limpet hemocyanin (KLH) in humans demonstrated that ingestion or nasal application of this antigen led to a humoral mucosal immune response but suppressed T-cell mediated proliferation while systemic administration led to systemic immune responses (Mestecky et al. 2005). More importantly the humoral and cellular immune responses elicited from systemic administration could not be suppressed by subsequent ingestion of large quantities of KLH. Numerous studies (Sun et al., 1996; Yoshino et al. 1995; Moldoveanu et al., 2004) have all observed that following systemic immunization tolerization by long term ingestion of the antigen does not occur. These findings are important in developing the sequence of administration of potential oral vaccine candidates (Mestecky et al. 2005). While oral administration of an edible vaccine may carry a risk of inducing tolerance that would need to be carefully investigated the use of such a vaccine as a booster would appear to be indicated.

4.8 Summary

The truncated form of the Hepatitis B virus nucleocapsid was successfully expressed in *E. coli*, baculovirus, GM-tobacco, GM-rice and GM-carrots. In all systems it self-assembles into virus-like particles, which are highly immunogenic. The different plant constructs produced varying levels of HBc153. The H4 construct, containing a plant codon-optimized sequence and an extensin signal, produced the highest amount of HBc153 in both tobacco and carrots. HBc153 had a similar immunogenicity whether it was produced in transgenic tobacco plants or in the *E .coli* expression system as assessed

in BALB/c mice. Mice that were fed GM-plant products, such as rice seed, tobacco seed and carrot root did not produce a detectable serum IgG or fecal IgA immune response. While the lack of success in the feeding trials was unexpected it was demonstrated that a larger quantity of antigen (eHBc153) could be delivered orally by gavage and induce both a systemic and local immune response. Using eHBc153 it was demonstrated that while an immune response could be generated when antigen was delivered orally, intranasally and subcutaneously that the oral and intranasal routes were much less efficient. These results show that HBc153 antigen can illicit an immune response when delivered by a oral, intranasal and subcutaneous immunization; however, the GM-plants created for feeding trials did not contain a sufficient quantity of antigen in order to induce an immune response when fed to animals.

4.9 Future Directions

While results with edible vaccines have not been overly encouraging there are indications that effective subunit oral vaccines are possible. Moreover, it is possible that production of such vaccines could occur in plants. Our results show that particulate antigens such as HBc153 can induce an immune response, without the use of adjuvants, when delivered orally if there is sufficient quantity. However, the use of unprocessed plants to deliver edible vaccines to humans seems unlikely as most groups have the similar problem we observed – there is not enough antigen per plant mass. Currently, there are a number of difficulties in delivering subunit vaccines orally that need to be addressed. Without effective and safe oral adjuvants plant material will need to be concentrated in order to achieve immunogenic levels of antigen in a reasonable amount of plant material. The plant-to-plant variation in antigen expression does not appear to

have a solution and remains a large regulatory obstacle. Despite these difficulties Hepatitis B core protein appears to be a suitable candidate for use as a vaccine vehicle and further study should be done to determine its effectiveness at presenting foreign epitopes when delivered orally. While local and humoral tolerance was not observed we could not discount the presence of systemic T cell tolerance, further research should investigate this more thoroughly.

Before oral subunit vaccines can be used it must be confirmed that vaccines delivered through this route do not induce oral tolerance (Webster et al., 2002). Oral tolerance is cause for considerable concern if antigens are delivered orally in a food product. However, continuous or repeated exposure is generally needed to induce tolerance and this would not be part of a vaccine regimen. While the expression of vaccine antigens has generally occurred in commonly consumed foods this does not mean that they should become a part of a regular diet. The vaccine producing GM-plants should be treated as a pharmaceutical and administered appropriately.

4.10 Will We See Edible Vaccines in the Near Future?

It seems unlikely that an edible vaccine produced in GM-plants will be available anytime soon although the production of vaccines in plant systems certainly seems possible in the near future. There are two primary difficulties that must be overcome. First, despite using a wide range of antigens and delivering them in a number of different types of plants there has not been a lot of success at eliciting an immune response from any animal fed plants expressing a foreign protein. Our results, using a strong antigen reinforce this observation. In many cases it is just not possible to deliver enough antigen orally in the plant when foreign protein are expressed at 1-5% TSP. The second difficulty

lies in the plant-to-plant and field-to-field variability of GM-plants (Smith et al., 2003). However, freeze-drying the plant to create powders or pellets may help to overcome both of these obstacles – creating a more concentrated amount of protein and allowing for batch quantification. Following processing, the ability to store the antigen at room temperature remains and the concentration of the antigen can be increased by 20-30 fold. So far the only human trials with edible plants containing hepatitis B surface antigen have demonstrated the ability to boost an already present immune response. While freeze-drying adds to the production cost the technology of crop production and food processing and storage are already developed in most countries (Bonetta, 2002).

The environmental impact of GM-plants is still unknown. It is not expected that the expression of bacterial or viral antigens would confer any selective advantage to the plant. However, the concern of transfer of genes to non-target organism would have to be addressed. In most plant species the chloroplast genome is maternally inherited. Therefore, with the advent of chloroplast transformation the transgene and any expressed protein would not be present in the pollen thereby reducing the risk of transmission to neighboring crops or weeds by cross-pollination (Webster et al., 2002). However, to satisfy regulators and environmentalist it may be necessary for GM-plants to be grown in some sort of biological containment setting to avoid introduction into wild species (Arntzen *et al.* 2005). While this would increase production cost it is not difficult to construct containment greenhouses as this already routinely done.

It seems that the first commercial introduction to plant-based vaccines may be in livestock. This would provide a clear demonstration that such a vaccine system can be effective which may lead to the use of these types of vaccines in humans. Edible vaccines

for humans may have the greatest utility as a booster following primary vaccination with traditional vaccines. This would still result in a cost reduction over delivering booster vaccination by the traditional route.

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