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CHAPTER SIXTEEN

Toward Mucosal DNA Delivery: Structural Modularity in Vaccine Platform Design

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Contents

16.1 INTRODUCTION

The hepatitis E virus (HEV) is a nonenveloped, positive-sense RNA virus that causes viral hepatitis in humans and infects several other animals. Like other hepatitis viruses, it mainly replicates in the liver and causes acute and self-limiting viral hepatitis.[1]

HEV is feco-orally transmitted and has caused large waterborne epidemics in various parts of the developing world. Recently, its status as a zoonosis has been realized. Pigs are

known to be reservoirs of the virus, and zoonotically transmitted hepatitis E occurs due to consumption of contaminated pork.[2]

HEV virions are ~34 nm in size, are nonenveloped, and have a 7.2 kB single-stranded positive-sense RNA genome that is capped and poly(A) tailed. Four genotypes—Gt 1, 2, 3, and 4—exist with only a single serotype, and these have geographically distinct distributions. Of these, Gt 3 and 4 are found in pigs as well as humans. The genome encodes three proteins from three open reading frames (ORFs): 1, 2, and 3. ORF1 and 3 proteins have early and late nonstructural functions, respectively. ORF2 encodes a 660 amino acid-long capsid protein that self-assembles into virus-like particles (VLPs) when expressed in mammalian, insect, or bacterial cells.[3] Like the feco-orally transmitted virus, VLPs exhibit great stability in the harsh environment of the gut. This and various other properties of these VLPs make them very attractive tools for developing orally deliverable nanoparticles carrying foreign epitopes, heterologous DNA, and small molecules. These properties have been outlined in this chapter in the context of our knowledge about the virus.

16.2 DISCOVERY AND CRITICAL LANDMARKS IN HEV BIOLOGY

A large outbreak of jaundice occurred in New Delhi in 1955–66 as a result of contamination of drinking water with raw sewage. It was initially thought to be caused by the hepatitis A virus (HAV), although HAV was not detected upon retrospective testing of stored serum samples of patients. Further, the clinical signs and symptoms were different from HAV infection, and it was suggested that a novel infectious agent was responsible for the outbreak.[4] This disease was first called enteric non-A, non-B hepatitis (ET-NANBH).

In 1983 Dr. Mikhail Balayan studied an outbreak of hepatitis among Soviet troops in Afghanistan. Through voluntary self-inoculation of an infected stool sample, he developed clinical signs of non-A non-B hepatitis and monitored the progression of the disease by examining his stool and serum.[5] Immune electron microscopy showed presence of a virus, and inoculation of this into cynomolgus macaques resulted in enzymatically and histopathologically confirmed hepatitis, with shedding of VLPs in the feces and antibodies reactive to the VLPs. Independently, in India, a large epidemic of waterborne jaundice and concomitant sporadic outbreaks showed characteristic patterns: self-limiting disease that did not progress to chronicity, affected young adults, was enterically transmitted, and a high mortality in pregnant women.[6–9]

The genome of the virus was cloned and sequenced upon isolation from the bile of experimentally infected cynomolgus monkeys.[10,11] Following the identification and sequencing of its etiological agent, the disease became known as hepatitis E and its agent as HEV.[12] The *E* may stand for *enteric* (in the gut), *endemic*, or *epidemic*, all of which are features that adequately describe the epidemiology of HEV.

16.3 HEV BIOLOGY AND THE CAPSID PROTEIN

A major hindrance in the study of the HEV replication cycle has been difficulty in culturing the virus. Surrogate systems, such as transfection of infectious genomic replicons, and VLPs have been partially successful in helping understand HEV biology. The genome organization is given in Fig. 16.1, and the three ORFs are partially overlapping. These encode three proteins—ORF1, 2, and 3—which carry out all the functions of the virus life cycle.

16.3.1 Early nonstructural ORF1 protein

The HEV PORF1 is 1693 amino acids long and is a nonstructural early viral protein that is responsible for various steps in HEV life cycle. Based on homology with similar proteins from other plus-sense RNA viruses,[13] pORF1 is proposed to be posttranslationally cleaved into four functional domains: the methyltransferase (MeT) (amino acids 1–110), papain-like cysteine protease (PCP) (amino acids 433–592), RNA helicase (Hel) (amino acids 960–1204), and RNA-dependent RNA polymerase (RdRp) (amino acids 1207–1693). Two additional domains, the X (macro) and Y, are poorly characterized. Expression of pORF1 or HEV genomic replicons in various systems showed the presence of multiple smaller peptides,[14–16] and these peptides reacted with anti-MeT, Hel, and RdRp antibodies. The activities of MeT, Hel,[17,18] and RdRp[19,20] have been demonstrated in vitro, but that of PCP has not. The X domain of other RNA viruses, such as the SARS coronavirus, bind viral poly(A) tails and recruit poly(ADP-ribose)-modified cellular factors to the site of replication.[21]

16.3.2 Late nonstructural ORF3 protein

PORF3 is a late nonstructural protein that modulates and optimizes the host cell environment for viral replication. Although ORF3 deletion does not interfere with viral

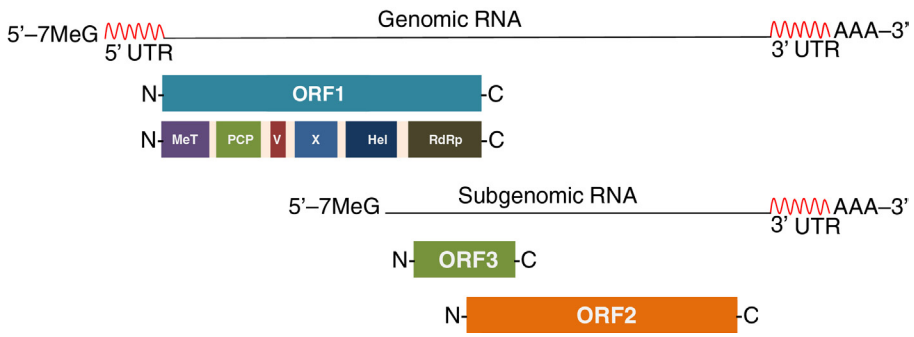

Figure 16.1 ***Genome organization, open reading frames (ORFs), and their encoded proteins.*** *(Adapted from Ahmad et al., 2011).*

replication in cell lines,[22] it prevents infection in monkeys. The 13 kDa ORF3 protein has two large *N*-terminal hydrophobic domains (D1: amino acids 7–23 and D2: amino acids 28– 53) and two proline-rich regions (P1: amino acids 66–77 and P2: amino acids 95–111) and is phosphorylated at serine 71.[23] pORF3 binds to cytoskeletal elements and MAPK phosphatase via its cysteine-rich D1 domain,[24,25] while the P1 domain has substrates for certain cellular kinases.[26] The P2 domain has two overlapping PXXP domains through which it interacts with several Src homology region 3 (SH3) domain-containing proteins.[27] pORF3 plays two broad roles in infected cells: it pushes the cell toward a prosurvival phenotype and attenuates antiviral immune signaling.

Recent studies have shown that ORF3 plays important roles in virus morphogenesis and egress.[28,29] Indeed, its status as a nonstructural protein has also been recently questioned because viral particles produced in culture are coated with ORF3 protein and host-derived lipid.[30] Virus shed in the feces of HEV-infected patients is not associated with lipid or pORF3.[31] Recently, an intraviral interactome was created through yeast two hybrid screens, which showed that ORF3 protein interacts with several functional units of the ORF1 protein and probably forms a densely connected hub in the interactome.[32]

16.3.3 Major component of the capsid protein ORF2

The only structural protein of HEV is encoded by ORF2 (nucleotides 5145–7125). It produces a 660 amino acid–long polypeptide that forms an icosahedral shell encapsidating the HEV genome.[33]

In mammalian cultured cells ectopically expressed pORF2 presents as two proteins of ~74 kDa and ~88 kDa. By treating with tunicamycin and endoglysidase, these were shown to correspond to the nonglycosylated and glycosylated forms, respectively.[34] The glycosylation is *N*-linked on three conserved asparagine residues (Asn 137, 310, and 562).[35]

The capsid is a major determinant of host cell tropism and the mechanism of infection. Full length and various truncated versions of ORF2 protein have been expressed in a variety of surrogate expression systems, and these self-assemble into VLPs that morphologically and antigenically resemble the virus. The baculovirus system was first used to express an N-terminally truncated ORF2 protein (amino acids 112–660). The recombinant protein of 58 kDa was produced, along with a 50 kDa protein secreted into the culture medium, which formed VLPs of 23–24 nm.[36] Expression of full-length pORF2 yields three proteins of 63 kDa, 56 kDa, and 53 kDa, and these correspond to amino acids 112–660, 112–607, and 112–578, respectively.[37]

The critical VLP-forming region is between amino acids 126 and 601, and truncated forms of the ORF2 protein (n111c52) were secreted as VLPs in both Sf9 and Tn5 insect cells. Interestingly, when only the n111 amino acids were truncated, VLPs were not released into the culture medium in Sf9 cells but formed inclusion bodies in

the cytoplasm, indicating the requirement for some posttranslational modification to assemble into particles, although it is not clear whether assembly takes place in the cell or after secretion.

The icosahedral viral structure has two-, three- and fivefold axes of symmetry. Dimeric, trimeric, and pentameric interactions around two-, three- and fivefold icosahedral axes lead to assembly into VLPs. Broad depressions are located around the fivefold axis. The HEV pORF2 has features of a typically secreted protein: an N-terminal signal sequence and conserved glycosylation sites. Interestingly, the N-terminal 111 amino acids show maximum sequence divergence among HEV genotypes, and expressing full length ORF2 in insect cells usually results in proteolytic cleaving of this region. The virion has a $T = 3$ symmetry, with 180 monomers, while truncated pORF2 (amino acids 112–608) folds into a $T = 1$ particle with 60 subunits and 30 protruding spikes.[38]

The first study to describe the structure of the insect cell–expressed VLP was through cryo-EM at a resolution of 22 Å. Sixty monomeric units clustered into dimers that formed a $T = 1$ lattice with surface protrusions that have spherical rings. The structure was hollow, with the solvent having easy access to the center of the particle.[38] A subsequent X-ray crystal structure of a recombinant VLP expressing amino acids 70–123 of ORF3 protein followed by amino acids 112–608 of ORF2 was made available at a resolution of 8.3Å.[39] This used the previous 22A cryo-EM map for initial phasing of the X-ray data. When expressed in insect cells, the amino acids 14–608 yielded a VLP that was considerably larger than the $T = 1$ VLP (diameter: 410Å; inner radius:170Å).[40] STEM measurements revealed that this particle was composed of 180 subunits, thus resembling the native virion and forming a $T = 3$ particle with 90 protruding spikes. Further, X-ray photoelectron spectroscopy found a signal from phosphorous from the large VLP, indicating the presence of nucleic acids. This was corroborated by the detection of a 2 kB RNA from these particles.

Three linear domains form distinct structural elements: S, the continuous capsid shell; M, the threefold protrusions; and P, the twofold spikes. The S domain (amino acids 118–317) adopts a jelly-roll β-barrel fold commonly observed in small RNA viruses. The M (amino acids 318–451) and P domains (amino acids 452–606) both adopt β-barrel folds. The P domain contains binding sites for neutralizing antibodies and receptors. Nineteen divergent amino acids exist in the P domain across the four HEV genotypes, but only nine are surface exposed, and only one falls in the antibody-binding region, resulting in a single HEV serotype. Binding to sugars at the capsid protein interface may lead to capsid disassembly and facilitate cell entry. The $T = 3$ capsid structure docks well with the $T = 1$ particle; the additional N domain in the $T = 3$ particle corresponding to the N-terminal 111 amino acids.

The subunits of the VLP are grouped into three unique monomers (A, B, and C) based on their unique geometric environments, where A and B subunits dimerize with

bent conformation around the fivefold axis, while C monomers dimerize with flat conformation at the twofold axis. There is a 90-degree difference in the C-C dimers in the P domain of the $T = 3$ particle compared with the A–B dimer and the dimer of HEV-LP/$T = 1$.

Various monoclonal antibody fragments, or fused B cell tag at the C terminus of the capsid protein, have been characterized structurally to reveal the major antigenic sites on the VLP surface.[41] One major epitope of HEV on the protrusion domain is spatially mapped to be conformational and corresponds to amino acids 459–607 (Fig. 16.2). This study was also critical in pointing out the ability of foreign epitopes to be inserted at the C terminus of the ORF2 protein by cloning rather than at other random sites. An insertion at the C terminus allows for proper quaternary structure of the VLP to be formed as well as expression of the foreign epitopes in correct conformation.

Shorter constructs of ORF2 protein have also been expressed in *E. coli* and seen to self-assemble into higher-order structures. The p239 (amino acids 459–606) folds into globular protein with aggregates of an average size of ~23 nm, while the E2 (amino acids 394–606) and E2a (amino acids 459–660) construct forms of mainly hexamers.[42] Under partially denaturing conditions, these proteins exist as dimers that mimic the dominant antigen determinants and neutralization site of HEV.[43] The p239 aggregates purified from *E. coli* have proven efficacious as a preventive vaccine (HEV 239) against hepatitis E[44] and been shown to bind and penetrate cell lines susceptible to HEV infection.[45] The

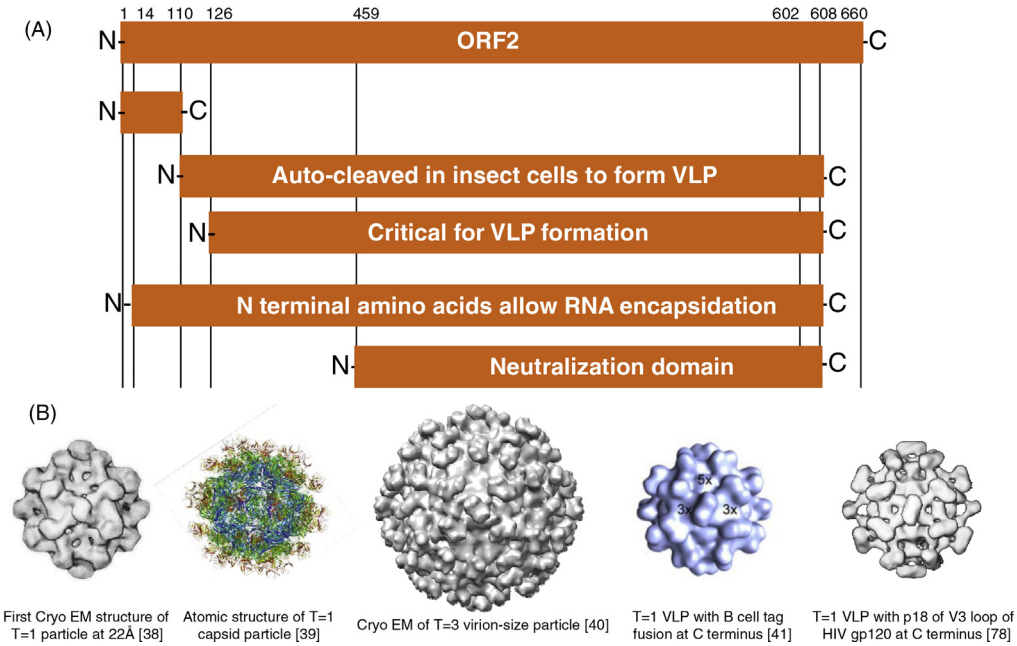

Figure 16.2 (A) HEV constructs and (B) structures in chronological order of their deduction.

E2S domain (amino acids 459–606) forms the minimum region that induces neutralizing antibodies, thus making it a crucial target for HEV immunology. Recently, Zhao et al. created a toolbox of 12 neutralizing antibodies that cover most of the conformational epitopes of the E2S.[46] The majority of the protective antibody produced was found to be against the 8G12 epitope in the E2S region.[47]

16.4 LIFE CYCLE OF HEV MAKES IT AMENABLE TO BEING USED AS AN ORALLY DELIVERED DRUG

Based on genomic similarities with other positive-sense RNA viruses, HEV is proposed to replicate in the cytoplasm via a negative-sense intermediate. A schematic representation is provided in Fig. 16.3. A better understanding of the HEV life cycle will enable us to fully appreciate the potential of this virus to be used as a model of VLP-based vaccine and drug delivery systems.

16.4.1 Entry

Hepatoma cell lines, such as Huh-7 and PLC/PLRF/5, and the colorectal carcinoma cell line Caco-2 are generally considered permissive for HEV replication. Interestingly, the lung carcinoma cell line A549 also supports replication of the virus. A specific receptor for HEV has not been identified, although heparin sulfate proteoglycans (HSPGs) on cell surface syndecans have been shown to act as nonspecific attachment factors.[48] On account of being feco-orally transmitted, the virus is expected to interact and transgress the oral, stomach, and gut mucosa to reach the hepatic sites via blood circulation. It is interesting that a nonenveloped RNA virus shows such high stability to extreme pH, bile, and blood before undergoing specific receptor engagement, entry, and replication in liver cells. Recently, Holla et al.[49] have shown that HEV entry in liver cell lines is dependent on clathrin, dynamin-2, and membrane cholesterol. By studying the uptake of FITC-labeled p239 VLP in cells treated with various small molecule inhibitors, siRNA-mediated knockdown and transfection of dominant negative/constitutively active constructs, they have shown that the VLP goes into the Rab5 positive early endosomes soon after entering cells. Previously, Kapur et al.[50] used a GFP-labeled VLP to show that entry is dependent on clathrin but not membrane cholesterol.

16.4.2 Uncoating and escape from endosomes

RNA viruses that are internalized via endocytosis need to be released into the cytosol, and trafficking to the site of replication is dependent on several viral and cytoskeletal components. Endosome acidification is a trigger for the uncoating of the viral capsid of many viruses, although this doesn't seem to be the case for HEV entry in liver cells.[49] This is understandable in the context of HEV being enterically transmitted and thus being acid stable. The mechanism of uncoating and targeting to the site of replication is

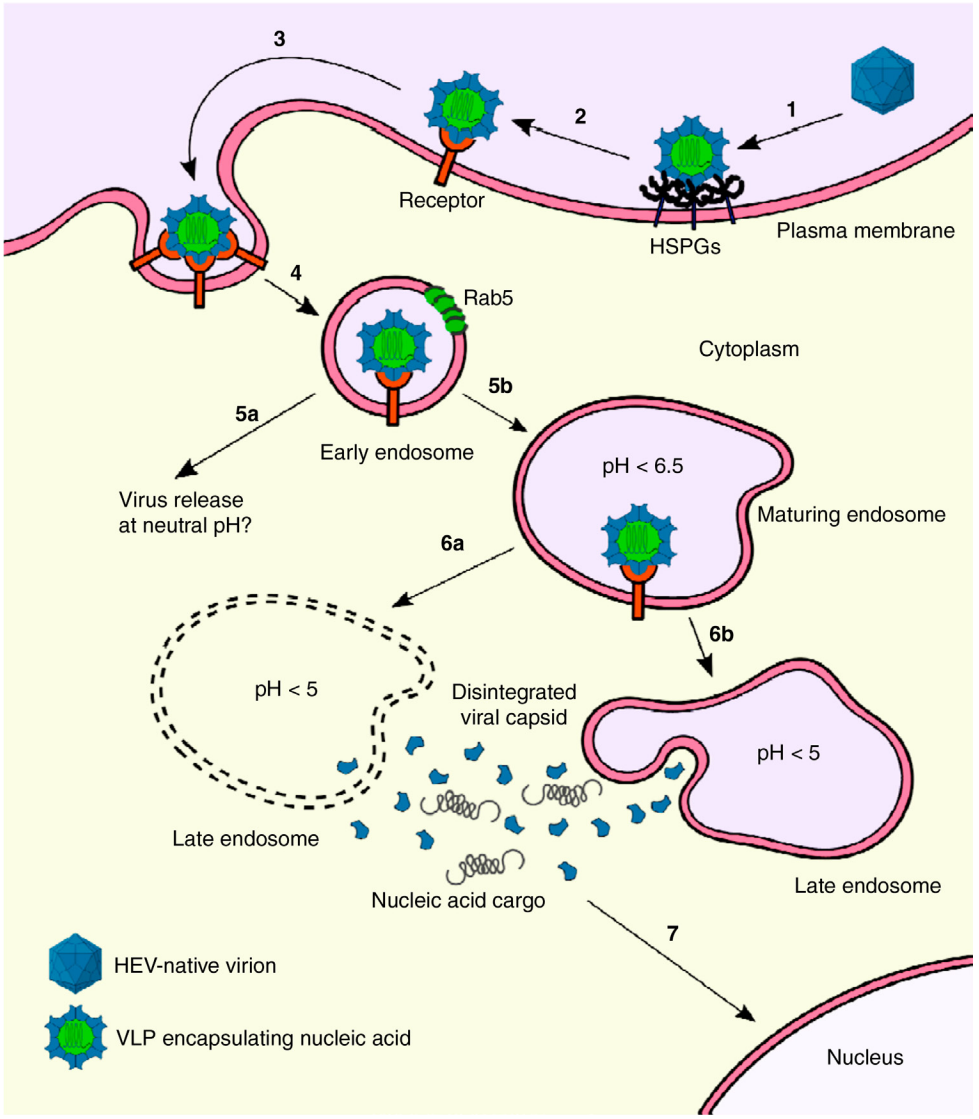

Figure 16.3 ***Proposed life cycle of HEV or HEV VLP carrying nonspecific DNA cargo.*** (1) The virus/VLP binds to the cell surface through nonspecific attachment molecules such as heparan sulfate proteoglycans. (2) Subsequent binding to a specific receptor (or receptors) facilitates (3) viral entry by endocytosis into membrane-bound vesicles. (4) The vesicles fuse with early endosomes. (5a) Escape from the early endosome maybe independent of endosome acidification or (5b) may occur when the endosomal compartment becomes more acidic. Further, escape from the maturing or late endosome may involve (6a) lysis of endosomal compartment or may not involve lysis (6b). Finally, virion RNA or cargo DNA is released, and the DNA cargo may be transported to the nucleus for expression.

still not understood, although inhibitors of tubulin and actin polymerization arrest p239 uptake and reduce HEV infection.

16.4.3 Replication

Viral RNA that is released into the cytoplasm is rapidly translated to yield ORF1 polyprotein, which is cleaved into smaller functional units. Of these, the RdRp initiates replication of the genomic RNA into a negative-sense intermediate. Negative-sense intermediates are templates for the synthesis of full-length genomic plus-sense RNAs (to be packaged into progeny virions) and plus-sense subgenomic RNAs that are translated into structural and nonstructural late proteins (ORF2 and ORF3 proteins).[51,52] The full-length genomic RNA is infectious in cells and in vivo in animals, implying that subgenomic RNAs are not packaged with visions or required for initiation of replication.

16.4.4 Assembly

RNA viruses assemble on internal membranes, which are sometimes modified in composition and morphology by viral proteins.[53] Self-assembly of HEV capsid protein into VLPs has been well studied in mammalian, insect, and bacterial systems. Xing et al.[40] showed that the $T = 3$ HEV VLP was able to encapsidate a subgenomic HEV RNA, while a smaller $T = 1$ particle did not. This indicates a role of the genomic RNA in assembly of the virus. Further, a potential packaging signal was identified using a yeast three-hybrid approach toward the 5' end of the genome within the ORF1 gene.[54] The implications of this translocation are not clear, although it may have a role in virus assembly.

16.4.5 Egress

Not much is known about how virion particles are released from cells, although recent studies in replicon-transfected cells show that egressed viral particles are associated with a host-derived lipid membrane and the ORF3 late protein. These lipid membranes are not derived from the cell surface but retain markers like the trans-Golgi network protein 2 (TGOLN2).[55] pORF3 has been shown to play a critical role in egress.[29,56] Interestingly, virus shed in feces of infected animals lacks associated lipids, indicating that this is probably shed off as the virus traverses the gut and is exposed to bile salts.

16.5 VIRUS-LIKE PARTICLES AS DRUG DELIVERY SYSTEMS/ VACCINE EPITOPE CARRIERS

The mammalian immune system has evolved to elicit strong and specific protective responses against viruses. This is, in part, due to viral capsid structure, which allows multiple, repeated epitopes to be expressed on the viral surface. Additionally, viruses are

large enough to be engulfed by dendritic cells (DCs), which are the first responders of the surveillance system and connect innate and adaptive branches of immunity.

Classically, infectious virions have been "attenuated" to make them replication incompetent before administering as vaccines. There are many examples of successful live, attenuated vaccines.[57] The oral polio vaccine (OPV), for example, has been highly effective in eradicating polio from most parts of the world.[58] Since poliovirus transmits fecoorally, a vaccinated person is able to evade infection due to high levels of secreted IgA antibody that prevent the virus from crossing the intestinal barrier and into the blood. A major concern with such vaccines is, however, the risk of reversion to wild-type, replication-competent virus. Other concerns like the integration of viral genome into the host genome can consequently disrupt the host gene in the cell life cycles. This has largely been overcome by the use of VLPs. The native configuration of the viral capsid and/or envelope proteins is maintained when expressed using yeast, bacterial, insect, or mammalian expression systems. The self-assembly of the viral structural proteins displays the immunological epitopes in a way that would resemble the proper conformations of viruses without the risk of infection by voiding the viral nucleic acid completely for replication. VLP-based vaccine formulations are unique because they can be modified to change the epitopes on the exposed surface and thus be used as a platform for presenting immunogens from heterologous sources and other pathogens. Several VLP-based vaccines are in clinical trials or are already commercially available (Table 16.1).[59,60] There have been several recent advances in the development of mucosally deliverable vaccine formulations. While many of these use various natural and synthetic polymers,[61,62] there are several that make use of VLPs based on naturally pathogenic viruses (Table 16.1).

16.6 HEV VLPS ARE WELL SUITED AS NANOCARRIERS FOR ORAL DELIVERY

Among other VLP based nanocarrier systems, HEV VLPs expressed in insect cells have several unique structural properties that make them particularly well suited. When orally administered in nonhuman primates, the HEV VLP was found to be protective in challenge studies with infectious virus (Fig. 16.4). This has exciting implications in terms of the oral bioavailability and immune potential of the VLP in generating protective responses in the mammalian system. Various structural properties of the VLPs make them amenable to modification, and this provides an exciting platform for the development of mucosal vaccines.

16.6.1 Modularity and modifiability of HEV capsid structure

PORF2 has a three-domain tertiary structure. Each of the domains—S, M, and P—carries out its functions independently of the others. Upon multimerization, the S and M domains form the icosahedral base and threefold plateau, while the P domain is

Table 16.1 VLP-based vaccine formulations for oral/intranasal delivery

Backbone	Formulation	Administration route (animal)	Description	Response	Status	References
Mu-PyV	VLP-MuPyV	Nasal (mice)	The J8i peptide antigen of group A streptococcus (GAS) is fused to be expressed on the VLP This construct is self-adjuvating	Systemic IgG and mucosal IgA responses were observed; mice were protected against a lethal dose challenge	Preclinical	[63]
Norwalk virus	VLP-NV	Oral/nasal (human)	VLP + adjuvants/ excipients like chitosan, mannitol, and sucrose	CT will evaluate adverse effects, systemic (IgG) and secretory (IgA) responses	Phase I clinical trial (NCT008069762)	[64]
	LT-enhanced VLP-NV	Nasal (mice)	rNV VLPs administered with mutant *E. coli* labile toxin LT(R192G)	Systemic IgG and mucosal (fecal and vaginal) IgA	Preclinical	[65–67]
	MB enhanced Norwalk virus (NV) VLP	Nasal (mice)	The immunomodulator murabutide (MB) as an adjuvant enhances immune response	Systemic antiviral IgG and IgA at distal mucosal sites	Preclinical	[68]
Influenza	Subunit-eurocrine VLP formulation	Nasal (mice)	Influenza subunit antigens with lipid-based adjuvants (eurocine)	Hemagglutination inhibition with live virus	Preclinical	[69–72]
	M2e-VLPs	Intraperetonial/ nasal (human)	On the backbone of HBV-protective response against influenza in mice; VLPs contained nucleic acids	Th1-based antibody response	Phase I (NCT00819013)	[73–75]

(*Continued*)

Table 16.1 VLP-based vaccine formulations for oral/intranasal delivery (*cont.*)

Backbone	Formulation	Administration route (animal)	Description	Response	Status	References
HEV	Recombinant HEV VLPs	Oral (mice, cynomolgus monkeys)	The HEV capsid with N terminal 111 amino acids truncated were expressed and purified from insect cells infected with baculovirus	Systemic and intestinal immune responses observed in mice; monkeys were protected against infection with HEV	Preclinical	[76,77]
	p18 HEV VLPs	Oral (mice)	The HEV capsid protein expressing the p18 peptide from the V3 loop of HIV gp120 elicited anti-HIV response	Fecally shed anti-gp120 IgA, anti-HIV CTL	Preclinical	[78,79]
	DNA encapsidating HEV VLP	Oral/ subcutaneous (mice)	Positively charged residues on the exposed inner surface allow for binding to DNA	Anti-HIV CTL and gp120 expressed on the surface of intestinal epithelial cells two days post oral immunization	Preclinical	[79]
	P-P18/NGag capsule	Oral (mice)	Chimeric HEV VLP expressing P18 peptide + encapsidating entire HIV gag DNA	CTL in spleen, mesenteric lymph nodes, and Peyer's patches	Preclinical	[79]
HIV	Whole HIV gp120/140 envelope protein	Nasal (female Rhesus macaques)	The VLP formulation was based on an Ugandan clade A field isolate; VLP was administered either intranasally or intramuscularly	Systemic humoral IgG was strong, but intestinal and vaginal responses were not significant	Preclinical	[80]

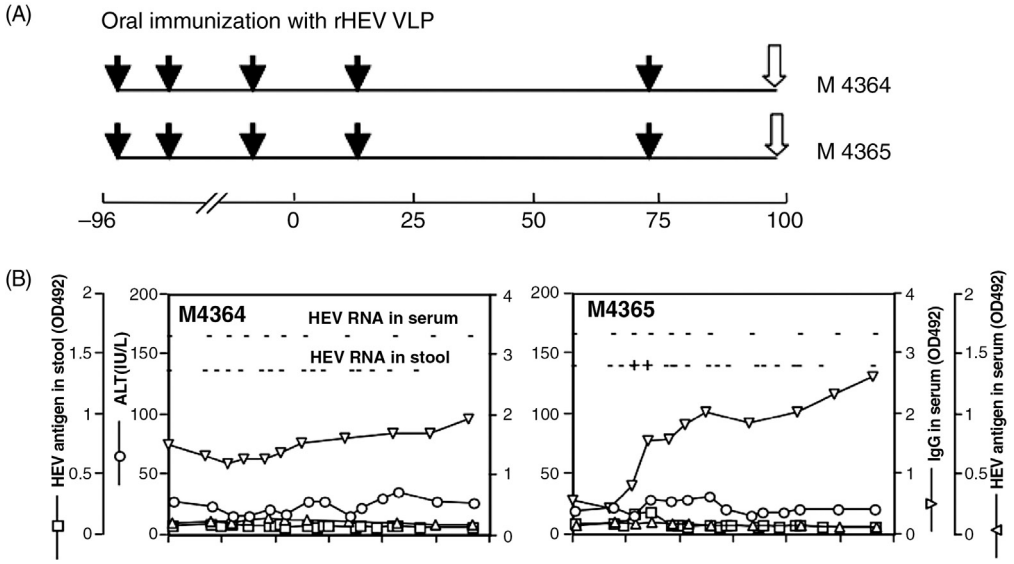

Figure 16.4 (A) Immunization schedule. Two monkeys (M4364 and M4365) were inoculated with 10 mg rHEV VLPs on days 0, 7, 21, 36, and 80; on day 100 both monkeys were challenged with 2 mL of the challenge virus. (B) Kinetics of biochemical, serological, and virological markers after the challenge. Monkeys (M4364 and M4365) orally immunized with HEV VLPs were challenged with infectious HEV at day 0. HEV antigen in the serum (Δ), HEV antigen in the stool (□), IgG antibody in the serum (∇), and ALT elevation (○) were determined. HEV RNA in the sera and stool was monitored by RT-PCR. (+) positive; (−) negative.[76]

responsible for antigenicity, dimerization, and host–cell interaction (Fig. 16.5). The M domain connects the S and P domains. This structural modularity allows the particle to be disassembled and reassembled in vitro without any change to the capsid morphology. Further, this ensures that reassembly is independent of the requirement of a DNA or RNA strand to be encapsulated, thus implying that the $T = 3$ and $T = 1$ particles have the same antigenic properties. However, the most important advantage of the structure modularity of HEV capsid is that the P domain can be extensively modified by genetic engineering or chemical cross-linking without affecting the self-assembly and stability of the VLPs.

A chimeric VLP expressing the p18 peptide from the V3 loop of HIV gp120 near the C terminus of HEV ORF2 peptide self-assembled into VLPs (Fig. 16.5A) with P18 expressed correctly on the surface protrusions.[78] This peptide induces cytotoxic T lymphocyte response (CTL) that is HIV-1 specific.[80] There was no difference in the icosahedral shell of chimeric and wild-type VLPs, although the orientation of the P domains was slightly thicker and rotated (Fig. 16.4B). The p18-VLP cryo-EM density map fit well with the crystal structure of the HEV VLP (Fig. 16.5C–F).

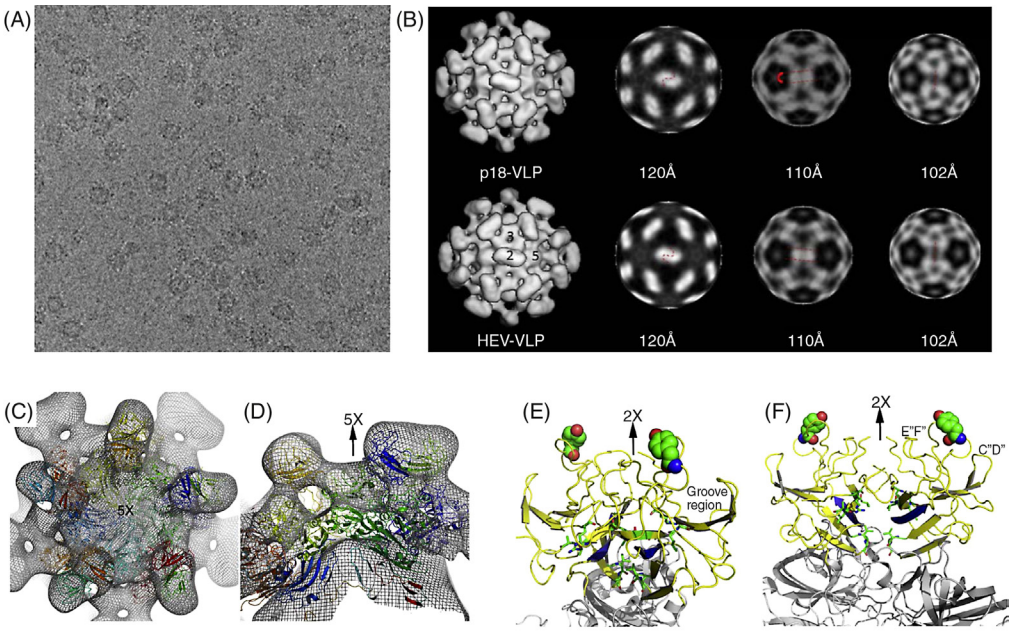

Figure 16.5 (A) Cryo-electron micrograph of frozen-hydrated p18-VLP. (B) Three-dimensional density maps of p18-VLP (top panel) and the wild-type HEV VLP (*bottom panel*). Particles were sliced into thin sections to show the density distribution at radii of 120Å (*the P domain*), 110Å (*the M domain*), and the 102Å (*the S domain*). The red dashed lines indicate differences between the p18-VLP and the wild-type HEV VLP. (C) Fitting of p18-VLP cryo-EM density map with the crystal structure of the HEV VLP. The coordinates of PORF2 decamer at fivefold axis agreed well with the cryo-EM density map (A) and with the separation of S, M, and P domains (D). Ribbon presentation of a pORF2 dimer showing the position of surface groove region (E) and the hydrophobic residues (stick presentations) at the P domain dimeric interface (F).[78]

When injected orally in mice, the p18-VLP induced a strong and specific immune response (Fig. 16.6). Both IgG and secretory IgA antibodies were elicited, along with cell-mediated immune response. Interestingly, these administrations were made without the need for an external adjuvant. A major limitation of a nonreplicating oral delivery system is crossing the gut epithelial barriers. By further chemical modification or reengineering, the HEV VLP can be made to express cell-specific targeting ligands. Our lab is currently trying to develop one such ligand that would allow the chimeric HEV VLP to zone to immune cells of the gut mucosal system, thereby increasing rapid access of the VLP to T and B immune cells of the nearest lymphoid organs.

16.6.2 Resistance to gut environmental conditions and protease cleavage

The HEV-VLP, owing to its resemblance to the feco-orally transmitted virus, shows high stability and resistance to proteolytic enzymes.[78] The solvent-exposed part of

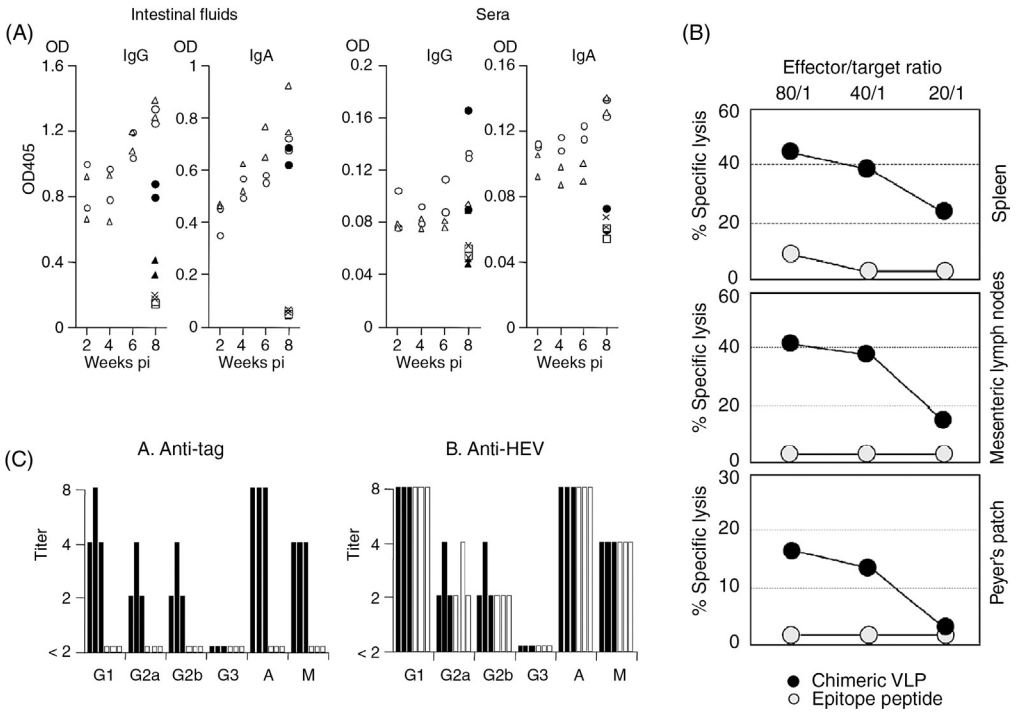

Figure 16.6 (A) Humoral responses against the chimerized epitopes (p18-VLP) in orally inoculated mice. Three oral immunizations were carried out with either of the following: p18 VLPs (*open circles and triangles*) or wild-type HEV VLP (*closed circles and triangles*). HEV-specific antibody responses are depicted by circles, while tag-specific responses are shown as triangles. Background antibody levels to HEV and tag in nonimmunized mice are shown by squares and crosses respectively. Sera samples were diluted 1:100, and fecal suspensions (200 mg/mL) were diluted 1:2. Specific anti-HEV responses were measured by enzyme-linked immunosorbent assay (ELISA) using a synthesized peptide antigen. (B) Cell-mediated immunity in terms of specific cell lysis (cytotoxic T lymphocyte) from three immune organs was measured in mice orally treated with p18-VLP (closed circles) or control mice (open circles). As a control, a synthetic p18 peptide was used. (C) Antibody isotype titers IgA (A), IgM (M), and IgG subclass (G1, G2a, G2b, and G3) against tag and HEV were measured in the intestinal fluids of orally immunized mice using isotype-specific secondary antibodies and shown as end-point titers. Solid and open bars indicate antibody levels of each mouse immunized with the chimeric VLP and VLP without tag epitope insertion, respectively.[78,79,81]

the protrusion domain does not contain features that make it susceptible to trypsin cleavage. However, chimerization of the P domain with the arginine-rich p18 peptide increases susceptibility to action by intestinal proteases. This could be a major pitfall in the context of using the VLPs as nanocarriers or vaccines. Interestingly, we found that p18 VLPs remained intact when treated with trypsin and pepsin and observed by electron microscopy (Fig. 16.7A). Proteolytic cleavage did take place, likely at the end of the C terminal end of the P18 insertion, and this was evidenced by the 42 kDa band

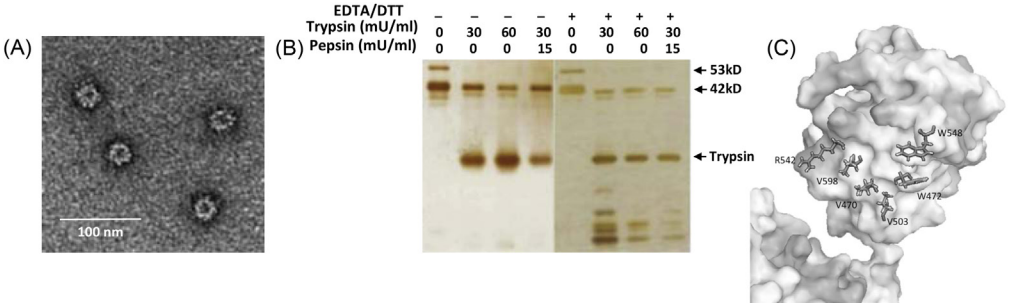

Figure 16.7 (A) Electron micrograph of negatively stained p18-VLPs after treatment with 60 mU trypsin. Bar = 100 nm. (B) Silver-stained reducing SDS PAGE gel of the p18-VLP pretreated with EDTA/DTT, 30 mU/mL or 60 mU/mL trypsin, and 15 mU/mL pepsin and then immunoblotted with anti-HIV-1 antibody 447-52D. (C) Surface potential representation of pORF2 monomer showing dimeric contact regions of the P domain. The blue and red represent positively and negatively charged regions, respectively. White are nonpolar. Critical residues involved in dimerization are shown by ball-and-stick representation.[78]

observed on trypsin treatment by SDS-PAGE that was reactive to HIV antibodies (Fig. 16.7B). Trypsin treatment did not cause surface spikes to be dissociated form the VLP, and this is due to the hydrophobic interactions that hold the quaternary structure of the VLP together and also stabilize the dimeric interactions of two P domains in the same spike. Even after trypsin cleavage, the hydrophobic residues in the A beta strand of the P domain form a strong interaction with the C terminal region, thereby maintaining the integrity of VLP structure (Fig. 16.7C).

16.6.3 Self-immunity to HEV is preventable by modification of the P domain

Sequence diversity is seen only in 19 amino acids of the P domain across the genotypes and, of these, where only nine of these residues are accessible from the capsid surface. Intriguingly, only one divergent amino acid lies within the antibody binding region, and this is why, despite four genotypes, HEV presents as a single serotype.

A major consideration for protein-based oral delivery systems is that proteins elicit immune response, thereby making the nanocarrier unreusable. We have seen that modification by genetic engineering within the P domain of HEV VLP can be done without effecting particle formation. Further, HEV VLP antigenicity lies exclusively in the P domain. Therefore the conformational epitope that binds the monoclonal antibody HEP224 is made up of three loops around Tyr485 of pORF2. Insertion of a foreign epitope at this residue disrupts the conformational epitope bound by HEP224 without altering VLP properties and antigenicity of the foreign epitope (Fig. 16.8A, B).[78] This eliminates the issue of self-immunity that arises with many other mucosal VLP systems, such as the polyoma virus.[82]

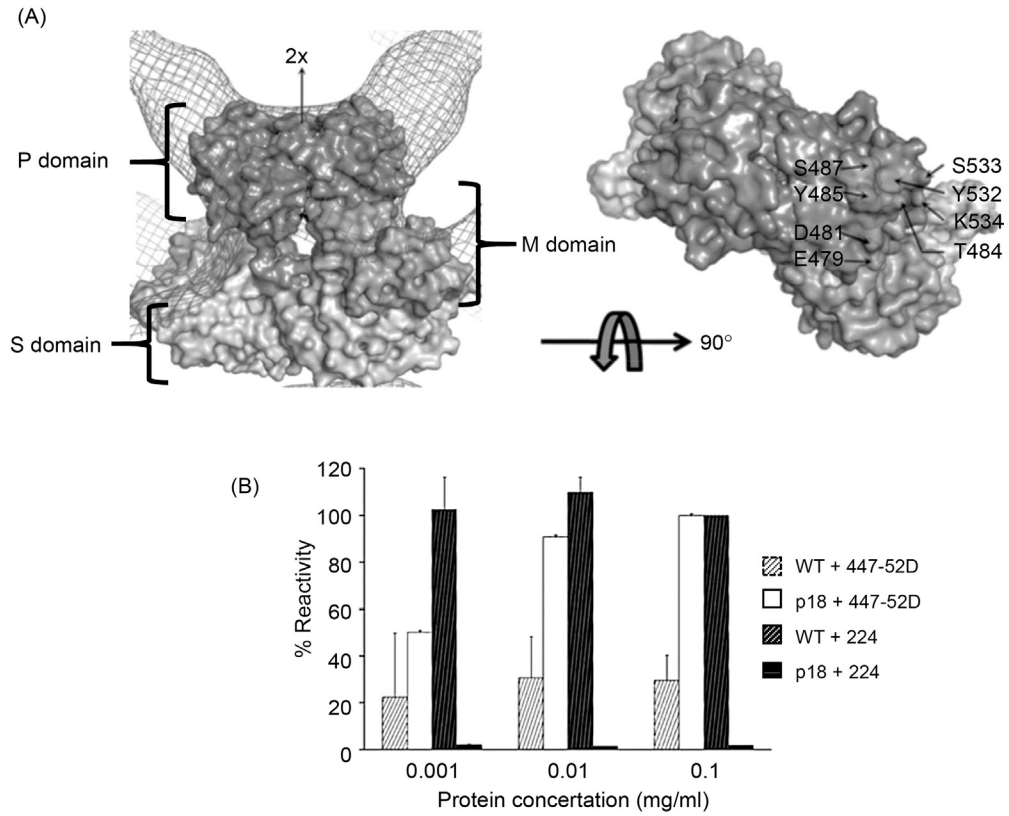

Figure 16.8 (A) The binding of the Fab region of HEP224 antibody (Fab224) to HEV VLP. The PORF2 dimer is presented as a solid surface and colored in gray, violet, and light magenta for the P domain, the M domain, and the S domain, respectively. The residues along the Fab binding interface are colored according to element, with green for carbon, blue for nitrogen, and red for oxygen. The cryo-EM density map of VLP-Fab224 was fitted with the crystal structure of PORF2 and viewed along a bound Fab molecule. The neighboring dimers are drawn in ribbon mode and colored wheat. Top right—Side view of a PORF2 dimer fitted into the cryo-EM density map. Bottom left—A pORF2 dimer viewed along the twofold axis and overlapped with the cryo-EM density map. Bottom right—Top view of a pORF2 dimer viewed along the twofold axis. The amino acids in pORF2 responsible for binding to Fab224 are labeled. (B) Chimerization of HEV VLP abrogates HEV antibody binding. ELISA-based determination of reactivity of antibodies HIV447-52D *(white bars)* and HEV224 *(black bars)* to the p18-VLPs (*nonstriated*) and WT-VLPs (*striated*). The data represent triplicate experiments and are expressed as mean ± standard deviation.[40,78]

16.6.4 HEV VLPs can encapsulate foreign DNA plasmids and release them in cells

The hollow core of the VLP can be utilized to carry foreign DNA. Critical positively charged residues on the inner surface of the VLP allow encapsulation of negatively charged, nonsequence-specific DNA to be incorporated. This can be achieved by

disassembling the particles into dimers using DTT and EGTA and subsequently allowing reassembly in the presence of the DNA in a calcium-rich environment. Thus, the HEV VLP has potential to be exploited as a carrier for orally deliverable DNA vaccines. We encapsulated HIV-1 gag DNA plasmid into HEV VLPs and orally administered them in mice. A strong and specific humoral as well as cell-mediated immune response was generated in local and systemic lymphoid organs (Fig. 16.9A, B), indicating that this oral DNA vaccine not only was successful in being delivered to lymphoid organs but also allowed for the particles to enter immune cells, undergo uncoating, and release the encapsulated DNA. Further, gag gene expression took place successfully and the Gag protein was presented successfully as a foreign antigen, as can be seen from the anti-Gag

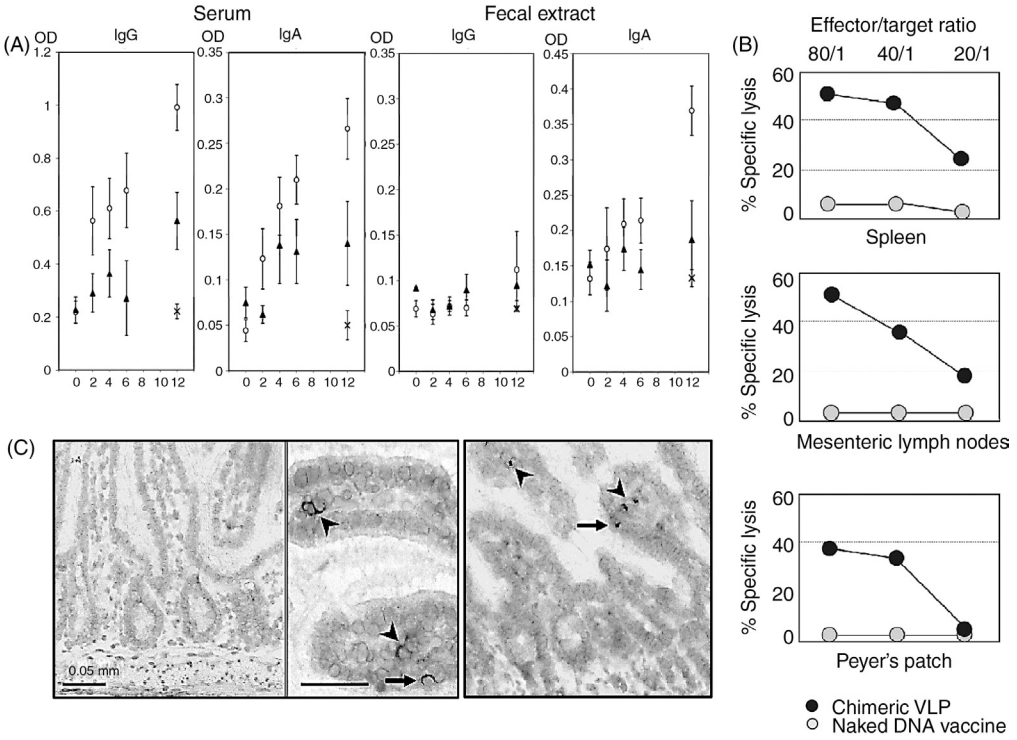

Figure 16.9 (A) Humoral immunity in mice orally inoculated with gag DNA–encapsulating chimeric HEV VLPs. Circles and triangles represent immune response against Gag protein from VLPs and naked DNA vaccine, respectively. (B) Cell-mediated immunity in terms of specific cell lysis (cytotoxic T lymphocyte response) from three immune organs was measured in mice orally treated with gag DNA–loaded p18-VLP *(closed circles)* or control mice *(open circles)*. As a control, naked gag DNA vaccine was used. (C) Immuno-gold labeling with anti-Gag antibody of gut epithelial sections from mice orally immunized with gag DNA–encapsulating VLPs.[79]

immune-gold labeled electron micrographs of gut epithelial sections of orally immunized mice (Fig. 16.9C).

16.6.5 Ease and economy of scalability and administration

The insect cell culture–based protein expression system is inexpensive and easy to scale up. Being eukaryotic, insect cells allow correct folding and posttranslation modification, and purified protein can be used therapeutically. A high degree of purity is achievable with an easy purification process, particularly when recombinant protein is secreted into the cell culture medium. HEV capsid protein PORF2, when expressed in *Spodoptera frugiperda* (Sf9) or *Trichoplusia ni* (Tn5) insect cells, self-assembles into VLPs and is secreted into the culture medium. Further, being natural human pathogens, viruses have evolved such that their capsid proteins are recognized by the immune system and internalized by antigen-presenting cells (APCs), processed and presented in the context of major histocompatibility (MHC) molecules. Viral antigens are exposed to CD8+ and CD4+ T lymphocytes to generate primary and memory responses. This feature allows chimeric HEV VLP–based vaccines to be administered without the need for an external adjuvant for enhancing the immune response. Most artificial adjuvants, such as alum, have various side effects related to irregular translocation to nonspecific sites and are unsuitable for use with specific vaccine antigens, such as those requiring a Th1 response.[83] VLPs, on the other hand, are easily encountered by the innate immune system, which in turn primes the adaptive immune system to generate a specific and strong response against the chimerized antigen or DNA vaccine. Thus, the chimerized HEV VLP is uniquely positioned in terms of its ability not only to express correctly folded foreign antigens but also to carry heterologous DNA to be used as a DNA vaccine. This has been summarized in Fig. 16.10.

In order to fully capitalize on the use of HEV VLP as a drug delivery system/vaccine candidate, it is important to understand the exact way in which it is perceived and taken up by the host cell and how specificity and tropism play a role in specific targeting.

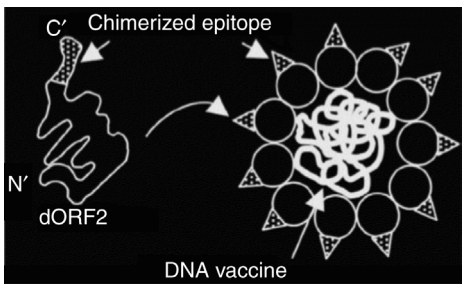

Figure 16.10 HEV VLP with a chimerized epitope folds to correctly express the epitope on its surface. It can also incorporate a DNA vaccine within its core.

Since the first site of entry for the VLP would be the oral/buccal or gut mucosa, it is critical to study the receptors present on the cells of gut immunity that facilitate HEV/VLP entry. Further, when exposed to DCs, the VLP would be expected to be taken up and may utilize a different set of cell surface molecules to bind and be engulfed. Although a receptor for HEV is not known, it binds to heparan sulfate proteoglycans (HSPGs) on the hepatocyte cell surface.[48]

It is crucial to understand how these nanocarriers enter the cell and where they deliver their cargo. Many cytoplasmically replicating RNA viruses enter cells by endocytosis and then are released from the endosomal system into the cytosplasm, from where they travel to their site of replication by exploiting the cytoskeletal system. In liver cells, HEV enters via endocytosis into early endosomes and uses clathrin, dynamin-2, membrane cholesterol, and the microtubular network to facilitate successful infection.[49] Further, escape from endosomes is not triggered by lowered endosomal pH, and further exploration into the mechanism of endosomal escape has to be carried out. This is particularly relevant in the context of HEV VLP for DNA delivery because the ultimate goal is the use of these orally active VLPs of gene therapy. As a proof-of-concept, we have already seen that HEV VLPs carrying DNA vaccines are successful in gaining exposure and access to the immune system and subsequently generating an immune response.

The HEV VLP has a unique position among VLP-based orally delivered nanocarriers. Despite being a nonreplicating, protein-based particle, its structural features make it more ideally suited than many other available systems.

ACKNOWLEDGMENTS

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