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A Dual Vaccine Candidate against Norovirus and Hepatitis E Virus

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Abstract

Norovirus (NoV) and hepatitis E virus (HEV) are both enterically-transmitted viruses causing gastroenteritis and hepatitis, respectively, in humans. While a vaccine against HEVs recently became available in China, there is no prophylactic or therapeutic approach against NoVs. Both NoV and HEV have surface protrusions formed by dimers of the protruding (P) domains of the viral capsids, which is responsible for virus-host interactions and eliciting viral neutralizing antibody. We developed in this study a bivalent vaccine against the two viruses through a recently developed polyvalent complex platform. The dimeric P domains of NoV and HEV were fused together, designated as NoV P⁺-HEV P, which was then linked with the dimeric glutathione-S-transferase (GST). After expression and purification in *E. coli*, the GST-NoV P⁺-HEV P fusion protein assembled into polyvalent complexes with a mean size of 1.8 μ m, while the NoV P⁺-HEV P formed oligomers ranging from 100 to 420 kDa. Mouse immunization study demonstrated that both GST-NoV P⁺-HEV P and NoV P⁺-HEV P complexes induced significantly higher antibody titers to NoV P⁺ and HEV P, respectively, than those induced by a mixture of the NoV P⁺ and HEV P dimers. Furthermore, the complex-induced antisera exhibited significantly higher neutralizing activity against HEV infection in HepG2/3A cells and higher blocking activity on NoV P particles binding to HBGA receptors than those of the dimer-induced antisera. Thus, GST-NoV P⁺-HEV P and NoV P⁺-HEV P complexes are promising dual vaccine candidates against both NoV and HEV.

Keywords

Polyvalent complex; immune response; vaccine development; bivalent vaccine; vaccine platform; norovirus; hepatitis E virus (HEV)

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INTRODUCTION

Noroviruses (NoVs), members of the family *Caliciviridae*, are the major cause of epidemics of viral acute gastroenteritis with significant morbidity and mortality, affecting millions of people in both developed and developing countries [1]. NoVs are highly contagious, often leading to large outbreaks in closed or semi-closed settings, including healthcare centers, nursing homes, military camps and cruise ships. Each year NoVs cause around 23 million cases of disease in the United States and more than 200,000 deaths worldwide [1]. On the other hand, hepatitis E viruses (HEVs), members of the families *Hepeviridae* [2], cause enterically-transmitted non-A, non-B viral hepatitis [3]. Generally, hepatitis E is a self-limiting disease that prevails mainly in developing countries with poor sanitation and hygiene, although chronic hepatitis E has recently become an emerging clinical problem in immunocompromised individuals, such as organ transplant recipients [4, 5]. Additionally, severe and fulminant hepatitis E can occur in pregnant women with a mortality rate of up to 20% [6, 7]. Thus, both NoVs and HEVs are threats to public health.

Despite their differences in genetic make-ups, NoVs and HEVs share a number of similarities. In fact, HEV was originally classified in the family of *Caliciviridae* based on superficial similarity in morphology and genomic organization [8]. They both are small (27-37 nm), nonenveloped viruses containing a single-stranded, positive-sense RNA genome of ~7.4 kb that contains three open reading frames (ORFs). Both viruses are highly infectious, transmitted through fecal-oral route, often causing large outbreaks. Structurally, both NoVs and HEVs are encapsulated by icosahedral protein capsids that are formed by a single major structural protein [9, 10], the capsid protein, encoded by ORF2. Both viruses have surface protrusions that are formed by dimers of the protruding (P) domain of the capsid proteins [11-15]. These protrusions play an important role in virus-host receptor interaction, viral attachment and entry [reviewed in [16-20], can elicit neutralizing antibodies [21-23], and therefore, are excellent targets for vaccine development against these two viruses [22, 24].

The first subunit HEV vaccine (HEV 239/Hecolin®) that was developed by Xiamen Innovax Biotech has recently been approved by Chinese health authorities for commercial use in China, where genotypes 1 and 4 HEVs are prevalent [25]. The vaccine has not yet been available in other countries and its efficacy against other HEV genotypes, especially the emerging zoonotic HEV strains remains unknown. However, there is no vaccine or antiviral available against NoVs, partially due to the lack of an effective cell culture and a small animal model for NoVs. Subunit vaccines against NoVs are under intensive development [24, 26], in which a VLP-based vaccine has been in phase II clinical trial [27]. In this study we set off to develop a dual vaccine against both NoV and HEV through the polyvalent complex platform that we developed recently [28] and examined its immune responses and neutralizations. The dimeric P domains of NoV and HEV were fused together (NoV P⁺-HEV P) that was then fused with the dimeric glutathione-s-transferase (GST) (GST-NoV P⁺-HEV P), resulting in large polyvalent complexes for improved immunogenicity. Our data from this study demonstrated that the GST-NoV P⁺-HEV P and NoV P⁺-HEV P complexes exhibited significantly increased immunogenicity and neutralization against both HEVs and NoVs compared with the dimeric proteins, and thus are promising dual vaccine candidates.

MATERIALS AND METHODS

Plasmid constructs

The plasmid for expression of GST-NoV P⁺-HEV P protein was created based on the GST-NoV P⁺ and the GST HEV P constructs that were made previously [28]. A 12-glycine linker

was added between NoV P⁻ and HEV P. NoV P⁻ is the P domain of a GII.4 NoV (VA387) without the last five residues to prevent P particle formation [29], while the HEV P is the P domain (residue 452 to 617, AC#: DQ079627) of a zoonotic genotype 3 HEV from a pig) [30] that is a part of the E2 protein of HEV [11, 31]. A peptide (CDCRGDCFC) was added to the C-terminus of the HEV P to stabilize the protein [28]. The NoV P⁻- and HEV P-encoding cDNA sequences were PCR-amplified using two primer pairs (gcacggatcctcaagaactaaaccattcacc/atatcgctcctccgctccgctccgctccgccctcgctccgcccccgctccatttcc, for NoV P⁻ and tattcgctcccggatcctccggctccatctcgctcgtttctctgttc/catcgcgccgcttagcaaaagcaatcgccacggcaatcgcacgggtagtcaacgggtgc, for HEV P) with BamHI/BsmBI and BsmBI/NotI sites, respectively, ligated and cloned into pGEX-4T-1 after enzyme digestion. The NoV P⁻-HEV P was a thrombin-cleaved product of GST-NoV P⁻-HEV P (Figure 1). The dimeric NoV P⁻ and HEV P were made using the GST-NoV P⁻ and the GST-HEV P constructs as described previously [28].

Production and purification of recombinant proteins

The recombinant proteins were expressed in *E. coli* (BL21, DE3) as described previously [28, 32-34]. GST fusion proteins were purified using Glutathione Sepharose 4 Fast Flow resin (GE Healthcare Life Sciences). GST was removed from the interested proteins by thrombin (GE Healthcare Life Sciences) digestion.

SDS-PAGE and protein quantitation

Purified proteins were examined SDS-PAGE using 10% separating gels. Proteins were quantitated by SDS-PAGE using serially diluted bovine serum albumin (BSA, Bio-Rad) as standards on same gels [35].

Gel filtration chromatography

This was performed as described elsewhere [28, 32–34] using an Akta Fast Performance Liquid Chromatography system (model 920, GE Healthcare Life Sciences) through size exclusion columns (Superdex 200, 10/300 GL, GE Healthcare Life Sciences). The column was calibrated using gel filtration calibration kits (GE Healthcare Life Sciences) and purified NoV P particles (~830 kDa) [33], small P particles (~420 kDa) [36] and P dimers (~69 kDa) [32] as described previously [28]. The protein identities in the peaks were further characterized by SDS-PAGE.

Size analysis of polyvalent complexes by light scattering

The sizes of GST-NoV P⁻-HEV P and NoV P⁻-HEV P proteins were analyzed by light scattering using the high definition digital particle size analyzer (Saturn DigiSizer 5200, Micromeritics) with measurement range from 100 nm to 100 μ m. 1x phosphate buffer saline (PBS, pH7.4) were used to prewash the instrument.

Immunization of mice

Female BALB/c mice (Harlan-Sprague-Dawley, Indianapolis, IN) at 3-4 weeks of age were divided into three groups (N = 6-7) that were immunized with: 1) GST-NoV P⁻-HEV P (14.4 µg/mouse), 2) NoV P⁻-HEV P (10 µg/mouse), and 3) a mixture of NoV P⁻ (5 µg/mouse) and HEV P (5 µg/mouse) to insure same molar amount (~0.143 nanomole in 50-µl) of NoV P⁻ and HEV P for each mouse. Another group that was immunized with 50-µl PBS was included as negative control. Mice were immunized three times intranasally without adjuvant in 2-week intervals as described previously [28, 35]. Blood was collected by retro-orbital capillary plexus puncture before each immunization and two weeks after the final immunization. Sera were processed from blood via a standard protocol.

Enzyme immunoassay (EIA)

EIA was performed to determine the antibody titers of mouse antisera after immunization, as described elsewhere [35]. Gel-filtration purified NoV P⁻ and HEV P proteins were used as antigens to measure the NoV- and HEV-specific antibodies, respectively. Antigens (1 µg/ml) were coated on 96-well microtiter plates and incubated with serially diluted mouse sera. Bound antibodies were detected by goat-anti-mouse secondary antibody-HRP conjugates (MP Biomedicals, Inc). Antibody titers were defined as the end-point dilutions with a cutoff signal intensity of 0.15. Mouse sera after immunization with PBS were used as negative controls.

Histo-blood group antigen (HBGA) binding and blocking assays

The saliva-based binding assays that mimic NoV-HBGA attachment were performed as described elsewhere [37, 38]. Briefly, diluted saliva samples with defined HBGA were coated on 96-well microtiter plates and incubated with diluted NoV P proteins. The bound NoV P proteins were measured by guinea pig anti-NoV VLP antiserum, followed by an incubation of HRP-conjugated goat anti-guinea pig IgG (ICN Pharmaceuticals). The blocking assay that mimics neutralization of NoV-HBGA attachment by specific mouse antisera was basically a binding assay with an extra step of pre-incubation of NoV P particles (NoV surrogates) with mouse sera for 1 h before the P particles were incubated with the coated saliva. The blocking rates were defined as reduction rates by comparing the optical density with and without blocking. The 50% blocking titer (BT₅₀) was defined as the highest serum dilution causing a 50% reduction on the binding of NoV P particle to HBGA. The negative binding of HEV P with saliva was measured by the above binding assay using HEV P protein and HEV P specific antibody.

HEV neutralization assay

The HEV neutralization by mouse sera was measured essentially as previously described [39, 40] using the Kernow P6 strain (genotype 3, kindly provided by Dr. S.U. Emerson, NIAID) and HepG2/C3A cells. Infectious titers of HEV expressed as focus forming units (FFU) were determined by a fluorescent-focus assay (FFA). ~50,000 HepG2/C3A cells/well were seeded in 96-well plates. The viruses (100 FFU/well) were mixed with the 2-fold serially diluted mouse sera for 2 hours at 37°C and then added to the cells. After a 2-hour incubation, the inocula were discarded and replaced with maintenance medium. After further incubation for 5 days, the infected cells were fixed with 80% acetone and incubated with rabbit anti-HEV ORF2 antibody, washed with PBST (1xPBS with 0.2% tween-20), and then incubated with Alexa Fluor® 488 Goat Anti-Rabbit IgG Antibody. The stained cells were visualized via a fluorescence microscope. The neutralization titers of the sera were defined as the highest serum dilution that can reduce at least 60% of infected cells compared with no serum controls.

Statistical analysis

Statistical differences among data sets were calculated by softwares GraphPad Prism 6 (GraphPad Software, Inc) using an unpaired, non-parametric *t* test. *P*-values were set at 0.05 (*P* < 0.05) for significant difference, and 0.01 (*P* < 0.01) for highly significant difference.

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (23a) of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Cincinnati Children's Hospital Research Foundation (Animal Welfare Assurance no. A3108-01).

RESULTS

Production of GST-NoV P⁻-HEV P and NoV P⁻-HEV P complexes

Our previous study [28] showed that fusion of two to three dimeric proteins covalently formed polyvalent complexes with high immunogenicity. Based on this principle we fused the dimeric P domains of NoV [13] and HEV [11] together, designated as NoV P⁻-HEV P, and then fused it with the dimeric glutathione S-transferase (GST) [41], referred as GST-NoV P⁻-HEV P (Figure 1A). When expressed in *E. coli*, the GST-NoV P⁻-HEV P (~79 kDa) can be purified as a soluble protein at a yield of ~10 mg/liter of bacterial culture. NoV P⁻-HEV P protein (~54 kDa) can be obtained by thrombin digestion of the GST-NoV P⁻-HEV P protein (Figure 1, A and B).

The two fusion proteins were further purified and analyzed by gel filtration chromatography (Figure 1, B to D). The complex sizes of the GST-NoV P⁻-HEV P range from ~200 kDa to void (>800 kDa), being eluted in fractions 16 to 25, while the sizes of the NoV P⁻-HEV P range from ~100 to ~420 kDa, being eluted in fractions 23 to 28 (Figure 1, C and D). The accurate size distributions of the two proteins were further analyzed by light scattering using a high definition digital particle size analyzer (Saturn DigiSizer 5200, Micromeritics). A minor and a major peak were observed at 0.45 μ m and 1.8 μ m, respectively, while the sizes of the NoV P⁻-HEV P was outside the measuring range (100 nm to 100 μ m) of the instrument (Figure 1F and data not shown). These data showed that the GST-NoV P⁻-HEV P formed large polyvalent complexes, while the NoV P⁻-HEV P formed much smaller oligomers, most likely ranging from dimers (108 kDa) to octamers (432 kDa).

The complex formation increased ligand-binding of NoV P⁻

The P domain is the HBGA binding domain of NoVs and the free NoV P⁻ dimers is known to bind HBGA weakly [29, 32], while the polyvalent complex increases the binding activity of NoV P⁻ [28]. As expected, both GST-NoV P⁻-HEV P and NoV P⁻-HEV P exhibited significantly increased binding activity to HBGA than that of the free NoV P⁻ dimers (Figure 2, P s < 0.01). These data indicated that complex formation is an effective approach to increase the ligands binding of NoV P domain and that the NoV P⁻, most likely also the HEV P, retains the functional conformation necessary for a vaccine candidate.

The large complexes increased HEV P-specific antibody responses

Same molar amount of the two large complexes (GST-NoV P⁻-HEV P and NoV P⁻-HEV P) were immunized to mice intranasally using a mixture of NoV P⁻ and HEV P dimers for comparison and PBS as negative control. Mice after immunization with GST-NoV P⁻-HEV P and NoV P⁻-HEV P complexes developed significantly higher titers of HEV P-specific antibody than those immunized with the dimeric HEV Ps (P s < 0.01, Figure 3A). While the GST-NoV P⁻-HEV P induced higher HEV P antibody titer than that induced by the NoV P⁻-HEV P, the difference was not significant (P > 0.05). Thus, both polyvalent GST-NoV P⁻-HEV P and oligomeric NoV P⁻-HEV P significantly increased the immunogenicity of the HEV P antigen compared with that of the dimer.

The large complex-induced antisera exhibited increased HEV neutralizing titers

The antisera were further tested for their neutralizing titers against HEV (Kernow P6) infection in HepG2/C3A cells through fluorescent-focus assays. As shown in Figure 3B, the antisera after immunization of the polyvalent GST-NoV P⁻-HEV P complexes exhibited highest neutralizing titer among all experimental groups, which is significantly higher than that induced by the oligomeric NoV P⁻-HEV P (P < 0.01) or by the dimeric HEV Ps (P > 0.01). It was also noted that, while the neutralizing titer of the antisera after immunization of

the oligomeric NoV P⁻-HEV P is higher than that of sera induced by dimeric HEV P, their difference was not statistically significant ($P > 0.05$). These data suggested that the polyvalent GST-NoV P⁻-HEV P is a promising vaccine candidate against HEV.

The large complexes increased NoV P-specific immune responses

The mouse antisera were also examined for their NoV P-specific antibody titers. The data (Figure 4) showed that mice after immunization of polyvalent GST-NoV P⁻-HEV P and oligomeric NoV P⁻-HEV P resulted in significantly higher titers of NoV P-specific IgG than that induced by the dimeric NoV P⁻ antigen (both P s < 0.05 , Figure 4A). Interestingly, unlike the HEV P-specific antibody, the NoV P⁻-HEV P induced the highest NoV P antibody among all experimental groups, even higher than that induced by the polyvalent GST-NoV P⁻-HEV P complexes ($P < 0.05$). These data indicated that different components of a large complex may have different immunogenicity, probably owing to their differences in location or steric conformation within the complexes.

The mouse antisera blocked NoV-HBGA attachment

Due to the lack of a cell culture system, the blocking assay of NoV-HBGA attachment has been used as a surrogate neutralization assay for human NoVs [27, 42]. The mouse antisera were examined for their blocking activity, revealing that, corresponding to their NoV P-specific antibody titers, the antisera after immunization with NoV P⁻-HEV P exhibited the highest neutralization titer with a BT₅₀ of ~1:2400 to both type A and B salivas, followed by the antisera after immunization with GST-NoV P⁻-HEV P with a BT₅₀ of ~1:1000 to both salivas, while the antisera induced by the P dimer mixture exhibited the least blocking activity with a BT₅₀ of ~1:300 to both saliva samples (all P s < 0.05). These data suggested that both GST-NoV P⁻-HEV P and NoV P⁻-HEV P are promising vaccine candidates against NoVs.

DISCUSSION

In this study we designed and produced two novel chimeric protein complexes containing the neutralizing antigens of NoV and HEV and characterized them as bivalent vaccines against the two viruses. The complexes were designed based on the recently proved principle that fusion of two to three dimeric proteins covalently forms polyvalent complexes with high immunogenicity [28]. Both gel-filtration and light scattering confirmed the formations of the polyvalent complexes of GST-NoV P⁻-HEV P and oligomeric NoV P⁻-HEV P. Mouse immunization experiments demonstrated that both GST-NoV P⁻-HEV P and NoV P⁻-HEV P significantly increased the immunogenicity of the NoV P⁻ and HEV P antigens compared with those of their dimeric P antigens. The resulting mouse antisera exhibited high titers of NoV P- and HEV P-specific antibodies and specifically neutralized the two viruses. Thus, our data strongly suggest that the GST-NoV P⁻-HEV P and NoV P⁻-HEV P are promising dual vaccine candidates against both NoVs and HEVs.

The driven force of formation of the two types of large complexes in this study is the intermolecular dimerization between the dimeric partners of the fusion proteins. This principle has been proven previously [28]. Based on the sizes of NoV P ($5.7 \times 6.3 \times 6.9$ nm) [13], HEV P ($5.4 \times 3.0 \times 1.5$ nm) [11], and GST [41] dimers, a polyvalent GST-NoV P⁻-HEV P with a size of ~1.8 μ m should contain hundreds to thousands of each antigen. We noted that the NoV P⁻-HEV P fusion protein did not form polyvalent complexes as expected, instead, it formed a bunch of oligomeric complexes from dimers to octamers. Clearly, the oligomers were formed through the same intermolecular dimerization of NoV P⁻s and HEV Ps. However, unknown factors prevented the further enlargement of the complexes, probably due to the relatively lower affinity of dimerization between the HEV P

proteins. A previous study shown that dimerization of HEV P protein can be inhibited by a single residue mutation at the dimerization interface [11], suggesting that the interaction between the two HEV P is weak. Thus, a fusion of HEV P to NoV P may further weaken the dimerization of HEV P. If this is true, the polymer formation of the GST-NoV P⁻-HEV P may rely mainly on the dimerization of GST and NoV P⁻, as GST-NoV P⁻ has been shown to form polymer previously [28].

Although the NoV P⁻-HEV P proteins did not form polyvalent complexes, the resulting oligomeric complexes (100 to 420 kDa) are much larger than their monomeric forms (34.5 kDa and 19 kDa, respectively) and, most importantly, the oligomeric NoV P⁻-HEV P were able to induce significantly higher immune responses against NoV P and HEV P antigens compared with those induced by the dimeric antigens. In case of the NoV P antigen the oligomeric NoV P⁻-HEV P induced even significantly higher immune response than that induced by the polyvalent GST-NoV P⁻-HEV P. While the exact reason for this outcome remains unclear (see below), these new data extend the usefulness of our complex vaccine platform because it also works for oligomers for bivalent vaccine development. Further study is ongoing to replace the GST of the GST-NoV P⁻-HEV P with another antigen to make a trivalent vaccine.

The immune response against the NoV P induced by the polyvalent GST-NoV P⁻-HEV P being lower than that induced by the oligomeric NoV P⁻-HEV P was unexpected, which differed from that against the HEV P antigen. The factor(s) that affected these different outcomes remain unknown. Since the copy numbers of the two antigens are the same in each type of complex and equal molar amount of complexes were used in the immunization experiments, the different immune responses may be due to the different steric locations of the two antigens in the complexes. Further study is necessary to clarify this issue, which may also help to illustrate the underlying mechanism and help to prevent such issues. In addition, we noted that GST-NoV P⁻-HEV P and NoV P⁻-HEV P induced similar titers of HEV P-specific antibodies (Figure 3A, $P>0.05$), but the antisera after immunization of GST-NoV P⁻-HEV P showed significantly higher neutralization against HEVs than those induced by NoV P⁻-HEV P (Figure 3B, $P<0.01$). While the reason for these differences remains to be defined, one possibility may be due to different HEV strains used for antibody titer determination (a zoonotic genotype 3 HEV from a pig) and neutralization assay (Kernow P6 strain, genotype 3).

The observed increase of binding signals of the GST-NoV P⁻-HEV P and NoV P⁻-HEV P to HBGAs compared with that of the NoV P⁻ dimer may be due to the avidity effects of the large complexes, in other words, the combined strength of multiple bond interactions. Correlation between the binding signals and the valence of the NoV P protein was observed before. For example, the order of the binding signals of different NoV VP1 complexes to HBGAs are virus-like particles (VLPs, 180mer) > P particles (24mer) > small P particles (12mer) > P dimers [32, 36], although other studies suggested that the binding affinity of the binding site of these dimers, oligomers, polymers may remain the same [43-45]. In addition, the large complexes may provide more reactive regions for the detective antibody, which may also contribute to the increased binding signals in an EIA-based NoV-HBGA binding assay. Most importantly, the binding of the two complexes to HBGA ligands suggested that the NoV P⁻ and HEV P antigens in the complexes retained functional structure and conformation required for vaccine candidates. In fact, the data that both GST-NoV P⁻-HEV P and NoV P⁻-HEV P induced neutralizing antibodies against the two viruses supported this hypothesis. Since both HBGA binding activity detected by an Elisa assay and immune response of NoV P domain are correlated with valence and complexity of the complexes, the increase of ligand binding appeared to correlate with the increase of immune response, as shown by the results that both large complexes (GST-NoV P⁻-HEV P and NoV P⁻-HEV P)

revealed significantly higher HBGA binding signals and increased immunogenicity of the NoV P antigen than those of the NoV P dimers.

It was noted that bacterial GroEL (~60kDa), a member of chaperonin family, co-purified with the affinity column purified recombinant proteins, which can be removed from our target proteins by gel-filtration chromatography (Figure 1). In addition, our ongoing study showed that the contaminated GroEL can be removed by anion exchange chromatography through a Resource Q or Mono Q column (GE Healthcare life sciences) (unpublished data). Most importantly, our previously studies using bacteria-expressed recombinant proteins with more or less contaminated GroEL did not reveal an effect of GroEL on the immunogenicity of the studied proteins [28, 35, 46, 47]. For an accurate quantitation, immunogens were loaded on SDS PAGE gels with known amount of BSA as standards on the same gel. The purpose of this study is to compare the immunogenicity of recombinant proteins with similar components but in different complexity or valences and they had similar level GroEL contamination. Thus, our results should be reliable. Finally, while cell culture-based neutralization assays of the vaccines were performed for HEVs, a cell culture for human NoVs is lacking and thus surrogate neutralization (a blocking assay of HBGA-NoV interaction) of the vaccine was carried out for NoVs. Further evaluation of the efficacy of the vaccines through challenge studies is necessary in the future.

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Figure 1.

Expression, purification and characterization of the GST-NoV P⁻-HEV P and the NoV P⁻-HEV P complexes. (A) Schematic illustration of the GST-NoV P⁻-HEV P fusion and its thrombin digestion (+T) into NoV P⁻-HEV P (blue and purple) and GST (green). The thrombin digestion site (T) between the GST and the NoV P⁻-HEV P is indicated. (B) Affinity column-purified (left panel) and gel-filtration-purified (right panel) GST-NoV P⁻-HEV P and NoV P⁻-HEV P proteins are analyzed by SDS PAGE. Positions of the GST-NoV P⁻-HEV P, the NoV P⁻-HEV P and a co-purified protein, bacterial GroEL, are indicated. M represents pre-stained protein markers, with bands from top to bottom representing 113, 92, and 54 (left panel) or 52 (right panel) kDa. (C) and (D) The elution

curves of gel filtrations of GST-NoV P⁻-HEV P (C) and NoV P⁻-HEV P proteins (D). The GST-NoV P⁻-HEV P fusion protein (C) showed a collection of large complexes as indicated by the major peaks at and near the void volume of the size-exclusion column Superdex 200 (10/300 GL, GE Healthcare Life Sciences), while NoV P⁻-HEV P (D) forms peaks between ~420 kDa and ~100 kDa, suggesting formation of polymer (C) and oligomers (D), respectively. The gel filtration columns were calibrated by the Gel Filtration Calibration Kit (GE Healthcare Life Sciences) and the purified recombinant P particle, small P particle, and P dimer of norovirus (VA387). The elution positions of blue Dextran 2000 (~2000 kDa, void), P particle (~830 kDa), small P particle (~420 kDa), P dimer (~69 kDa), and aprotinin (~6.5kDa) were indicated. The major protein peaks of the gel-filtrations were analyzed by SDS-PAGE shown below the corresponding elution curves. Fraction #15 represents the beginning of the void volume, fractions #31/32 represent the elution position of NoV P dimers (~69 kDa). The elution ranges of the GST-NoV P⁻-HEV P (C) or NoV P⁻-HEV P (D) are indicated by blue bars. Inp, input protein. (E) The size distribution curve of the GST-NoV P⁻-HEV P protein determined by a high definition digital particle size analyzer (Saturn DigiSizer 5200, Micromeritics). A minor and a major peak were seen centering at ~0.45 μ m and 1.8 μ m, respectively.

Figure 2.

Large complexes of GST-NoV P⁻-HEV P and NoV P⁻-HEV P increased binding activity to HBGA ligands compared with that of the NoV P⁻ dimers. Boiled and diluted saliva samples with defined type A (A) or B (B) antigens, respectively, were used for the binding assays. GST-NoV P⁻-HEV P (NoV P-HEV P) and NoV P⁻-HEV P (NoV P-HEV P), NoV P⁻ (NoV P) and HEV P proteins at different concentrations are indicated in X-axis, while the signal intensities of binding (OD) are shown in Y-axis. The statistical significances of the binding activity between the GST-NoV P⁻-HEV P or NoV P⁻-HEV P and the NoV P⁻ are shown by * symbols (** $P < 0.01$).

Figure 3.

The polyvalent and the oligomeric complexes significantly increased immunogenicity of the HEV P antigen in mice. (A) HEV P-specific antibody responses of mice after immunization with equal molar amount of polyvalent GST-NoV P⁻-HEV P (GST-NoV P-HEV P, blue), oligomeric NoV P⁻-HEV P (NoV P-HEV P, red), a mixture of dimeric NoV P⁻ and HEV P (NoV P + HEV P, green), or phosphate buffer saline (PBS, black). (B) Neutralizing titers of the mouse antisera after immunization with different immunogens against HEV (Kernow P6 strain) infection in HepG2/C3A cells. The statistical significances between the data groups in (A) and (B) are shown by * symbols (** $P < 0.01$).

Figure 4.

The polyvalent and the oligomeric complexes significantly increased the immunogenicity of the NoV P antigen in mice. (A) NoV P-specific antibody responses of mice after immunization with equal molar amount of polyvalent GST-NoV P⁻-HEV P (GST-NoV P-HEV P, blue), oligomeric NoV P⁻-HEV P (NoV P-HEV P, red) and a mixture of dimeric NoV P⁻ and HEV P (NoV P + HEV P, green). Mice immunized with phosphate buffer saline (PBS, black) served as negative controls. The statistical differences between the data groups are shown by * symbols (* $P < 0.05$, ** $P < 0.01$). (B) and (C) Blocking activities of the mouse antisera after immunization with GST-NoV P⁻-HEV P (NoV P-HEV P, blue), NoV P⁻-HEV P (NoV P-HEV P, red), or a mixture of dimeric NoV P⁻ and HEV P (NoV P + HEV P, green), or PBS (PBS, black). The levels of BT₅₀s for all experimental groups are

indicated. Blocking experiments were performed using five to seven mouse sera independently for each immunization group and the average values are shown. The statistical *P* values among the BT₅₀s of all three experimental groups are < 0.05.