Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Vaccine 25 (2007) 3328–3337
Vaccine
www.elsevier.com/locate/vaccine

Non-toxic *Pseudomonas aeruginosa* exotoxin A expressing the FMDV VP1 G-H loop for mucosal vaccination of swine against foot and mouth disease virus

Sreerupa Challa [a,b], Roger Barrette [a,b], Debra Rood [a,b], John Zinckgraf [a,b,1], Richard French [c], Lawrence Silbart [a,b,d,*]

[a] *Department of Animal Science, University of Connecticut, Storrs, CT 06269, United States*
[b] *Center of Excellence for Vaccine Research, University of Connecticut, Storrs, CT 06269, United States*
[c] *Department of Pathobiology & Veterinary Science, University of Connecticut, Storrs, CT 06269, United States*
[d] *Department of Allied Health Sciences, University of Connecticut, Storrs, CT 06269, United States*

Received 8 October 2006; received in revised form 1 January 2007; accepted 3 January 2007
Available online 12 January 2007

Abstract

Synthetic peptides derived from the G-H loop of the foot and mouth disease virus (FMDV) capsid protein VP1 are relatively poor at recapitulating the native conformation present in the virus, and thus are often poor immunogens. We hypothesized that a candidate mucosal vaccine against FMDV could be developed using the non-toxic *Pseudomonas aeruginosa* exotoxin A (ntPE) to deliver the G-H loop in its native conformation. An added benefit of this approach is the potential for ntPE to serve as an effective carrier/adjuvant molecule for delivery of the fusion protein across the epithelial barrier by virtue of its capacity to bind to CD91. A chimeric protein (ntPE-GH) was generated by inserting the coding sequence of the G-H loop into an expression plasmid encoding ntPE, in place of the native Ib loop. Recombinant ntPE-GH and wild-type ntPE were each expressed in *Escherichia coli*, purified over a nickel resin, then administered intranasally to the pigs, with or without the mucosal adjuvant cholera toxin (CT). Both the ntPE and ntPE-GH induced mucosal and systemic immune responses against ntPE; moreover, ntPE-GH administered without CT induced anti-GH loop serum IgG antibodies. In conclusion, these data demonstrate that ntPE can be used as a mucosal carrier/adjuvant to induce an immune response against the VP1 G-H loop of FMDV.
© 2007 Elsevier Ltd. All rights reserved.

Keywords: Mucosal vaccine; Foot and mouth disease virus; Swine; Porcine; Vaccine

1. Introduction

Foot and mouth disease (FMD) is one of the most economically devastating and highly contagious diseases of cloven hoofed livestock. FMD outbreaks are usually pandemic, resulting in the slaughter of millions of animals and catastrophic economic consequences. Many industrialized nations including the US have adopted a “non-vaccination” policy toward FMDV, favoring aggressive import restrictions and surveillance to maintain their FMDV-free status. Thus, vaccination is relegated to one arm of a more comprehensive emergency response strategy in the event of an outbreak.

FMD is caused by a single stranded RNA virus bearing the same name (FMDV) which belongs to the family Picornaviridae and the genus *Aphthovirus*. It is a small, icosahedral, non-enveloped virus with a single stranded positive sense RNA genome of approximately 8400 nucleotides [1]. The FMDV capsid is composed of 60 copies of four structural proteins; the external VP1, VP2, VP3 and the internal VP4 [1]. X-ray crystallographic analysis of the virus suggests that all three external capsid proteins have a similar

* Corresponding author at: University of Connecticut, Center of Excellence for Vaccine Research, 1390 Storrs Road, ABL Room 302E, Unit 4163, Storrs, CT 06269-4163, United States. Tel.: +1 860 486 6073; fax: +1 860 486 5067.
E-mail address: Lawrence.Silbart@uconn.edu (L. Silbart).
[1] Present address: Antigen Express Inc., Worcester, MA 01606, United States.

0264-410X/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2007.01.006

Author's personal copy

structure consisting of an eight stranded β-barrel with a jelly roll topology, with the internal capsid protein VP4 bearing an N terminal myristic acid [2]. In serotype O, there are five known antigenic sites containing epitopes that elicit neutralizing antibodies of which only one antigenic site within site A of the G-H loop on the VP1 protein is continuous while the others are discontinuous (conformational). Antigenic site A comprises two highly mobile regions, the G-H loop, spanning residues 141–161, and the C-terminal residues 200–213 corresponding to the *major* and *minor* antigenic sites, respectively [2]. The G-H loop is highly mobile and exists as a stable quasi-circular structure on the capsid that undergoes hinge like movements due to the disulphide bond between Cys134 of the VP1 and Cys130 of the VP2 [3]. Additionally, the G-H loop contains an arginine–glycine–aspartic acid (RGD) triplet at positions 145–147 which is responsible for attachment of the virus to susceptible cells through integrin receptors such as $\alpha_v\beta_6$ [4,5]. Within the loop, the RGD triplet shows an open turn conformation preceded by an extended region, followed by a 3_{10} helix that extends to the anchoring point at the end of the loop [3,6]. Evidence from previous studies suggests that the G-H loop contains an important, immunodominant, linear B-cell epitope [7,8]. Neutralizing antibodies recognize this epitope as an independent unit and antibody binding immobilizes the loop in an orientation that depends on steric and structural constraints of the specific antibody binding [9–11]. Moreover, the G-H loop may adopt different serotype-specific orientations during antibody binding based upon sequence and conformational differences within the loop or in other capsid-associated elements [12]. Therefore, retention of the native G-H loop structure is important for inducing protection. Synthetic linear peptides representing the G-H loop have in some instances been shown to elicit neutralizing antibodies and protection against challenge [13,14], but have failed in other instances [15,16]. In general, peptide vaccines are poorly immunogenic, which may be due in part to their small size, rapid clearance from the body, lack of strong helper T cell epitopes or low proportion of the peptides adopting the native G-H loop conformation found on the virus. One strategy for improving the efficiency and immunogenicity of these peptide vaccines is to incorporate the G-H loop sequence into an easily expressed carrier protein, through genetic fusion. To date, a number of partner molecules including β galactosidase [17], and the bacterial outer membrane proteins Omp A [18] have been used to produce fusion proteins linked to the G-H loop.

FMDV is a mucosal pathogen with its primary route of entry through the oro- or naso-pharyngeal route, with virus replication taking place initially in epithelial or lymphoid cells [19]. Hence, the best prophylactic defense against FMD would be a mucosal vaccine capable of inducing both systemic and mucosal immunity. Unfortunately, stimulation of mucosal immune responses by non-viable antigens is often inefficient and may in some instances result in immunological tolerance [20]. Therefore, generation of an effective immune response requires addition of a carrier/adjuvant molecule such as bacterial exotoxins like cholera toxin (CT), heat labile enterotoxin (LT), pertussis toxin (PT) and exotoxin A (PE) secreted by *Vibrio cholera*, *Escherichia coli*, *Bordetella pertussis* and *Pseudomonas aeruginosa*, respectively. Each of these toxins contain ADP-ribosylating enzymes, and their non-toxic forms can be used as mucosal adjuvants because of their ability to bind to receptors on the epithelial cell surface and reach underlying mucosal tissue [21].

PE secreted by *Pseudomonas aeruginosa* in response to diminished availability of environmental iron, is a 66 kDa single protein with NAD^+-diphthamide ADP-ribosyl transferase activity. The crystal structure of wild-type PE reveals that the tertiary structure of the molecule consists of three domains; domain I (residues 1–252, "Ia"; residues 365–404, "Ib"), domain II (residues 253–364) and domain III (residues 405–613), required for receptor binding, transmembrane targeting and ADP-ribosylation, respectively [22–24].

PE binds to α2-macroglobulin receptor/low density lipoprotein receptor-related protein (LRP1)/CD91 present on epithelial cells and APC plasma membranes [25–28]. After binding, the toxin enters the host cell through receptor-mediated endocytosis. A portion of domain II, domain Ib and domain III translocate into the cytosol due to the presence of REDLK at the carboxy terminus after proteolytic cleavage by furin within domain II in the endocytic compartments. Domain III, which is a toxic moiety, catalyzes the inactivation of eukaryotic elongation factor 2 (eEF-2) via ADP-ribosylation resulting in an inhibition of protein synthesis and cell death [29,30]. However, deletion of GLU553 of domain III eliminates the ADP-ribosylating activity and renders the PE non-toxic (ntPE) [31]. Moreover, previous studies have shown induction of both salivary and serum antibody responses upon mucosal immunization of mice with ntPE-V3 loop of HIV, when the eight amino acid Ib loop of ntPE was replaced with the HIV V3 loop [32,33].

In the present study, ntPE was used as a carrier/adjuvant to deliver the G-H loop of the FMDV and present it to target cells in its native conformation. We constructed a chimeric ntPE-GH protein by inserting the coding sequence of the G-H loop into an expression plasmid encoding ntPE, in place of the native, non-essential sub-domain Ib loop. ntPE-GH was evaluated for the display of the G-H loop, and tested as a mucosal immunogen by immunizing pigs intranasally, then assessing the anti-peptide immune response. We show that ntPE-GH induced anti-G-H loop serum IgG antibodies along with anti-ntPE serum IgG and mucosal IgA antibodies, confirming the merit of this approach.

2. Materials and methods

2.1. Gene synthesis of G-H loop

A synthetic, *E. coli* biased gene sequence encoding the G-H loop (75 bp) of $O1_{BFS}$ strain was synthesized by

Author's personal copy

Table 1
Primers used in making recombinant fusion proteins

Primer name	Primer sequence (5′–3′)	Restriction sites (underlined)
SDMF	gcggcgggcgaggtccggctgatcggccatc	–
SDMR	gatggccgatcagccggacctcgcccgccgc	–
GH-synFP	ggtaccagcgccgacgtggtgagcctgacc**tgc**cgttatagt agaaacgcggtgccaaatttgaggggagat	*Xcm*1
GH-synRP	ggttgggcccgc**gca**cagtgtgcgagcaactttctgggctac agacttgcagatctcccctcaaatttggcac	*Apa*1
ExpFP	gcacatatgagcggccgcgccgaggaagccttt	*Not*1
ExpRP	gccaaccccg ggttacttcaggtcctcgcgcggcgg	*Xma*1

SDMF and SDMR were the primers used for site directed mutagenesis to delete Glu553 in PE. GH-synFP and GH-synRP were the primers used to synthesize the gene encoding G-H loop of FMDV. ExpFP and ExpRP were the primers used to amplify ntPE and ntPE-GH and subsequently insert into the protein expression plasmid pIVEX 2.3d.

Author's personal copy

polymerase chain reaction to encode the following amino acid sequence, RYSRNAVPNLRGDLQVLAQKVART as follows: TGC AGG TAT AGT AGA AAC GCT GTG CCA AAT TTG CGA GGA GAT TTA CAA GTA CTA GCG CAG AAA GTT GCA CGT ACA TGC. The sequence also incorporated *Xcm*1 and *Apa*1 restriction sites in the forward and reverse primers, respectively (Table 1). PCR was performed as follows: 94 °C for 1 min, followed by 5 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 1 min and 10 cycles of 94 °C for 15 s, 72 °C for 1.5 min and 72 °C for 1 min.

2.2. *Generation of non-toxic P. aeruginosa exotoxin A (ntPE)*

The plasmid pMJ21 was generated from plasmid pUC18 and genomic PAO1 (pseudomonas exotoxin A (PE)). pMJ21 was used to generate non-toxic PE (ntPE) by removing glutamic acid at position 553 through site directed mutagenesis (QuickChange^R XL Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA) according to the manufacturer's instructions using overlapping primers (Table 1). The deletion of glutamic acid GAA was confirmed by nucleotide sequencing (Retrogen Inc., San Diego, CA).

2.3. *Construction of ntPE-GH*

The nucleic acids encoding the Ib loop between domains II and III of ntPE were removed by restriction enzyme digestion using the restriction enzymes *Xcm*1 and *Apa*1 (New England Biolabs, Inc., Ipswich, MA). Both the digested ntPE and G-H loop were ligated using T4 DNA Ligase (Invitrogen, Carlsbad, CA) and transformed into Top10 *E. coli* cells (Invitrogen). Thus, the coding sequence for the G-H loop was inserted into ntPE, thereby replacing the Ib loop between *Xcm*1 and *Apa*1 restriction sites which was later confirmed upon nucleotide sequencing (Retrogen Inc.).

2.4. *Cloning ntPE-Ib and ntPE-GH into expression vector pIVEX2.3d*

Both ntPE-Ib (with Ib loop intact) and ntPE-GH (with the G-H loop in place of the Ib loop) were amplified from PMJ21 by PCR. The forward and reverse primers used for amplification included *Not*1 and *Xma*1 restriction sites, respectively (Table 1). The PCR reaction was carried out as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 65 °C for 30 s, 68 °C for 2 min and finally followed by elongated extension at 68 °C for 7 min. The amplified ntPE and ntPE-GH were each inserted into pIVEX 2.3d, RTS pIVEX His-tag vector with C terminus His6 tag (Roche Applied Sc., Indianapolis, IN) between the *Not*1 and *Xma*1 restriction sites within the multiple cloning site.

2.5. *Expression of fusion proteins*

The plasmids pIVEX 2.3d-ntPE-GH and pIVEX 2.3d-ntPE-Ib were transformed into Rosetta 2(DE3) pLysS cells (Novagen, San Diego, CA) and plated on 25 μg/ml carbenicillin and 20 μg/ml chloramphenicol containing Lauria Broth (LB) agar plates. The positive clones were grown in 25 ml LB media containing carbenicillin and chloramphenicol for 16 h at 37 °C in a shaker incubator. Cultures were transferred to 475 ml LB media with carbenicillin alone and incubated at 37 °C for 4 h in a shaker incubator until cultures had reached 0.5–0.6 O.D. units. IPTG (1 mM) was then added to induce protein synthesis, followed by incubation for 4 h at 37 °C in a shaker incubator. The cells were centrifuged at 2605 × *g* for 10 min in Sorvall Superspeed RC2-B centrifuge and pellets were stored at −20 °C overnight. The pellets were thawed and 12 ml of CelLytic^{TM} B reagent (Sigma, St. Louis, MO) and 20 μl of Benzonase nuclease (Novagen) were added and vortexed until samples were no longer viscous, then incubated at room temperature (RT) for 10 min. The solutions were transferred to 35 ml centrifuge tubes and inclusion bodies pelleted upon centrifuging at 23,360 × *g* for 10 min using the Sorvall super T 21 Tabletop Superspeed centrifuge. The soluble

supernatants were saved and inclusion bodies resuspended in 6 ml of Cellytic B reagent and 300 μl of working Lysozyme solution (Sigma), vortexed and incubated at RT for 10 min. Following incubation, 10 ml of 1:10 CelLytic B reagent was added and centrifuged at 23,360 × *g* for 10 min and the supernatant discarded. Inclusion bodies were washed three times with an additional 10 ml of 1:10 CelLytic B reagent, after which the inclusion bodies were solubilized in 10 ml of 8 M urea buffer.

2.6. Purification of fusion proteins

2.0 ml of His-select Ni^{2+} resin (Sigma) was centrifuged in a 15 ml Falcon tube at 200 × *g* for 5 min to remove EtOH. The resin was washed 1× with 13 ml ddH_2O and equilibrated using 13 ml inclusion body buffer with 8 M urea and centrifuged at 200 × *g* for 5 min. Resin was incubated with solubilized inclusion bodies at RT for 1 h followed by centrifugation at 200 × *g* for 5 min and the supernatant was saved for analysis. The resin with bound protein was then washed 2 times with 13 ml 1× wash buffer and purified protein was eluted after incubating the resin with 10 ml of elution buffer at RT for 30 min.

2.7. SDS-PAGE

Samples were mixed with 2× Laemmli Sample buffer (Sigma) in a 1:1 ratio and boiled at 70 °C in water bath for 10 min before electrophoresis through 4–20% ready gel Tris–HCl gels (Bio-Rad Laboratories Hercules, CA) using SDS-running buffer (25 mM Tris, 192 mM Glycine, 0.1%, w/v, SDS, pH 8.3) for one and half hour at 100 V. Gels were stained with Bio-safe ™ coomassie stain (Bio-Rad) overnight for protein visualization.

2.8. Western blot

Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 1 h. The membrane was then blocked for 1 h at RT using 3% non-fat dry milk in PBS with 0.05% Tween 20 (PBST), washed three times with PBST. Proteins were then probed with a 1:1000 dilution of pig anti-GH serum that was obtained from a pig immunized with G-H loop consensus peptide UBITh®-O (United Biomedical, Inc., Hauppauge, NY) for one hour at room temperature followed by washing five times with PBST. The membrane was then incubated for one hour at room temperature with horseradish peroxidase-conjugated goat anti-pig IgG antibody (Bethyl Laboratories, Inc, Montgomery, Texas). Antibody binding was detected upon incubation with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate per the manufacturer's instructions (Vector laboratories, Burlingame, CA) after the final five washes.

2.9. Circular dichroism

The far-UV circular dichroism spectra were recorded using a JASCO 810 (Jasco, MD) instrument at room temperature using default parameter settings. The spectra, in the 195–250 nm range, in a 1 mm path length cuvette, were collected, averaged, and converted to molar ellipticity using the instrument software. Both ntPE and ntPE-GH were used at a concentration of 100 μg/ml in PBS for all spectrophotometric readings.

2.10. Animals

Twenty mixed gender, 6–8 weeks old Yorkshire pigs (Arnold Yorkshires, Womellsdorf, PA) were used for the immunization as four groups of five each by the following vaccination regimen using ntPE and ntPE-GH with or without cholera toxin (CT) as mucosal adjuvant via the intranasal (i.n.) route; and ntPE-GH with Incomplete Freund's adjuvant via the intramuscular (i.m.) route of administration.

Group 1: ntPE (200 μg) + CT (20 μg) intranasally on weeks 0–3 and boosted i.m with 100 μg ntPE with IFA on week 5.
Group 2: ntPE-GH (100 μg) intramuscularly + IFA on weeks 0 and 3.
Group 3: ntPE-GH (200 μg) + CT (20 μg) intranasally on weeks 0–3 and boosted i.m with 100 μg ntPE-GH with IFA on week 5.
Group 4: ntPE-GH (200 μg) intranasally on weeks 0–3 and boosted i.m with 100 μg ntPE-GH with IFA on week 5.

Sera and nasal washes were collected from all animals weekly until the animals were sacrificed (Table 2). For all intranasal vaccinations, the antigens were administered as drops using a pipette, and the adjuvant CT was administered by mixing with the antigen in 1:1 ratio.

Table 2
Immunization regime: all groups had five animals each

Group	Priming week	Priming treatment	Adjuvant	i.n Boosting treatment	i.n. Boosting week	i.m Boosting treatment	i.m. Boosting week
I	0	200 μg ntPE i.n	20 μg CT	200 μg ntPE	1–3	100 μg ntPE	5
II	0	100 μg ntPE-GH i.m	IFA			100 μg ntPE-GH	3
III	0	200 μg ntPE-GH i.n	20 μg CT	200 μg ntPE-GH	1–3	100 μg ntPE-GH	5
IV	0	200 μg ntPE-GH i.n	–	200 μg ntPE-GH	1–3	100 μg ntPE-GH	5

Sera, nasal washes and saliva were collected on a weekly basis for 7 weeks.

Author's personal copy

2.11. ELISA

2.11.1. ntPE/ntPE-GH/G-H loop peptide specific IgG responses

The ntPE, ntPE-GH and G-H loop specific IgG antibodies in sera were determined using an indirect ELISA. The 96-well polystyrene Immulon-4 HBX microtiter plates (Dynex Technologies, Chantilly, VA) were coated with 50 μl of a 10 μg/ml solution of either ntPE, ntPE-GH, G-H loop peptide or BSA (negative control) in coating buffer (0.05 M Na_2CO_3 buffer, pH 9.6), tightly wrapped in Parafilm^{TM} (Pechiney Plastic Packaging, Menasha, WI) and incubated overnight at room temperature, then stored at 4 °C until needed. Plates were washed three times with 200 μl of wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). The plates were then incubated with 100 μl of blocking solution (50 Mm Tris, 0.14 M NaCl, 1% BSA, pH 8.0) for 1 h at room temperature (RT) and then washed three times with wash buffer. Serum samples and nasal washes from weeks 0 to 7 were diluted at 1:50 and 1:4, respectively, in sample buffer (50 Mm Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0), and 50 μl of each sample was applied in duplicate to the microtiter plate both on ntPE and BSA coated wells. After 1 h incubation at RT, the plates were washed 5 times with wash buffer, followed by addition of goat anti-pig IgG antibody conjugated to horse-radish peroxidase (Bethyl Laboratories) at a dilution of 1:50,000 in sample buffer and incubated for 1 h at RT. The plates incubated with nasal wash samples were also washed 5 times with wash buffer, followed by addition of goat anti-pig IgA conjugated to horse-radish peroxidase (Bethyl Laboratories) at a dilution of 1:20,000 in sample buffer and incubation for 1 h at RT. A final wash was performed followed by the addition of TMB substrate (KPL, Inc., Gaithersburg, Maryland) followed by addition of 2 M H_2SO_4 stop solution. The final O.D. reading was taken at 450 nm with an EL-311 Bio-Tek plate reader (Winooski, Vermont). The net anti-ntPE/G-H loop peptide responses for each sample was calculated by subtracting the mean O.D. of the BSA coated wells from the mean O.D. of specific antigen coated wells, and all IgG values were calculated by interpolation from the standard curve included on each plate.

2.12. Statistical analysis

Data were analyzed using SAS 9.3.1 for Microsoft Windows. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

In the present study we expressed, purified and characterized non-toxic *P. aeruginosa* exotoxin A (ntPE) bearing the G-H loop of FMDV VP1 protein in place of the native Ib loop. This chimeric protein was then tested as a potential vaccine candidate for production of humoral antibodies, both in serum and nasal washes, directed against the G-H loop epitope.

3.1. Gene synthesis of G-H loop

To produce the G-H loop of FMDV $O1_{BFS}$ strain in its native form, the gene was synthesized with an additional cysteine residue flanking the gene (Fig. 1A and Table 1). Synthesis of this 75 bp DNA sequence was confirmed by agarose gel electrophoresis (Fig. 2A) and DNA sequencing.

3.2. Construction of ntPE-GH

P. aeruginosa exotoxin A (PE) is a 67 kDa polypeptide composed of 613 amino acids. PE was rendered non-toxic (ntPE) by deleting the glutamic acid residue at position 553 through site-directed mutagenesis and deletion was confirmed by DNA sequencing. To construct ntPE-GH, a small portion of the gene encoding the Ib domain of ntPE was removed and subsequently replaced with the coding sequence for VP1 G-H loop (Fig. 1B). Insertion of the G-H loop coding sequence was confirmed by DNA sequencing the PCR amplified ntPE-GH product (Fig. 2B).

3.3. Expression and purification of ntPE and ntPE-GH

Both the control ntPE, with its intact native Ib loop, and recombinant ntPE with G-H loop were subcloned into the prokaryotic expression vector pIVEX2.3d which contained a C-terminal His6 tag. The fusion proteins were highly expressed after IPTG induction for 4 h at 37 °C and the recombinant protein was purified from inclusion bodies upon solubilisation in 8 M urea buffer and passage over a nickel resin. SDS-PAGE analysis confirmed that a single major protein band of the expected 67 kDa size was expressed in inclusion bodies and successfully purified using this approach (Fig. 3A and B).

3.4. Antigenicity of ntPE-GH

The antigenicity of the G-H loop within the fusion protein ntPE-GH was confirmed by immunoblotting. After SDS-PAGE, both ntPE and ntPE-GH were transferred onto a membrane and probed with hyperimmune antisera from pigs immunized with the G-H loop containing peptide UBITh® [37]. The G-H loop specific polyclonal antibodies recognized only ntPE-GH fusion protein, but not the native ntPE (Fig. 3C).

3.5. Circular dichroism

The secondary structural characteristics of the ntPE and ntPE-GH recombinant proteins were compared using circular dichroism spectroscopy to determine if both proteins had refolded properly. The secondary structure of the native ntPE was compared with that of ntPE-GH. The CD spectra for ntPE

Authors personal copy

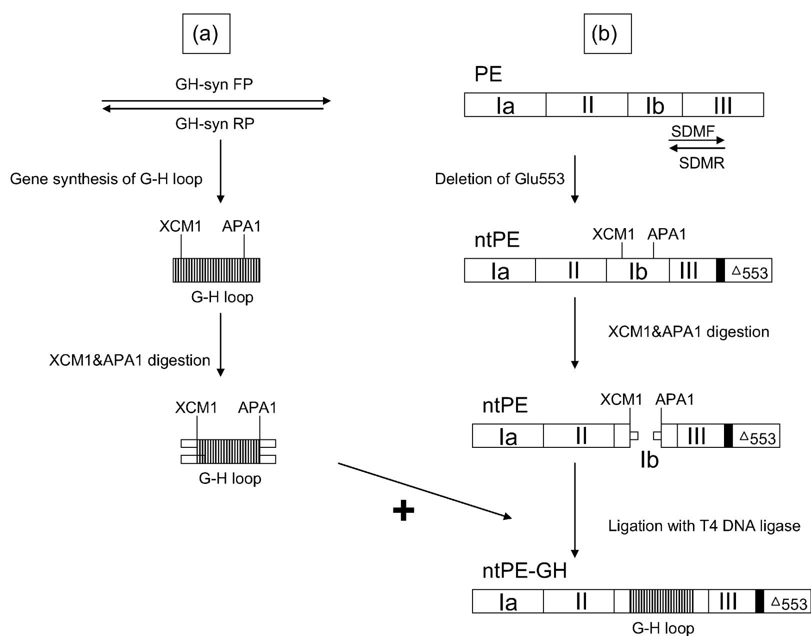

Fig. 1. Cloning strategy for generation of ntPE-GH. (a) The coding sequence for the G-H loop was synthesized by PCR using 72-mer overlapping primers GH-syn FP and GH-syn RP with *Xcm*1and *Apa*1 restriction enzyme sites at 5′ and 3′ end, respectively. (b) The coding sequence for *Pseudomonas aeruginosa* exotoxin A (PE) with all the domains, i.e., domain Ia, II, Ib and III in pUC18 vector was used to generate ntPE-GH. PE was made non-toxic (ntPE) by deleting the ADP-ribosylating amino acid glutamic acid at position 553 in domain III by site-directed mutagenesis using primers SDMF and SDMR. A small portion of Ib domain was removed by digesting ntPE with *Xcm*1 and *Apa*1. Digested ntPE and G-H loop were ligated with T4 DNA ligase to generate ntPE-GH.

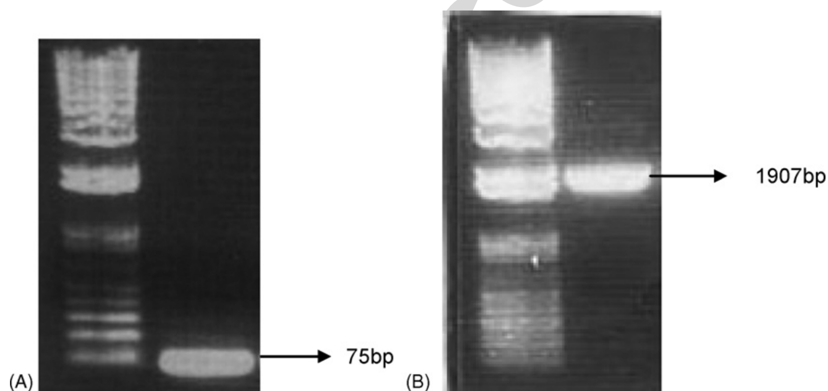

Fig. 2. Generation of ntPE-GH. (A) The 75 bp sequence encoding the VP1 G-H loop was synthesized by PCR using overlapping primers. (B) ntPE-GH amplified from a clone that was positively selected for the presence of plasmid after ligation and transformation. The presence of the G-H loop sequence in ntPE was later confirmed by DNA sequencing.

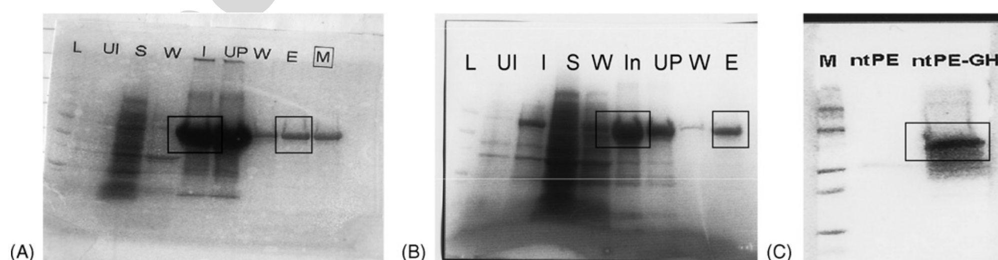

Fig. 3. Coomassie blue staining of expressed and purified ntPE and ntPE-GH. pIVEX2.3d-ntPE and pIVEX2.3d-ntPE-GH were transformed into Rosetta 2(DE3) pLysS cells and ntPE and ntPE-GH were expressed after induction with 1 mM IPTG. The proteins were expressed in inclusion bodies. The inclusion bodies were solubilized in 8 M urea. The proteins were purified by passing solubilized inclusion bodies over Ni^+ columns. Approximately 13 mg of ntPE per liter and 4 mg of ntPE-GH per liter culture of cells was obtained. (A) Expression and purification of ntPE. (B) Expression and purification of ntPE-GH (UI, uninduced; S, soluble protein fraction; I, inclusion bodies fraction; UP, unbound protein after passing over Nickel column; E, eluted purified protein). (C) Western blot analysis of ntPE-GH. After transfer of proteins to PVDF membrane, proteins were probed with anti-GH sera from G-H loop peptide immunized pig. Only ntPE-GH but not ntPE was immunoreactive to anti-GH sera. The bands in the marked boxes correspond to 67 kDa molecular weight.

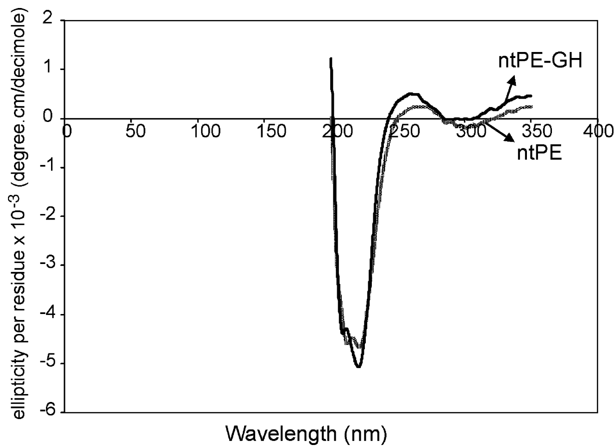

Fig. 4. Far-UV CD spectra of ntPE and ntPE-GH. The spectra in the 195–250 nm range in a 1 mm path length cuvette were collected, averaged, and converted to molar ellipticity using the instrument software.

and ntPE-GH were nearly identical, confirming the proper refolding of ntPE-GH (Fig. 4).

3.6. Antibody responses to recombinant fusion proteins

The purified ntPE and ntPE-GH fusion proteins were intranasally and intramuscularly administered to pigs as described in materials and methods (Table 2). Sera and nasal washes collected weekly were assayed for antigen specific antibodies by indirect ELISA. Nasal immunization with ntPE, ntPE-GH with or without CT, and parenteral immunization with ntPE-GH emulsified in IFA induced serum IgG antibodies directed against ntPE in all pigs (Fig. 5A). G-H loop specific antibody responses were significantly higher in group IV (group that received ntPE-GH without CT intranasally) than group III (group that received ntPE-GH with CT intranasally) indicating that the presence of CT did not show any additional effect in inducing anti G-H loop antibody responses (Fig. 5B). Nasal IgA anti-ntPE responses were detected in all intranasally vaccinated animals from as early as week 4 until the animals were sacrificed (Fig. 5C).

4. Discussion

Foot and mouth disease is widely reputed to be the most contagious and infectious disease of livestock, affecting both domesticated and wild cloven-hoofed animals. Typically, once an index animal is infected, there is a high probability that all other animals in the herd will be infected as exemplified by FMD outbreaks that occurred in Taiwan (1997) and the UK (2001). There are three reasons that prophylactic vaccination is often avoided in countries that are currently free of disease: (i) vaccinated animals, when exposed to live virus, may become persistently infected carriers; (ii) vaccination programs are expensive; and (iii) once animals have seroconverted to FMDV epitopes, differentiating vaccinated

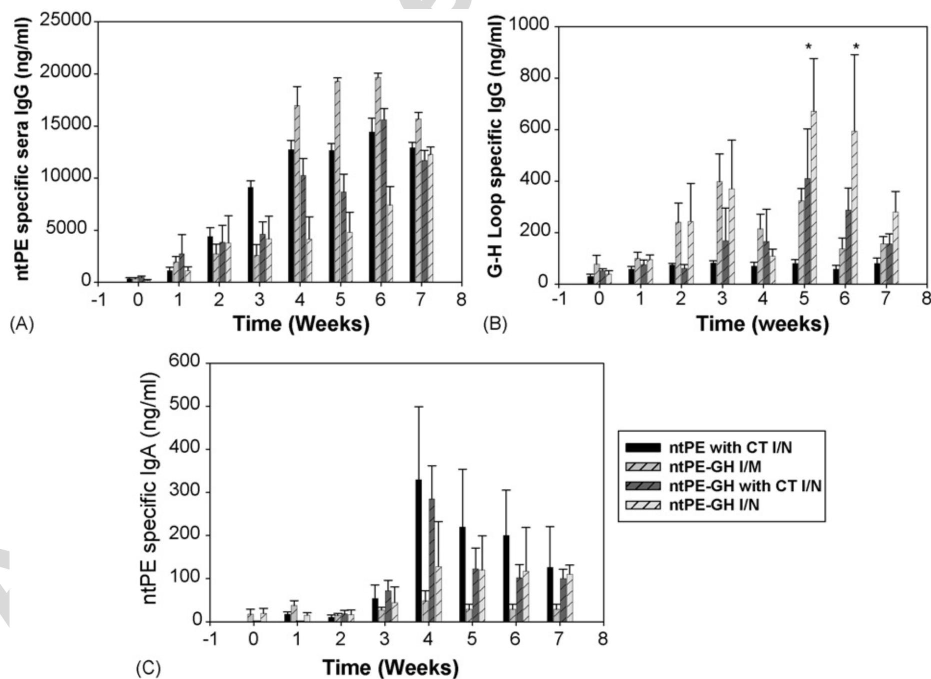

Fig. 5. Sera IgG and Nasal wash IgA responses. Four groups of five animals were used to study antibody responses to fusion proteins. Pre- and post-vaccination sera samples and nasal washes collected weekly for 8 weeks were assayed at 1:50 dilution and 1:4, respectively. All groups were primed on week zero. The groups that received ntPE or ntPE-GH i.n. were boosted i.n. on weeks 1–3 and i.m. on week 5 and were sacrificed on 7th week. The group that received ntPE-GH i.m. was boosted i.m with IFA on week 3 and assayed for anti-ntPE response by ELISA. (A) ntPE-specific IgG response in sera. (B) G-H loop peptide specific IgG response in sera. (C) ntPE-specific IgA response in nasal washes.

Author's personal copy

from infected animals can be difficult, resulting in export embargoes.

Although many mucosal vaccines have been developed against a variety of viral pathogens (e.g. poliovirus [34], rotavirus [35] and influenza virus [36]), there is no mucosal vaccine available for FMDV. Many studies have investigated the use of various G-H loop and VP1 peptides [13] as vaccine candidates for preventing FMD, which have been shown to elicit FMDV neutralizing antibodies and confer protection in guinea pigs [7] and pigs [37]. However, peptide vaccines are often poorly immunogenic, inducing poor memory responses as well as short durations of immunity. One promising approach to increase the immunogenecity of peptide vaccines is to incorporate the amino acids corresponding to specific linear epitopes into an easily expressed mucosal delivery protein by genetic fusion. We tested this approach by inserting the 25 amino acid G-H loop in place of the 8 amino acid Ib loop in ntPE, with two cysteine residues flanking the loop, constraining the C–C bond distances by formation of the native disulfide bond (Fig. 1 and Table 1). We hypothesized that the resulting fusion protein would have several distinct advantages to the linear peptide approach, including; providing appropriate molecular size, promiscuous helper T_h cell epitopes and also preserving the native conformation of the G-H loop.

In general, mucosal vaccination induces antigen-specific immune response at mucosal surfaces by virtue of lymphocyte trafficking to numerous tissues that comprise the common mucosal immune system (CMIS). This approach offers several advantages over parenteral immunization, including needle-less administration, reduced side effects and the potential for frequent boosting without the need for trained personnel. Despite the advantages of mucosal vaccination, success has been limited due to induction of low antibody titers, transient immune responses requiring multiple doses of antigen, a short duration of immunity, inefficient establishment of memory responses and mucosal tolerance. Nonetheless, it is well established that co-administration of bacterial enterotoxins like cholera toxin (CT), shiga toxin (ST), pertussis toxin (PT) and heat labile enterotoxin (LT) of *E. coli* can induce strong mucosal immune response and prevent the induction of oral tolerance [21,38], however, their use is restricted due to their toxicity.

PE is an ADP-ribosylating toxin that ribosylates elongation factor 2 and inhibits protein synthesis leading to cell death. Fortunately, PE can be made non-toxic by deleting the glutamic acid at position 553, a mutation that does not decrease its receptor-binding activity or modify any of the intracellular events that normally occur after internalization. Previously PE was shown to mediate the delivery of influenza peptides to MHC class I [39], to transport various peptides and enzymes to the cytosol [40], and to bind CD91 receptors and enter cells through receptor-mediated endocytosis [27,32]. CD91 was also shown to play an important role in binding to Heat Shock protein complexes and antigenic peptides, thereby facilitating antigen presentation in MHC class I molecules [41]. ntPE also induced a mixed Th1/Th2 response and sustained secretory IgA and systemic IgG responses because of its ability to present exogenous antigens to both MHC classes I and II molecules [32,42].

In the present study, we demonstrate that non-toxic pseudomonas exotoxin A can be used as a carrier/adjuvant to deliver the FMDV VP1 G-H loop motif across the mucosal barrier and induce both mucosal and systemic immune responses against this epitope. This was accomplished by first constructing a chimeric expression vector composed of non-toxic pseudomonas exotoxin A containing the VP1 G-H loop of FMDV, then testing the purified protein as an antigen for mucosal immunization.

Our results demonstrate that immunization with an ntPE-GH chimeric fusion protein induced strong ntPE specific and modest G-H loop specific IgA and IgG responses in nasal washes and serum, respectively. Fortunately, the intranasal route of immunization with ntPE-GH was effective at inducing both systemic (Fig. 5A) and mucosal (Fig. 5C) antibody responses. Furthermore, similar serum IgG responses were observed following either systemic or mucosal administration of ntPE-GH, suggesting that the chimeric protein was able to achieve systemic deposition following mucosal administration. Moreover, all animals vaccinated with ntPE-GH raised comparable levels of anti-GH antibodies in serum (Fig. 5B). However, there was a high degree of variability observed between animals within each group, possibly due to the out-bred genetics of pigs used in this study. One group of pigs that was immunized intranasally with the ntPE-GH along with mucosal adjuvant CT showed higher levels of anti-ntPE antibodies when compared to the group that was immunized with ntPE-GH without CT but surprisingly, the group that did not receive CT showed significantly higher levels of anti-GH antibodies than those receiving ntPE along with CT. Curiously, CT did not appear to act as an adjuvant to induce higher levels of antigen specific antibodies when co-administered with ntPE-GH but was effective in inducing higher levels of anti-ntPE antibodies. Unfortunately, in this study the ntPE-GH fusion protein did not induce virus neutralizing activity [data not shown] which is surprising in view of the encouraging results obtained in inducing anti-GH antibodies. Thus, further work is necessary to better understand the adjuvant action of ntPE and the conformational constraints it may have placed on the G-H loop.

In summary, we tested the response of pigs to a chimeric protein composed of a non-toxic form of pseudomonas exotoxin A containing 25 amino acid residue G-H loop of FMDV. We have shown that mucosal application of this chimera stimulated the production of ntPE and G-H loop specific serum and nasal IgA immune responses. Weekly immunization via the intranasal route induced a more rapid immune response than a single parenteral immunization, with serum IgG antibodies evident within 2 weeks and nasal IgA antibodies within 4 weeks of vaccination; however, coadministration of ntPE-GH with the mucosal adjuvant CT was not effective. At present, it is unclear how ntPE-GH induces these immune

Author's personal copy

responses whether by facilitating the delivery of the G-H loop or serving as an adjuvant, or both. In any case, ntPE may prove useful for inducing an efficient immune response when applied mucosally to swine.

Acknowledgements

We thank Drs. Randy Mrsny and David Fitzgerald for helpful discussions during the formative stages of the project, and Dr. Lynn Rust for providing the PE-encoding plasmid. We also thank Dr. Luis L. Rodriguez and Edward Kramer, USDA Plum Island Animal Disease Center, for performing the virus neutralization assays, Dr. Vijaya Kumar Challa for assistance with the CD experiments, Dr. Steven J. Geary for the critical review of the manuscript, and Roger Zemek for assistance with animal care and handling. We gratefully acknowledge the support of the USDA National Research Initiative Competitive Grants Program (Animal Health; NRICGP 2002-02833). This work was also supported in part by USDA grant 58-1940-0-007.

References

[1] Grubman MJ, Baxt B. Foot-and-mouth disease. Clin Microbiol Rev 2004;17(2):465–93.

[2] Acharya R, Fry E, Stuart D, Fox G, Rowlands D, Brown F. The three-dimensional structure of foot-and-mouth disease virus at 2.9 Angstroms resolution. Nature 1989;337:709–16.

[3] Jackson T, King AMQ, Stuart DI, Fry E. Structure and receptor binding. Virus Res 2003;91:33–46.

[4] Fox G, Parry N, Barnett PV, McGinn B, Rowlands DJ, Brown F. The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). J Gen Virol 1989;70:625–37.

[5] Brown JK, McAleese SM, Thornton EM, Pate JA, Schock A, Macrae AI, et al. Integrin-$\alpha_v\beta_6$, a putative receptor for foot-and-mouth disease virus, is constitutively expressed in ruminant airways. J Histochem Cytochem 2006;54:807–16.

[6] Logan D, Abu-Ghazaleh R, Blakemore W, Curry S, Jackson T, King A, et al. Structure of the major immunogenic site on foot-and-mouth disease virus. Nature 1993;362:566–8.

[7] Bittle JL, Houghten RA, Alexander H, Shinnick TM, Sutcliffe JG, Lerner RA, et al. Protection against FMD by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. Nature (Lond) 1982;298:30–3.

[8] Pfaff E, Mussgay M, Bohm HO, Schultz GE, Schaller H. Antibodies against a pre-selected peptide recognize and neutralize foot-and-mouth diesease virus. EMBO J 1982;1:869–74.

[9] Verdaguer N, Mateu MG, Andreu D, Giralt E, Domingo E, Fita I. Structure of the major anitgenic loop of foot-and-mouth disease virus complexed with a neutralizing antibody: direct involvement of the Arg-Gly-Asp motif in the interaction. EMBO J 1995;14(8):1690–6.

[10] Verdaguer N, Mateu MG, Bravo J, Domingo E, Fita I. Induced pocket to accomodate the cell attachment Arg-Gly-Asp motif in a neutralizing antibody against Foot-and-Mouth-Disease Virus. J Mol Biol 1996;256:364–76.

[11] Hewat EA, Verdaguer N, Fita I, Blakemore W, Brookes S, King A, et al. Structure of the complex of an Fab fragment of a neutralizing antibody with foot-and-mouth disease virus: positioning of a highly mobile antigenic loop. EMBO J 1997;16:1492–500.

[12] Brown F. Antibody recognition and neutralization of foot-and-mouth disease virus. Virology 1995;6:243–8.

[13] Fischer D, Rood D, Barrette RW, Zuwallack A, Kramer E, Brown F, et al. Intranasal immunization of guinea pigs with and immunodominant foot-and-mouth disease virus peptide conjugate induces mucosal and humoral antibodies and protection against challenge. J Virol 2003;77(13):7486–91.

[14] Rodriguez LL, Barrera J, Kramer E, Lubroth J, Brown F, Golde WT. A synthetic peptide containing the concencus sequence of the G-H loop region of foot-and-mouth disease virus type-O VP1 and a promiscuous T-helper epitope induces peptide-specific antibodies but fails to protect cattle against viral challenge. Vaccine 2003;21(25–26):3751–6.

[15] Taboga O, Tami C, Carrillo E, Nunez JI, Rodriguez A, Saiz JC, et al. A large-scale evaluation of peptide vaccines against foot-and-mouth disease: lack of solid protection in cattle and isolation of escape mutants. J Virol 1997;71(4):2606–14.

[16] Tami C, Taboga O, Berinstein A, Núñez JI, Palma EL, Domingo E, et al. Evidence of the coevolution of antigenicity and host cell tropisim of foot-and-mouth disease virus in vivo. J Virol 2003;77(2):1219–26.

[17] Broekhuijsen MP, van Rijn JM, Pouwels PH, Enger-Valk BE, Brown F, Francis MJ. Fusion proteins with multiple copies of the major antigenic determinant of foot-and-mouth disease virus protect both the natural host and laboratory animals. J Gen Virol 1987;68(Pt 12):3137–43.

[18] Ruppert A, Arnold N, Hobom G. Omp-A-FMDV VP1 fusion proteins: production, cell surface exposure and immune responses to the major antigenic domain of foot-and-mouth disease virus. Vaccine 1994;12:492–8.

[19] Prato Murphy ML, Forsyth MA, Belsham GJ, Salt JS. Localization of foot-and-mouth disease virus RNA by in situ hybridization within bovine tissues. Virus Res 1999;62(1):67–76.

[20] Holmgren J, Czerkinsky C, Eriksson K. Mharandi A: mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. Vaccine 2003;1(21 Suppl 2):S89–95.

[21] Lycke N. ADP-ribosylating bacterial enzymes for the targeted control of mucosal tolerance and immunity. Ann N Y Acad Sci 2006;1029:193–208.

[22] Allured S, Allured R, Collier J, Carroll SF, McKay DB. Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0-angstrom resolution. PNAS 1986;83:1320–4.

[23] Guidi-Rontani C, Collier RJ. Exotoxin A Pseudomonis aeruginosa: evidence that domain I functions in receptor binding. Mol Microbiol 1987;1(1):67–72.

[24] Hwang J, Fitzgerald DJ, Adhya S, Pastan I. Functional domains of Pseudomonis exotoxin identified by deletion analysis of the gene expressed in *E. coli*. Cell 1987;48(1):129–36.

[25] Mrsny RJ, Daugherty AL, McKee ML, Fitzgerald DJ. Bacterial toxins as tools for mucosal vaccination. DDT 2002;7(4):247–58.

[26] Smith DC, Lord JM, Roberts LM, Tartour E, Johannes L. 1st class ticket to class I: protein toxins as pathfinders for antigen presentation. Traffic 2002;3(10):697–704.

[27] Pastrana DV, Hanson AJ, Knisely J, Bu G, Fitzgerald DJ. LRP 1B functions as a receptor for Pseudomonas exotoxin. Biochim Biophys Acta 2005;1741(3):234–9.

[28] Kounnas MZ, Morris RE, Thompson MR, Fitzgerald DJ, Strickland DK, Saelinger CB. The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. J Biol Chem 1992;267(18):12420–3.

[29] Ogata M, Fryling CM, Pastan I, Fitzgerald DJ. Cell-mediated cleavage of Pseudomonas exotoxin between Arg279 and Gly280 generates the enzymatically active fragment which translocates to the cytosol. J Biol Chem 1992;267(35):25396–401.

[30] Armstrong S, Yates SP, Merrill AR. Insight into the catalytic mechanism of *Pseudomonas aeruginosa* exotoxin A: studies of toxin interaction with eukaryotic elongation factor-2. J Biol Chem 2002;277:46669–75.

[31] Killeen KP, Collier RJ. Conformational integrity of a recombinant toxoid of *Pseudomonas aeruginosa* exotoxin A containing a dele-

Author's personal copy

tion of glutamic acid-553. Biochim Biophys Acta 1992;1138(2): 162–6.
[32] Mrsny R, Daugherty A, Fryling C, Fitzgerald DJ. Mucosal administration of a chimera composed of Pseudomonas exotoxin and the gp120 V3 loop sequence of HIV-1 induces both salivary and serum antibody responses. Vaccine 1999;17:1425–33.
[33] Fitzgerald DJ, Fryling C, McKee ML, Vennari JC, Wrin T, Cromwell MEM, et al. Characterization of V3 loop-pseudomonas exotoxin chimeras. J Biol Chem 1998;273:9951–8.
[34] Ogra PL, Karzon DT, Righthand F, MacGillivray M. Immunoglobulin response in serum and secretions after immunization with live and inactivated poliovaccine and natural infection. N Engl J Med 1968;279:893–900.
[35] Barnes GL, Lund JS, Mitchell SV, DeBruyn L, Piggford L, Smith AL, et al. Early phase II trial of human rotavirus vaccine candidate RV3. Vaccine 2002;20(23–24):2950–6.
[36] Beyer WE, Palache AM, deJong JC, Osterhaus AD. Cold-adapted live influenza vaccine versus inactivated vaccine: systemic vaccine reactions, local and systemic antibody response, and vaccine efficacy. A meta-analysis. Vaccine 2002;20(9–10):1340–53.
[37] Wang CY, Chang TY, Walfield AM, Ye J, Shen M, Chen SP, et al. Effective synthetic peptide vaccine for foot-and-mouth disease in swine. Vaccine 2002;20:2603–10.
[38] de Haan L, Hirst TR. Cholera toxin and related enterotoxins: a cell biological and immunological perspective. J Nat Toxins 2000;9(3): 281–97.
[39] Donnelly JJ, Ulmer JB, Hawe LA, Friedman A, Shi X, Leander KR, et al. Targeted delivery of peptide epitopes to class I major histocompatability molecules by a modified Pseudomonas exotoxin. PNAS 1993;90:3530–4.
[40] Goletz TJ, Klimpel KR, Leppla SH, Keith JM, Berzofsky JA. Delivery of antigens to the MHC class I pathway using bacterial toxins. Hum Immunol 1997;54(2):129–36.
[41] Basu S, Binder RJ, Ramalingam T, Srivastava PK. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunity 2001;14(3):303–13.
[42] Lippolis JD, Denis-Mize KS, Brinckerhoff LH, Slingluff Jr CL, Galloway DR, Engelhard VH. Pseudomonas exotoxin-mediated delivery of exogenous antigens to MHC class I and class II processing pathways. Cell Immunol 2000;203(2):75–83.

Author's personal copy