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Induction of mucosal immune responses and protection against enteric viruses: rotavirus infection of gnotobiotic pigs as a model

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Abstract

Enteric viruses are a major cause of diarrhea in animals and humans. Among them, rotaviruses are one of the most important causes of diarrhea in young animals and human infants. A lack of understanding of mechanisms to induce intestinal immunity and the correlates of protective immunity in neonates has impaired development of safe and effective vaccines against enteric viruses. Studies of candidate vaccines using an adult mouse model of subclinical enteric viral infections often do not predict vaccine efficacy against disease evaluated in neonatal large animals. A series of studies have been conducted using a neonatal gnotobiotic pig model of rotavirus infection and diarrhea to identify correlates of protective immunity and to evaluate traditional and novel vaccine approaches for the induction of mucosal immune responses and protection to enteric viruses. Gnotobiotic pigs recovered from infection with virulent Wa human rotavirus (HRV) (mimic natural infection) had high numbers of intestinal IgA rotavirus-specific primary antibody-secreting cells (ASCs) and memory B-cells (to recall antigen) measured by ELISPOT assay, which correlated with complete protection against rotavirus challenge. Most short-term IgA memory B-cells were resident in the ileum, the major site of rotavirus replication. Spleen, not the bone marrow, was the major resident site for longer-term IgG memory B-cells. Candidate rotavirus vaccines evaluated in pigs for their ability to induce intestinal or systemic ASC and protection against rotavirus infection and diarrhea included attenuated live virus, inactivated virus, and baculovirus-expressed double-layered rotavirus-like particles (2/6-VLPs). In combination with those candidate vaccines, various adjuvants, delivery systems, and immunization routes were tested, including incomplete Freund's adjuvant for i.m. immunization, and a mutant *Escherichia coli* heat labile enterotoxin R192G (mLT) for i.n. immunization. It was shown that orally administered replicating vaccines were most effective for priming for intestinal IgA ASC and memory B-cell responses, but i.n. administered non-replicating 2/6-VLPs plus mLT were effective as booster vaccines. We conclude that protective immunity depends on the magnitude, location, viral protein-specificity, and isotype of the antibody responses induced by vaccination. Therefore highly effective enteric viral vaccines should: (i) induce sufficient levels of intestinal IgA antibodies; (ii) include viral antigens that induce neutralizing antibodies; and (iii) require the use of effective mucosal adjuvants or antigen delivery systems for non-replicating oral or i.n. vaccines. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Enteric viruses infect and damage intestinal epithelial cells leading to diarrheal disease (Saif, 1999b). Enteric viral infections remain a major cause of morbidity and mortality for neonatal farm animals and children (Saif and JackWood, 1990). A lack of understanding of mechanisms to induce intestinal immunity and the correlates of protective immunity in neonates has impaired development of safe and effective vaccines against enteric viruses for farm animals and children. Protection induced by candidate vaccines observed in studies using lab animals, such as mouse and rabbit models of rotavirus subclinical infections often do not predict the protective efficacy of these vaccines against clinical infections in studies of neonatal large animals (Fernandez et al., 1996; O'Neal et al., 1998; McNeal et al., 1999a,b; Yuan et al., 1998, 2000; Welter and Welter, 1990). Efforts to identify immunologic correlates of protective immunity to rotavirus infection using knockout mice for selected immune-related genes, passive lymphocyte transfer experiments, and immunodepletion of selected populations of lymphocytes by the injection of specific monoclonal antibodies have shown that none of the known effectors for acquired immune responses (CD4⁺ or CD8⁺ T-cells, antibodies, interferon- γ , perforin, etc.) are essential for protective immunity in adult mice (Franco and Greenberg, 1999; O'Neal et al., 2000). The outcome of vaccine efficacy studies using the adult mouse model of rotavirus infection also largely depends on the genetic background of the mouse strain (Franco and Greenberg, 2000). Uncharacterized innate immune mechanisms were suggested to play a major role in protective immunity against rotavirus infection in some mouse strains (Choi et al., 1999; Franco and Greenberg, 1999; McNeal et al., 1999b). The uncertainty of the roles of known immunologic effectors in protective immune responses to rotavirus in adult mice raises questions of the relevance of studies using adult mice as models for rotavirus immunity in neonatal farm animals and human infants.

Studies of mucosal immune responses and protective immunity induced by experimental rotavirus infections and candidate vaccines using a gnotobiotic pig model of rotavirus diarrhea (Chen et al., 1995; Yuan et al., 1996, 1998, 2000, 2001a,b) or trans-

missible gastroenteritis coronavirus (TGEV) infection in conventional piglets (VanCott et al., 1993, 1994) have consistently demonstrated that protective immunity against diarrhea from enteric viral infections depends upon the production of intestinal IgA antibody-secreting cell (ASC) and memory B-cell responses at the sites of virus replication (Saif, 1996, 1999a). Studies of natural rotavirus infections in children showed a similar positive correlation of IgA antibodies and protective immunity against rotavirus infection and diarrhea (Matson et al., 1993; Velazquez et al., 2000). Strategies to enhance production of virus-specific IgA antibodies to the neutralizing viral antigens in the small intestine (intestinal lamina propria) may be the most effective approach to induce protection against viral diarrheas. This paper will: (i) review vaccination strategies for induction of mucosal immune responses and protection against enteric viral infections and disease with emphasis on candidate rotavirus vaccines evaluated in a gnotobiotic pig model of rotavirus disease; (ii) discuss comparative vaccine protective efficacies evaluated using the neonatal gnotobiotic pig model of rotavirus disease versus the adult mouse model of rotavirus infection; (iii) compare the distribution pattern of memory B-cells in pigs to that of T- and B-memory cells in mice and their role in the protective immunity; and (iv) briefly summarize the features of three novel mucosal vaccine delivery systems (coronavirus derived expression systems, microencapsulation and immune stimulating complexes (ISCOMs)) and their potential for improving the efficacy of enteric virus vaccines.

2. Vaccination approaches evaluated in gnotobiotic pigs for induction of intestinal antibody-secreting cells and memory B-cells to rotavirus and protection against rotavirus diarrhea

The characteristics of the mucosal immune system and the pathogenesis of enteric viruses, including rotavirus have been reviewed (Mowat and Viney, 1997; Saif, 1999b). Using neonatal gnotobiotic pigs, a unique model to study rotavirus immunity (Saif et al., 1997), a series of studies have been conducted to identify the immune correlates of protective immunity to rotavirus infection and disease (Chen et al., 1995;

Ward et al., 1996b; Yuan et al., 1996, 1998, 2000, 2001a,b) and to evaluate various vaccination approaches (vaccine types, immunization routes and adjuvants) for their ability to induce intestinal and systemic immune responses and protection against rotavirus challenge (Ward et al., 1996b; Yuan et al., 1996, 1998, 2000, 2001b). The questions addressed were: What are the basic parameters of the host mucosal and systemic antibody responses (isotype and tissue distribution) to infectious rotavirus (virulent or attenuated) or non-replicating (inactivated or VLP) vaccines in naïve pigs? How do these responses correlate with protection? How does the type of vaccine and route (oral, i.m., i.n., or sequential combinations of oral and i.n.) of administration influence the mucosal and systemic antibody responses to rotavirus and the degree of protection?

2.1. Immunity induced by oral inoculation of pigs with live viruses

We first investigated the ASC and memory B-cell responses to a virulent human rotavirus (HRV) Wa strain (to mimic natural infections) and an attenuated Wa HRV strain (to mimic live oral vaccines), the degree of protection induced against virulent Wa HRV challenge, and potential correlates of protection (Yuan et al., 1996, 2001a). Four groups of gnotobiotic pigs were orally inoculated with one dose of virulent Wa HRV at 3–5 days of age, or 2 or 3 doses of attenuated Wa HRV (AttHRV 2 \times , 3 \times) at 10-day intervals, or mock-infected with cell-culture fluids, respectively, and challenged at 21–28 postinoculation days (PIDs) with virulent Wa HRV. The virus-specific ASC and memory B-cell responses in the intestinal (duodenum, ileum, and mesenteric lymph nodes) and systemic (spleen, peripheral blood, and bone marrow) lymphoid tissues were enumerated using an isotype-specific ELISPOT assay at PID 21–28 and post-challenge days (PCDs) 7 (PID 28–35). To quantitate virus-specific ASC, an ELISPOT assay was performed on the day of mononuclear cells (MNCs) extraction (Yuan et al., 1996). To detect virus-specific memory B-cells, an ELISPOT assay was performed after the MNC from each tissue had been stimulated *in vitro* with semipurified Wa HRV antigen in cell culture for 5 days (Yuan et al., 2001a). The ASC and memory B-cell responses in three representative tissues at

PCD 0 and protection rates against challenge are summarized in Table 1. Pigs recovered from virulent rotavirus infection were completely protected from virus shedding upon challenge. These pigs developed significantly higher numbers of virus-specific IgA and IgG ASC and memory B-cells in the intestinal lamina propria compared to the attenuated Wa HRV inoculation groups. Oral inoculation of pigs with 2 or 3 doses of attenuated Wa HRV induced lower numbers of IgA and IgG ASC and memory B-cells in the intestinal lamina propria, but higher numbers of memory B-cells in the spleen compared to the virulent Wa HRV inoculated pigs. The attenuated Wa HRV inoculated pigs were partially protected against virus shedding and diarrhea after challenge (Table 1). The protection rates from the virulent, AttHRV2 \times , and AttHRV3 \times inoculation groups were positively correlated with the magnitude of IgA ASC and memory B-cell responses in the intestinal lamina propria. A positive correlation between intestinal IgA antibody responses and protection was also shown in a study of mice comparing the differences in the mucosal and systemic immune responses generated by oral inoculation of mice with heterologous (non-murine) and homologous (murine) rotaviruses and the ability of these infections to produce protective immunity against subsequent reinfection upon challenge (Feng et al., 1994). Sucking mice were inoculated orally with live rhesus (RRV), bovine (NCDV), or murine (EHP) rotavirus and challenged at PID 42 with virulent murine rotavirus (ECW). Conclusions from this study using the adult mouse model of rotavirus infection agreed with the findings from studies of pigs infected with virulent or attenuated Wa HRV and challenged with virulent Wa HRV; i.e., the ability of a heterologous rotavirus to stimulate a detectable intestinal IgA antibody response correlated with its ability to generate protective immunity.

2.2. Non-replicating vaccines (inactivated virus and VLPs)

Due to the nature of enteric virus infections (superficial infections of the intestinal epithelial cells), IgA antibodies present in the intestine at the time of exposure provide the most important first line of protection by neutralizing virus infectivity in the lumen or intracellularly (Burns et al., 1996), preventing the viruses from attaching to and penetrating the

Table 1
Intestinal and systemic IgA and IgG ASC and memory B-cell responses at PCD 0 and protection induced by virulent and attenuated Wa HRV in gnotobiotic pigs^a

Virus inoculation group	Mean no. ASC/ 5×10^5 MNC						Mean no. memory B-cells/ 5×10^5 MNC						Protection rate against challenge	
	Intestinal lamina propria		Spleen		Bone marrow		Intestinal lamina propria		Spleen		Bone marrow		Diarrhea (%)	Shedding (%)
	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG		
Virulent Wa HRV	171	114	4	13	1	1	364	3637	58	2288	0	10	87	100
Attenuated Wa HRV 3 \times	8	3	2	10	0	1	8	55	135	3700	0	4	62	67
Attenuated Wa HRV 2 \times	6	41	1	2	ND ^b	ND	ND	ND	ND	ND	ND	ND	34	19

^a Data summarized from Yuan et al. (1996, 2001a).

^b Not determined.

epithelium, and excreting bound antigens (immune complexes) through the epithelium back into the lumen (Saif, 1999a). To stimulate intestinal IgA antibody responses, oral immunization is preferable because it mimics the route of natural infection. However, oral vaccines have shown insufficient or inconsistent efficacy in the field and clinical trials (Bresee et al., 1999; Saif and JackWood, 1990). The reduced immunogenicity of the vaccine strain compared to the virulent strain of the virus (Yuan et al., 1996; Ward et al., 1996b), the potential risk of adverse effects of high vaccine doses or live vaccines in infants (intussusception) (Anonymous, 1999), and the interference of preexisting antibodies in the intestine of neonates (mainly maternal antibodies derived from colostrum and milk) with replicating virus vaccines (Hodgins et al., 1999; Parreno et al., 1999) are major obstacles to improving the efficacy of live oral rotavirus vaccines.

To circumvent the obstacles for oral live vaccines, non-replicating vaccines have been evaluated for their immunogenicity and protective efficacy. Studies of adult mice and rabbits have shown that non-replicating rotavirus vaccines (inactivated virus or VLPs) administered via extraintestinal immunization routes (i.m., i.n. or i.p.) induced complete or significant partial protection against infection with or without adjuvants (Conner et al., 1993; McNeal et al., 1992, 1998, 1999a,b; Offit and Dudzik, 1989; O'Neal et al., 1997, 1998). We evaluated the immunogenicity and protective efficacy of non-replicating rotavirus vaccines in combination with different adjuvants, including inactivated Wa HRV given by the oral or i.m. route with incomplete Freund's adjuvant (IFA) (Yuan et al., 1998) and 2/6-VLP vaccines given by the i.n. route with or without a mutant *Escherichia coli* heat labile enterotoxin R192G (mLT) as a mucosal adjuvant in gnotobiotic pigs (Yuan et al., 2000). The mLT and other LT or *Cholera toxin* (CT) derivatives have been shown to retain the adjuvant effects of the native LT or CT to enhance immune responses to unrelated antigens when coadministered orally or i.n. through induction of both Th1- and Th2-type immune responses (LT) or by promoting Th2-type immune responses (CT) (Freytag and Clements, 1999; Pizza et al., 2001). The ASC and memory B-cell responses and the protection rates induced by the non-replicating rotavirus vaccines are summarized in

Table 2. Binary-ethyleneimine inactivated Wa HRV administered orally alone or by the i.m. route with IFA twice to gnotobiotic pigs induced few IgA ASC and memory B-cells in the intestinal lamina propria and conferred minimal protection (0–6%) against challenge. Substantially higher numbers of IgG ASC and memory B-cells were induced in the systemic lymphoid tissues (spleen and peripheral blood) by i.m. inoculation of pigs with inactivated Wa HRV; however, IgG ASC or memory B-cells in the systemic lymphoid tissues were not associated with protective immunity. Thus, inactivated rotavirus vaccines administered via either the oral or i.m. route were significantly less efficacious in inducing intestinal IgA antibody responses and conferring protective immunity than the oral live rotavirus vaccines. The segregation of the mucosal and systemic immune responses appears to be the cause of the failure of i.m. vaccination to induce intestinal immune responses. It was shown in a study of humans that T- and B-cells activated by the oral and parenteral routes differ markedly with regard to their respective homing potentials (Kantele et al., 1997). The majority of lymphocytes activated by parenteral inoculation expressed L-selectin, the principal peripheral lymph node homing receptor; and the majority of this population of B-cells were committed to IgG production (Quiding-Jarbrink et al., 1995), which explains the high numbers of IgG ASC and memory B-cells detected in the spleen of pigs inoculated i.m. with inactivated rotavirus (Table 2). The observations on the isotype and tissue distribution of antibody responses and protection in pigs inoculated i.m. with inactivated rotavirus concur with the findings from studies of mice showing a relationship between the homing receptor expression pattern of memory B-cells and protective immunity (Rose et al., 1998; Rott et al., 1997; Williams et al., 1998) whereby protective immunity for an intestinal pathogen, such as rotavirus, resides in the subsets of memory B- and T-cells expressing the intestinal homing receptor $\alpha 4\beta 7$, and this phenotype is not shared with memory cells elicited by i.m. immunization.

Lymphocytes sensitized in the nasal-associated lymphoid tissues (NALTs) can relocate to distant effector sites through the common mucosal immune system (Brandtzaeg et al., 1999). The advantages of NALT as an inductive site for vaccine antigens, particularly

Table 2
Intestinal and systemic IgA and IgG ASC and memory B-cell responses at PCD 0 and protection induced by non-replicating vaccines in gnotobiotic pigs^a

Vaccine group	Mean no. ASC/5 × 10 ⁵ MNC				Mean no. memory B-cells/5 × 10 ⁵ MNC				Protection rate against challenge	
	Intestinal lamina propria		Spleen		Intestinal lamina propria		Spleen		Diarrhea (%)	Shedding (%)
	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG		
Inactivated Wa HRV PO 2×	5	1	0	0	0	0	4	16	0	0
Inactivated Wa HRV + IFA IM 2×	3	4	0	34	2	0	5	2017	6	0
SA11 2/6-VLPs IN 2×	6	4	0	1	9	3	1	3	0	0
SA11 2/6-VLP + mLTIN 2× and 3×	29	16	0	1	75	149	4	26	0	10

^a Data summarized from Yuan et al. (1998, 2000).

for non-replicating antigens, include the presence of antigen-retaining crypts and the absence of antigen-degrading digestive enzymes. Although the i.n. route has been shown to be more efficient for the induction of IgA antibody responses in the upper respiratory and genitourinary tract, and less efficient for the intestinal lamina propria in rodents and pigs (Brandtzaeg et al., 1999; McDermott and Bienenstock, 1979; VanCott et al., 1994), the i.n. vaccination route has been shown to be effective for induction of protective immunity against rotavirus infection in adult mice (O'Neal et al., 1997, 1998; McNeal et al., 1999b). In gnotobiotic pigs, double-layered 2/6-VLP vaccines derived from co-expression of the bovine RF core protein VP2 gene and the inner capsid protein VP6 gene of simian SA11 or human Wa rotavirus strains were evaluated using the i.n. route (Yuan et al., 2000). The VP2 core and VP6 inner capsid rotavirus proteins did not induce virus neutralizing antibodies which are induced only by the rotavirus outer capsid proteins VP4 and VP7. Four groups of gnotobiotic pigs received 250 µg per dose SA11 2/6-VLPs in 2 or 3 i.n. dose regimens (SA11 2/6-VLP + mLT IN 2× and 3×), two doses of SA11 2/6-VLPs alone (SA11 2/6-VLPs IN 2×), or mLT alone as controls, respectively. Intranasal inoculation of pigs with SA 11 2/6-VLPs plus mLT induced higher numbers of intestinal IgA and IgG ASC and memory B-cell responses than three oral doses of live attenuated Wa HRV, but lower IgA and IgG ASC and memory B-cells in the spleen (Tables 1 and 2). The mLT adjuvant enhanced intestinal ASC and memory B-cell responses to the VLP vaccines, as evident by the greatly increased numbers of intestinal IgA and IgG ASC and memory B-cells in the pigs vaccinated with SA11 2/6-VLPs + mLT compared to the SA11 2/6-VLPs alone at PID21 (PCD 0). However, SA11 2/6-VLPs administered i.n. in two or three dose regimens with or without mLT did not confer protection to pigs against diarrhea upon challenge (Table 2). The failure of 2/6-VLP vaccines suggests that protective immunity to rotavirus diarrhea in pigs depends not only on the location, the magnitude, and the isotypes of antibodies, but also on the protein-specificity (VP4 and VP7 neutralizing antibodies) of the B-cell responses. Although a similar SA11 2/6-VLP vaccine conferred complete protection against infection when adult mice were inoculated i.n. (O'Neal et al., 1998), protection against diarrhea in neonatal pigs probably requires the

presence of IgA neutralizing antibodies in the intestine to the outer capsid rotavirus proteins, VP4 and VP7.

Most non-replicating antigens (e.g. inactivated virus, recombinant proteins, and VLPs) are usually poor immunogens for inducing mucosal immune responses when administered without adjuvants, and may even result in immunological tolerance instead of immunity (Czerkinsky et al., 1999). To induce sufficient levels of immune responses, an effective mucosal adjuvant or delivery system that can greatly enhance the immunogenicity of the vaccine antigens is of critical importance. However, in a study of mice inoculated i.n. with purified inactivated double- or triple-layered rotavirus particles, similar rates of protection were induced by the vaccines with or without adjuvant; although mLT significantly increased antibody responses and reduced antigen concentrations needed for full protection (McNeal et al., 1999b). The discrepancies in rotavirus vaccine efficacies in studies of adult mice and neonatal pigs will be discussed further in a later section.

2.3. Combined vaccination regimens

Gut-associated lymphoid tissues (GALTs) and NALT are two major inductive sites for mucosal immune responses. The use of combined oral and i.n. vaccination routes stimulates multiple mucosal inductive sites, i.e., both GALT and NALT in contrast to the individual oral or i.n. vaccination route alone. We postulated that by exploiting the advantages of multiple mucosal immunization routes (oral and i.n.), and vaccine types (replicating virus and non-replicating VLPs), a combined vaccination regimen may optimally stimulate the mucosal immune system and increase the protective efficacy of rotavirus vaccines. Two combined vaccine regimens were evaluated in gnotobiotic pigs. In the first combined vaccine regimen group, gnotobiotic pigs were inoculated with one oral dose of attenuated Wa HRV at 3–5 days of age to prime the GALT inductive sites by live replicating virus. This was then followed by two i.n. boosting doses of Wa 2/6-VLP plus mLT (AttHRV/VLP2×) at 10-day intervals. In the second combined vaccine regimen group, pigs were inoculated with two i.n. doses of Wa 2/6-VLP plus mLT followed by one oral dose of live attenuated Wa HRV (VLP2×/AttHRV1×). A single oral dose of attenuated

Wa HRV (AttHRV1 \times) and three i.n. doses of Wa 2/6-VLP plus mLT (Wa 2/6-VLP + mLT 3 \times) groups were included as controls. The ASC responses and protective efficacy of the combined regimens are summarized in Table 3. The AttHRV/VLP2 \times regimen induced similar mean numbers of intestinal IgA ASC as induced after virulent Wa HRV infection (135 versus 171 ASC per 5×10^5 MNC, respectively, Tables 1 and 3) and the IgA ASC numbers were significantly higher than those induced by a single oral dose of attenuated Wa HRV or three i.n. doses of Wa 2/6-VLP plus mLT at PID 28 (PCD 0). The AttHRV/VLP2 \times regimen induced the highest mean numbers of intestinal IgA ASC at challenge among all rotavirus vaccines tested to date in gnotobiotic pigs including one to three oral doses of live attenuated Wa HRV, two or three i.m. doses of inactivated Wa HRV with IFA, and two or three i.n. doses of SA11 or Wa 2/6-VLPs with or without mLT (Yuan et al., 1996, 1998, 2000, 2001a,b). These results suggest that the combined immunization routes, oral priming followed by intranasal boosting, and vaccine types, live attenuated Wa HRV for priming followed by 2/6-VLPs plus mLT for boosting, was more effective in stimulating intestinal IgA ASC responses than each individual vaccine. The protective efficacy of the AttHRV/VLP2 \times regimen was slightly lower than that of three doses of attenuated Wa HRV (Table 1) with a 58% protection rate against virus shedding and a 44% protection rate against diarrhea upon challenge of pigs with virulent Wa HRV. Although the AttHRV/VLP2 \times regimen did not confer a higher rate of protection compared to the AttHRV3 \times vaccine, the potential advantage of the

combined AttHRV/VLP2 \times vaccine was indicated by the significantly higher ASC responses induced in the intestinal lymphoid tissues and the potential greater safety of using a non-replicating vaccine as boosters to reduce the risk of intussusception associated with a live oral reassortant HRV vaccine in infants (Anonymous, 1999). The absence of the outer capsid VP4 and VP7 rotavirus neutralizing antigens from the i.n. boosting doses of 2/6-VLPs in the AttHRV/VLP2 \times regimen was again presumably a determinant in its failure to confer higher protection rates as it was for the complete failure of i.n. 2/6-VLP vaccines tested in pigs to confer any protection. If a triple-layered 2/4/6/7-VLP vaccine were used for boosting in the combined regimen, a much higher protection rate might have been achieved based on the high numbers of IgA ASC in the intestinal lymphoid tissues induced by this vaccine approach and based on the positive correlation between the numbers of IgA ASC in the intestinal lymphoid tissues induced by live rotavirus and the degree of protection (Yuan et al., 1996, 2001a). The VLP2 \times /AttHRV1 \times regimen was less efficacious than the AttHRV/VLP2 \times regimens in inducing intestinal ASC responses and protection against virus shedding or diarrhea, but more efficacious than the i.n. Wa 2/6-VLP + mLT alone (Table 3). The ineffectiveness in inducing intestinal ASC responses by the VLP2 \times /AttHRV1 \times vaccine regimen was possibly partially due to the functional compartmentalization of the primary mucosal inductive site (NALT) involved in this vaccination approach, suggesting that oral priming (at the portal of infection and the site of virus replication) with live replicating vaccine is more

Table 3
Intestinal and systemic IgA and IgG ASC responses at PCD 0 and protective immunity induced by combined vaccine regimens in gnotobiotic pigs^a

Vaccine group	Mean no. ASC/ 5×10^5 MNC				Protection rate against challenge	
	Intestinal lamina propria		Spleen		Diarrhea (%)	Shedding (%)
	IgA	IgG	IgA	IgG		
Combined regimens						
AttHRV/VLP2 \times	135	67	6	4	44	58
VLP2/AttHRV1 \times	16	11	4	15	25	17
Controls						
Wa 2/6-VLP + mLT IN 3 \times	2	52	0	0	0	0
AttHRV1 \times oral	3	2	1	6	33	0

^a Data summarized from Yuan et al. (2001b).

effective for inducing intestinal IgA responses. Taken together, the use of a replicating vaccine to prime lymphocytes in the major inductive site (GALT) followed by boosting with a non-replicating vaccine at a second mucosal inductive site (NALT) may be a highly effective approach to stimulate the mucosal immune system and induce protective immunity against enteric infections.

3. Possible mechanisms for discrepancies in vaccine efficacies evaluated in the neonatal gnotobiotic pig model of rotavirus disease versus the adult mouse model of rotavirus infection

There are numerous reports of protective immunity against rotavirus infection in adult mice induced by different types of rotavirus antigens (e.g. live or inactivated, homologous or heterologous rotavirus (Feng et al., 1994; McNeal et al., 1992, 1998, 1999a,b; Offit and Dudzik, 1989); recombinant rotavirus proteins or VLPs (Conner et al., 1996; Choi et al., 1999; O'Neal et al., 1997, 1998); and DNA plasmids (Chen et al., 1997, 1998; Herrmann et al., 1996)], using various routes of inoculation (oral, i.n., i.m., i.p., or i.d.). When comparing studies of mice, there are discrepancies in the protective efficacies of rotavirus vaccines possibly due to the use of different strains of in- or out-bred adult mice or murine rotaviruses. The genetic background and virulence of murine rotavirus strains used for challenge plays a critical role in the outcome of an efficacy study in mice (Franco and Greenberg, 2000). The results often differ even more dramatically between studies of rotavirus vaccines in adult mice and neonatal pigs. It is difficult to compare data derived from studies of adult mice with neonatal pig studies due to species genetic differences, the body size, age, and in-bred (mice) or out-bred (pigs) animals, in addition to their different susceptibilities to enteric viruses or the different pathogenesis of the viruses in mice versus pigs (Conner and Ramig, 1997; Saif and Greenberg, 1997; Yuan et al., 2000). Inoculation of mice i.m. with inactivated rotavirus or i.n. with 2/6-VLPs + mLT or purified inactivated native rotavirus particles induced complete protection against infection in adult mice. However, similar vaccines did not confer protection against rotavirus disease in neonatal pigs. There are many possible

reasons for the discrepancies; however, the difference in the pathogenesis of rotaviruses between pigs and mice is likely one of the most critical factors. The pathogenesis of rotavirus disease in pigs, calves and probably in human infants is similar; but it differs from that in mice (Conner and Ramig, 1997; Saif et al., 1994; Saif and Greenberg, 1997). Rotavirus infections of young pigs, calves, and human infants induces watery diarrhea, anorexia, depression, and dehydration (Conner and Ramig, 1997; Saif et al., 1994). Pronounced histologic changes (e.g. villous atrophy as a result of virus replication and epithelial cell lysis) occur in gnotobiotic pigs infected with virulent Wa HRV (Ward et al., 1996a) and similar intestinal villous atrophy has been reported for human infants (Conner and Ramig, 1997; Davidson and Barnes, 1979; Saif and Greenberg, 1997). In mice under 15 days of age, homologous rotavirus infection causes diarrhea and lethargy, but only mild intestinal lesions consisting of vacuolization of villous tip epithelial cells with little or no villous atrophy (Little and Shaddock, 1982). In adult mice, rotavirus infection occurs without causing disease or histopathologic changes (Ward et al., 1990). High levels of intestinal IgA antibodies to the neutralizing rotavirus antigens are required to prevent rotavirus disease in neonatal pigs. In contrast, protection against infection or clearance of an established infection in mice apparently can be mediated by any one of the immune effectors (IgA or IgG; CD4⁺ or CD8⁺ T-cells) or some unknown innate immune effectors. The redundancy of immune mechanisms (innate or acquired) in mice may be better developed than that in pigs or humans. Such differences are very important to consider when comparing the protective efficacy of a vaccine evaluated in the adult mouse model of infection with the neonatal pig model of rotavirus infection and disease.

4. Distribution pattern of memory B-cells in pigs compared to that of T- and B-memory cells in mice and their role in the protective immunity against enteric viral infections

Vaccination induced protective immunity depends on the induction of memory B- and T-cells. To investigate the distribution and sites of residence of memory B-cells, and their role in protective immunity to

reinfection against an enteric virus, we assessed the memory B-cells responses and protection in pigs inoculated with virulent and attenuated Wa HRV (Yuan et al., 2001a). Short-term rotavirus-specific IgA and IgG memory B-cells were detected in the intestinal lamina propria (duodenum and ileum), MLN, and spleen, but with much higher numbers in the ileum and slightly higher numbers in MLN than in the other tissues at PID 28 and 35. Complete protection against HRV infection was associated with significantly higher numbers of IgA and IgG effector and memory B-cells in the intestinal lamina propria (especially in the ileum) of virulent HRV-inoculated pigs at challenge (Table 1). Our findings clearly demonstrate that the short-term memory B-cells induced by virulent Wa HRV reside primarily in the ileum, the major site of rotavirus replication, up to PID 35. Our findings parallel the recent findings for both memory CD4⁺ and CD8⁺ T-cells (Mackay and von Andrian, 2001; Masopust et al., 2001; Reinhardt et al., 2001) that a substantial subset of memory T-cells reside in extra-lymphoid tissues (e.g. skin, lung, and intestinal lamina propria) and this subset of memory cells acts rapidly to execute effector activity when exposed to recall antigen. It is logical that a similar distribution pattern of memory T- and B-cells would facilitate the cognate interaction between T- and B-cells required for the successful establishment of the T-cell-dependent immune response. Based on the study of CD4⁺ and CD8⁺ memory T-cells (Masopust et al., 2001; Reinhardt et al., 2001), Mackay and von Andrian (2001) proposed that immunological memory could be divided into two phases—a short-term phase followed by a long-term phase. The short-term memory depends on the survival and activation of the extra-lymphoid tissue-origin memory T-cells, whereas long-term memory depends on secondary lymphoid tissue-origin memory T-cells. In gnotobiotic pigs infected with virulent Wa HRV (Yuan et al., 2001a), the numbers of IgA and IgG memory B-cells in all the tissues, but especially IgA in the ileum declined substantially from PID 28 to PID 83. Significant waning of IgA memory B-cell responses in the intestine from PID 28 to PID 83 indicates a short IgA B-cell memory to rotavirus infection in neonatal pigs. Our findings agree with previous observations that IgA memory to mucosal pathogens is short-lived and requires periodic boosting to be maintained, as often

occurs after repeated reexposure to endemic enteric pathogens in the environment (Bernstein et al., 1989; Saif and Fernandez, 1996). At PID 83, the spleen had the highest numbers of IgG memory B-cells compared to the other tissues, including the bone marrow, suggesting that the spleen, and not the bone marrow was the major resident site for longer-term IgG memory B-cells after an intestinal viral infection (Yuan et al., 2001a). Our findings for the resident sites for the short-term (up to PID 35) and longer-term (at PID 83) memory B-cells agree with the two-phase theory for memory T-cells (Mackay and von Andrian, 2001). However the time scale of the two phases observed for memory B-cells in pigs orally infected with virulent or attenuated Wa HRV was shorter compared to that investigated for memory CD8⁺ T-cells in mice infected with vesicular stomatitis virus (Masopust et al., 2001), and in the latter studies the short-term phase was proposed to last for weeks to months (up to PID 296) and the long-term phase supposedly lasts for years (Mackay and von Andrian, 2001). The difference in the observed persistence of memory cells is consistent with the different lengths of protective immunity induced by local (short-term) versus systemic infections (longer-term) with viral or bacterial pathogens.

According to the conventional immunological dogma based on observations in mice, bone marrow was considered to be the major site for long-term antibody production (Benedetti et al., 1998). In contrast to this concept, our study demonstrated that bone marrow is not a major site for IgA and IgG rotavirus-specific antibody production or for memory B-cells in pigs from PID 28 up to at least PID 83 after oral inoculation with HRV, as indicated by the lowest mean numbers of IgA and IgG memory B-cells and ASC in bone marrow compared to the other tissues tested (Yuan et al., 2001a). Studies of mice also showed that no IgA and IgG ASC or memory B-cells were detected in bone marrow after adult mice were orally inoculated with the heterologous rhesus rotavirus strain RRV from PID 21 to PID 126 (Moser and Offit, 2001). Williams et al. (1998) reported that rotavirus-specific, FACS-sorted $\alpha 4\beta 7^{+}$ IgA memory B-cells adoptively transferred from donor mice seven months after oral murine EC strain rotavirus inoculation into RAG-2 mice preferentially resided in the lamina propria with ASC numbers at least 250-fold higher than that in

bone marrow. It was shown that the memory B-cell subset responsible for the secretory IgA response and protective humoral immunity to rotavirus expressed the intestinal homing receptor, $\alpha 4\beta 7$. In conclusion, after rotavirus infection of pigs or mice, a substantial subset of memory B-cells migrate to and reside in the lamina propria of the small intestine and another major subset of memory B-cells reside in the spleen. Memory B-cells resident in the lamina propria of the small intestine likely mediate protective immunity upon reinfection with rotavirus. The memory B-cells resident in the spleen may be a source for long-term antibody production (mainly IgG antibody) in the circulation, but were not associated with protective immunity to reinfection with rotavirus. Bone marrow was not a major site for rotavirus-specific antibody production or for memory B-cells in pigs and mice orally infected with rotavirus.

5. Features of novel vaccine delivery systems for enhancement of the immunogenicity of mucosal vaccines

Novel delivery systems, such as use of recombinant attenuated bacteria as carriers of heterologous antigens (Gentshev et al., 2001), encapsulation of antigens into microspheres, or ISCOMs have been investigated for enhancing the immunogenicity of mucosal vaccines. The goal of these novel delivery systems is to deliver the antigens more efficiently to mucosal lymphocyte populations (i.e., Peyer's patch) after inoculation, or to increase the persistence of antigen in lymphoid tissues at mucosal inductive sites. A newly developed coronavirus expression system (Enjuanes et al., 2001) showed attractive features and great potential for use as a virus vector for delivery of mucosal vaccines. Since coronaviruses infect the mucosal surfaces of the respiratory and/or enteric tracts, they can target the vaccines to the mucosal inductive sites like the natural infections. Non-pathogenic coronavirus strains infecting most species of interest, including humans, swine, and cattle are available to develop expression systems. In addition, the tissue and species tropism of coronavirus may be manipulated through engineering the S gene which is the tropism determinant; thus the expressed vaccine antigens may target to specific tissues in specific

animal species (Enjuanes et al., 2001). The use of the coronavirus expression system as a potential delivery vehicle for rotavirus vaccines is of interest to evaluate in the gnotobiotic pig model of rotavirus diarrhea.

Microencapsulation has been extensively studied for mucosal vaccination because of its attractive features (O'Hagan et al., 1993; O'Hagan, 1998), including delivery of encapsulated antigens to provide protection from stomach acidity and the myriad proteases present in the gastrointestinal tract. Microencapsulation has the potential to disseminate antigen to various lymphoid tissues dependent upon the size of the microsphere; therefore, manipulation of the size of the microcapsules allows targeting of vaccine antigens to specific sites. Varying the ratio of lactide to glycolide allows antigen release over a long or short period of time. Thus it may be possible to deliver a primary immunization plus a booster immunization with a single dose of vaccine. Inoculation of mice orally with microencapsulated rotavirus enhanced the frequencies of virus-specific IgA ASC and the quantities of virus-specific IgA antibodies produced in the intestinal lymphoid tissues (Moser et al., 1998). Use of microencapsulated Wa HRV in the combined AttHR/VLP2 \times vaccine regimen may further improve vaccine efficacy by providing the prolonged presence of rotavirus antigen in the inductive sites of the small intestine and enhancing the homing of antigen-specific T- and B-lymphocytes after i.n. boosting.

ISCOMs are another attractive approach for delivery of mucosal vaccines. The ISCOMs are 30–40 nm highly structured particles composed of antigen, cholesterol, phospholipids and adjuvant *Quilaja saponins* (Rimmelzwaan and Osterhaus, 1995). The advantages of ISCOM vaccines include their potential to induce high local and systemic antibody responses and CD4⁺ T-cell responses, even in the presence of pre-existing antibodies; and their capacity to induce MHC class I restricted CD8⁺ CTL responses, which are important in the clearance of established virus infections (Osterhaus and Rimmelzwaan, 1998). ISCOMs have been shown to induce antibody responses and activate Th cells and cytolytic T lymphocytes in a number of animal species, including non-human primates and humans (Sjolander et al., 2001). The immune modulating effects of ISCOMs for an oral non-replicating rotavirus vaccine was evaluated in gnotobiotic lambs

(van Pinxteren et al., 1999). ISCOMs and ISCOM-matrix preparations enhanced virus-specific IgG ASC responses after vaccination and significantly increased IgA and IgG ASC responses in the blood after challenge compared to lambs given inactivated virus alone. This study showed that different oral ISCOM vaccines could induce different types of immune responses, either Th1-like (recombinant UK VP6 ISCOMs) or Th2-like (inactivated rotavirus mixed with ISCOM-matrix). Besides the oral route, ISCOMs have been also evaluated for i.m. and i.p. immunization routes. Intraperitoneal injection of ISCOMs recruited and activated many components of the innate immune system, including neutrophils, macrophages, and dendritic cells (Smith et al., 1999). It was suggested that ISCOMs act by targeting antigen and adjuvant to macrophages and/or dendritic cells (Smith et al., 1999). This pathway may be important to exploit for vaccine development, especially if combined with another vaccine type or vaccination route, plus a different mucosal adjuvant, such as mLT. We are currently evaluating the efficacy of oral or i.n. VLP + ISCOM vaccines in the neonatal gnotobiotic pig model, alone or combined with priming by oral live attenuated Wa HRV. Further exploring the combination of multiple vaccination routes, vaccine types, adjuvants, and new mucosal delivery systems may lead to the optimal stimulation of the intestinal mucosal immune system and the maximal efficacy of mucosal vaccines against enteric viruses.

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