Old Herborn University Seminar Monograph 24:
Development of strategies to overcome barriers to effective
mucosal immunization of infants in developing countries.
Editors: Peter J. Heidt, Richard I. Walker, and Volker Rusch.
Old Herborn University Foundation, Herborn-Dill, Germany: 111-118 (2010).

DEVELOPMENT OF STRATEGIES TO OVERCOME BARRIERS TO EFFECTIVE MUCOSAL IMMUNIZATION OF INFANTS IN DEVELOPING COUNTRIES

SUMMARY OF THE SEMINAR DISCUSSION

RICHARD I. WALKER, A. LOUIS BOURGEOIS, and
LILLIAN VAN DE VERG

Vaccine Development Global Program, PATH, Washington, DC, USA

INTRODUCTION

Nearly half of the world's six billion people exist on less than US$ 2.00 per day. Infectious diseases play a major role in perpetuating poverty and suffering among these people. Vaccines offer one important means to help break this cycle of poverty and disease. The ease and safety of needle-free vaccination make the oral route of immunization an attractive option for the delivery of many current and future vaccines, especially among young children in poverty-stricken populations. Furthermore, mucosal immunity may improve or be essential for obtaining the maximum efficacy of vaccines against certain pathogens.

Unfortunately the efficacy of orally administered vaccines is often reduced among children living in many resource-limited settings. For example, polio eradication is stalled in the Northern Indian states of Uttar Pradesh and Bihar due largely to the reduced efficacy of oral polio vaccine (*Paul*, 2009). In another example, the efficacy of oral rotavirus vaccines may be reduced by up to 50 percent in the countries most severely impacted by the disease (*Sack* et al., 2008). In order to address this problem, the Old Herborn University Foundation (www.old-herborn-university.de), with support from the Bill & Melinda Gates Foundation and PATH, held a workshop bringing together experts in this area for a two-day meeting on June 24-25, 2010, in Herborn, Germany. Their task was to discuss possibilities for better understanding the mechanisms that may contribute to this poor response to oral vaccines and to suggest potential near-term approaches to achieving more effective mucosal immunization in the disease-vulnerable populations that are less responsive to oral vaccination. A summary of these discussions is presented below.

A QUESTION OF NUMBERS?

Most orally administered vaccines are live attenuated products, so many of the examples demonstrating poor responsiveness involve live vaccines. In addition to the polio and rotavirus vaccines mentioned above, poor responsiveness has also been observed with attenuated bacterial vaccines. For example, a live attenuated *S. flexneri* 2a vaccine (SC602) was immunogenic and protective when administered to North American volunteers at a dose of 10^4

cfu. This same dose given to Bangladeshi children, however, did not colonize or stimulate detectable serological responses (*Sack* et al., 2008). Even raising the vaccine dose to 10^6 cfu did not increase responsiveness among Bangladeshi children, although this dose induced some dysentery in North American volunteers (*Sack* et al., 2008).

Experience with the live attenuated oral cholera vaccine (CVD103HgR) has been similar to the results obtained with the *Shigella* vaccine. A dose of $5x10^8$ cfu of the cholera vaccine stimulated vibriocidal responses in North American volunteers. This response was rarely seen when the same dose was given to Indonesian subjects. Raising the dose to $5x10^9$ cfu did increase the take rate, but even this dose did not offer protection against cholera (*Richie* et al., 2000) in this endemic setting.

One of the benefits of live oral vaccines is that they are usually immunogenic in the intestines of North Americans or Europeans with the application of fairly small doses as described above. These live vaccines presumably replicate in the intestine that they transiently colonize, and the stimulus associated with the greater numbers achieved through replication yields a protective immune response. This can be illustrated by one study's administration of $3x10^9$ wild type enterotoxigenic *Escherichia coli* (ETEC) to North American volunteers. Sixty-five percent of the volunteers developed a serum ELISA titre $\geq$155 (*McKenzie* et al., 2008). When the same amount of an attenuated ETEC vaccine strain, ACAM 2017, was given to volunteers, none of them reached an ELISA titre $\geq$155 (*Daley* et al., 2007). When given $3x10^{10}$ ACAM 2017, 54 percent of the volunteers mounted the threshold response (*Daley* et al., 2007). Therefore, the normal barriers to colonization found in the human intestine were able to restrict the mucosal immunizing potential of the vaccine strain whose colonizing ability had been compromised by attenuation. The intestines of children living in developing countries may have even more barriers than those found in the intestines of North Americans. Thus, starting with relatively low-level inoculations of oral vaccine such as these may be effective in individuals living in developed countries, but they may not achieve as strong an immunizing effect among infants in the developing world.

POSSIBLE STEPS TO IMPROVE VACCINE EFFECTIVENESS

To date, there has been too little research on this important aspect of vaccine development. The workshop participants identified two generic approaches to addressing the problem of unresponsiveness to oral vaccines. The first is to gain a better understanding of the nature of the barriers to immunization and seek ways to remove or reduce them. The second is to maximize antigenic stimulation by practical means to override or circumvent the barriers (Table 1).

Remove or reduce barriers

Shortly after birth, the infant intestine begins a dynamic process of colonization by microorganisms and adaptation to the environment. Poor vaccine responsiveness is seen early in infants vaccinated on the World Health Organization's (WHO's) Expanded Program

Table 1: Possible strategies to enhance mucosal responses to orally administered vaccines in infants in developing countries

Remove or reduce barriers	Override or circumvent barriers
a. Withhold breastfeeding (not recommended)	a. Raise vaccine dose administered
b. Reduce parasitic load	b. Increase antigenicity associated with vaccines
c. Provide micronutrient supplementation	c. Use a mucosal adjuvant
d. Incorporate prebiotics/probiotics into diet	d. Develop intestine-specific formulations
e. Improve sanitation and reduce exposure to potentially toxic chemicals in the environment	e. Use alternate routes of vaccination to avoid poorly responding mucosal areas
f. Improve maternal and infant nutrition	

on Immunization schedule. A variety of factors have been implicated as contributing to this diminished response to orally administered vaccines, and these may change over time. For example, breast milk, transplacental antibodies, and altered development of the infant intestinal microbiota may be major factors in very young children. After weaning, poor nutrition, micronutrient deficiencies, microbial colonization, and helminth infections may become more pronounced and are associated with histologic abnormalities termed "environmental enteropathy," represented by an inflamed intestinal mucosa with shortened villi.

Although a combination of factors likely causes poor responsiveness to oral vaccines, the contributing importance of some of these factors has been suggested by attempts to reduce or eliminate them. These approaches include:

a. *Withhold breast-feeding*
   Although conflicting data exist in this area, some research has indicated that withholding breastfeeding for three hours before immunization may improve oral vaccine responsiveness (*Ahmed* et al., 2009). This may not be accomplished easily in large-scale programs. Further there is concern that the practice of breastfeeding should be encouraged. Due to the latter reason, this approach is not recommended.
b. *Reduce parasitic load*
   Geohelminths may have deleterious effects on immunity induced by oral vaccines. This is suggested in part by the observation that anti-helmintic treatment before vaccination may partially reverse deficits in responses to the live attenuated oral cholera vaccine, CVD 103-HgR (*Cooper*, 2009).
c. *Provide micronutrient supplementation*
   A prolonged (42 days) administration of zinc has been associated with stronger antibody responses (*Ahmed* et al., 2009). Similarly, neonatal supplementation with vitamin A may also help regulate/modulate responses to enteric vaccines, since Retinoic acid (a metabolite of vitamin A) has been shown to promote gut homing of T and B cells and to regulate the balance between regulatory T cells and IL-17 producing T cells (*Serazin* et al., 2010).

d. *Incorporate prebiotics/probiotics into diet*
   Probiotics may provide antibacterial and immunological benefits to the health of the intestine, and they may play an important role in helping to ensure that the gut microbiota in young infants develops appropriately. This is an extremely new field, but it is clear that the gut microbiome has an essential role in shaping the maturation, quality, and duration of mucosal immune responses (*Prescott* et al., 2008; *Sezarin* et al., 2010). Probiotics may also have intrinsic properties that are immunostimulatory (probiotic-derived CpG motifs) and thus may also help to improve the immunogenicity of enteric vaccines (*Ménard* et al., 2020). More research is needed to determine the most opportune time in infant development to intervene with probiotics (pre- and/or post-natal) and whether the administration of representative members of our own indigenous bacterial microflora is the best approach for maximizing the potential beneficial effects. Studies are also needed to determine how prebiotics may be best added to the diet to enhance indigenous flora or administered probiotic flora.
e. *Improve sanitation and reduce exposure to potentially toxic chemicals in the environment*
   Improving sanitation may reduce some of the intestinal abnormalities associated with poor responsiveness to oral vaccines. Faecal contamination is ubiquitous in some locations, where flush toilets are unavailable. It is estimated that people living under these conditions may ingest about 10 grams of human waste every day containing approximately 100 million viruses, 10 million bacteria, 10 thousand parasites, and 1,000 worm eggs (*George*, 2008). This microbial contamination may affect maternal immunity and contribute to environmental enteropathy in infants. In addition, in some areas of the developing world, with South Asia as a prime example, environmental contamination with toxic chemicals like arsenic may also have a negative impact on the immunological response capabilities of infants born into this environment. For example, Bangladeshi infants born to mothers exposed to arsenic during pregnancy have a smaller thymus as well as a higher overall mortality rate (D. Sack, personal communication).
f. *Improve maternal and infant nutrition*
   Although improving the nutritional status of developing-world infants is clearly important in enhancing their ability to respond effectively to enteric vaccines, a growing body of evidence suggests that nutritional supplementation of mothers prenatally may also have profound effects on later immune function. Some of the most interesting evidence in this area comes from prenatal zinc supplementation studies in Bangladesh and Peru that demonstrated a reduced risk of developing diarrhoea among infants born to mothers receiving daily zinc supplements (*Iannotti* et al., 2010; *Osendarp* et al., 2001).

Override or circumvent barriers

A number of strategies could be pursued to override or circumvent identified barriers. Instead of targeting the improvement of general intestinal health, these approaches aim to improve the immune response that is induced upon vaccination in poorly responsive children. They may also exploit interconnections of the mucosal immune system to immunize the targeted mucosal area through routes

other than oral administration. These approaches include:

a. *Raise vaccine dose administered*
   It is possible that additional or higher doses could at least partially overcome the issue of poor responsiveness. In many cases, live vaccines have only been given as a single dose, and it is possible that two to three doses could be more effective. It may also be useful to develop vaccine candidates that are safe in volunteers when administered at higher dosage levels, such as 10^9 to 10^{10} cells. Even one log difference in dose may determine whether protective mucosal immune responses can be achieved. The magnitude of the immune response induced may be even more important in areas with poor sanitation where the challenge dose may be particularly high.
   Evidence with the inactivated cholera vaccine Dukoral shows that 10^{10} non-replicating cells can induce mucosal immunity in the human intestine. Although this vaccine was protective in trials in Africa, Asia, and South America, antibacterial responses were less frequent in children two- to five-years of age than in older children or adults (*Holmgren* and *Berquist*, 2004). Further, more doses of Dukoral achieved a higher take rate. No data are available for younger children.
b. *Increase antigenicity associated with vaccines*
   An inactivated vaccine candidate against ETEC, while immunogenic and protective in adults, provided no protection when given to 6- to 18-month-old children in Egypt (*Svennerholm* and *Savarino*, 2004). Although the infants mounted an immune response against colonization factor antigens, the response was not as high as has been previously associated with protection. In reviewing these data, a WHO committee (*WHO weekly epidemiology record*, 2006) recommended the use of adjuvants and/or genetic enhancement of the amount of antigen expressed by the cells to hopefully reach a protective threshold. Subsequently *E. coli* was modified to express large amounts of colonization factor antigens, more than was associated with the wild-type ETEC cells used in the original vaccine evaluated in Egyptian children. Higher titres to enhanced antigens were seen when the improved vaccine was given to mice, but clinical evaluation remains to be done (J. Holmgren, personal communication).
c. *Use a mucosal adjuvant*
   The possibility that the mucosal immune system in children can be induced to respond more strongly needs to be evaluated. This approach could be practical for a vaccination program, but it has been held back by the lack of a suitable adjuvant. Recently a double mutant of the heat labile enterotoxin (LT) of ETEC has been developed and, if found safe, could be used to potentially improve responsiveness to vaccines. This material is based on the original single mutant of LT reported by Dickenson and Clements (*Dickenson* and *Clements*, 1995) which has a lysine replaced by alanine at position 211 in addition to the arginine substitution by glycine at position 192 in the single mutant. Both mutations are in the toxic A subunit of the toxin. Animal studies have indicated that the double mutant of LT (dmLT) may be safe and that it retains its adjuvant properties when co-administered orally with various antigens (J. Clements, personal communication). Oral co-administration of dmLT and an inactivated ETEC vaccine in mice led to much higher

titres against the ETEC colonization factor antigens than in the mice receiving vaccine alone. However, trials with infants in developing countries are needed to determine whether their immune responses can benefit from adjuvants such as dmLT.

d. *Develop intestine-specific formulations*
Live vaccines may benefit from administration in buffer formulations that enhance their survival during gastric transit. An example of this is a study of the Peru-15 vaccine that obtained greater titres in volunteers when it was delivered in CeraVacx buffer instead of conventional bicarbonate buffer (*Sack* et al., 1997). Another approach could be to promote transient survival of mutant vaccine organisms by supplying some needed growth factor in the buffer.

e. *Use alternative routes of vaccination*
Mucosal immunity may not require immunization of the intestinal mucosal surface. It may be possible to achieve mucosal immunity in the intestine using delivery routes other than oral. While much work remains to pursue this approach, it has been demonstrated that intestinal immunity can be achieved by transcutaneous (*Hickey* et al., 2009) and sublingual immunization (*Cuburu* et al., 2007). Rectal immunization is a little-studied approach that also could be useful in developing countries as the rectal area is responsive to immunization in normal humans and animals (*Haneberg* et al., 1995) and may remain so in infants in developing countries. In addition, mucosal-parenteral prime boost strategies have been found effective at improving mucosal immune responses in normal individuals (*Ramirez* et al., 2010), and this approach could also be used with children in developing countries. The drawback with this latter approach may be a logistical one rather than a scientific one because it would require critical record keeping as well as two formulations of vaccine.

Reduce or override the barriers to more effective immunization?

Complex factors evolving over time have been implicated in contributing to vaccine hypo-responsiveness. As described above, some of the approaches now available for improving intestinal responsiveness to vaccines involve steps to reduce some of the barriers to more effective immunization. While it seems wise to do everything possible to improve the intestinal health of children living in developing countries, unfortunately many of these approaches are impractical for implementation within the context of a vaccine program. Instead, perhaps, intestinal health should be promoted on its own, which would benefit health and resistance to disease in general and, in the process, may result in a stronger response to vaccination.

Workshop participants felt that there may be a number of yet to be identified host or environmental factors that could significantly contribute to the barriers against effective mucosal immunization in developing-country infants. They urged researchers to take advantage of existing vaccine field-trials data for younger age groups to carry out retrospective case-control studies to identify additional biomarkers or predictors of poor responsiveness, which could be explored more fully in prospective studies of vaccine immunogenicity in traditional poor-responder populations.

The consensus of the workshop participants was that the most effective approach to improving responsiveness

to vaccines would be to focus on increasing the amount of antigenic stimulation to effectively override or circumvent barriers to immunization that may be present at a given point in infant development. This approach can be accomplished in vaccine design or delivery to ensure that the maximum amount of antigen reaches lymphoid tissues affecting the mucosal surface. While much work remains to evaluate the means to achieve this approach, the strategies to override or circumvent impediments to immunization, as outlined above, would be practical to include as part of an immunization program.

ACKNOWLEDGEMENTS

The authors appreciate the support provided for this meeting by the Bill & Melinda Gates Foundation and the Old Herborn University Foundation. We also grateful for the review of this document provided by the session chairmen at this workshop, Dr. John Clements and Dr. James Versalovic, and editing by Ms. Allison Clifford.

LITERATURE

Paul, Y.: Why polio has not been eradicated in India despite many remedial interventions? Vaccine 27, 3700-3703 (2009).

Sack, D.A., Qadri, F., and Svennerholm A.-M.: Determinants of Responses to Oral Vaccines in Developing Countries. Ann. Nestlé [Engl.] 66, 71-79 (2008).

Richie, E.E., Punjabi, N.H., Sidharta, Y.Y., Peetosutan, K.K., Sukandar, M.M., Wasserman, S.S., Lesmana, M.M., Wangsasaputra, F.F., Pandam, S.S., Levine, M.M., O'Hanley, P.P., Cryz, S.J., and Simanjuntak, C.H.: Efficacy trial of single-dose live oral cholera vaccine CVD 103-HgR in North Jakarta, Indonesia, a cholera-endemic area. Vaccine 18, 2399-2410 (2000).

McKenzie, R., Darsley, M., Thomas, N., Randall, R., Cvarpenter, C., Forbes, E., Finucane, M., Sack, R.B., Hall, E., and Bourgeois, A.L.: Double-blind, placebo-controlled trial to evaluate the efficacy of PTL-003, an attenuated enterotoxigenic *E. coli* (ETEC) vaccine strain, in protecting against challenge with virulent ETEC. Vaccine 26, 4731-4739 (2008).

Daley, A., Randall, R., Darsley, M., Choudhry, N., Thomas, N., Sanderson, I.R., Croft, N.M., and Kelly, P.: Genetically modified enterotoxigenic *Escherichia coli* vaccines induce mucosal immune responses without inflammation. Gut 56, 1550-1556 (2007).

Ahmed, T., Svennerholm, A.-M., Tarique, A.A., Sultana, G.N.N., and Qadri, F.: Enhanced immunogenicity of an oral cholera vaccine in infants in Bangladesh obtained by zinc supplementation and by temporary witholding breast-feeding. Vaccine 27, 1433-1439 (2009).

Cooper, P.J.: Mucosal immunology of geohelminth infections in humans. Mucosal Immunology 2, 288-299 (2009).

Serazin, A.C., Shackelton, L.A., Wilson, C., and Bhan, M.K.: Improving the performance of enteric vaccines in the developing world. Nature Immunol. 11, 769-773 (2010).

Prescott, S.L., Wickens, K., Westcott, L., Jung, W., Currie, H., Black, P.N., Stanley, T.V., Mitchell, E.A., Fitzharris, P., Siebers, R., Wu, L., Crane, J., Probiotic Study Group: Supplementation with *Lactobacillus rhamnosus* or *Bifidobacterium lactis* in pregnancy increases cord blood interferon-

gamma and breast milk transforming growth factor-beta and immunoglobulin A detection. Clin. Exp. Allergy 38,1606-1614 (2008).

Menard, O., Gafa, V., Kapel, N., Rodriguez, B., Butel, M.J., and Waligora-Dupriet, A.J.: Characterization of immunostimulatory CpG-rich sequences from different Bifidobacterium species. Appl. Environ. Microbiol. 76, 2846-2855 (2010).

George, R.: The big necessity. The unmentionable world of human waste and why it matters. Henry Holt and Company, New York (2008).

Iannotti, L.L., Zavaleta, N., León, Z., Huasquiche, C., Shankar, A.H., and Caulfield, L.E.: Maternal zinc supplementation reduces diarrheal morbidity in Peruvian infants. J. Pediatr. 156, 960-964 (2010).

Osendarp, S.J., van Raaij, J.M., Darmstadt, G.L., Baqui, A.H., Hautvast, J.G., and Fuchs, G.J.: Zinc supplementation during pregnancy and effects on growth and morbidity in low birth weigh infants: A randomized placebo controlled trial. Lancet; 357, 1080-1085 (2001).

Holmgren, J. and Berquist, C.: Oral B subunit-killed whole cell cholera vaccine. In: New Generation Vaccines, 3rd edition (Levine, M.M., Kaper, J.B., Rappuoli, R., Liu, M.A., and Good, M.F., Eds.). Marcel Dekker, New York (2004).

Svennerholm, A.-M. and Savarino, S.J.: Oral inactivated whole cell B subunit combination vaccine against enterotoxigenic Escherichia coli. In: New Generation Vaccines, 3rd edition (Levine, M.M., Kaper, J.B., Rappuoli, R., Liu, M.A., and Good, M.F., Eds.). Marcel Dekker, New York (2004).

WHO: Future directions for research on enterotoxigenic Escherichia coli vaccine for developing countries. WHO weekly epidemiology record 81, 97-104 (2006).

Dickenson, B.L. and Clements, J.D.: Dissociation of Escherichia coli heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. Infect. Immun.; 63, 1617-1623 (1995).

Sack, D.A., Shimko, J., Sack, R.B., Gomes, J.G., MacLeod, K., O'Sullivan, D., and Spriggs, D.: Comparison of alternative buffers for use with a new live oral cholera vaccine, Peru-15, in outpatient volunteers. Infect. Immun. 65, 2107-2111 (1997).

Hickey, D.K., Aldwell, F.E., Tan, Z.Y., Bao, S., and Beagley, K.W.: Transcutaneous immunization with novel lipid-based adjuvants induces protection against Helicobacter pylori infection. Vaccine 27, 6983-6990 (2009).

Cuburu, N., Kweon, M.N., Song, J.H., Hervouet, C., Luci, C., Sun, J.B., Hofman, P., Holmgren, J., Anjuère, F., and Czerkinsky, C.: Sublingual immunization induces broad-based systemic and mucosal immune responses in mice. Vaccine 25, 8598-8610 (2007).

Haneberg, B., Kendall, D., Amerongen, H.M., Apter, F.M., and Neutra, M.R.: The colon and rectum as inductor sites for local and distant mucosal immunity. Adv. Exp. Med. Biol. 371A, 107-109 (1995).

Ramirez, K., Ditamo, Y., Galen, J.E., Baillie, L.W., and Pasetti, M.F.: Mucosal priming of newborn mice with S. typhi Ty21a expressing anthrax protective antigen (PA) followed by parenteral PA-boost induces B and T cell-mediated immunity that protects against infection bypassing maternal antibodies. Vaccine 28, 6065-6075 (2010).