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
VOLKER RUSCH

Old Herborn University

Old Herborn University Seminar Monograph 24

ISBN 3-923022-35-2
ISSN 1431-6579

EDITORS:

Prof. Dr. Peter J. Heidt,
Foundation for Microbiology and Gnotobiology
Vlietweg 60A
2323 LE - Leiden
The Netherlands

Richard I. Walker, Ph.D.
Enteric Vaccine Initiative
PATH Vaccine Development Global Program
1800 K Street NW
Washington, DC 20006
USA

Dr. rer. nat. Volker Rusch,
Stiftung Old Herborn University
Postfach 1765
D-35727 Herborn-Dill
Germany

Old Herborn University

Old Herborn University Foundation
Postfach 1765
D-35727 Herborn-Dill
Germany
Telephone: +49 - 2772 - 921100
Telefax: +49 - 2772 - 921101

Supported by

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Participating Authors

John D. Clements, Ph.D., Department of Microbiology and Immunology, Tulane University School of Medicine, 1430 Tulane Avenue (SL-38), New Orleans, LA 70112, USA.
(E-mail: jclemen@tulane.edu)

Philip J. Cooper, M.D., Ph.D., Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, United Kingdom; and Colegio de Ciencias de la Salud, Universidad San Francisco de Quito, Quito, Ecuador.
(E-mail: pcooper@ecnet.ec)

Denise Kelly, M.D., Ph.D., Gut Immunology Group, Rowett Institute of Nutrition & Health, University of Aberdeen, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB, Scotland.
(E-mail: d.kelly@abdn.ac.uk)

Dennis Lang, Ph.D., Senior Program Coordinator, MAL-ED Consortium, Fogarty International Center, National Institutes of Health, 16 Center Drive, Bethesda, MD 20892, USA; and The Foundation for the National Institutes of Health, 9650 Rockville Pike, Bethesda, MD 20814, USA.
(E-mail: lang4@fnih.org])

Andrew S. Neish, M.D., Department of Pathology and Laboratory Medicine, 105-F Whitehead Building, Emory University School of Medicine, 615 Michaels Street, Atlanta, GA 30322, USA.
(E-mail: aneish@emory.edu).

Pearay L. Ogra, M.D., Women and Children's Hospital, 219 Bryant Street, Buffalo, NY 14222, USA.
(E-mail: pogra@upa.chob.edu).

Marcela F. Pasetti, Ph.D., Department of Pediatrics, Center for Vaccine Development, University of Maryland School of Medicine, 685 W. Baltimore Street, Baltimore, MD 21201, USA.
(E-mail: mpasetti@medicine.umaryland.edu).

Firdausi Qadri, Ph.D., Immunology Unit, International Centre for Diarrhoeal Disease Research (ICDDR,B), Bangladesh, Dhaka, Bangladesh.
(E-mail: fqadri@icddrb.org).

David A Sack, M.D., Department of International Health, Johns Hopkins Bloomberg School of Public Health, 615 N Wolfe Street, Baltimore, MD 21205, USA.
(E-mail: dsack@jhsph.edu).

James Versalovic, M.D., Ph.D., Department of Pathology, Texas Children's Hospital, Feigin Center Suite 830, 1102 Bates Avenue, Houston, TX 77030, USA.
(E-mail: jamesv@bcm.edu).

Richard I. Walker, Ph.D., Enteric Vaccine Initiative, Vaccine Development Global Program, PATH, 1800 K Street, NW, Suite 800, Washington, DC 20006, USA.
(E-mail: rwalker@path.org).

MUCOSAL IMMUNE RESPONSES IN INFANCY AND EARLY CHILDHOOD: IMPLICATIONS IN SUCCESSFUL ORAL IMMUNIZATION

PEARAY L. OGRA

Department of Pediatrics, University at Buffalo,
State University of New York, Buffalo, NY, USA

INTRODUCTION

Since the introduction of routine childhood immunization nearly 4 decades ago, at least 10 major childhood infectious diseases have been either eliminated or effectively controlled with significant decline in associated mortality and morbidity in most developed parts of the world (Table 1). Yet, many vaccine preventable infections continue to pose major public health problems in many countries. In some disease situations, such as paralytic poliomyelitis, limited areas of endemic infection have continued to persist, often in spite of repeated immunization with otherwise highly effective vaccines. There is also evidence to suggest that current immunization approaches against several gastrointestinal infections, including cholera, polio, typhoid, rotavirus in the developing world and other economically under-privileged settings is either inferior or discernibly less effective in prevention of disease in children on re-exposure than observed in other, developed, countries (*Centers for Disease Control and Prevention (CDC)*, 1999).

The mucosal defences in the human and mammalian neonates, although quite competent at the time of birth of the neonate, continue to evolve and acquire functional maturity at varying intervals after birth. The mucosal barrier is a reflection of multiple host and environmental interactions. The major contributors to the mucosal barrier include a number of biophysical and non-immunologic factors, as well as specific soluble and cellular components representing both innate and adaptive immune function. Acquisition of mucosal microbial flora after birth serves a major role in the maturational process of mucosal defence. The functional maturation of the mucosal barrier is critically influenced by the gestational age at birth (pre-term vs. full-term birth), host genetic background, neuroanatomic components and intact epithelium (neuropeptide mast cells), quality and quantity (microbial load) of the mucosal microflora, and the development and nature of specific mucosal immune functions.

This brief review will focus on the nature and biologic characteristics of mucosal immune system and specific immune response in external mucosal surfaces in early childhood. The potential implication of these observations to the use of existing and the development of mucosal vaccines in the future will also be considered.

MUCOSAL IMMUNE SYSTEM: BASIC FRAMEWORK

Mature immunologic repertoire of mucosal surfaces, especially of the respiratory and gastrointestinal tracts constitutes the largest antibody-producing

Table 1: Provisional morbidity for certain infectious diseases in the United States in 1998 compared to peak morbidity prior to 1990, before universal use of vaccines in children (*Centers for Disease Control and Prevention [CDC]*, 1999)

Disease	Annual morbidity (Peak yr)	1998 Provisional morbidity	% Decrease
Smallpox	48,164 (1900)	0	100
Diphtheria	175,885 (1920)	1	100
Pertussis	147,271 (1922)	6,279	95.7
Tetanus	1,314 (1922)	34	97.4
Paralytic poliomyelitis	16,316 (1952)	0	100
Measles	503,282 (1958)	89	99.8
Mumps	152,209 (1968)	606	99.6
Rubella	47,745 (1967)	345	99.3
H. influenzae type B	20,000 (1985)	54	99.7

site in the human body. The important sites of the mucosal associated lymphoid tissue (MALT) include the gut (GALT), broncho-epithelium (BALT), and nasopharynx (NALT), sublingual tissue (SLLT), and possibly the skin (SALT), and larynx associated lymphoid tissues (LALT) (*Ogra* et al., 2008; *Thibeault* et al., 2009).

The lymphoid tissues of GALT, BALT, NALT and SLLT represent major inductive sites for mucosal responses. The GALT and the NALT possess a wealth of B cell follicles with well-defined T cell zones, and are repleted with dendritic cells and macrophages as the principal antigen presenting cells (APC). The mucosal lymphoid follicles do not possess afferent lymphatics. Over 80% of activated B cells in the human host reside in the gastrointestinal tract, and belong to IgA isotype, associated with J-chain, and secretory component (SC). The B cell differentiation in the mucosa is driven by a diverse spectrum of innate immune factors including pathogen recognition receptors (Toll-like receptors [TLR]), retinoic acid, cytokines, resident microflora, and other environmental macromolecules.

The mucosal sites destined for eventual expression of the effector functions of mucosal immune responses are the lamina propria of the gastrointestinal and respiratory tracts and sub-epithelial sites as well as other distant mucosal sites such as genital tract, mammary glands and products of lactation. This information has been extensively reviewed in several recent publications (*Ogra*, 2010; *Holmgren* and *Czerkinsky*, 2005; *Ogra* and *Welliver*, 2008).

Briefly, the functional activation of mucosal immune system is initiated by a cascade of events beginning with the exposure to an antigen and its sampling by the mucosal epithelial M cells. This is followed by participation of intra-epithelial lymphocyte (IEL), dendritic cells and regulatory T cells and several cytokines in the mucosal associated lymphoid tissue, such as IL-5, TGF-β, IL-6, IL-10, IL-23, IL-27, and retinoic acid. These events are followed by T cell activation and induction of regulatory T cells, and ultimately by the expansion of IgA plasma cell differentiation via IL-5, IL-6 and IL-10 by activation of the T-helper cell population in the specific mucosal tissue.

Exposure to specific antigens in different inductive sites has been shown to elicit a widespread but somewhat compartmentalized site-specific response in different effector sites. For example, oral administration of an antigen is associated with specific response in the intestine, genital tract and the mammary glands. Administration of antigen via sublingual or intranasal route has been shown to induce high levels of antibody response in the lung, upper airways as well as in genital tract. On the other hand, intra-rectal or intra-colonic, or intra-vaginal immunization appears to result in antigen-specific response restricted to the sites of immunization (*Ogra* and *Karzon*, 1969). The restricted nature of mucosal response in different effector sites appears to be related to the expression of specific homing ligands in activated B lymphocytes in different inductive sites. Lymphocyte trafficking and homing after respiratory tract immunization has been shown to be dependent on expression of α4β1 integrin and presence of VCAM-1, CCL28, and CCR10. On the other hand, homing after immunization in the GALT appears to be related to CCL25, CCR9, MAdCAM1 and expression of α4β7 integrin (*Kiyono* et al., 2008; *Holmgren* and *Czerkinsky*, 2005).

The principal functions of specific mucosal immune responses are:

1) barrier function, to prevent microbial translocation across mucosal surfaces and thus modulate antigen uptake via immune exclusion,
2) to regulate systemic immune responsiveness and limit uptake and presentation of dietary, microbial and other antigens by DCs to the regional lymph nodes (oral tolerance),
3) to facilitate development of regulatory cells (T-reg ($CD4^+$, $CD25^+$), and other regulatory cells in regional lymph nodes, and
4) to induce polyclonal T-cell-independent IgM responses against commensals, but not against pathogens, and thus provide a shield against specific adaptive immune responses to commensals and other established resident microflora in the mucosal surfaces (*Brandtzaeg*, 2005; *Ogra* and *Welliver*, 2008).

IMMUNE FUNCTIONS IN THE NEONATAL PERIOD AND EARLY CHILDHOOD

The development of immune function in the foetal, neonatal and early childhood years has also been discussed extensively in several recent reviews (*Levy*, 2007; *Adkins* et al., 2004; *Lewis* and *Wilson*, 2006). This information is briefly summarized here.

The neonatal period is characterized by reduced levels of innate mechanisms of defence, including reduced complement levels, lower number and function of leukocytes and macrophage, IFNγ, IL-10, and unpaired superoxide production. The neonate also exhibits reduced APC, CMI, altered antibody-producing patterns and enhanced eosinophilic responses (*Lewis* and *Wilson*, 2006).

Human neonates exhibit impaired Th1 T cell response, with a strong bias towards Th2 T cell responses, delayed maturation of IL-12 producing dendritic cells, reduced IFN-γ production by $CD4^+$ T cells, and NK cells, reduced $CD4^+$ T cells (but normal $CD8^+$ T cell responses), reduced DTH, but normal graft regulation, and reduced intracellular killing of cell associated organisms such as mycobacteria and DNA viruses. Recent studies have suggested

that neonates exhibit robust primary response with both Th1 and Th2 T cells. However, secondary responses are mostly of Th2 phenotype. Such shift to Th2 T cell response may be related to increased apoptosis of Th1 T cell by IL-4. Neutralization of IL-4 has been found to prevent apoptosis of Th1 cells and subsequent re-establishment of IFN-γ responses. Most T cells in the neonatal period are naive in phenotype and function (over 90% are $CDRA45^{+}$), and exhibit high activation threshold and co-stimulation dependence for IL-2 production. These cells initially express lower production levels of IFN-γ and IL-4, but these return to normal after activation-induced proliferation. In general neonates exhibit high overall T cell numbers for $CD4^{+}$ and $CD8^{+}$ cells than observed in older children and adults (*Holt*, 2003; *Zaghouani* et al., 2009). The unique nature of T cells and their functional characteristics in the neonatal period and early infancy may be the principal mechanisms underlying the delayed onset of T independent B cell response, delayed cell associated (HSV, CMV) viral specific $CD4^{+}$ response after perinatal infection, inverse temporal relationship between virus shedding and viral antigen load and CD4 specific response. However, Th1 specific responses have been observed in the neonatal and early infancy after immunization with BCG, whole cell pertussis (but not after cellular pertussis), and after neonatal HSV infection (*Wilson* and *Kollman*, 2008).

MUCOSAL IMMUNE RESPONSE TO VACCINES IN INFANCY AND CHILDHOOD

As pointed out above, the mucosal lymphoid tissue and the immunologic framework is well-developed and fairly competent at birth. However, there is significant lack of expression of effector function in the neonatal period and early childhood. Such functional maturation occurs after exposure to postnatal environment. It is now clear that the nature of early environmental exposure after birth is critical for specific programming and subsequent functional spectrum of mucosal lymphoid tissue as well as systemic immunoregulatory functions. This is exemplified by age related changes in the quantitative and qualitative aspect of Peyer's patches and GALT, NALT, IgA_1, and IgA_2, and expression of HLA-DR in respiratory mucosal tissue. Rudimentary lymphoid structures containing $HLA\text{-}DR^{+}$ and $CD4^{+}$ cells are seen in the intestine by 10-11 weeks of gestation as Peyer's patches. $CD8^{+}$ T cells $CD5^{+}$ IgA^{+} B cells are observed by 16-18 weeks of gestation. However, visible Peyer's patches in about 45-70 in number are first detected after 20 weeks of gestation. Subsequently, the number of Peyer's patches increase significantly between 24 hours and 6 weeks after birth, with significant expression of germinal centre only after exposure to external environment (*MacDonald* and *Spencer*, 1994; *Cornes*, 1985).

The highest number of Peyer's patches ranging between 185-325 is observed between 12-15 years of age. After 20 years of age the number of Peyer's patches begins to exhibit significant decline and only about 100 Peyer's patches are visualized after 70 years of age. Limited information is available about age related changes in BALT or NALT in humans. IgA and HLA-DR expression in tracheal wall tissue has been reported only after 1-month postnatal age. No IgA has been

detected at birth, but begins to appear in mucosal secretions after 1-2 weeks of age in over 90-95% of infants. However, adult concentrations of IgA in secretions are attained only after 5-7 years of age (*Cripps* et al., 2005).

Animal studies have suggested that while GALT exhibits involution in a manner similar to man as a function of aging, the NALT begins to develop only after birth and does not exhibit significant involution with ageing. In contrast to the development of Peyer's patches and GALT, the rodent NALT development appears to be independent of IL-7R and LTβR. It does not follow programmed inflammation model and cell development appears to be dependent only on Id2 gene expression (*Kiyono* and *Fukuyama*, 2004; *Kunisawa* et al., 2008).

Since the introduction of cowpox virus in 1798 for immunization against human smallpox, over 30 additional viral and bacterial vaccines have been introduced for routine immunization against many childhood infectious diseases (Table 2). Of these, only Sabin live oral poliovirus (OPV) reassortant rotavirus, attenuated influenza virus, adenovirus, cholera and typhoid vaccines are available for use by mucosal route of administration (Table 2).

Numerous observations over the past 30-40 years have amply demonstrated that mucosal administration of replicating or non-replicating microbial agents often result in the development of mucosal and frequently serum antibody and cell mediated immune responses. Induction of secretory IgA antibody responses provides microbial specific protection against many respiratory, enteric, genital and many systemic infections and reduces severity of clinical disease. Passive transfer of specific monoclonal IgA antibodies have been found to provide significant protection against re-infection challenge with influenza, rotavirus, respiratory syncytial virus, poliovirus, Salmonella, Helicobacter, and cholera in several experimental and human infection models (*Ogra* and *Welliver*, 2008).

Many earlier investigations have demonstrated that the development of mucosal antibody response is dependent on the route of vaccine administration, nature of vaccine antigens, age at the time of immunization and possibly the frequency of immunization. These studies employed priming and booster immunization with several bacterial and viral agents such as adenovirus, rubella, varicella-zoster, rotavirus cholera and polio (*Ogra*, 2984; *von Ginkel* et al., 2000; *Ogra* et al., 1989).

Many European countries and certain provinces in Canada in the past and more recently the U.S. have relied solely on the intramuscular use of Salk IPV or the more immunogenic enhanced potency IPV (EIPV). Carefully controlled studies with EIPV have demonstrated that intramuscular immunization with inactivated virus can provide sufficient protection against natural polio. The high degree of success with IPV has been largely attributed to the inclusion of entire populations in the primary vaccination programs and the ability to deliver booster immunization at regular intervals to large segments of the population. Such immunization programs have been found to maintain effective levels of specific circulating antibody over long periods.

In order to examine the nature of mucosal antibody responses after initial (primary) immunization by systemic or mucosal routes, groups of infants were immunized with trivalent OPV (TOPV) administered orally or trivalent IPV (TIPV) inoculated intramuscularly or intranasally. The subjects were two months of age when first immunized and received three doses of the vaccine

Table 2: Available vaccines listed by year of first vaccine development or licensure in the United States (1700-2009)

Period	Vaccine	Efficacy by recommended route of administration: Mucosal	Systemic
1700-1799	Smallpox	-	++
1800-1899	Rabies	-	++
	Typhoid	-	+
	Cholera	-	+
	Plague	-	++
1900-1959	Diphtheria	-	++
	Pertussis	-	++
	Tetanus	-	+++
	Tuberculosis	-	+
	Influenza	-	++
	Yellow fever	-	++
	Poliomyelitis (IPV)	-	+++
1960-2000	Poliomyelitis (OPV)	++	-
	Measles	-	++
	Mumps	-	++
	Rubella	-	++
	Anthrax	-	++
	Meningococcus (Aac)	-	++
	Streptococcus pneumoniae	-	+++
	Adenovirus[a]	++	-
	Hepatitis B	-	+++
	Haemophilus influenzae B	-	+++
	Japanese encephalitis	-	++
	Hepatitis A	-	++
	Varicella-zoster	-	++
	Lyme disease	-	±
	Rotavirus rhesus[b]	++	-
2001-2009	Typhoid[a]	++	-
	Cholera[a]	++	-
	Influenza A[c]	++	-
	HPV[c]	-	++
	Meningococcus	-	++
	Zoster (shingles) [c]	-	++
	Rotavirus[c]	++	-

[a]Not available for routine use in U.S.
[b]Discontinued
[c]Recently developed
+ to +++: Effective to highly effective

at monthly intervals. All subjects were subsequently immunized with a booster dose of the TIPV administered intramuscularly or intranasally or, with TOPV administered orally at 12 months of age. The IgG antibody in the serum and SIgA antibody responses in the nasopharynx were measured at various intervals (*Ogra*, 1984). The outcome of immunization relative to the route and type of primary vs. secondary immunization is summarized below.

Mucosal priming and mucosal challenge

Primary oral administration of TOPV resulted in the appearance of significant serum IgG and nasopharyngeal SIgA poliovirus antibody response in a predictable manner. Booster immunization with TOPV at 12 months of age (approximately eight months after the last dose of primary immunization) resulted in no significant change in the pre-existing serum IgG or nasopharyngeal SIgA activity. Primary intranasal administration of TIPV resulted only in the transient appearance of nasopharyngeal antibody activity, without any detectable antibody response in the serum. The level of pre-existing maternal IgG antibody continued to decline within its expected half-life. Booster or re-immunization with TIPV administered intranasally elicited the reappearance of SIgA antibody in the nasopharynx. Several subjects also manifested low levels of IgG antibody in the serum. Re-immunization with TOPV in subjects previously primed intranasally with TIPV manifested a booster effect for serum IgG as well as for nasopharyngeal SIgA. It should, however, be pointed out that the mean SIgA titres after mucosal challenge with TOPV in individuals previously primed with TIPV or TOPV by the mucosal route (intranasally) were remarkably similar.

Systemic priming and mucosal challenge

Primary immunization with TIPV administered IM resulted in high levels of poliovirus-specific serum IgG antibody response in all subjects studied. However, no SIgA response was observed in the nasopharynx. Re-immunization with TIPV via the intranasal route in such parenterally primed subjects resulted in the appearance of SIgA in the nasopharynx, but no significant booster effect on the SIgA response or on the levels of pre-existing serum IgG was observed. On the other hand, re-immunization challenge with TOPV in such individuals resulted in a significant boost of SIgA antibody in the nasopharynx and of pre-existing IgG antibody in the sera.

Other forms of immunization

Although no SIgA poliovirus antibody response was observed in the nasopharynx after primary IM administration of TIPV, re-immunization with TIPV administered IM elicited a modest SIgA activity and a predictable booster effect on pre-existing IgG in the serum. A similar booster effect on serum IgG antibody response was observed after IM challenge with TIPV in subjects who had received primary immunization with TOPV administered orally (*Ogra*, 1984).

No booster effect was observed for SIgA response after IM challenge with TIPV in subjects previously immunized with TOPV or intranasally with TIPV.

These observations suggest that mucosal priming may not significantly influence the outcome of specific SIgA immune response in the nasopharynx to subsequent challenge with antigen administered by the mucosal route. On the other hand, parenteral priming followed by parenteral challenge resulted in minimal enhancement of SIgA response in the nasopharynx. More sig-

nificantly, parenteral priming resulted in significant enhancement of poliovirus-specific SIgA response in the nasopharynx to subsequent oral administration of the vaccine virus. In subsequent more extensive studies on priming by mucosal vs. systemic routes, employing immunization with polio vaccines, serum neutralizing, nasopharyngeal neutralizing, and IgA antibodies were determined in 123 infants immunized with one of four schedules containing live oral vaccine (OPV), inactivated vaccine (IPV), or combinations of the two trivalent poliovirus vaccines: OPV-OPV-OPV, IPV-IPV-IPV, IPV-OPV-OPV, or IPV-IPV-OPV. Nearly 100% of individuals formed serum-neutralizing antibodies. The highest geometric mean titre (GMT) of antibody to polioviruses 1, 2, and 3 occurred in groups IPV-IPV-OPV, IPV-OPV-OPV, and IPV-IPV-IPV, respectively. Local neutralizing and IgA antibody responses were detected in 41%-88% and 75%-100%, respectively. Peak GMT of nasopharyngeal antibodies differed minimally between immunization groups. The data suggest that incorporation of at least one dose of IPV at the start of the immunization schedule tends to increase systemic as well as local antibody production. Over 70% of the subjects were monitored serologically over the subsequent 4 years and challenged with OPV at 5 years of age. Each of the immunization groups exhibited an initial 10- to 100-fold decline in neutralizing antibody to poliovirus types 1, 2, and 3 during the first 2 years of follow-up; thereafter antibody titres stabilized. The IPV-IPV-OPV group maintained the highest antibody levels throughout the observation period, including the response to OPV challenge at 5 years of age. These data suggest that immunization with OPV, IPV, and combinations of the two vaccines confer long-term immunity. Optimal systemic immunity was associated with two or more doses of IPV (*Faden* et al., 1990, 1993).

No discernable suppression of IgG response in the serum or SIgA response in the nasopharynx was observed with either the mucosal or the systemic form of administration in these children. However, studies by Svennerholm (*Svennerholm* et al., 1981) demonstrated a significant suppression of pre-existing SIgA activity in the milk after oral administration of OPV in women who were previously infected, presumably as a result of prior natural exposure to wild poliovirus. These studies were carried out in groups of lactating women in Sweden, a country with little or no wild poliovirus infection and in Pakistan, where poliovirus infection was endemic at that time. At the beginning of these studies, the Swedish women lacked significant titres of SIgA poliovirus antibody in the milk. Subsequent parenteral immunization with IPV in these women resulted in a low and a transient increase in the titres of SIgA activity in the milk. On the other hand, Pakistani women had significant SIgA titres in their milk before any active immunization was carried out. Parenteral immunization with IPV resulted in a significant increase in SIgA titres in the milk of 45% of the subjects tested. On the other hand following oral administration with OPV given in conjunction with subcutaneously administered killed *Vibrio cholerae* vaccine, the pre-existing titres of poliovirus antibody in the milk manifested a significant (as much as 40-fold) decline. However, when OPV was used alone, some subjects appeared to manifest a mild enhancement of SIgA titre, while other manifested a modest drop in pre-existing SIgA titres (*Svennerholm* et al., 1980).

It is apparent that the extent of serum and secretory immune responses

may be determined by the functional homeostasis of the regulatory T cell subsets, other immunoregulatory cells, immune complexes, histocompatibility, and the nature, physicochemical characteristics, and route of delivery of antigens. The possible synergism or antagonism of different organisms or antigens on the network of immunoregulatory mechanisms must be considered in the explanation of the diverse spectrum of changes in the systemic and SIgA immune responses with different antigens administered by different routes. The efficacy of IPV as well as OPV in the prevention and control of poliomyelitis has been conclusively demonstrated by their routine use over the past three decades, especially in the technologically developed countries (*Centers for Disease Control and Prevention [CDC]*, 2005; *Hayman*, 2004). However, serious concern has been raised about the effectiveness of immunization with OPV in the developing countries where paralytic poliomyelitis continues to exist in endemic albeit very small proportions. Even large-scale repeated immunization with OPV in these countries has been associated with a high rate of failure in several communities to seroconvert for poliovirus-specific antibody (*Mittal* and *Mathew*, 2007). In fact, several major outbreaks of paralytic poliomyelitis from community-acquired wild poliovirus infection have continued to exist in these countries in children previously immunized with high-potency OPV given in standard dosage schedules at appropriate intervals (*Chandrakant* and *Pradhan*, 2007). A number of possible explanations have been offered for this phenomenon. These include co-existing enteric viral infections interfering with implantation of vaccine virus, loss of potency of vaccine during transportation in the tropical heat, presence of other inhibitory factors such as interferon, or co-existing infection with wild polioviruses. However, none of these mechanisms can be clearly implicated in most if not all cases of OPV failure in such settings.

It is possible that the microbial ecology or specific environmental antigens in the alimentary tract of vaccinees in the developing countries may under certain situations have a profound influence on the activation of immunoregulatory mechanisms in the gut-associated lymphoid tissue, notably on functional activity of immunoregulatory T cell subsets. Such alterations may in turn determine the degree of systemic or secretory antibody response to vaccine-induced and, possibly, naturally acquired poliovirus infections (*Sack*, 2008).

MUCOSAL IMMUNIZATION AND ORAL TOLERANCE

Oral exposure to non-replicating antigens may significantly influence the outcome of systemic immune response to subsequent re-exposure to the same antigen. The phenomenon of systemic hypo-responsiveness observed following oral ingestion of an antigen gained scientific credence in the early 1940s with the classic experiments of Chase employing simple chemicals (Chase, 1946). Since then, oral sensitization with a number of non-infectious antigens has been observed to induce suppression of the systemic immune response to the homologous antigen following subsequent systemic challenge. These include picryl chloride, sheep red blood cells (SRBC), ovalbumin (OVA), ragweed antigen E, dinitrophenylated human gammaglobulin

Table 3: Effect of the route of priming on the outcome of immune response to subsequent challenge with soluble proteins and other non-infectious agents (*Ogra*, 1984)

Route of priming/challenge	Antigen	Effects on immune response: Systemic IgG	Mucosal SIgA
Mucosal/systemic	Picryl chloride	S	NA
	SRBC	S	NA
	OVA	S	NE
	Ragweed-E	S	NE
	DNP-HGG	S	NA
	Transplantation	S	NA
Mucosal/systemic	OVA	S	NE
	BSA	S	NE
Systemic/systemic	Haptens	S	NA
	Hapten-syngeneic cell complex	S	NA

SRBC = sheep red blood cells
OVA = ovalbumin
DNP-HGG = dinitrophenylated human γ-globulin
BSA = bovine serum albumin
SIgA = secretory IgA;
S = suppression (tolerance)
NE = no effect
NA = no available data

(DNP-HGG), and transplantation antigens as shown in Table 3 (*Ogra*, 1984). In addition, suppression of the systemic immune response has also been observed for OVA and bovine serum albumin (BSA) after mucosal challenge in animals previously primed via the mucosal route and for certain haptens after systemic sensitization. In virtually all experiments with such non-infectious antigens reported to date, no suppressive effect has been observed on the mucosal immune responses. On the basis of these observations subsequently Tomasi proposed the concept of "oral tolerance" as a mechanism of possible defence by which certain mucosally introduced antigens will result in systemic hypo-responsiveness, thus reducing the risk of the development of systemic immunologically mediated disease states.

While the observations on systemic tolerance with many soluble protein antigens and other macromolecules are clear-cut, an extreme degree of variation seems to exist for the induction of or the levels of systemic hypo-responsiveness to non replicating and possibly replicating infectious agents. The available data on the effects of mucosal vs. systemic priming on the outcome of subsequent re-exposure challenge with infectious organisms or specific microbial antigens are reviewed in Table 4. A careful examination of these observations suggests that with most infectious agents, the effect of mucosal or systemic priming on subsequent challenge is, in fact, in favour of a booster effect on the systemic immune response rather than tolerance. Similarly, the SIgA response to infectious agents does not manifest a consistent pattern

Table 4: Effect of route of priming on the outcome of immune response to subsequent challenge with infectious microorganisms or their antigens (*Ogra*, 1984; *Faden* et al., 1990; *Svennerholm* et al., 1981)

Route of priming/challenge	Agent	Effects on immune response: Systemic IgG	Mucosal SIgA
Mucosal/systemic	OPV/IPV	B	NE, B(S)
	IPV/IPV	B/NE	NE, B
	Streptococcus mutans	S	NE
	Vibrio cholerae LPS	B	B, NE
Systemic/mucosal	IPV/OPV	B	B
	V. cholerae	B	(S)B
	V. cholerae toxoid	NA	S
	IPV	NE	NE
Mucosal/mucosal	IPV/OPV	NE	B
	OPV/OPV	B	B
	Live wild-type poliovirus/OPV	NE	S*
	RSV	B	B
	Rubella	B	B
	V. cholerae	NE, B	B
Systemic/systemic	IPV	B	NE
	V. cholerae LPS	B	(S)NE

OPV = live attenuated (oral) poliovirus vaccine
IPV = inactivated poliovirus vaccine
LPS = lipopolysaccharide
RSV = Respiratory syncytial virus
SIgA = secretory IgA
B = booster effect
NE = no effect
S = suppression (tolerance)
NA = no data available.

of suppression after mucosal or systemic priming and subsequent challenge. It would seem that the pre-existing SIgA responses to most replicating agents exhibit a booster effect or in certain situations no discernible change in pre-existing immunity.

CONCLUDING REMARKS

Possible approach to enhance mucosal immune response in childhood

Mucosal administration of vaccine antigens especially replicating agents and for organisms whose portal of entry are the external mucosal surfaces of the respiratory, enteric or genital mucosa in general mimic the development of immunity following natural infection. The observations summarized in the preceding sections of this review suggest that factors that favour devel-

Table 5: Status of existing non-replicating vaccines delivered by mucosal routes

- Antigen mass limited to the amount administered (no replication)
- Inactivated antigens used alone, not highly immunogenic
- Induce effective but transient secretory and little serum antibody and cellular immune response often exhibit:
  1. Lack of memory
  2. Require appropriate adjuvants, mucosal delivery formulations and immunogenic epitopes for effective immune responses and disease protection (cholera)
  3. Less efficient immune response and disease protection in the developing world
     - Minimal or no untoward side effects in vaccinees or in contacts
     - No community spread. Evidence of herd immunity with some? No potency loss in field setting

opment of mucosal antibody and cell-mediated immune response include mucosal route of immunization and the replicating nature of the vaccine antigen. However, to date, very few replicating vaccines have been available for mucosal immunization. The paucity of available mucosal vaccines is related in part to potential danger currently perceived with replicating agents, especially when the risk of continued vaccination may exceed the risk of disease following naturally acquired infection. This is best illustrated by the withdrawal of OPV from routine immunization from most of the developed world. In countries where wild poliovirus infection has been effectively eliminated, the reasons for limited use of other mucosal vaccines is related to the observations that it has also been difficult to induce mucosal protection consistently after mucosal administration of many candidate non-replicating antigens. The mechanisms underlying such poor mucosal responses include, poor antigenicity, rapid elimination, inactivation by mucosal enzymes or interference by existing mucosal environment, including gut microflora. Other potential limitations include lack of optimal contact of vaccine components with antigen presenting or processing mucosal cells including M cells, DC and mucosal macrophages (*Ogra* et al., 2001).

Based on the experience with existing vaccines, the development of mucosal immunity by administration of vaccines via the mucosal routes is clearly not a pre-requisite for the effective control of most infections. With the exception of oral rotavirus vaccine, cholera, and intranasal influenza virus vaccine, most newly developed vaccines (such as HPV, pneumococcal and meningococcal conjugates), and other vaccines currently under development, are designed solely for parenteral use. As the use of parenterally administered vaccines continues to remain single major option for new vaccine development; the average infant will have received over 25 vaccine doses by intra-dermal or intramuscular injections by 18-24 months of age of the infant. The availability of mucosally deliverable vaccines will provide simpler relatively painless approach for as frequent a delivery as necessary, and for multiple vaccine-antigens. The benefits and potential limitation encountered with currently available replicating and non-replicating vaccines are listed in Tables 5 and 6.

The possible approaches suggested to address the difficulties encountered in the development of effective mu-

Table 6: Status of Existing Replicating Vaccines Delivered by the Mucosal Route

- Induce amplification of antigen mass (based on level of replication).
- Induce of effective serum and secretory antibody and cellular (CTL) immune responses.
- Prolonged responses and induction of memory.
- Protection against both mucosal and systemic infection and or illness.
- Development of herd immunity and community spread, relative to the level of replication. However, immunization may be associated with:
  1. Development of untoward and sometime serious side effects in vaccinees and contacts
  2. Loss of potency in field settings
  3. Less efficient immune response and disease protection even after multiple immunization doses in many parts of the developing world

cosal vaccines are listed in Table 7. A number of approaches are being considered to reduce microbial virulence and enhance antigen load with replicating vaccines. These include use of recombinant protein and use of live vectors, subunit vaccines and use of specific antigen containing transgenic edible plants. The use of micro-particles, viral-like particles is being explored more extensively to improve delivery of antigens into the mucosa. Currently efforts are underway to employ safer mucosal adjuvants and, consider routes of mucosal immunization other than oral or intranasal. These include sublingual and trans-cutaneous routes to enhance development of effective mucosal response at desired target effector site (*Belyakov* et al., 2004; *Song* et al., 2008). Ample evidence has suggested that mucosally delivered vaccines could also be more effective in preventing systemic illness and mucosal infection during subsequent natural re-exposure to the virulent pathogen.

The induction of tolerance is possibly an important limitation to the use of non-replicating antigens by the mucosal route especially in the absence of appropriate adjuvants. However development of mucosal tolerance has not been demonstrated for replicating or non-replicating microbial vaccine antigens in man. It is not known whether the failure to develop effective immunity against polio after repeated immunization with OPV described earlier in this review in some countries reflects induction of oral tolerance. One of the goals of vaccine delivery by the mucosal route should include approaches to examine the development of tolerance and to overcome such potential threats that may exist prior to exposure especially in the neonatal period or early infancy. Interestingly enough, in an earlier publication on immune response to Leishmania antigen in an experimental animal model infection, it was proposed that induction of tolerance to potentially harmful population of Leishmania antigens may permit development of protective immune response to other Leishmania antigens and thus prevent development of disease. The author proposed induction of oral tolerance may be a possible immunization approach in preventing disease with other cell associated pathogens such as Candida, Schistosoma and microflora (*McSorley* and *Gaside*, 1999).

Numerous recent observations have suggested that the acquisition and the nature of mucosal microbial flora in early childhood especially in the neonatal period is critical in later develop-

Table 7: Approaches to enhancement of mucosal immunity to vaccines (*Ogra* et al., 2001)

Goal	Approach
Reduce virulence and enhance antigen load	Recombinant proteins, live vectors Subunit vaccines DNA vaccines Transgenic edible plants
Improve delivery into the mucosa	Non-living micro-particle carriers VLP
Improve mucosal interaction with antigens	Adhesive antigens Adjuvants
Enhancement of immune response	Mucosal adjuvants Combination systemic-mucosal immunization Trans-cutaneous, sublingual and other routes of immunization

ment and regulation of the mucosal immune responses and their functions. Depending on the nature of microbial-host mucosal interactions, the functional nature of mucosal immune response is protective against disease producing microorganisms, and other environmental macromolecules. Such protection is mediated by colonization by commensals, development of protective B and T cell responses, possible activation of specific innate immune mechanisms and induction of tolerance to dietary antigens and other macromolecules. The nature of mucosal immune responses may also be pathogenic and facilitate development of immunologically mediated diseases and induction of autoimmunity. Such effects may be related to altered microbial colonization in early life, diet, use of antibiotics and failure to develop tolerance to dietary antigens and other external agents (*Ogra* and *Welliver*, 2008). The symbiotic relationship between the microbial flora and the host has evolved over millions of years of balanced co-existence, in which the host as well as the "normal" microbial flora contribute to each others functional integrity and survival. An exciting recent series of investigations has provided a new and unique dimension to the evolution of mammalian intestinal microbial flora. These studies have obtained convincing evidence to suggest that mammals, including the humans are "composed of not only their own gene pool, but also of all their associated microbes". Both the host diet and microbial phylogeny influence the nature of bacterial diversity in the mammalian gut (*Ley* et al., 2008). These observations open up new avenues for the development of effective mucosal vaccines against human microbial pathogens whose primary portal of entry represents the external mucosal surfaces.

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DETERMINANTS OF RESPONSIVENESS TO ORAL VACCINES IN DEVELOPING COUNTRIES

DAVID A. SACK

Department of International Health,
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

SUMMARY

Vaccines can be a life saving tool to prevent infectious diseases. Oral vaccines are now being used for polio, typhoid, and cholera, and have recently been introduced for rotavirus. New oral vaccines are also being developed for other enteric infections. Unfortunately, oral vaccines tend to stimulate less consistent immune protective responses in children living in very poor countries. Several mechanisms have been proposed to explain this insufficient immune response, but the reasons for the poor response is not fully understood and no practical methods have yet been developed to correct this problem. Future studies are needed to insure that life saving vaccines can be developed which will be effective for children living in areas where the disease burden is the highest.

INTRODUCTION

Oral vaccines have been developed and are being used for polio, rotavirus, typhoid, and cholera. Others are under development for enterotoxigenic *E. coli*, Shigella and others. The vaccines for enteric bacterial infections are targeted to benefit children in developing countries where these diseases are endemic and most life-threatening, but children in the poorest countries tend to respond in a manner that is less than optimal, relative to those in industrialized countries. Enteric infections are major causes of deaths in children, and such vaccines have the prospect of preventing millions of deaths if they are able to induce protective immunity. However, it appears that that the children who are most at risk of severe infections do not respond well to these vaccines. Unless this problem is understood and corrected, the potential life-saving benefit of these vaccines will not be reached.

Figure 1 illustrates this point through a cartogram in which the area of each country is proportional to the annual number of children who die under the age of 5 years (*Sack*, 2008). The newer vaccines for rotavirus, RotaTeq and RotaRix, provide high levels of protection in children in the low mortality countries, but are much less protective in the high mortality countries, the ones which appear most prominently on the cartogram (*Madhi* et al., 2010). This paper reviews observations illustrating the problems of the relatively poor immune response in children who are at highest risk and examines the various explanations for the poor immune responses and lower protection.

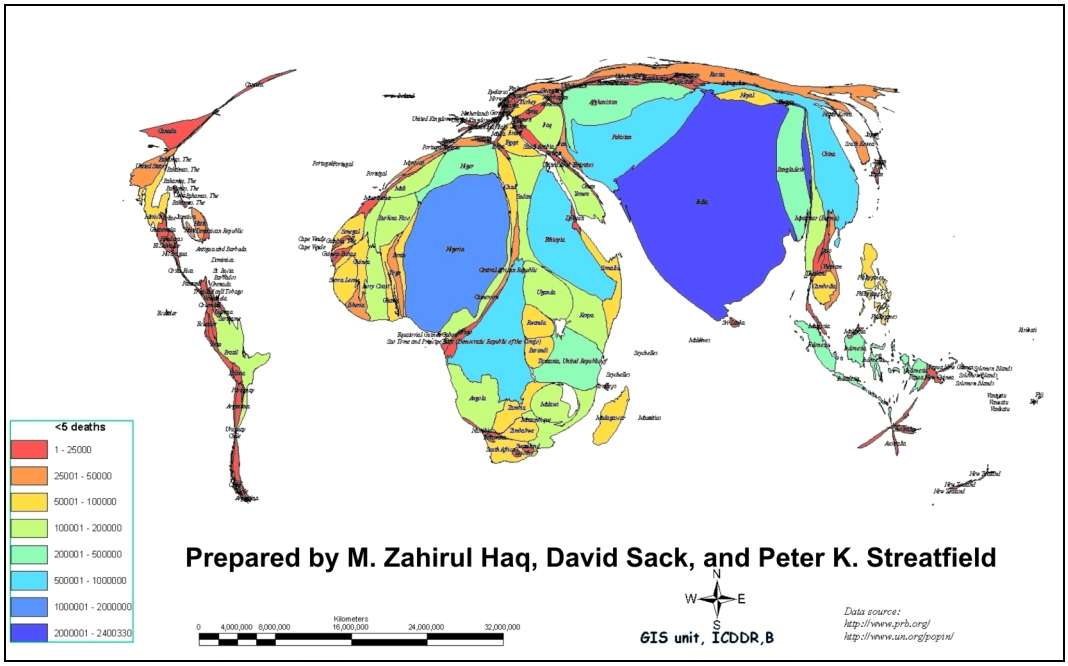

Figure 1: Under the age of 5 years deaths adjusted map of the world (2003).

This paper concentrates on oral vaccines rather than injectable vaccines since most evaluations of injectable vaccines find that children in developing and industrialized countries respond in a similar manner to injectable vaccines. Measures of the lowered immunogenicity include lowered take rate, lower geometric mean titres following immunization, higher doses required to induce an immune response, reduced efficacy against disease, and shorter durations of protection.

EXAMPLES OF SUB-OPTIMAL VACCINE RESPONSES

It appears that most vaccines given orally yield sub-optimal responses when given to children in developing countries. Some children who have received multiple doses of oral polio vaccine have developed paralytic polio, and many other immunized children may be infected with wild type virus, even though asymptomatic (*Grassly* et al., 2010). In a recent study, only 70% of Bangladeshi infants had a serological take to polio serotype 3 following immunization with OPV even though they responded to the other serotypes (*Zaman* et al., 2009). The sub-optimal responses to the vaccine has impeded efforts to totally eradicate polio from certain geographic areas where wide-type virus continues to circulate, such as India, Pakistan, Afghanistan and Nigeria (*Paul*, 2009; *Hasan* et al., 2004).

Rotavirus vaccines have had lower rates of protective efficacy when tested in countries in sub-Saharan Africa and South and South East Asia, as well as lower take rates and lower geometric mean titres following immunization (*Madhi* et al., 2010; Zaman, K., personal communication). Although the

protective efficacy and immunogenicity of these rotavirus vaccines is lower in these poor countries, they still have the potential to be important public health tools because of the large numbers of cases of severe diarrhoea which can be averted. Still, their public health effectiveness is lessened by the sub-optimal immune responses.

A live attenuated Shigella vaccine (SC602) which was immunogenic and protective in North America volunteers did not colonize or stimulate detectable serological responses in Bangladeshi children (*Katz* et al., 2004). Doses of vaccine, from 10^4 to 10^6 were given to children in Bangladesh but with no detectable responses, even though a dose of 10^6 induced some dysentery symptoms in North American volunteers (Sack, D., unpublished data,). Though a higher dose might have been immunogenic in Bangladeshi children, it was felt that a higher dose would not have been acceptable.

Besides inducing a lesser serological response, sub-optimal vaccination may also be exhibited by a shorter duration of protection. This was seen in the trial of Dukoral (killed oral cholera vaccine) when given to subjects in Bangladesh. Children <5 years of age were protected against cholera for the first six months, but then protection was lost during the second six months. By contrast, the older children and adults continued to be protected for up to three years (*Clemens* et al., 1990).

Another oral cholera vaccine is the live attenuated oral vaccine (CVD103HgR). Among North American volunteers, a dose of $5x10^8$ bacteria was adequate to stimulate vibriocidal responses, but among Indonesian subjects, this same dose induced such responses rarely. Thus, the dose was increased to $5x10^9$ to stimulate an adequate take rate. Even with this higher dose, the vaccine did not protect against cholera (*Richie* et al., 2000).

Thus, there are several examples of reduced immune responses to many types of oral vaccines when used in developing countries.

RELEVANCE OF THE SUB-OPTIMAL RESPONSES TO ORAL VACCINES

These vaccines are intended to be "life-saving" interventions. The deaths, which could potentially be prevented with these vaccines, occur among the poor groups within these poor countries. For example, it is estimated that between 500,000 and 600,000 children die from rotavirus diarrhoea annually. However, these deaths are not equally distributed among the world's children; rather, they nearly all occur within the poorest countries and within these poor countries, they occur most often in the poorest families. These same groups are the ones who appear to respond in the least consistent manner. Although a rotavirus vaccine with an efficacy of 50% in the poor countries might be estimated to reduce rotavirus deaths by 50%, in fact, the reduction could be much less. This is because children who are most at risk of a rotavirus death are likely the same as the ones who respond less well to the vaccine.

Generally, vaccines are among the most equitable health interventions because they can be given to many who may not receive treatment if they do become ill. Depending on treatment for a treatable illness is much less equitable because, in reality, treatment many not be available or provided. Thus, prevention of illness is especially critical for those without access to care.

However, when a vaccine is less effective among the most vulnerable groups, there is a "mismatch" between those who respond to the vaccine and those whose lives are at most risk. Such vaccines, which provide high efficacy to the groups, which are the least vulnerable, but lower efficacy to the most vulnerable, might be said to be "inequitable vaccines." This mismatch emphasizes the critical importance of finding a solution to the problem of sub-optimal responses to immunization.

APPROACHES TO SOLVING THE PROBLEMS OF SUB-OPTIMAL RESPONSES

Because the experience with past vaccines, vaccine programs have adopted empiric strategies to correct for sub-optimal vaccine responses. In the case of polio, national programs have provided additional doses of OPV to children through "national immunization days (NIDS)" (*Centers for Disease Control and Prevention [CDC]*, 2004, 2005, 2008). These NIDS aim to provide OPV vaccine to all children regardless of previous receipt of OPV. Many children end up receiving 10 to 15 doses of OPV over a lifetime. From a programmatic perspective, these NIDS have been very successful in reaching a very high proportion of all children. Due to the wide and massive coverage in countries with inadequate sanitation, the live vaccine virus spreads in the environment and immunizes many others who may not have received vaccine directly, thereby enhancing herd immunity. This strategy has essentially stopped transmission of wild type virus in many countries. Unfortunately in some areas, asymptomatic transmission of wide-type polio virus has continued in spite of this wide scale immunization through the NIDS (*Grassly* et al., 2010). Thus, this strategy of giving additional doses on a massive scale has been successful in many areas, but has not been adequate to eradicate the disease as was hoped it would.

For rotavirus, RotaRix is now recommended as a two-dose vaccine given during the first two immunization visits. Is it possible that a third dose as a potential way to increase protection? Unfortunately, one study from Africa did not show an improvement in protection with a third dose (*Madhi* et al., 2010).

In the case of the live attenuated cholera vaccine (CVD103HgR), the strategy used was to give a ten-fold higher dose. In theory, it may be possible to have a specific formulation with a higher dose for use in developing countries; however, this is certainly not optimal and could only be used if the vaccine was shown to be extremely safe. It would seem more logical to adopt the higher dose, appropriate for use in developing countries, as the "standard" dose and use this dose in all countries.

Whether giving higher doses or more doses will improve vaccine performance is not clear. In the case of live attenuated vaccines, the effectiveness of additional doses may be blocked by "immune exclusion" resulting from the first dose, so it is not clear that simply giving additional doses will result in more robust immune responses.

The underlying mechanism responsible for the sub-optimal immune response is not known. Since children living in tropical countries often have an inflamed intestinal mucosa with shortened villi it is possible that "tropical enteropathy" contributes to the

problem (*Lagos* et al., 1999). Malnutrition, including specific micronutrient deficiencies have also been implicated, as have intestinal parasites (*Cooper* et al., 2001). It seems unlikely that a single factor will provide an explanation with all vaccines. Some vaccines, e.g. rotavirus, are given at a very young age, prior to the age where malnutrition, micronutrient deficiency or intestinal worms are commonly found. By contrast, other vaccines, e.g. cholera vaccine, are given at 1 or 2 years. By this time, tropical enteropathy, malnutrition, micronutrient deficiency, and infestations are frequent.

Studies have been carried out to determine if supplements with vitamin A, zinc or a combination of these would improve immunogenicity of killed cholera vaccine. In a group of children who were not vitamin A deficient, supplemental vitamin A did not change the immunogenicity, but zinc did stimulate a higher titre of vibriocidal antibodies (*Albert* et al., 2003). Vitamin A is thought to be critical for healthy mucosa as well for immunity; however, when a routine vitamin A distribution program is providing vitamin A, it appears that additional vitamin A is not helpful.

Maternal antibodies via placental route or via breast milk may inhibit vaccine responses. This is well established in the case of measles vaccine, but is unclear in the case of oral vaccines (*Griffin* et al., 2008; *Triki* et al., 1997). There is some evidence that breast milk may neutralize antigens and that the immune response may be blunted (*John* et al., 1976). Studies are ongoing to determine the extent to which withholding breast feeding temporarily may improve rotavirus vaccine immunogenicity. Results from this study will be informative; however, a strategy of withholding breast-feeding is neither practical nor feasible, and could interfere with messages in favour of breast-feeding more generally.

MATERNAL INTERVENTIONS

There is increasing interest in attempting to improve the health of the infant through interventions that are directed toward the mother during or prior to pregnancy. When examining risk factors that are associated with high infant mortality, many of these are related to mother's education and health. Mothers of children who are most vulnerable are frequently underweight, suffer from frequent illnesses, have short birth intervals between children, are anaemic and under stress. It seems likely that some of these factors can influence the immune system and the health of the infant.

An example of the relation between mother's toxic stress and the infant's immune system is the information showing that women who are exposed to arsenic during pregnancy have a smaller thymus (*Raqib* et al., 2009). While this is only one example, addressing the health needs of women before and during pregnancy may be more effective than focusing only on the infant.

INTERACTION BETWEEN IMMUNITY AND ENVIRONMENT

The focus on poor immune response of the infant should not exclude consideration of the lack of sanitation in the environment in which the infant lives.

Intestinal immunity can be overwhelmed if an inoculum is very high, at least for bacterial infections and possibly for viral infections. Areas where protection is lower tend to be areas of very poor sanitation. Is it possible that the heavy environmental faecal contamination results in a very high inoculum, and that this results in vaccine failures and increased transmission of the enteric pathogens? The cycle of immune and environmental failure could be a key concept to reducing the predisposing factors for hyporesponsiveness to vaccination as well as continued transmission of pathogens.

RESEARCH AGENDA

The cause of oral vaccine hyporesponsiveness is not known, but solving the puzzle is clearly critical if the effectiveness of vaccines for rotavirus, polio and other enteric diseases are to be improved and be more equitable. Risk factors for vaccine and immunological failure need to be identified. A limitation of past efficacy studies for rotavirus has been their study designs which did not allow for identifying many risk factors for vaccine failure. Data on potential risk factors such as birth weight, illnesses in the infants, maternal nutrition and micronutrient deficiency, birth interval, chemical exposures, and maternal illnesses need to be correlated with vaccine failures. Based on data from such case control studies, rational interventions can be devised to test hypotheses.

Pending data from such case control studies, potential interventions can be attempted such as micronutrients and calories for mothers, maternal immunizations, and micronutrients for the infant. Breast milk can be withheld for a period during the time of immunization to understand the role of breast milk antibody, but this will not be a practical strategy for the future.

While attempting to understand vaccine hyporesponsiveness, it may be that a practical solution is not possible and that a different approach is needed. For polio it seems clear that children who do not respond to OPV will respond to injectable polio vaccine (IPV) (*Sutter* et al., 2000). Until now, the very low cost of OPV and the relatively high cost of IPV has favoured the use of OPV for developing countries. For limited areas where polio has not been able to be eradicated with OPV, use of IPV may need to be reconsidered.

An injectable vaccine for rotavirus was never tested in children, but in view of the effectiveness of IPV, this might be considered. Many years ago, it might have been possible to have a parallel track to evaluate an injectable vaccine for rotavirus, but this was not attempted because of the belief that local intestinal immunity was best stimulated with an oral vaccine. In the field, it seems that the current oral rotavirus vaccines protect against symptomatic rotavirus disease, but they are much less efficient in protecting against rotavirus infection even in populations where high-level protective efficacy is seen. It would seem that an injectable vaccine could be prepared in relatively straightforward manner and could be tested in humans.

Other vaccine strategies could also be attempted with the current rotavirus vaccine. These might include a booster dose at 7 to 9 months of age. Giving rotavirus vaccine at an older age is not currently approved because of the fear of intussusceptions from the previous vaccine, RotaShield (*Peter* and *Myers*, 2002). The current vaccines, RotaTeq and RotaRix have not been associated with any increased risk for this compli-

cation, and it would seem that this strategy might be evaluated. An obvious limitation to a booster dose at 9 months is that it would not prevent the cases which occur earlier in life. The proportion of cases occurring prior to 9 months of age varies depending on the geographic region, though with a successful rotavirus vaccine program, the median age may increase as the disease burden lessens.

Other vaccination methods are being considered as well, such as sublingual or transdermal approaches. These are currently experimental approaches and have not been attempted, but they do appear promising.

Finding strategies to overcome the sub-optimal immune responses to oral vaccines among children in poor countries is a challenge. Finding solutions to the problem will be critically important if these oral vaccines are to accomplish their role as life saving interventions for the most vulnerable.

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IMPACT OF NUTRITION AND INTESTINAL MICROBIOTA ON DEVELOPMENT OF MUCOSAL IMMUNITY

DENISE KELLY and IMKE MULDER

Rowett Institute of Nutrition & Health, University of Aberdeen, Bucksburn, Aberdeen, Scotland

SUMMARY

It is now widely accepted that early life nutrition and the commensal gut microbiota play a key role in driving immune development, and maintaining immune homeostasis, but also in contributing to inflammatory and autoimmune diseases. Although much progress has been made on dissecting the various levels by which gut bacteria modulate gut barrier function and immunity, little is known about the host mechanisms and bacterial effector molecules and signals which drive these major physiological effects. Clearly microbial composition has a profound impact on gut health and disease. The quest to identify health-promoting gut bacteria and to unravel their mode of action continues to advance at a rapid rate. The commercial and clinical opportunities surrounding the exploitation of such research activities are significant, particularly in the development of functional foods and probiotics which promote, maintain, and restore gut health.

INTRODUCTION

During the last decade there has been a major surge in interest in the role of the gut microbiota in human health and disease. Since the publication by Eckburg and Relman (*Eckburg* et al., 2005), describing the diversity of the human gut microbiota, there have been significant advances in our understanding of how specific commensal gut bacteria influence mucosal immunity. The beneficial effects of commensal bacteria, particularly in promoting T regulatory cells (Tregs) and anti-inflammatory signalling pathways, have been scrutinised in an attempt to understand at a detailed mechanistic level how gut bacteria promote and maintain immune homeostasis.

Commensal microbes interact with the mucosal immune system in many ways and recent research has revealed that their beneficial effects can arise from specific interactions with epithelial cells which line the gut wall, mucosal dendritic cells, B cells and ultimately gut T cells within the human gut. New studies have also unveiled an important role for gut bacteria in regulating the enteric nervous system of the gut (*Rhee* et al., 2009). The accumulating data on host microbe interactions presents significant opportunities for exploiting the biological actions of live gut microbes and their component parts as mucosal adjuvants, anti-inflammatories and treatments for autoimmune, allergic and atopic diseases.

THE HUMAN GUT MICROBIOTA

The human gut is home to approximately 10^{14} gut bacteria, numbers of bacteria which outnumber human cells by a factor of 10 and human genes by a factor of 100 and whose collective genome is referred to as the gut microbiome. The diversity of the human gut microbiota, which results from strong host selection and co-evolution, was first comprehensively described by Eckburg and Relman (*Eckburg* et al., 2005). Using approximately 13,000 16S ribosomal RNA gene sequences they revealed that the human gut microbiota is dominated by three major bacterial phyla namely Bacteroidetes, Firmicutes and Actinobacteria all of which are highly diverse at both species and strain level. These authors also verified that mucosa-associated bacteria are distinct from those in the gut lumen and faeces, suggesting that mucosal-associated bacteria may in fact perform different host functions. A total of 395 phylotypes were identified of which 80% had never been cultivated. This astonishing finding serves to highlight the level of work required to fully appreciate the biological properties and function of individual members of the human gut microbiota.

Microbial diversity appears to vary between individuals and at different sites within the gastrointestinal tract (*Zoetendal* et al. 2008). However within an individual it is generally quite stable (*Ley* et al. 2008). The level of inter-individual variation probably reflects the functional redundancy among the constituent members of the human gut microbiota (*Turnbaugh* et al., 2007). This has been confirmed by additional studies which show that among family members, the human gut microbiome (microbial genes) is shared, but the gut microbial community within each individual varies in terms of the specific bacterial lineages. Different microbial diversities can in fact give rise to a core microbiome, supporting the concept of functional redundancy (*Turnbaugh* et al., 2009; *Qin* et al., 2010). Clearly diet, whether herbivore, omnivore, or carnivore has a highly significant impact on microbial diversity composition and tree-based clustering and appears to accounts for the diversity differences between unrelated host species (*Ley* et al., 2008). The gut microbiota of humans living a modern life-style appears typical of omnivorous primates (*Ley* et al., 2008).

The gut is sterile at birth and microbial colonisation is influenced by such factors as mode of delivery, environment, diet and antibiotics. The major microorganisms colonising the newborn gut are derived from both maternal (vaginal and faecal) and environmental sources. Mode of delivery has a major effect on the composition of intestinal microbiota in early infancy, and it has been shown that infants born by Caesarean section have lower numbers of Bifidobacteria and Bacteroides compared with vaginally born infants (*Biasucci* et al., 2010). Furthermore, preterm infants have very different microbiotas, mainly characterised by a low diversity of culturable microorganisms (*Rouge* et al., 2010). Due to the accumulating evidence that the human gut microbiota in early life impacts on subsequent adult health, the issue of gestational age at birth and how it impacts on microbial composition and function in adult life is an important consideration worthy of further investigation. Furthermore, the ageing process is thought to affect the structure of the human gut microbiota, and disturbed immune homeostasis and age-related differences in gut microbiota composition may contribute to the

progression of disease and frailty in the elderly population (*Biagi* et al., 2010). As with early life, the composition and alteration of the gut microbiota in the elderly and the implications for immune status and health is also an important topic requiring more research.

In the first years of life the gut microbiota is highly dynamic and appears not to reach an adult phenotype until around 2 to 3 years of age. Immediately after birth the gut microbiota is characterised by high number of facultative anaerobes including lactobacteria, enterobacteria and streptococci. As oxygen levels begin to deplete within the gut other more obligate bacteria colonise and become established and these bacteria include clostridia and bacteroides (*Marques* et al., 2010). The factors which influence the specific gut bacteria which establish themselves as members of the gut microbiota are not fully understood. In addition to the influences of diet and environment other factors including host genotype and epithelial glycobiology are thought to be important. More specifically, the mucus gel layer overlying the intestinal epithelium has been proposed as a key contributor to the structural and functional stability of the microbiota and tolerance by the host (*Sonnenburg* et al., 2004). This notion gave rise to the view that microbial biofilms form within the gut and that these biofilms are stable communities composed of microorganisms able to utilised and degrade gut mucins as well as recognise mucin-associated glycan structures/carbohydrates as attachment structures (*Sonnenburg* et al., 2004).

COMMENSAL BACTERIAL GENOMES

The first microbial genome was published in 2003 by the Gordon lab (*Xu* et al., 2003). The decoding of the genetic make-up of *Bacteriodes thetaiotaomicron*, a Gram-negative bacterium which is a dominant member of the human gastrointestinal tract, provided the first opportunity to study the molecular mechanisms by which gut microbes shape human physiology. This bacterium containing a 4779-member proteome including an elaborate apparatus for hydrolyzing indigestible dietary polysaccharides and an associated environment sensing system presented the first opportunity to identify commensal-derived effector molecules that could regulate important functions of the host gut including its immune status. Since this significant advancement, the human microbiome project (HMP) has provided reference genomes for a large number of gut commensals, including multiple bacterial isolates belonging to the same species (*Turnbaugh* et al., 2009). Following on, other projects including MetaHIT (Metagenomics of the Human Intestinal Tract) and more recently the comprehensive publication of 3.3 million non-redundant human gut microbial genes (*Qin* et al., 2010) have provided the means to define microbial gene functionality in the context of host response and physiology. The rapid identification of bacterial gene products which influence host immunity, either by augmenting (adjuvants) or, attenuating (anti-inflammatories) specific mucosal immune responses is anticipated.

MUCOSAL IMMUNE DEVELOPMENT

Development of the mucosal immune system starts *in utero* and continues at a dynamic pace in early life, stabilises in adult life and then declines with advancing age through processes of senescence or cellular death (*Ogra*, 2010). The mucosal immune system is highly complex consisting of diverse populations of innate and adaptive immune cells as well as memory cells. These various cell populations are present in organised gut associated lymphoid structures including Peyer's patches, lamina propria, lymphoid aggregates and mesenteric lymph nodes. In addition to the structural complexity of the mucosal immune system, the functionality is also highly complex and involves processes of microbial and antigen recognition, presentation, and response. These processes critically differentiate between harmful (pathogenic) and harmless challenges; the mucosal immune system must be able to defend rigorously against infectious agents whilst maintaining oral tolerance to self-antigens as well as those derived from the diet and the commensal microbiota.

Epithelial cells and dendritic cells (DCs) are the first cells involved in recognising and sampling commensal gut microbes. Epithelial cells undergo maturation in terms of their digestive and absorptive capabilities but also in aspects of their glycobiology, defence properties and synthesis and secretion of membrane bound and soluble mediators, all which affect their interactions with gut microbes and cells of the innate immune system. Important secreted epithelial factors include thymic stromal lymphopoietin (TSLP), IL-10 and TGFβ which promote the initiation and maintenance of oral tolerance (*Ziegler* and *Artis*, 2010). Gut bacteria are recognised by various classes of recognition receptors including Toll-like receptors (TLRs) expressed on the apical and basolateral surfaces of epithelial cells (*Abreu*, 2010). Following TLR recognition and ligation of specific bacterial structures referred to as microbial associated molecular patterns (MAMPS), a number of signalling cascades are activated which collectively influence host epithelial gene expression. These epithelial gene products operate in a paracrine and autocrine fashion to regulate the functional properties of both epithelial cells and neighbouring immune cells.

Gut DCs also respond to gut microbes through similar recognition events but as highlighted above they respond to factors secreted by intestinal epithelial cells such as TSLP and TGFβ which exert a profound effect on DC function within the gut (*Grainger* et al. 2010). Very significant progress has been made in recent years describing the role of intestinal DCs either in activating protective immune responses by engaging naive T cells or in promoting tolerance responses (*Rescigno* and *Di*, 2009). These divergent end points are managed by distinct DC subsets. An important DC subset has recently been defined within the gut which promotes the differentiation and expansion of Treg cells and responds to conditioning signals received from epithelial cells including TGFβ and TSLP (*Sun* et al. 2007; *Coombes* et al. 2007). Defective immunity in the neonatal gut has partly been explained by immaturity of antigen presenting cells including DCs. The precise developmental profiles of DC subsets within the neonatal gut, and the factors which influence their activation and maturation, are not currently known but clearly this information is essential to establishing the factors that influence tolerance and active immu-

nity and hence the susceptibility to allergic and infectious diseases.

In response to antigen presentation by DCs, T cells develop into a number of distinct subsets with associated effector functions depending on the specific cytokine milieu. Specifically T helper (Th)1 cells produce mostly interferon gamma (IFNγ), an inflammatory cytokine important in responses against microbial infections, while Th2 cells secrete interleukin (IL)-4 and IL-13, which participate in immunity against parasites but also play major roles in allergic reactions. Other T cells subsets include Th17 and T regulatory cell (Tregs), the former involved in driving inflammatory and autoimmune conditions and the latter functioning to suppress immune responses and induce tolerance.

Although neonates possess an immature immune system, as revealed by their under-developed lymphoid architecture, low numbers of T and B cells as well as DCs and memory cells, they are still able to mount immune responses. It has been suggested for some time that neonatal immunity is characterised by a dominance of Th2 responses, with a lower prevalence of Th1 responses thus contributing to the so called Th2 bias. Furthermore, although capable of mounting both Th1 and Th2 (mixed T cell responses) the overall response appears to default to a predominant Th2 response upon antigen re-challenge (*Zaghouani*, et al., 2009). Since many gut pathogens require robust Th1 immune responses for efficient clearance and immune protection, this may explain why the neonate is particularly susceptible to a number of important pathogens, resistant to the effects of certain vaccines as well as predisposed to allergic diseases.

More recently the subject of Th1 and Th2 balance has been investigated using a murine neonatal model of infection. Low doses of virulent *Yersinia enterocolitica* were found to induce strong inflammatory Th1 and Th17 cell responses with large quantities of IFNγ and IL-17 supporting the view that the neonate is perfectly capable of mounting a diverse range of T cell responses under certain circumstances (*Echeverry* et al., 2010). However, the enhanced susceptibility to infection in early life suggests that immune immaturity or defective Th1 immunity may be contributing factors. Recent data also suggests that low immune cell populations may not in fact fully account for the decreased immune responsiveness of neonates. An alternative mechanism may be due to an intrinsic "default" mechanism that neonatal $CD4^{(+)}$ T cells have to become Treg cells in response to T cell receptor (TCR) stimulations (*Wang* et al., 2010). This finding provides intriguing insights into Treg cell generation and the predisposition towards tolerance during early life. Equally it may explain the increased susceptibility of neonates to infectious diseases as well as the inadequate response to certain vaccines since neonatal Tregs could impair the specific T cell responses required for pathogenic clearance and account for the premature death of millions of human infants world-wide.

BREAST FEEDING AND IMMUNE DEVELOPMENT

It has long been suggested that breastfeeding confers protection against infections, diarrhoea, inflammatory and allergic diseases, but the mechanisms involved have remained elusive. Breast milk promotes strong anti-inflamma-

tory effects mediated by TGFβ, IL-10 and lactoferrin which serve to limit inflammation in the developing gut, and it also augments host defences through presentation of diverse antimicrobial factors (*Walker*, 2010). The protection against inflammatory and autoimmune diseases may also be related to the induction of oral tolerance mediated by milk antigen immunoglobulin immune complexes that promote antigen-specific FoxP3 regulatory T cells (*Mosconi* et al., 2010). As for B cell development and expansion within the gut, the recent identification of syntenin-1 which preferentially induces IgA production by B cells together with the biological effects of TGFβ may be significant (*Ogawa* et al., 2004; *Sira* et al. 2009)

LIVING ENVIRONMENT AND IMMUNE DEVELOPMENT

Environment during early life has for many years been considered to influence immune development and susceptibility to childhood allergies and asthma. This viewpoint was first postulated by Strachan in 1989 in the form of the hygiene hypothesis (*Strachan*, 1989) and was further endorsed with the notion that improved hygiene associated with decreased infectious agents in early life is a significant factor in the aetiology of atopic allergy disorders (*Sheikh* and *Strachan*, 2004). The latest version of this hypothesis suggests that exposure to farm animals, pets and non-pasteurized milk or fermented beverages may promote healthy development of the immune system (*Gern* et al., 2009). A recent study referred to as Urban Environment and Childhood Asthma (URECA) analysed 560 families from 4 urban areas who were at high risk of allergy along with 49 families without atopic diseases. This study revealed some associations between early life environment and subsequent risk of asthma but more studies are required (*Gern* et al., 2009). Experimental models mimicking the hygiene concept add additional support to the hypothesis that early life environment influences both microbial diversity and immune development and susceptibility to disease (*Mulder* et al., 2009).

COMMENSAL BACTERIA AND INNATE IMMUNITY

Barrier effects

Microbial colonisation is critical for the development and optimal functionality of the mucosal immune system. Beneficial effects on gut barrier function are one of the important biological actions of the colonising microbiota. These effects are thought to be induced down-stream of commensal-mediated TLR signalling through the production of interferon alpha which prevents intestinal epithelial apoptosis (*Mirpuri* et al., 2010).

Epithelial cells

Commensal bacteria also interact with epithelial cells to mediate and regulate NF-κB signalling (*Kelly* et al., 2004). Mice defective in NF-κB signalling developed gut barrier defects suggesting that NF-κB plays an important cytoprotective role in the gut in addition to its role in driving inflammatory responses (*Wullaert*, 2010). The ways in which commensal bacteria regulate host signalling are likely to be complex involving recognition recep-

tors such as TLRs and NODs but potentially other receptor systems that modulate NF-κB signalling responses in favour of immune homeostasis and cytoprotection.

TLRs and commensals

The interactions between commensals and TLRs are not always positive in terms of immune homeostasis and health. For example, Type 1 diabetes (T1D) is a debilitating autoimmune disease and its incidence has increased significantly during the past several decades particularly in Westernised countries. In addition to T1D other autoimmune, inflammatory and atopic diseases are steadily increasing leading to a growing view that environment and in particular microbial exposure is playing a significant role in enhancing susceptibility to immune-mediated diseases. Scientific evidence strengthening this link reveals that the innate immune recognition of gut microbes can in fact promote T1D and elimination of MyD88, an important adapter molecule mediating bacterial TLR signalling protects against T1D. These findings indicate that interaction of the intestinal microbes with the innate immune system is a critical factor modifying T1D predisposition (*Wen* et al. 2008). Furthermore, a reduction in commensal microbiota by antibiotic treatment has been documented to impair the development of autoimmune encephalomyelitis (*Ochoa-Reparaz* et al., 2009). This protection was associated with reduced pro-inflammatory cytokines and increased IL-10 and IL-13. These cases of autoimmune disease clearly indicate that alterations in microbial composition can dramatically impact on disease susceptibility and outcome. They also highlight that not all interactions between the gut and the commensal microbiota are beneficial and that manipulation of microbial diversity profiles can have very significant impact on both intestinal and extra-intestinal diseases.

COMMENSAL MICROBIOTA AND ADAPTIVE IMMUNITY

Commensal Microbiota and T cells

Recent advances investigating the impact of the commensal microbiota in relation to T cell differentiation have revealed that certain bacteria belonging to the class Clostridia are potent inducers of Th17, Th1 and Treg responses (*Gaboriau-Routhiau* et al., 2009). The ability to influence mucosal T cells seems to be restricted to a relatively small group of gut colonising bacteria. Currently the features and biological actions of gut bacteria which can regulate T cell events are unknown but one bacterium shown to be effective, namely the Segmented Filamentous Bacteria, is firmly attached to the gut epithelium and may engage in signalling through a unique class of intestinal epithelial receptors. The identification of other bacteria that influence T cell differentiation is likely and insight into their mechanisms of action will be extremely helpful in developing strategies for manipulation of T cell responses during early life presenting obvious therapeutic benefits and opportunities.

Commensal Microbiota and B cells

The early gut microbiota is dominated by bifidobacteria and lactobacilli particularly if maternal milk is the main nutrient supply. It has recently been suggested that elevated Bifidobacterial diversity enhances the maturation of SIgA levels (*Sjögren* et al., 2009). As infants with higher levels of SIgA are less likely to develop allergic diseases, the presence of bifids in the early gut microbiota is thought to be beneficial.

EDUCATING THE IMMUNE SYSTEM THROUGH MICROBIAL SUPPLEMENTS

Many intestinal diseases are associated with dysregulated immune responses and include the inflammatory bowel diseases Crohn's Disease and Ulcerative Colitis. With both diseases, exaggerated immune responses directed against the commensal microbiota are a common feature and the normal function of cells of the innate (DCs) and adaptive immune (Th) systems are disrupted. Clearly, identification of bacteria which can restore the tolerogenic and regulatory functions of 'defective' DCs and Tregs in IBD patients would be an exciting outcome. Furthermore, the notion that immune protection can be induced in early life as a means of preventing or reducing the incidence of immune-mediated diseases in adult life is even more attractive.

As the beneficial effects of commensal bacteria and probiotics on health and disease prevention become increasingly more defined the application of live microbial supplements to promote and restore gut health will gain much more attention in many aspects of human health. Robust scientific evidence based on human studies is required for EFSA/FDA approved health claims. The future of probiotics as human health products lies with mechanistic studies which prove mode of action and efficacy in human subjects and consumers. One important outcome will be new food products designed to promote gut health at key life stages.

ACKNOWLEDGEMENTS

The authors would like to thank the Scottish Government, Rural and Environment Research and Analysis Directorate for financial support of their work.

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IMPACT OF THE INTESTINAL MICROBIOTA ON THE DEVELOPMENT OF MUCOSAL DEFENCE

ANDREW S. NEISH

Department of Pathology and Laboratory Medicine,
Emory University School of Medicine, Atlanta, GA, USA

SUMMARY

The resident microbiota of the mammalian intestine influences diverse homeostatic functions of the gut, including regulation of cellular growth, restitution after injury, maintenance of barrier function, and modulation of immune responses. Normal acquisition of the microbiota in early infancy has inductive effects on these processes. However, it is unknown how commensal prokaryotic organisms mechanistically influence gut biology. We have shown that epithelia contacted by enteric commensal bacteria *in vitro* and *in vivo* rapidly generate reactive oxygen species (ROS), and distinct microbial taxa have markedly different potencies in stimulating this response. This physiologically generated ROS is known to participate in a variety of cellular signal pathways via the rapid and transient oxidative inactivation of a spectrum of regulatory enzymes. We show that these oxidant sensitive enzymes include key control points in the pro-inflammatory NF-κB pathway, regulation of cytoskeletal dynamics and activation of proliferative signals. Accordingly, we demonstrate various commensal bacteria have the ability to suppress inflammatory signalling and stimulate cell motility both in cell culture and in animal models. These events are consistent with known effects of the microbiota and selected probiotics. Collectively, our studies outline a molecular mechanism that may account for aspects of microbial-host cross-talk in the intestine in normal physiology and during therapeutic intervention with probiotics. These data illustrate that the normal flora, particularly in its initial acquisition in the neonatal period, can influence innate and structural defences and have consequences in adaptive immune development.

EUKARYOTIC/PROKARYOTIC INTERACTIONS IN THE GASTROINTESTINAL TRACT

Commensal host-microbe interactions have coevolved over millennia in many animals, with the human luminal ecosystem representing a highly medically relevant example (*Neish*, 2009). The vast majority of the human microbiota is represented by about 500 genera of bacteria, broadly grouped into two taxonomic divisions, the Bacteroidetes and Firmicutes. An accurate census of the microbiota is not practical by culture based microbiological techniques. However, recent high-throughput sequencing and molecular taxonomic

methodologies have greatly increased our understanding of the population composition, dynamics, and ecology of the gut microflora (reviewed in: *Hooper* and *Gordon*, 2001; *Xu* et al., 2007; *Dethlefsen* et al., 2007; *Gill* et al., 2006; *Backhed* et al., 2005). The gut is sterile *in utero* and is colonized immediately after birth, rapidly developing into a diverse and stable community, though marked variations in microbial composition between individuals is typical (*Eckburg* et al., 2005). Total numbers vary from 10^{11} cells/ gram luminal content in the ascending colon, 10^{7-8} in the distal ileum, and 10^{2-3} in proximal ileum and jejunum. Most members of the microbiota are autochthonous, meaning indigenous and stable, though allochthonous, or transient members are known (certainly most enteric pathogens fall into this category).

The microbiota is separated from the systemic compartment of the host by only a single layer of epithelial cells (or epithelial derived component, e.g. mucus layer). Impressively, epithelia and the complete mucosa perform vital fluid and nutrient absorptive functions, and must do so in presence of the microbiota and their products. Epithelial cells, by definition, act as interfaces between the host and the environment, and are equipped with apical surface specializations (microvilli, mucus production, vectorial ion secretion, intercellular junctions) to permit physiological function while contacting the microbiota -thus comprising a barrier. However, studies with germ-free mice have revealed that the microbiota is not functionally insulated from the mucosa, but in contrast, gut bacteria can fundamentally influence epithelial metabolism, proliferation and survival, and barrier function (*Ismail* and *Hooper*, 2005; *Madsen* et al., 2001; *Smith* et al., 2007; *Hooper* and *Gordon*, 2001; *Hooper* et al., 2001). For example, the small intestinal villi of the germ-free gut are elongated, while crypts are atrophic, show a slower turnover of the epithelial cells (*Pull* et al., 2005) and defective angiogenesis (*Stappenbeck* et al., 2002). Such mice mono-colonized with a single gut symbiont species (*Bacteriodes thetaiotaomicron*) exhibit robust host transcriptional responses, indicating that host perception of the microbiota occurs (*Hooper* et al., 2001).

Intestinal bacteria thrive in a stable, nutrient rich environment but also serve beneficial functions to the host including energy salvage of otherwise indigestible complex carbohydrates, vitamin and micronutrient syntheses, stimulation of immune development and competitive exclusion of pathogenic microorganisms (*Hooper* and *Gordon*, 2001; *Marchesi* and *Shanahan*, 2007). Thus there is a dynamic interaction between the microbiota and the host, where the epithelia form the major interface, allowing for the most part a mutually beneficial relationship. However, in other cases, the normal flora of the intestine may be sufficient to provoke intestinal inflammation, such as that seen in IBD [which includes Ulcerative colitis (UC) and Crohn's disease (CD)] (*Sartor*, 2008). There is much current interest in quantitative and/or qualitative abnormalities of the flora that may be associated with other systemic metabolic, infectious and particularly, immune and allergic disorders (*Wills-Karp* et al., 2001; *Noverr* and *Huffnagle*, 2004). The microbiota is clearly involved in the anatomic and functional development of mucosal immunity (*Slack* et al., 2009). Peyer's patches are grossly hypoplastic, and IgA responses are reduced in germ-free animals. It is also known that germ-free animals have reduced total CD4 T-cell populations and an inap-

propriate balance of T_H-cell subsets (*Macpherson* and *Harris*, 2004), which can be moderated within weeks upon colonization with a representative member of the normal flora (*Bacteroides fragilis*) (*Mazmanian* et al., 2005) via dendritic cell recognition of a specific polysaccharide (Polysaccharide A) component of *B. fragilis* (*Mazmanian* et al., 2008).

There is also increasing interest in potential therapeutic benefits of supplementing the normal flora with exogenous viable bacteria. This approach, termed probiotics, has been reported to dampen inflammation, improve barrier function, and augment adaptive immune processes and has shown promise as therapy in several inflammatory and developmental disorders of the intestinal tract (*Park* and *Floch*, 2007; *Hord*, 2008). Thus, there is increasing and compelling evidence that the gut flora beneficially affects intestinal -and systemic- homeostasis and thus health. However, little is known of how the host perceives non-pathogenic bacteria, or how the microbiota mechanistically influences gut biology.

PATTERN RECOGNITION RECEPTORS AND EPITHELIAL PERCEPTION OF BACTERIA

All eukaryotic cells have the ability to respond to and manage threats from bacterial pathogens -and by extrapolation, respond to and manage commensals. Transmembrane and intracytoplasmic receptors, such as the now well-studied Toll-like receptors and related Nod proteins, are designated "pattern recognition receptors" or PRRs. PRRs recognize and bind to conserved structural motifs present on the surface of a wide range of microbes, which are termed MAMPs, or "microbe associated molecular patterns". For example, TLR4 recognizes lipopolysaccharide and TLR2 binds specific peptidoglycans -both components of bacterial cell walls (*Sansonetti*, 2006). TLR5 detects the bacterial protein flagellin (*Zeng* et al., 2003). The now well known association of Crohn's disease with mutant forms of Nod2 clearly underscores the importance of PRR monitoring in intestinal health (*Sartor*, 2008).

PRRs are expressed in most cells; however, given the vast microflora, the dominant interaction of bacteria with host cells occurs in the intestine, especially the epithelia. PRRs and their downstream signalling pathways, such as the MAPK and NF-κB systems, have an ancient lineage, exhibiting impressive structural and functional homology even at the level of invertebrates and plants. These systems represent entwined cytoplasmic information relays, which when activated employ rapid post translational events (covalent protein modifications and regulated protein degradation) to transduce PRR binding into well defined inflammatory and apoptotic tissue responses that evolved to eliminate pathogenic threats (*Neish*, 2009; *Sansonetti*, 2004; *Abreu* et al., 2005). However, while PRR mediated signalling clearly has a central and dominant role in initiating cellular inflammation during infection, it is now also apparent that basal tonic TLR (and possibly other PRR) mediated signalling in response to the normal flora and their products is necessary for mucosal health. Murine models with defective PRR signalling are hypersensitive to a variety of intestinal insults and stressors, and supplementation of TLR ligands such as CpG DNA and

flagellin can have cytoprotective effects (*Rakoff-Nahoum* et al., 2004; *Burdelya* et al., 2008). Regenerative responses to colonic injury are markedly attenuated in germ-free animals, indicating a discernable role of the flora in stimulation of epithelial proliferation and response to injury, and restitution is reduced in MyD88 (a signalling intermediate required by multiple TLRs) null mice, reinforcing the notion that PRR mediated signalling is necessary for trophic/restitutive effects (*Pull* et al., 2005). These and related observations with mice null in epithelial NF-κB pathway components (*Zaph* et al., 2007; *Nenci* et al., 2007; *Ben-Neriah* and *Schmidt-Supprian*, 2007; *Chen* et al., 2003) support the hypothesis that a constitutive degree of PRR signalling is necessary for normal gut homeostasis, presumably because of the tonic upregulation of cytoprotective genes in either epithelial cells or lamina propria macrophages (gene products with anti-apoptotic, chaperone/stress response, and antioxidant effects) (*Zaph* et al., 2007) and underscores the importance of gut-prokaryotic interaction as a beneficial and necessary relationship.

FORMYLATED PEPTIDE RECEPTORS

Another type of PRRs are the formylated peptide receptors (FPR). Classically, the FPRs are seven membrane pass, G-protein linked surface receptors expressed on neutrophils and macrophages, where they perceive bacterial cell wall products and stimulate phagocyte function (*Migeotte* et al., 2006). The best characterized ligands are formylated peptides, which are modified prokaryotic translation products tagged with a bacterial specific amino acid N-formyl-methionyl-leucyl-phenylalanine (fMLP). Upon ligand recognition in phagocytes, the FPR receptors undergo a conformation change that allows binding of pertussis toxin sensitive G proteins of the Gi family. Subsequent signalling trifurcates to PI3K MAPK signalling pathways, calcium release, and GTPase activation which eventuate in:

1) changes in actin dynamics and initiation of chemotaxis,
2) transcriptional upregulation of inflammatory effectors and cytokines, and
3) the activation of NADPH dependant oxidase enzymes and ROS generation (respiratory burst).

Thus, the FPRs are a key PRR that controls the biological response of professional phagocytes to bacterial ligands.

The formylated peptide receptors are represented in humans by the originally characterized FPR and the closely related FPRL1 and FPRL2. FPR has been characterized as high affinity with an ED_{50} for fMLP in the nanomolar range, while the low affinity FPRL1/FPRL2 responds to the same agonist at micromolar ranges (*Le* et al., 2002). Importantly, immunohistochemical staining has shown the formylated peptide receptors are expressed on the apical surface of the intestinal epithelia, prompting interest that this and related epithelial receptors may mediate physiological responses in the gut (*Babbin* et al., 2007). We have found that live commensal contact mediated activation of the ERK MAPK signalling pathway in gut epithelial cells *in vitro* and *in vivo*. A range of commensal bacteria tested potently induced ERK phosphorylation without stimulating pro-inflammatory phospho-IκB or phospho-JNK. Interestingly, this pattern of signalling activation was

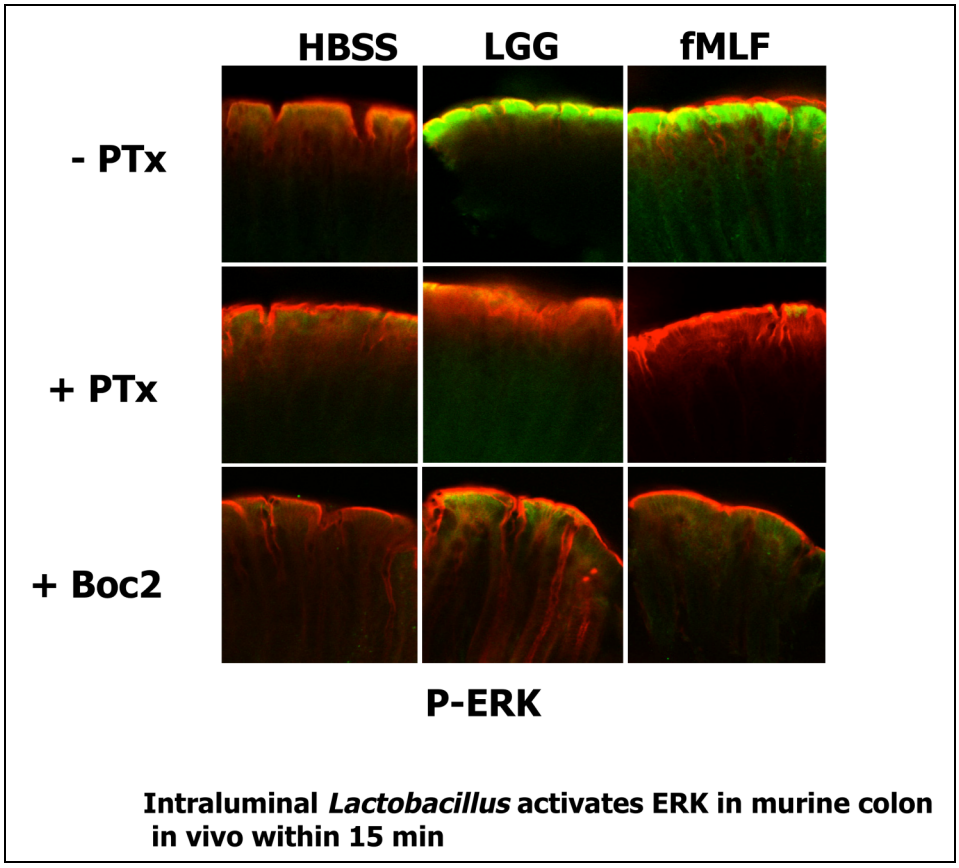

Figure 1: Commensal bacteria activate ERK MAPK *in vivo*. Immunostaining of murine intestine stimulated with addition of commensal bacteria by intra rectal instillation. tBOC and PTX and extracellular and intracellular inhibitors of FPR signaling, respectively. Activated ERK represents an example of non-inflammatory signaling stimulated by commensal bacteria.

recapitulated using the peptide, N-formyl-Met-Leu-Phe (fMLF), consistent with a role for formyl peptide receptors in activation. In addition, pretreatment of model epithelia and murine colon with Boc2 (a specific peptide antagonist) or pertussis toxin (a G_i-protein inhibitor) abolished commensal-mediated ERK phosphorylation (Figure 1). Together, these data show that commensal bacteria specifically activate the ERK MAPK pathway in an FPR-dependent manner, delineating a mechanism by which commensal bacteria contribute to cellular signalling in gut epithelia.

PHYSIOLOGICAL GENERATION OF REACTIVE OXYGEN SPECIES

The rapid generation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H_2O_2), hydroxyl radicals and a variety of their degradation products are a result of excitation or incomplete reduction of molecular oxygen. ROS are short-lived reactive molecules and at high levels are considered potently microbiocidal, necessary for the killing of engulfed organ-

isms. ROS production in response to FPR stimulation is a cardinal feature of the cellular response of phagocytes to both pathogenic and symbiotic bacteria. Phagocytes generate ROS via a very well studied enzymatic apparatus. The neutrophil NADPH oxidase, Nox2 (formerly gp120phox), is a constitutively inactive multi-subunit complex comprised of a membrane bound dimer of p22phox and gp91phox (*Lambeth*, 2004). The *in vivo* role of this enzyme in host defence is vividly illustrated by the fact that the genetic absence of Nox2 function results in chronic granulomatous disease (CGD), a condition where phagocytes fail to induce ROS and patients are predisposed to recurrent pyogenic infections. Invertebrate phagocytes stimulated by formylated peptides generate ROS (MAMPs) in the same manner as mammalian neutrophils, and plants also utilize induced ROS in response to bacterial pathogens and symbionts, continuing the theme of conversion of basic machinery of microbial perception and effector pathways (*Kotchoni* and *Gachomo*, 2006; *Pauly* et al., 2006; *Tanaka* et al., 2006; *Schneeweiss* and *Renwrantz*, 1993; *Lambeth*, 2004). Drosophila requires commensal microbe-induced hydrogen peroxide (H_2O_2) to maintain gut epithelial homeostasis (*Ha* et al., 2005a,b; *Pull* et al., 2005; *Abreu* et al., 2005). However, in the case of the fly, the ROS generation occurs in the epithelia, and is necessary for control of the luminal flora. This latter observation suggests a conserved role for epithelial ROS (as opposed to strictly phagocyte) generation in gut homeostasis and microbial control. Additionally, it is now apparent that the ROS generating enzymes activated by FPRs in neutrophils (Nox2) have functional paralogous enzymatic complexes in non-phagocytic cells (*Lambeth*, 2004). Indeed, a family of NADPH oxidase enzymes, the Nox's and Duox's is seen in many non-phagocytic tissues, with two, Nox1 and Duox2, strongly expressed in the intestinal epithelia (the inducible ROS observed in Drosophila intestine is produced by the fly ortholog of Duox). In general, the non-phagocytic NADPH oxidases exhibit similar, but not identical organization to the phagocyte enzyme.

Recently, we have shown that several species of normal human gut bacteria can induce rapid, "deliberate" generation of ROS within epithelial cells (*Kumar* et alo., 2007). Furthermore, these cells immediately show increased oxidation of soluble redox sinks, such as glutathione and thioredoxin, and exhibit an increase in redox stimulated transcriptional activation, both reflecting a cellular reaction to increased ROS. Interestingly, different strains of commensal bacteria can elect marked differences in ROS levels in contacted cells. We have found that the Lactobacilli are especially potent in ROS production in cultured cells and *in vivo*, though all bacterial tested have some ability to alter the redox environment of the cell. This is not surprising given that phagocytes can induce a respiratory burst regardless of whether they encounter nominal pathogens or stray commensals. As mentioned, Nox enzymes play a central role in ROS generation in phagocytes; whether the Nox's or Duox's are involved in the generation of ROS in mammalian epithelia or if this ROS also has microbiostatic functions is not known.

High ROS stimulating bacteria, such as Lactobacilli, may possess specific membrane components or even secreted factors that activate cellular ROS production. For instance Yan reported soluble factors of Lactobacilli that mediated beneficial effects in *in*

vivo inflammatory models (*Yan* et al., 2007). Alternatively, high ROS stimulating bacteria may simply possess enhanced adhesion or ability to penetrate mucin layers and gain more proximal access to cellular receptors such as TLRs and FPRs. As the FPRs are expressed on apical surfaces and are known to directly stimuli ROS production in phagocytes, these are interesting candidates for this function. Alternative possibilities include endogenous production of ROS from prokaryotic enzymes, though experiments showing potent ROS stimulation with non-viable and denatured bacterial components make this notion less likely. Additional sources of cellular ROS generation could include 5-lipoxygenase, xanthine oxidase and mitochondrial respiratory chain enzymes. Clearly, bacteria, unlike individual peptides and cytokines, are multifaceted biological stimuli and clearly would be expected to elicit a complex range of cellular receptors and influence diverse processes.

ROS MEDIATED SIGNALLING

ROS also have functions beyond microbial killing. Controlled generation of ROS by activation of receptors for various hormones, cytokines and growth factors mediate critical roles in the modulation of signal transduction pathways seen in all multi-cellular life, plants and animals alike (*Terada*, 2006; *Ogier-Denis* et al., 2008; *Kotchoni* and *Gachomo*, 2006; *Pauly* et al., 2006; *Tanaka* et al., 2006). The specificity of biological responses to altered levels of ROS can be modulated by the specific molecular species of ROS, the intensity/duration of the signal, the subcellular sites of production and the developmental stage of the cell (*Terada*, 2006; *Ogier-Denis* et al., 2008). ROS are short-lived molecules and can have a very small functional radius of action, which contributes to the selectivity of action. Indeed certain receptors physically interact with a ROS generating Nox enzyme, presumably to limit ROS mediated influences to the immediate vicinity of effector proteins (*Karrasch* et al., 2007).

A major mechanism by which ROS are thought to exert their effects on signal transduction pathways is by their ability to reversibly oxidize cysteine residues in specific target proteins (*Barford*, 2004). Only a subset of proteins can be modified by this reaction as oxidation of cysteine requires this amino acid to be present in the thiolate anion form (Cys-S-), whereas most cysteines (pK_a~8.5) are protonated (Cys-SH) at physiological pH. Only some cysteine residues exist as a thiolate anion at neutral pH as result of lowering of their pKa value by vicinal charged amino acids (*Rhee* et al., 2005). Specific examples of such oxidant sensitive proteins include protein tyrosine phosphatases (PTPs), the lipid phosphatase (PTEN), MAP kinase phosphatases (MAPK-P or DUSPs), and low-molecular-weight protein tyrosine phosphatases (LMW-PTPs) (*Tonks*, 2005; *Kamata* et al., 2005; *Chiarugi* and *Buricchi*, 2007). More recently examples of ROS mediated inactivation of enzymes have come from studies by Bossis and Melchior (*Bossis* and *Melchior*, 2006) and from our own laboratory (*Kumar* et al., 2007) with the sumoylation and the neddylation enzymes, respectively. Sumoylation and neddylation are the conjugation of ubiquitin-like proteins, Sumo or Nedd8, to target lysine resi-

dues of substrate proteins. The latter, Nedd8, plays a role in the control of the key inflammatory transcription factor, NF-κB, as is discussed next.

MICROBIAL EFFECTS ON INFLAMMATORY SIGNALLING

While it is obvious that the host must defend against threats posed by bacterial pathogens, the benefits conferred by the microbiota require that immune and inflammatory systems not eliminate them entirely. The epithelia can suppress TLR signalling or reduce TLR expression to moderate immuno-inflammatory signaling (*Sansonetti*, 2006; *Abreu* et al., 2005). Additionally, individual members of the microbiota are able to actively modulate signalling intensity (*Kelly* et al., 2005; *Iyer* et al., 2008; *Neish*, 2003). A variety of reports have described commensals -many employed as probiotics- are able to suppress eukaryotic inflammatory signalling pathways such as NF-κB and block inflammatory effector functions (*Yan* et al., 2007; *Menard* et al., 2004; *Pena* and *Versalovic*, 2003; *Madsen* et al., 1999). Several mechanisms have been described. The gut symbiont *Bacteroides thetaiotaomicron* has been elegantly shown to inhibit NF-κB pathways by regulating cytoplasmic to nuclear translocation of the p65 NF-κB subunit (*Kelly* et al., 2004). Several laboratories have demonstrated that intestinal bacteria are able to influence inflammatory pathways, and very likely other cellular regulatory processes, by manipulating the ubiquitin system (*Neish* et al., 2000; *Tien* et al., 2006; *Petrof* et al., 2004; *Iyer* et al., 2008). Ubiquitination is a covalent modification increasingly recognized to play a regulatory role in a wide spectrum of biochemical events, generally by targeting modified proteins for controlled degradation via the proteasome organelle. An example of a signalling component regulated by ubiquitination is the inhibitory component of the NF-κB pathway, IκB (*Karin* and *Ben-Neriah*, 2000), and there are numerous examples of pathogens that utilize preformed effector proteins to influence IκB ubiquitination and thus innate immunity (*Kim* et al., 2005; *Angot* et al., 2007; *Rytkonen* and *Holden*, 2007). Members of the microbiota interacting with epithelial cells *in vitro* are capable of blocking IκB ubiquitination and thus NF-κB activation by interference with the function of the IκB ubiquitination ligase, $SCF^{\beta TrCP}$(Skp1, Cdc53/Cullin, F box receptor) (Neish et al., 2000; *Collier-Hyams* et al., 2005; *Lee*, 2008). This enzymatic complex is activated by a second covalent modification, neddylation, on the regulatory subunit of the complex, cullin-1. Neddylation is the covalent modification of the SCF ubiquitin ligases by the ubiquitin-like protein Nedd8. The event is emerging as a central regulatory event in cellular processes that are controlled by protein degradation, including NF-κB and β-catenin. Neddylation occurs by an enzymatic series analogous to the ubiquitination reaction, specifically catalyzed by a Nedd8 ligase called Ubc12. We have shown that contact of commensal bacteria with epithelia *in vitro* and *in vivo* resulted in the rapid and reversible loss of the Nedd8 modification, accounting for the loss of overall SCF ubiquitin ligase function and consequent blockade of NF-κB activation (*Collier-Hyams* et al., 2005). Prompted by observations that other enzymes involved in modification of regulatory proteins by ubiquitin-like enzymes (the SUMOylation process) were controlled by transient oxidative inactivation, we

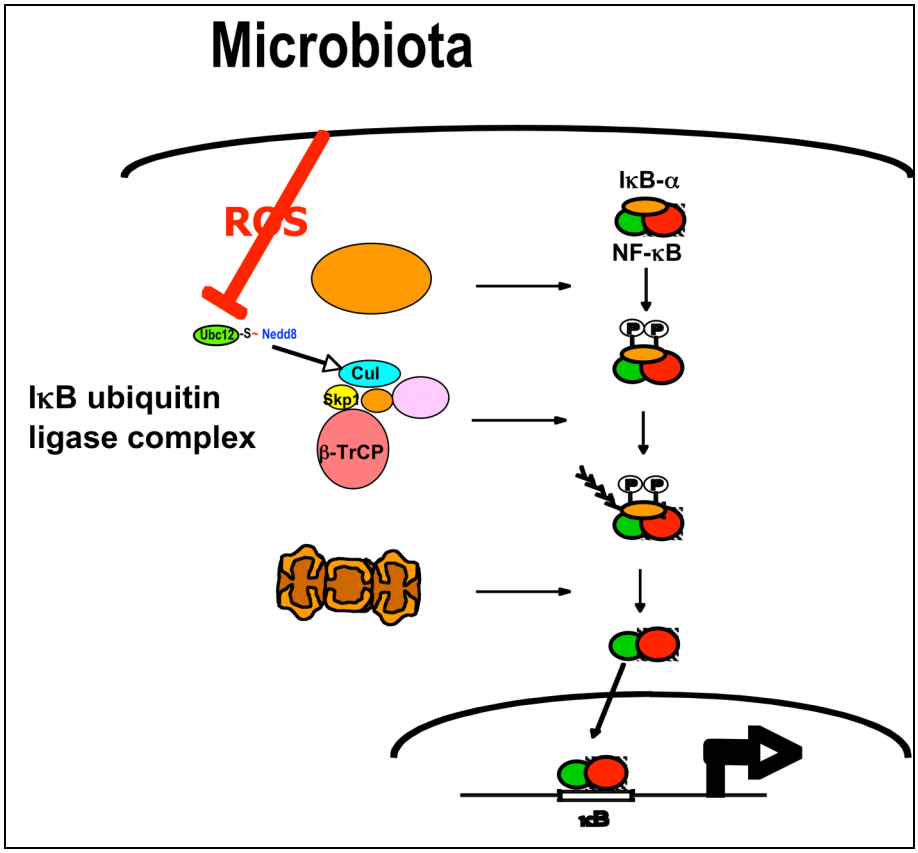

Figure 2: Diagram of the NF-κB pathway. NF-κB is activated by sequential modifications of IκB; phosphorylation (by IKK), ubiquitination (by the SCF complex) and degradation (by the proteasome). Free NF-κB dimer can then translocate to the nucleus and activate transcription. The SCF ubiquitin ligase (cullin subunit) must be modified by the ubiquitin-like protein Nedd8 for activity, and the neddylation reaction is mediated by the oxidant sensitive ligase Ubc12. Intracellular ROS from bacterial contact transiently inactivates Ubc12 and thus blocks activity of downstream functions, including IκB ubiquitination/degradation and NF-κB mediated signaling.

investigated if the neddylation reaction was influenced by oxidative signalling. We demonstrated that both endogenous ROS (H_2O_2) and ROS generation by bacterial contact was able to transiently inactive the Nedd8 ligase, Ubc12 (*Kumar* et al., 2007). These results demonstrated that commensal bacteria directly modulate a critical control point of the ubiquitin-proteasome system and is the first example of a eukaryotic signalling pathway influenced via bacterially stimulated ROS, and furthermore provides a detailed molecular mechanism for bacterial suppression of a key host inflammatory pathway (Figure 2). When considering the defences of the immature intestine, one must bear in mind that the gut is totally naive to bacteria and their products while *in utero*, and is instantly challenged by their presence at birth with the introduction of the normal flora. Potentially, an immature microbiota may be inadequate to modulate innate immune pathways with consequences on downstream events, including contribution to adaptive immunity.

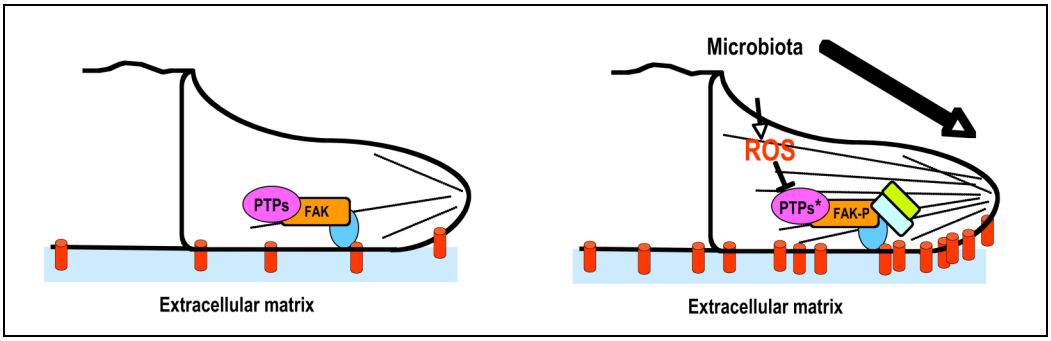

Figure 3: Diagram of the epithelial motility. In a resting state, oxidant sensitive protein tyrosine phosphatases (PTPases including LMW-PTPase) maintains focal adhesion kinase FAK in a dephosphorylated inactive state. Intracellular ROS from bacterial contact transiently inactivates PTPases and permits the autophosphorylation of FAK. Activated FAK acts as a nidus, recruiting other regulatory proteins and stimulating assembly of the actin cytoskeleton, eventuating in initiation of cellular movement.

MICROBIAL EFFECTS ON EPITHELIAL CELL FUNCTION, GROWTH AND SURVIVAL

As previously discussed, germ-free mice show defective epithelial proliferation and wound healing, indicating that commensal enteric bacteria are able to stimulate epithelial cell migration post-injury and during development, suggesting a mechanism by which the microflora could affect physical epithelia defences, such as barrier function. The single cell layer epithelium reconstitutes itself every 5 days from the crypt stem cell pool. Maintenance of this intestinal epithelial lining requires tight regulation of cell proliferation and migration. Epithelial cell migration depends on coordinated changes in actin cytoskeleton involving spatial and temporal changes in adhesion of the protruding membrane edge to the cell extracellular matrix at specialized signalling nidus points called focal adhesions (FA). FA assembly is regulated by focal adhesion kinase, a 125 kDa protein that is maintained in an inactive dephosphorylated form by the constitutive action of redox sensitive tyrosine phosphatases, LMW-PTPase and SHP-2 (*Mitra* et al., 2005). Past reports have shown that endogenous physiological stimuli, such as growth factors and integrin engagement with the epithelial basement membrane induced local ROS production via activation Nox1, resulting in rapid oxidative inactivation of these PTPase's, and consequent phosphorylation of FAK and initiation of cellular motility (*Chiarugi* et al., 2003). Accordingly, we have shown that interaction(s) of wounded intestinal epithelia with natural commensal bacterial strains is associated with rapid accumulation of ROS, especially at the leading edge of the migrating monolayer. Elicitation of ROS results in reversible oxidation of target low pKa cysteines in LMW-PTP and SHP-2, and thereby a consequent increase in phosphorylation of focal adhesion kinase (FAK). Concomitantly, commensal bacteria mediate an increase in number of FA at the migrating edge of the monolayer, and increased cell adhesion and velocity of epithelial migration (Figure 3). Functionally, commensal bacteria mediate enhanced wound closure in an *in vitro*

model of injury and enhanced resolution of dextran sodium sulfate-induced mucosal damage in a mouse model. Thus ROS production associated with commensal-epithelial contact can stimulate epithelial motility and likely contribute to epithelial barrier function. This data suggests another means for how the microbiota mediates physical defences in the gut.

Finally, the DUSPs are redox sensitive PTPases that serve as negative regulators of various MAPKinases, including ERK. Plausibly, FPR dependant activation of the ERK MAP-Kinase pathways may also be regulated by microbial induced redox events inactivating DUSPs. Experiments to address this hypothesis are in progress.

DISCUSSION

We have shown that epithelia exhibit increased ROS generation in response to commensal bacteria, in a manner similar to the events induced in phagocytic cells, suggesting a deep functional conservation. Indeed, recent data in invertebrates suggest that ROS generation for signalling and microbiocidal functions in the gut epithelia may represent the ancestral form of response to bacteria (*Ha* et al., 2005). We have shown ROS generated in epithelial in response to bacteria serves a signalling function (as in many non epithelial cells), and likely there are numerous ROS sensitive enzymes that could be influenced by changes in cellular redox status. As mentioned, reversible oxidative inactivation of a wide range of regulatory enzymes is an increasingly recognized mechanism of signal transduction (*Terada*, 2006; *Chiarugi* and *Buricchi*, 2007). Current protoeomic approaches that exploit reactive cysteines to label individual peptides may be employed as a high throughput system to screen for oxidant sensitive regulatory proteins (*Sethuraman* et al., 2004). Alternatively (but not contradictory), an epithelial antimicrobial function (as in phagocytes and the Drosophila gut) of bacterial elicited ROS, especially in limited locations such as the intestinal crypt is also plausible, and are questions to be resolved.

The source of ROS is an intriguing topic. Clearly the Nox enzymes, especially Nox1 and Duox2 are prime candidates given their pattern of tissue expression, but other sources such as mitochondria respiration chain enzymes, lipoxigenases and others could contribute to redox control in the cell. FPRs are attractive candidates for receptor stimulated ROS production, given that many of the same mechanisms that mediate FPR signalling in professional phagocytes are conserved in epithelial cells. Additionally, it is also unclear whether certain commensals could generate ROS by their own enzymatic machinery and influence eukaryotic signalling by exogenous ROS (conversely, some bacteria could achieve this result by producing anti-oxidants).

ROS mediated signalling may occur during rapid quantitative changes in microbial populations or qualitative changes in the composition in the gut, during development, or with probiotic therapy. The observation that different taxa of bacteria exhibit markedly different potencies in the ability to elicit/provide ROS supports the idea that qualitative changes in community composition can affect host biology. This notion may be relevant to the development and optimization of probiotics, and may explain a parameter that defines a healthy vs. “dysbiotic” mi-

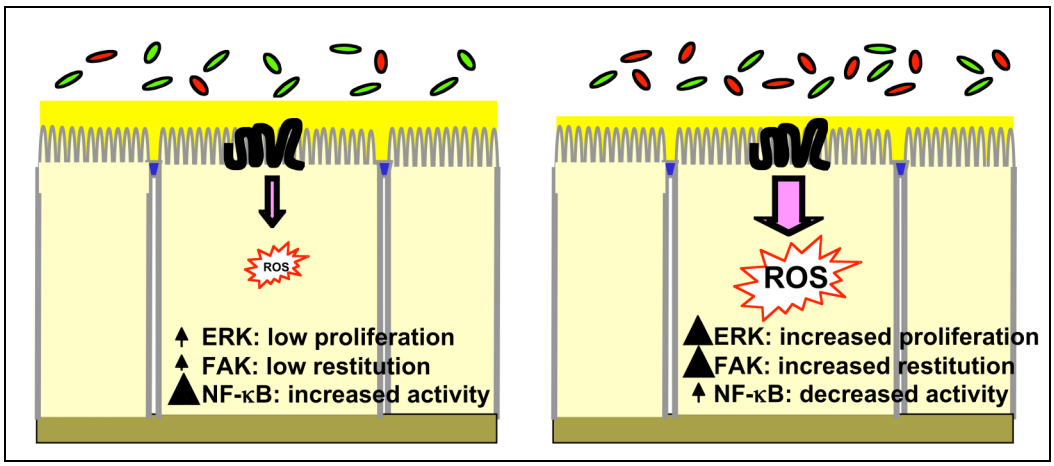

Figure 4: Possible scheme for ROS signaling in the gut. In conditions with low ROS generation, whether decreased total bacterial numbers or relative scarcity of high ROS stimulating bacteria, the NF-κB system is fully active while FAK dependant motility and ERK signaling pathway is relatively inactive. With increasing ROS signaling, inactivation of relevant enzymes leads to suppression of NF-κB and augmentation of motility and ERK. The long term consequences of these events are unknown, and clearly, other ROS sensitive enzymes could be influenced by ROS.

crobiota. Long-term biochemical accommodation to tonic bacterial presence, as in the colon, may affect different aspects of redox biology.

In conclusion, cellular ROS by microbe-epithelial contact is a conserved processes with many known, expected and plausible consequences, making this mechanism attractive as a general and non-species selective means by which a complex floral community could influence a wide range of host signalling and homeostatic processes (*Lee*, 2008). It is hoped that a fuller understanding of this mechanism may advance our understanding of the natural microbiota and exploitation of probiotic organisms.

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GEOHELMINTH INFECTIONS MAY HAVE DELETERIOUS EFFECTS ON IMMUNITY TO ORAL VACCINES

PHILIP J. COOPER

Liverpool School of Tropical Medicine, Liverpool, United Kingdom, and Colegio de Ciencias de la Salud, Universidad San Francisco de Quito, Quito, Ecuador

SUMMARY

There is compelling evidence that immune responses to mucosal vaccines are impaired in non-affluent populations living in the Tropics and enteric co-infections such as geohelminths may contribute to this effect. Geohelminths have been associated with impaired immune responses to the live attenuated oral cholera vaccine CVD 103-HgR and treatment for geohelminths prior to vaccination partially reversed the impaired immune responses. Other factors such as host nutrition and the presence of environmental enteropathy with which geohelminth infections are associated are likely to contribute also to this tropical barrier to mucosal immunization. There is a need for research on the mechanisms by which geohelminths may suppress immunity to mucosal vaccines and such research could contribute to the development of more effective mucosal vaccines.

INTRODUCTION

The geohelminth (also known as intestinal or soil-transmitted helminth infections) parasites, *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm, and *Stronglyoides stercoralis*, are common infectious diseases of childhood in tropical regions, particularly among populations living in poverty with poor access to sanitation and clean water. In endemic areas, geohelminth infections are chronic infections and individuals generally become infected during the second year of life and remain infected into adulthood through repeated infectious exposures. An estimated 2 billion humans are infected with geohelminths worldwide (*Savioli* et al., 2005). Infections are considered to cause significant morbidity particularly among pre-school and school-age children in whom infections are associated with adverse effects on nutrition, growth, and cognition (*Bethony* et al., 2006). The level of morbidity caused by geohelminth infections is strongly associated with parasite burden (*Anderson* and *May,* 1985) that is greatest among children.

Geohelminth infections induce an immune responses in humans characterized by elevated IgE and eosinophilia and the production of Th2 cytokines by peripheral blood leukocytes (PBLs) when stimulated with parasite antigen *in vitro* (*Cooper* et al., 2000a; *Cooper* et al., 2008). Chronic infections are associated with a tightly regulated inflammatory response in which anti-

parasite allergic reactions appear to be suppressed (*Maizels* and *Yazdanbakhsh*, 2003; *Cooper*, 2009a). Such a response reflects a stated of balanced parasitism allowing the parasite to survive but protecting the host from potentially damaging immunopathology.

There is evidence that the regulation of host immunity by chronic geohelminth infections may affect responses not just to parasite antigens but also other exogenous antigens such as the antigenic constituents of vaccines (*Malhotra* et al., 1999; *Cooper* et al., 2001; *Elias* et al., 2001). Because many mucosal vaccines are poorly immunogenic among poor populations living in the Tropics, an observation that has been referred to as a mucosal barrier to vaccination in such populations (*Czerkinsky* and *Holmgren*, 2009), there is growing awareness of how enteric parasites such as geohelminths may contribute to such an effect through their effects on the intestinal mucosa and mucosal immunity (*Czerkinsky* and *Holmgren*, 2009; *Cooper*, 2009b).

STUDIES OF EFFECTS OF GEOHELMINTH INFECTIONS ON MUCOSAL IMMUNITY IN CHILDREN

Geohelminth parasites have intimate contact with the mucosal immune system being separated from the intestinal tissues by a single layer of epithelium. Although there are extensive data available from experimental animals of the mucosal immune response to intestinal helminth infections, such data from human populations are limited. This is because of difficulties in accessing mucosal tissues in humans although useful data can be obtained by collection of mucosal secretions (e.g. faeces and saliva) and peripheral blood for sampling of B and T cells that traffic between mucosal sites after mucosal vaccination (*Lewis* et al., 1991; *Castello-Branco* et al., 1994; *Wasserman* et al., 1994). Developments such as wireless endoscopy will allow the easier sampling of intestinal mucosa in future studies although such technology is rarely available to researchers working in populations where geohelminth infections are present.

CHANGES IN THE INTESTINAL MUCOSA ASSOCIATED WITH GEOHELMINTH INFECTIONS

The expulsion of intestinal helminth parasites in animal models has been associated with marked changes in the intestinal mucosa characterized by villous atrophy, crypt hypertrophy, and increases in mucous-secreting goblet cells (*Finkelman* et al., 1997; *Anthony* et al., 2007). The intestinal epithelium proliferates so that parasites that live partly or completely in the epithelium (e.g. *Trichinella spiralis* and *Trichuris* spp.) are shed into the gut - the so-called epithelial escalator (*Artis* and *Grencis*, 2008). Such alterations make the intestinal lumen a hostile environment and reduce the surface area for parasite attachment. Both parasite expulsion and intestinal enteropathy are considered to be Th2-dependent processes (*Garside* et al., 2000; *Anthony* et al., 2007).

There are limited data from humans on the histological changes in the intestine associated with geohelminth

infections. Geohelminth parasites that dwell in the small intestine, *A. lumbricoides*, hookworm, and *S. stercoralis*, have been associated with enteropathy although generally the mucosa appears histologically normal (*Arean* and *Crandall*, 1971; *Burman* et al., 1970; *O'Brien*, 1975; *Garcia* et al., 1977) in individuals living in endemic areas. A minority with chronic infections show changes of partial villous atrophy, crypt hyperplasia and increased inflammatory infiltrate in the lamina propria (*Burman* et al., 1970). Humans infected experimentally with hookworm larvae develop eosinophilic enteritis (*Croese* et al., 2006), although this inflammation tends to largely resolve after repeated infections (*Croese* and *Speare*, 2006). *T. trichiura* that inhabits the large intestine has been more extensively studied because of the ease of sampling particularly of the rectal mucosa. Such infections may occasionally cause a dysentery-like syndrome (*Trichuris* dysentery syndrome [TDS]) (*Cooper* et al., 1991) associated with an increase in inflammatory cells in the lamina propria (*MacDonald* et al., 1991), and an increase in numbers and state of activation of mucosal mast cells (*Cooper* et al., 1991; *MacDonald* et al., 1994). The histological picture observed is likely to be determined by chronicity of infection, intensity of infections, and host genetic factors. Chronic infections may be associated with minimal inflammatory response in the mucosa and mild histologic alterations (e.g. partial villus atrophy) reflecting active immune regulation by host and or parasite. Chronic infections in a few individuals may be associated with severe inflammation (e.g. TDS) but most children are likely to be asymptomatic. Chronic infections down-regulate inflammatory responses in the intestinal mucosa to avoid the long-term consequences of an inflamed intestinal mucosa on host nutrition. During initial infections, benefit to the host may be obtained by mounting strong inflammatory responses to expel parasites. The findings of partial villus atrophy and crypt hypertrophy in the small intestine (*Keusch* et al., 1972; *Gracey*, 1979; *Fagundes-Neto* et al., 1984; *Haghighi* and *Wolf*, 1997; *Veitch* et al., 2001) and a non-specific inflammatory infiltrate in the small and large intestine (*Mathan* and *Mathan,* 1985) has been referred to as tropical or environmental enteropathy/colonopathy. Tropical enteropathy is a common histologic finding in apparently healthy individuals living in the Tropics (*Humphrey*, 2009) and may reflect a T-cell mediated inflammatory process (*Veitch* et al., 2001) to intestinal microbiota and pathogens such as geohelminths.

EFFECTS OF GEOHELMINTHS ON MUCOSAL VACCINATION

Current mucosal vaccines are designed to stimulate immune cells in the intestinal tract to induce both mucosal and systemic immunity. The most widely used are trivalent oral poliovirus (OPV) and oral rotavirus vaccines, both of which are live attenuated vaccines. There are several new oral vaccines under development, some of which may become available for widespread use during the next decade.

Several oral vaccines have been shown to be less immunogenic in populations in non-affluent compared to affluent regions including trivalent oral poliovirus vaccine, rotavirus vaccines (Rotashield, Rotarix, and RIT 4237 bovine vaccines), oral cholera

Table 1: Barriers to effective vaccination with oral vaccines in non-affluent populations living in the Tropics. Other factors include high cost and logistic considerations such as cold-chain and vaccine distribution and delivery systems.

- Nutritional deficiencies
  - Vitamin A
  - Zinc
- Tropical/environmental enteropathy
- Chronic diarrhoea
- Co-infections
  - Enteric bacterial infections
  - Intestinal protozoa (e.g. *Giardia intestinalis*)
  - Intestinal helminths
    - *Ascaris lumbricoides*
    - Hookworm
    - *Strongyloides stercoralis*
    - *Trichuris trichiura*
- Microbiota
- Previous exposures to natural infections (e.g. intestinal sIgA)
- Maternal antibodies in breast milk

vaccine (CVD-103HgR), and *Shigella flexneri* 2a SC602 vaccine (*Czerkinsky* and *Holmgren*, 2009). Effective vaccine immunity with such vaccines in non-affluent populations has required an increase in the dose or number of doses administered to achieve adequate vaccine immunity (*Patriarca* et al., 1991, *Perez-Schael* et al., 1997).

Geohelminth infections may have deleterious effects on immunity to oral vaccines. Children infected with geohelminths had reduced vibriocidal antibody levels (*Cooper* et al., 2000b) and IL-2 responses to cholera toxin B–subunit (*Cooper* et al., 2001) following vaccination with a single dose of live attenuated oral cholera vaccine (CVD 103-HgR), and these deficits were reversed partially by anthelmintic treatment before vaccination. Similarly, *Heligmosoisdes polygyrus* a natural and chronic infection of the mouse small intestine, was associated with impaired IFN-γ production to OVA following vaccination with a novel OVA-expressing Salmonella vaccine (*Urban* et al., 2007).

However, geohelminth infections alone are unlikely to explain impaired immunity to oral vaccines. A study investigating the impact of *A. lumbricoides* infection on responses to oral BCG Moreau, failed to demonstrate post-vaccination increases in the frequencies of tuberculin-stimulated PBMCs expressing IFN-γ among children with either active infections or those who had received either short or long courses of anthelmintics before vaccination (*Cooper* et al., unpublished data). The same vaccine showed strong boosting of post-vaccination IFN-γ responses in healthy UK adults (*Cosgrove* et al., 2006). These data indicate the presence of a mucosal barrier to

oral vaccination among children living in the rural Tropics that is present in the absence of geohelminth infections. Factors that may contribute to poor vaccine immune responses in populations living in non-affluent regions are listed in Table 1.

An important issue for evaluating the potential effects of enteric infections such as geohelminths on immune responses to oral vaccines is the age of acquisition of infection. Geohelminth infections, in most endemic settings, are acquired towards the end of the first year of life, and are unlikely to affect immune responses to vaccines given during the first 6 months of life (e.g. oral poliovirus and rotavirus vaccines). Geohelminth infections may have significant effects on oral vaccines given to children of pre-school or school age. However, there is evidence that maternal infections with geohelminths may modify the infant immune response (*Malhotra* et al., 1999; *Pit* et al., 2000; *Elliott* et al., 2005; *Guadalupe* et al., 2009) and such effects have been associated with impaired immunity to parenteral vaccines given during the first 6 months of life such as BCG (*Malhotra* et al., 1999), *Haemophilus influenzae* type B (*Labeaud* et al., 2009), and tetanus toxoid (*Cooper* et al., unpublished data). The extent to which effects of maternal geohelminth infections could contribute to impaired mucosal immune responses in infants is not known but is being investigated in birth cohorts being conducted in populations endemic for these parasites.

MECHANISMS OF MODULATION OF MUCOSAL IMMUNE RESPONSES BY GEOHELMINTHS

The limited inflammatory response observed in the intestinal mucosal in the presence of chronic geohelminth infections is likely to reflect potent immune regulation. The mechanisms by which such infections modulate mucosal immunity are not well understood. Findings from experimental murine models show that intestinal helminth infections suppress dendritic cell-responses to TLR ligands (*Balic* et al., 2004; *Segura* et al., 2007) and the production of IL-12 (*Balic* et al., 2004; *Cervi* et al., 2004), and induce the development of alternatively activated macrophages (*Kreider* et al., 2007) and IL-10-producing immune cells. Several studies have pointed to a central role for IL-10 in suppressing systemic inflammation associated with human helminth infections (*Fallon* and *Mangan*, 2007). Peripheral blood leukocytes from infected individuals produce elevated levels spontaneously of IL-10 (*Turner* et al., 2008; *Figueiredo* et al., 2010) and TGF-β (*Turner* et al., 2008). CTLA-4 is more highly expressed during chronic helminth infections (*Steel* and *Nutman*, 2003). Co-culture of peripheral blood leukocytes (PBLs) with hookworm antigen impaired PBL proliferation and cytokine production (*Geiger* et al., 2007) while dendritic cells show lower expression of CD86, CD1a, HLA-ABC, and HLA-DR and have a reduced capacity to promote cell proliferation (*Fujiwara* et al., 2009). Similarly, the co-culture of PBLs with parasite antigen has been shown to increased the expression of regulatory (e.g. CTLA-4, TGF-β, PD-1, and ICOS) and anergy-associated markers (e.g. cbl, Itch, and Nedd4), an effect that can be reversed at least partially by neutralization of CTLA-4 and TGF-β (*Babu* et al., 2006).

The modulation of intestinal mucosal immune responses by geo-

helminths may not only have adverse effects on immune responses to oral vaccines, but may increase susceptibility to infection with pathogenic bacteria (*Mansfield* et al., 2003; *Chen* et al., 2005). A study of severe cholera infection provided evidence that patients with concurrent intestinal helminth infections including *A. lumbricoides* had attenuated IgA responses to CTB in faeces and serum (*Harris* et al., 2009), although it is unclear if such effects were associated with an increased risk of severe illness. The potent regulatory effects of geohelminths on mucosal inflammation have been used therapeutically to treat inflammatory bowel diseases (*Summers* et al., 2005a,b; *Croese* et al., 2006) - although the efficacy of such treatment remains controversial it may be useful in specific sub-groups of patients (*Reddy* and *Fried*, 2009; *Cooper*, 2009b).

CONCLUSION

Chronic geohelminth infections have potent regulatory effects on intestinal immune responses and may contribute to the impaired immunogenicity of oral vaccines observed in non-affluent populations. The mechanisms by which geohelminth infections may suppress mucosal immune responses to vaccines are poorly understood. Under some circumstances, treatment with anthelmintic drugs before vaccination may improve such responses. The efficacy of new mucosal vaccines in infants from non-affluent populations will require detailed evaluation in geohelminth-endemic settings before widespread distribution. An understanding of the mechanisms by which geohelminths and other enteric infections may suppress mucosal vaccine responses could lead to the development of new interventions designed to enhance the effectiveness of mucosal immunization in non-affluent populations.

ACKNOWLEDGEMENTS

Philip J. Cooper is supported by Wellcome Trust grants no. 088862/Z/09/Z

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"EDUCATING" THE NEONATAL IMMUNE SYSTEM: IMPLICATIONS FOR MUCOSAL IMMUNIZATION EARLY IN LIFE

MARCELA F. PASETTI, GABRIELA MELLADO-SANCHEZ, and KARINA RAMIREZ

Department of Pediatrics, Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, MD, USA

SUMMARY

New-borns and infants under six months of age are highly susceptible to infectious diseases. Vaccines that can safely and effectively prevent life-threatening illnesses during the first year of life are sorely needed. Several challenges remain for successful early-life immunization, the two most important being: 1) understanding the structure of the neonatal immune system and the mechanisms underlying neonatal responses; and 2) identifying vaccine strategies that can efficiently engage the fully competent, yet "inexperienced," neonatal immune system. Vaccines and adjuvants that can stimulate innate immune defences, enhance the maturation of dendritic cells and promote Th1-type signals have shown great promise in animal models and humans. An ideal vaccine for this age group would be capable of inducing long-lasting systemic and mucosal immunity bypassing maternal antibodies and would require minimal dosing via a user-friendly route. Vaccines that can be administered mucosally hold great promise because they can mount an immune response at the site of infection and would be practical for large-scale immunization. Identifying the requirements for "educating" the infant immune system will be essential for developing safe and effective vaccines for paediatric immunization.

INTRODUCTION

New-borns and very young infants are highly susceptible to infectious organisms, which cause high rates of morbidity and mortality (*Siegrist*, 2007; *Wilson* and *Kollmann*, 2008). Safe and effective immunization early in life could significantly reduce this burden, but the development of vaccines for the very young has been beset by many challenges. New-borns do not respond to T cell-independent polysaccharide vaccines (*Mond* and *Kokai-Kun*, 2008) and mount modest and short-lived antibody responses to T cell-dependent antigens, which require booster immunizations up to the second year of life (*Adkins* et al., 2004; *Siegrist*, 2007). The Th2-type environment that remains after the gestation period makes it difficult to induce Th1-type cell-mediated immunity (*Wegmann* et al., 1993). Maternal antibodies, although helpful for preventing infections during the first months of life, interfere with vac-

cine take and even residual levels can abrogate responses to routine vaccines such as measles, polio, rotavirus, tetanus, diphtheria, pertussis and *Haemophilus influenzae* type b (Hib). This creates a window of vulnerability against these pathogens that spans up to nine months of age (*Siegrist* et al., 1998).

There has been significant progress in recent years in our understanding of the functional competency of the neonatal/infant immune system. Neonatal dendritic cells (DC) and the capacity of these cells for antigen presentation and cytokine production are at the centre of the debate (*Ridge* et al., 1996). Both in animals and humans, neonatal DC exhibit an "immature" phenotype, with low levels of expression of MHC class II and co-stimulatory molecules and a limited capability for production of IL-12 and pro-inflammatory cytokines. As a result, neonatal DC have a reduced capacity to present foreign antigens and to stimulate naïve T cells compared with their adult DC counterparts (*Gans* et al., 1999). In contrast, neonatal T cells are able to undergo expansion and differentiation into antigen-specific effector and memory cells, but they require fully functional DC and cytokine signals. Neonatal B cell responses are also compromised due to the suboptimal DC function. The inefficient stimulation of T cells limits $CD4^+$ T cell help, which impairs the normal processes of B cell stimulation, migration to the germinal centres, avidity maturation, isotype class switching, and differentiation into either long-lived plasma or memory B cells. As a consequence, the B cell responses are usually feeble, transient and markedly inefficient.

There is substantial evidence suggesting that the limitations discussed above can be overcome and that neonatal immune responses can be induced when vaccine antigens are administered in the appropriate immunologic context. Hence, current research efforts are aimed at identifying vaccines and adjuvants that are capable of providing immunostimulatory signals that would safely and efficiently engage the "inexperienced," but fully competent, neonatal immune system.

NEW-BORNS AND INFANTS RESPOND EFFICIENTLY TO MICROBIAL ANTIGENS

Immune responses to a variety of microbial antigens can develop *in utero* after maternal infection. Functionally mature $CD8^+$ T cell responses were found in new-borns exposed to congenital infection with cytomegalovirus (CMV) (*Marchant* et al., 2003; *Gibson* et al., 2004) and *Trypanosoma cruzii* (*Hermann* et al., 2002). Immune responses that originated *in utero* after maternal immunization have also been reported; new-borns from mothers vaccinated against influenza during pregnancy developed virus-specific IgM antibodies and $CD4^+$ T cells that were present at birth (*Rastogi* et al., 2007).

Similarly, new-borns and young infants can mount potent adaptive immunity when exposed to pathogens soon after birth. Cytotoxic $CD8^+$ T cell responses were reported in infants infected with respiratory syncytial virus (RSV) and human immunodeficiency virus (HIV) (*Collins* et al., 1990). Cytokine-secreting $CD4^+$ T cells were also found in infants infected with herpes simplex virus (HSV) at birth. In many instances, these responses were comparable in magnitude and quality to those found in adults (*Hermann* et al., 2002).

Live attenuated vaccines have proven to be highly immunogenic when they are administered during the neonatal period. The classical example is the bacillus Calmette-Guerin (BCG), which for decades has been given to new-borns at birth and is still widely used in the developing world (*Andersen* and *Doherty*, 2005). Immunization with BCG at birth or at two months of age elicits a $CD4^+$ Th1-type response to *M. tuberculosis* antigens that is similar to the response in adults (*Ota* et al., 2002; *Marchant* et al., 1999). The diphtheria-tetanus whole cell pertussis (DTwP) vaccine induces Th1-type responses in one- to four-month-old infants that are comparable with those induced by *B. pertussis* infection (*Mascart* et al., 2003). Oral polio vaccine has been given to human new-borns and was found to elicit intestinal and serum antibody responses (*Halsey* and *Galazka*, 1985).

In contrast to the responses induced by live organisms, subunit vaccines such as hepatitis B, diphtheria-tetanus toxoid and Hib-tetanus toxoid conjugates primarily elicit antibodies and Th2-type cytokine-secreting $CD4^+$ T cells in young infants. These responses are usually undetectable after the first dose, necessitating several doses to generate sustained immunity. Hepatitis B surface antigen (HBSAg) is the only vaccine given at birth in industrialized countries and represents the best example that neonatal vaccination is feasible and effective (*Delage* et al., 1993). In response to HBSAg, infants develop antibody levels above those of adults, but very poor cellular responses (*Ota* et al., 2004). Likewise, the diphtheria-tetanus-acellular pertussis (DTaP) vaccine elicits Th2-type responses (*Rowe* et al., 2001). Because they are limited in their ability to elicit robust cell-mediated immunity, subunit vaccines fail to adequately protect new-borns against intracellular organisms, and the Th2-biased responses increase the risk for allergy and other undesirable immunologic responses.

Collectively, these examples support the argument that there are no insurmountable intrinsic defects in the neonatal immune system that would prevent successful immunization and that new-borns have the capacity to respond to properly designed vaccines.

VACCINES CAN INDUCE POTENT ADAPTIVE IMMUNITY DURING THE NEONATAL PERIOD

Extensive studies in animal models have shown that new-borns can develop Th1-type immunity, including adult-like $CD8^+$ cytotoxic lymphocytes (CTL) in response to live replicating viruses (*Sarzotti* et al., 1996; *VanCott* et al., 2006), bacteria (*Eisenberg* et al., 2003; *Roduit* et al., 2002; *Rayevskaya* et al., 2002) and DNA vaccines (*Hassett* et al., 2000; *Zhang* et al., 2002; *Capozzo* et al., 2006). Vaccine antigens can elicit potent immune responses during the neonatal period if they are accompanied by immunomodulatory molecules and adjuvants that activate innate immunity and enhance DC maturation and function, for example LPS (*Dadaglio* et al., 2002; *Ismaili* et al., 2003), CpG oligonucleotides (*Kovarik* et al., 1999), Flt 3 (*Vollstedt* et al., 2003), polyriboinosinic:polyribocytidilic acid (*Ma* and *Ross*, 2005) and cytokines, including IL-12 (*Pertmer* et al., 2001; *Sabirov* and *Metzger*, 2006), GM-CSF (*Capozzo* et al., 2003) and IFN-γ (*Pertmer* et al., 2001).

Activation of neonatal DC is regarded as the "key" step to induce

adaptive immunity at early stages of life. Functionally mature neonatal DC can efficiently present vaccine antigens and stimulate naïve $CD4^+$ T cells. They can also trigger a cascade of cytokines that will further enhance and sustain the resulting response. In the presence of mature DC, both mouse and human neonatal T cell function increases to adult levels (*Adkins* et al., 2004). Interestingly, although neonatal human DC may have a limited capacity to present antigens to $CD4^+$ T cells through the class II pathway (*Canaday* et al., 2006), they are fully competent to process and present antigens to $CD8^+$ T cells through the MHC class I antigen processing pathway (*Gold* et al., 2007).

Our group has shown that new-born mice immunized intranasally with *S. typhi* or *S. typhimurium* carrying tetanus toxoid Fragment C developed adult-like Fragment C antibody responses, mucosal antibody-secreting cells and T cell responses even in the presence of maternal antibodies (*Capozzo* et al., 2006). Vigorous priming of the neonatal immune system was demonstrated in new-borns immunized intranasally with *S. typhi* expressing *Yersinia pestis* F1 antigen, and this priming was associated with the capacity of Salmonella to enhance DC maturation (*Ramirez* et al., 2009). The signalling of bacterial components (for example, LPS, OMP and CpG motifs) through toll-like receptors (TLR) facilitates DC recruitment, maturation and migration to secondary lymph nodes and synthesis of IL-12 and Type 1 IFN (*Salazar-Gonzalez* and *McSorley*, 2005). These mature and activated DC stimulate the production of IFN-γ and IL-2 by $CD4^+$ T cells, promoting Th1-type responses. Activated $CD4^+$ T-helper cells expressing CD40L interact more efficiently with B cells, supporting Ig isotype switching, affinity maturation and immunologic memory.

A well-configured vaccine for neonatal immunization would be one that could activate innate immunity, contributing immunostimulatory (danger-like) signals that would enhance DC function for proper activation of neonatal T cells.

VACCINES THAT ACTIVATE INNATE IMMUNITY AND ENHANCE DC FUNCTION CAN SUCCESSFULLY STIMULATE THE IMMUNE SYSTEM IN EARLY LIFE

The neonatal immune system has remarkable plasticity and a capacity to mount potent adaptive immunity when appropriately stimulated. Vaccines and adjuvants that engage TLRs, activate neonatal DC and evoke Th1-type cytokines seem to be promising tools for successful neonatal immunization. These vaccines can "educate" the neonatal immune system by allowing neonatal DC to become fully functional antigen presenting cells that will subsequently stimulate T and B cells. An additional advantage of Th1-type vaccines is their potential capacity to prevent exacerbated Th2-type responses, which have been associated with allergic reactions. The exposure to foreign/environmental antigens and the higher incidence of natural infections with a variety of organisms early in life have been linked with a lower prevalence of allergy. This has been the theory supported by the "hygiene hypothesis" (*Schröder*, 2009).

Conceivably, stimulatory signals can imprint a state of "activation" on neonatal DC that could lead to improved responses against unrelated antigens given at the same time or even

later in life. Human infants that received BCG together with the HepB vaccine soon after birth had increased antibody and $CD4^+$ T cell responses to the HBSAg (Ota et al., 2002), which was thought to be associated with the capacity of BCG to activate neonatal DC. We have shown that mucosal priming of new-born mice with *S. typhi* can enhance responses to a recombinant subunit protein given by the parenteral route at a later time point (*Ramirez* et al., 2009). Ty21a, the only licensed oral typhoid vaccine, was safe and well tolerated when given to toddlers (< 24 months old), even at high doses ($1x10^9$ CFU), and could conceivably be used, like BCG, in younger children (*Murphy* et al., 1991). A new generation of rationally attenuated strains harbouring stabilized plasmids and non-antibiotic selection markers are being pursued as safer live-vector vaccine alternatives for the paediatric population. Ghost-particles derived from Gram-positive and Gram-negative organisms have emerged as promising candidates that are safe while offering the immunostimulatory properties of a living organism. These particles can deliver foreign antigens and are amenable for mucosal immunization. We have shown that ghost particles from *L. lactis* displaying *Y. pestis* LcrV elicit potent mucosal and systemic immunity and protect neonatally immunized mice from lethal systemic plague infection (*Ramirez* et al., 2009).

Vaccines that can be given to infants through mucosal routes (i.e., orally or intranasally) are of special interest not only because of the ease of administration, but also to reduce the interference of maternal immunity because lower concentrations of maternal antibodies are present in the infant mucosa compared with the circulation and systemic lymphoid tissues (*Siegrist*, 2003).

IMMUNIZATION REGIMENS THAT CAN ENHANCE VACCINE-INDUCED PROTECTIVE IMMUNITY EARLY IN LIFE

In neonatal animal models, the route of immunization can make a significant difference in the capacity to induce protective immunity (*Sabirov* and *Metzger*, 2006). We argue that immune responses to vaccines could be enhanced by using more efficient immunization regimes, such as a heterologous prime-boost approach. An advantage of a two-step immune stimulation is that the final response will include distinct features of both the initial prime and the subsequent boost. Particularly attractive are prime-boost strategies that combine mucosal and parenteral immunization because they extend the breath of the responses. A sindbis-based measles DNA vaccine primed 1-2-month-old infant rhesus macaques to develop a vigorous neutralizing antibody response to a subsequent boost with an aerosolized live attenuated measles vaccine (*Pasetti* et al., 2007). New-born mice primed mucosally with *S. typhi* expressing *Y. pestis* F1 and boosted parenterally with F1-alum elicited a high-avidity F1-specific IgG response, mucosal antibody-secreting cells and T cell responses that surpassed those elicited by repeated immunization with *S. typhi* (F1) or F1-alum (*Ramirez* et al., 2009). A prime-boost strategy could also allow tailoring of the immune response to favour the responses necessary for protection. An obvious drawback of the prime-boost approach, however, is the requirement of more than one vaccina-

tion encounter, in which case priming in the form of an easily administered mucosal vaccine would advantageous. The success of prime-boost immunization in human new-borns and infants remains to be determined.

EARLY-LIFE IMMUNIZATION, TOLERANCE AND AUTOIMMUNITY: SHOULD WE BE CONCERNED?

A valid question related to neonatal vaccination is whether the introduction of foreign antigens early in life could induce tolerance or predispose recipients for hyporesponsiveness later in life. There is limited evidence indicating that this may actually occur in humans [Reviewed in (*Siegrist*, 2001)]. The manner, the context and the route by which antigens are introduced to the immune system are critical in determining whether an immune response or a lack of thereof will ensue. We argue that the development of tolerance is less likely when antigens are administered with a potent immune stimulation, in which case the Th1-type vaccines and adjuvants could lessen such a risk. Antigens delivered in particulate as opposed to soluble form are also less likely to induce tolerance. Reduced responses to DTaP have been reported when new-borns received a dose at birth followed by routine immunization at 2, 4, 6 and 17 months (*Halasa* et al., 2008). It has been noted, however, that this type of hyporesponsiveness likely reflects vaccine interference rather than neonatal immunological impairment (*Siegrist*, 2008) because a number of studies actually described enhanced vaccine effectiveness in new-borns immunized with acellular pertussis (aP) at birth prior to the routine DTaP immunization (*Knuf* et al., 2008; *Belloni* et al., 2003).

It has also been questioned whether neonatal vaccination could abrogate self-tolerance and lead to autoimmune diseases. Millions of new-borns have been vaccinated at birth with BCG, polio and HepB vaccines, and there is no evidence of an increased incidence of autoimmunity associated with perinatal immunization (*Belloni* et al., 2002). The mechanisms that induce tolerance to self-antigens (both systemically and mucosally) are not restricted to early life but are active throughout life. Thus, it can be argued that the neonatal period is not at higher risk for autoimmunity than any other stage in life. Regulatory T cells have a central role in the maintenance of tolerance. $CD4^{+}CD25^{+}$ Treg cells have been found in cord-blood (*Takahata* et al., 2004) and likely have a key role in controlling potentially harmful responses.

"EDUCATING" THE EARLY-LIFE IMMUNE SYSTEM TO OVERCOME THE TOLEROGENIC BARRIER FOR ORAL IMMUNIZATION

The "education" of the neonatal/infant immune system in the mucosal compartment has unique features that complement the education of immune cells in the systemic compartment. A state of hyporesponsiveness (which increases with age) prevails in the mucosal lymphoid tissue, particularly in the gastrointestinal tract, due to the massive and continuous exposure to foreign antigens. This tissue, however, can adapt to maintain a delicate balance between the

need to maintain immunologic silence or to respond to potentially harmful agents. Human milk promotes the development and maturation of the mucosal-associated lymphoid tissue and contains numerous immunomodulatory components (such as TGF-β and Vitamin A) that influence immunological competency. Exposure to food and other (non-self) antigens in breast milk favours the development of tolerance in rodents (*Verhasselt*, 2010). Infant breast-feeding has been associated with a reduced incidence of allergy and asthma, which could be explained by the presence of maternal antibodies that prevent respiratory viral infections, but also by the tolerogenic presentation of allergens [Reviewed in (*Verhasselt*, 2010)]. Interestingly, the commensal flora in the gut also enhances the development, maturation and functional capacity of immune cells in the gut (*Eberl* and *Lochner*, 2009). It is therefore conceivable that the processes that down-regulate immune responses to prevent disease in the intestinal environment may also interfere with vaccine take.

A number of routine (e.g., polio, rotavirus) and experimental (e.g., Shigella, cholera) vaccines have performed sub-optimally in developing countries compared with industrialized ones [Reviewed in (*Walker* et al., 2005, 2007; *Mirzayeva* et al., 2009)]. Several reasons have been proposed to explain this observation, including malnutrition of infants and mothers, micronutrient deficiencies, parasite and bacterial co-infections and maternal antibodies in breast milk (*Czerkinsky* and *Holmgren*, 2009). It has also been proposed that infants in developing countries (and their mothers) have a more diverse and frequent exposure to microbial antigens, and as a result, they have a more mature (and tolerogenic) immune system (*Czerkinsky* and *Holmgren*, 2009). The lower prevalence of allergy in the developing world compared with industrialized regions is also well known. If the "tolerogenic environment" of the gastrointestinal tract is indeed a factor contributing to the lower immune responses to enteric vaccines, administering these vaccines earlier (possibly at birth) could conceivably circumvent this limitation. Well-tolerated and effective mucosal adjuvants that could activate innate immune cells and trigger an adequate level of pro-inflammatory signals could also break this "tolerogenic" barrier. This concept is worth further study in animal models and *in vitro*, possibly using engineered 3D tissue-culture model systems.

CONCLUSION

Vaccination during the neonatal period could dramatically reduce disease burden and risk of infection during the first year of life. Since health care is sought at birth, perinatal vaccination can have a further outreach in areas where they are most needed. A challenge that remains is identifying vaccines and adjuvants that can provide immune stimulatory "danger" signals to efficiently activate the inexperienced neonatal immune system, without compromising safety. Neonatal immune cells have great plasticity and the potential to be "educated." Vaccines that hold great promise are those that stimulate innate immunity, promote activation and maturation of neonatal DC and allow for intracellular antigen delivery (to shield vaccine antigens from maternal antibodies). Ideally, such a vaccine could be given at birth,

through a mucosal route, and would be effective after a single dose. Neonatal immunization seems to pose a low risk for autoimmune disease and tolerance, but reactogenicity must be closely examined to achieve the appropriate risk/benefit balance. Early-life immunization and the use of well-tolerated and effective mucosal adjuvants could help overcome the tolerogenic barrier for enteric vaccine delivery.

ACKNOWLEDGMENTS

This work was supported in part by grant R01AI065760 to MFP. The authors thank Dr. Wilbur Chen for critically reading the manuscript.

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THE MAL-ED PROJECT: DECIPHERING THE RELATIONSHIPS AMONG NORMAL GUT FLORA, ENTERIC INFECTION AND MALNUTRITION AND THEIR ASSOCIATION WITH IMMUNE RESPONSE TO VACCINES

DENNIS LANG

Fogarty International Center, National Institutes of Health, and The Foundation for the National Institutes of Health, Bethesda, MD, USA

SUMMARY

It has been estimated that malnutrition affects 20% of children in the developing world (*Black* et al., 2008) and that poor nutrition is linked to more than half of all deaths worldwide in children under the age of five (*Caulfield* et al., 2004). In addition to its role in childhood deaths, malnutrition in early childhood may lead to cognitive and physical deficits and may cause similar deficits in future generations as malnourished mothers give birth to low birth weight children (*Victora*, et al., 2008). Malnutrition increases both susceptibility to and incidence of infections and mortality due to diarrhoea and other infectious diseases (*Campbell* et al. 2003); these effects may be mediated through a depressed immune response to natural infection or to administered vaccines.

The MAL-ED Consortium has been established in eight countries with a high incidence of both diarrhoeal disease and malnutrition. We are investigating the hypothesis that infection or co-infection with certain enteropathogens contributes to malnutrition by causing intestinal inflammation and/or by altering intestinal barrier and absorptive function which, in turn, leads to growth faltering, stunting, and deficits in cognitive development. In addition, the relationship of repeated enteric infection and malnutrition on the diminished protective immunity in children given orally delivered childhood vaccines is being examined at these sites. Our study also aims to shed light on relevant questions such as: (1) which pathogens or which combination of infectious agents are most frequently associated with growth faltering and poor development, and (2) at what periods during the first two years of life do specific infections produce the largest effect on growth and development? Among the factors being evaluated for their effects are: Enteric infections, micronutrient levels, diet, socio-economic status, composition of the gut microbial flora (the gut microbiome) and human genetic factors. Based on these new data we hope to be able to better define the problem and make both site specific and more generalized recommendations regarding the nature and timing of interventions aimed at improving child health in these resource poor settings.

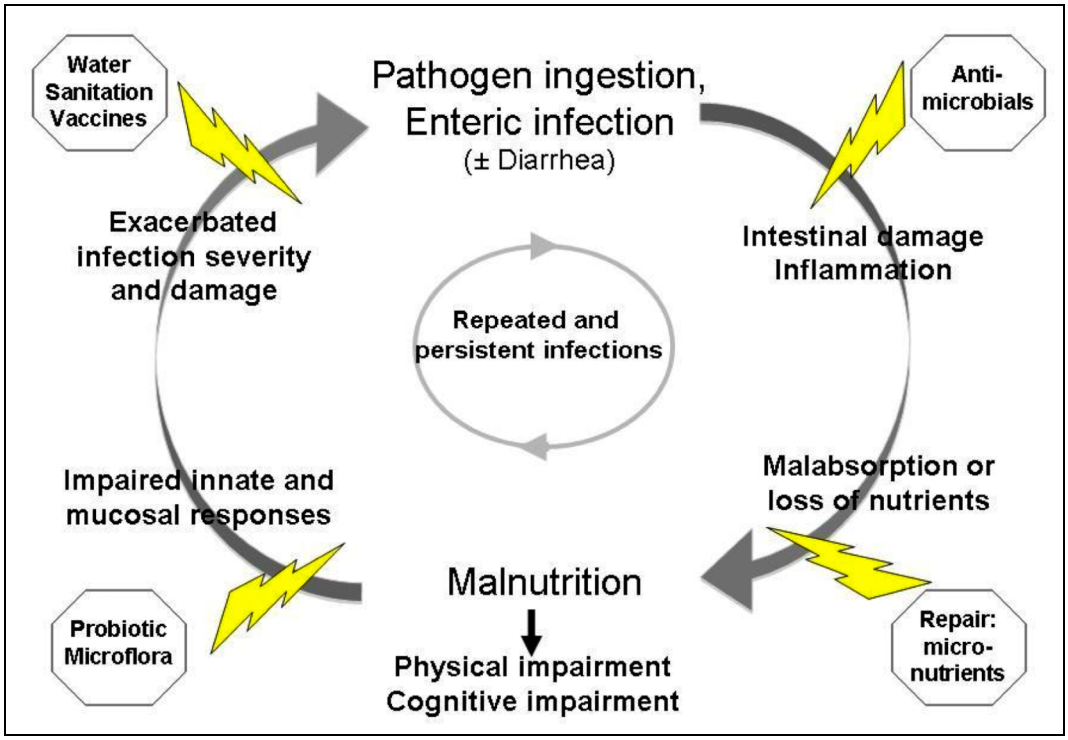

Figure 1. The cycle of malnutrition and enteric disease.
This figure, adapted from *Guerrant*, et al. 2008, depicts the cyclical nature of the synergistic relationship between infection with enteric pathogens and the development of malnutrition (undernutrition). Around the outer circle are indicated the expected physiological effects on children. The arrow pointing from "malnutrition" indicates the resulting impairment of both physical and cognitive development observed in other studies during the first two years of life that may extend into adulthood. Potential interventions that may be capable of interrupting this "vicious cycle" are depicted by the lightning bolts.

INTRODUCTION

It has been estimated that malnutrition affects 20% of children in the developing world (*Black* et al., 2008) and that poor nutrition is linked to more than half of all deaths worldwide in children under the age of five (*Caulfield* et al., 2004). Moreover, it is recognized that malnutrition increases susceptibility to and incidence of infections and is associated with diminished response to vaccines. Diarrhoeal infections and undernutrition synergistically contribute to morbidity and mortality. The combination of diarrhoeal infections and undernutrition has been demonstrated to have significant detrimental effects on growth during the first two years of life which can be observed as early as three months of age (*Victora* et al., 2010). This relationship between diarrhoea and malnutrition can be depicted as a "vicious cycle" (*Guerrant* et al., 2008) and is illustrated in Figure 1.

Among the factors that may lead to undernourishment in young children are: the lack of adequate amounts of food, insufficient breastfeeding, inadequate diversity of complementary foods which may lead to specific micronutri-

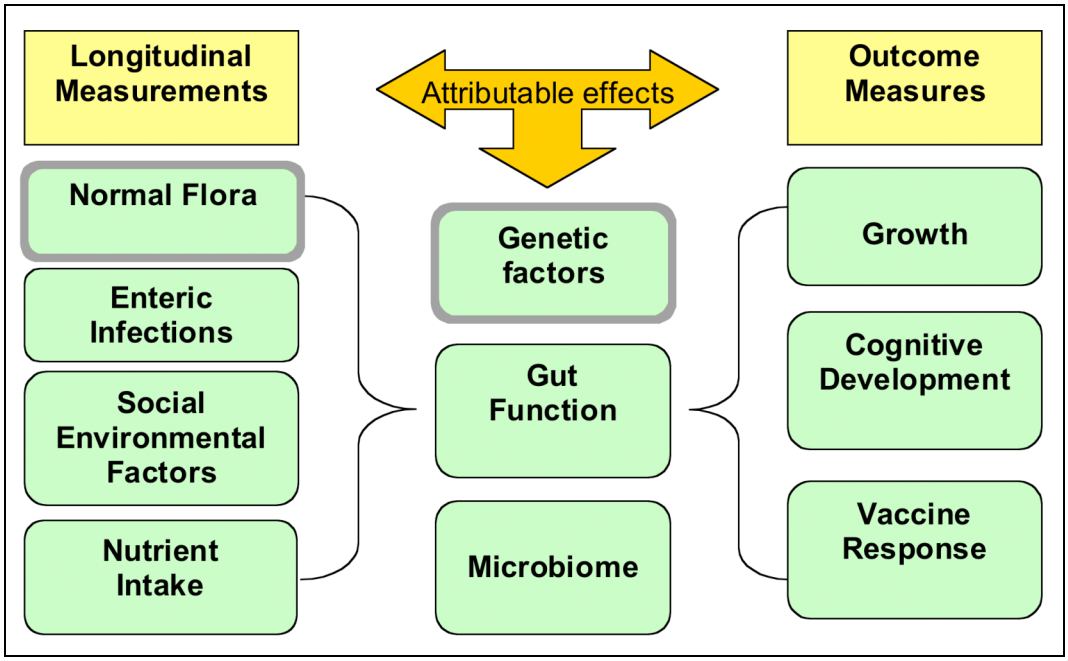

Figure 2: Proposed association among the factors being assessed during the MAL-ED study. We believe that the factors measured longitudinally will have their effects through alteration of gut function as indicated. We also recognize that gut function will be influenced by both genetic and epigenetic factors and by the composition of the gut normal flora (microbiome). These factors in combination, lead to effects on growth, cognitive development and immune response to vaccines indicated as outcome measures.

ent deficiencies, diets that are high in inhibitors of micronutrient absorption, catabolic states due to infection, and inadequate response of the host and the host's gut microbiome to caloric insufficiency. Pathogenic bacteria, viruses, and parasites in the gut also impact nutritional status through multiple mechanisms. First, enteric pathogens impair nutrient absorption by damaging the absorptive capacity of the intestine, causing protein-energy and micronutrient malnutrition. Second, enteric infections can compromise the intestinal barrier, causing increased intestinal permeability to pathogens, endotoxins, and other macromolecules that can result in the chronic stimulation of the immune system. Importantly, both micronutrient deficiencies and chronic immune stimulation have been found to impair growth and increase susceptibility to infectious diseases (*Black* et al., 2008). Additionally, altered gut flora and pathogens may also influence the effectiveness of orally-delivered vaccines. Understanding the complex and synergistic relationships between enteric infections and malnutrition, therefore, is fundamental to the design of better intervention strategies capable of reducing the infectious disease burden and improving the nutritional status of children born in these settings.

While it is likely that enteric infections contribute to malnutrition, data on the role of particular enteropathogens are limited by small sample sizes, limited geographic locales, and robustness of diagnostic tests employed. Additionally, there has been relatively little study of morbidity and mortality due to

Table 1a: MAL-ED network field site institutions and principal investigators

Performance sites	Principal investigators
Aga Khan University, Karachi, Pakistan[1]	Dr. Zulfiqar Bhutta
Christian Medical College Vellore, Vellore, India[1]	Drs. Gagandeep Kang, Sushil John
JHSPH Satellite Laboratory, Iquitos, Peru[1]	Dr. Margaret Kosek
Federal University of Ceara, Fortaleza, Ceará, Brazil[1, 2]	Drs. Aldo A. M. Lima, Reinaldo Oria
Walter Reed/AFRIMS Research Unit, Kathmandu, Nepal[1]	Dr. Sanjaya Kumar Shrestha
Institute of Medicine, Kathmandu, Nepal[1]	Dr. Prakash Sunder Shrestha
University of Venda, Limpopo, South Africa[1]	Dr. Pascal Bessong
International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh[1, 2]	Drs. Tahmeed Ahmed, Rashidul Haque
Haydom Lutheran Hospital, Haydom, Tanzania[1]	Dr. Erling Svensen

[1]Location of birth cohort studies.
[2]Location of case-control studies.

chronic and recurrent enteric microorganisms and parasites, their contribution to the global burden of disease in children under five, and the consequential long-term effects in adulthood. To date, there have been no systematic studies that help define particular windows of vulnerability in early childhood when specific pathogens or mixed infections could lead to greater deficits in developmental outcomes. Furthermore, there have not been conclusive studies that define the associations between enteric infections and undernutrition with intermediary indicators, such as measures of gut function, which would help explain the effects of enteric infections on growth and development. Likewise, there are also limited studies looking at the effects of normal gut flora, particular infectious agents, mixed infections, or micronutrient levels on the immune response in children. If we are to develop more effective vaccination strategies, it is important to elucidate how these factors interact to reduce the immune response to oral vaccines in particular, that is observed in the developing world.

ESTABLISHMENT OF THE MAL-ED NETWORK

The MAL-ED (pronounced mal-a-dee) Network has been established in order to better define the interrelationships among exposure to enteric pathogens, infection and diarrhoeal disease, diet, and socio-economic status (SES) affecting gut physiology, growth, immune response to vaccines and cognitive development in birth cohorts from eight resource poor communities (Figure 2).

MAL-ED's Scientific and Administrative Core was established at the Fogarty International Center, NIH (FIC) and at the Foundation for the National Institutes of Health (FNIH) to provide scientific and organizational management. Dr. Mark Miller, FIC and

Table 1b: MAL-ED collaborating institutions and investigators

Collaborating institutions	Principal investigators
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA[1]	Drs. Laura Caulfield, Laura Murray-Kolb, Robert Black
University of Virginia, Charlottesville, VA, USA[2,4]	Drs. Richard Guerrant, William Petri, Patrick Concannon, Stephen Rich Eric Houpt, Rebecca Dillingham
Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand[3]	Drs. Carl Mason, Ladaporn Bodhidatta
Washington University School of Medicine, St. Louis, MO, USA[4]	Dr. Jeffrey I. Gordon
University of Colorado at Boulder, Boulder, CO, USA[4]	Dr. Rob Knight

[1]Johns Hopkins Bloomberg School of Public Health is collaborating with the Peru field site.
[2] University of Virginia is collaborating with the Bangaladesh, Brazil, South Africa, and Tanzania field sites.
[3]Armed Forces Research Institute of Medical Sciences is collaborating with the Nepal field site.
[4]Location of Companion project.

Dr. Michael Gottlieb, FNIH serve as co-Principal Investigators of the project. This core also provides a data-coordinating centre (DCC) which receives de-identified data from all sites and will conduct, with participation of study investigators, a comprehensive analysis of the pooled data. The DCC may also assist sites in their analysis of site-specific data. We anticipate that these analyses will yield informative site-specific results and conclusions as well as ones that may transcend sites and geographic areas. The science/administrative core also conducts budget management and oversight activities and manages the activities and meetings of the advisory committees that have been established. The core also conducts site visits, organizes annual meetings and teleconference meetings with study investigators and has worked with our investigators, organized into technical sub-committees, to develop the common protocols used in the study.

Study sites from both urban and rural communities were chosen, in part, because of the high incidence of both diarrhoeal disease and malnutrition experienced by children less than five years of age and because of the scientific experience of the investigators at those sites. A list of the MAL-ED study sites and the Principal Investigators at each site are indicated in Table 1a while Table 1b indicates the collaborating institutions and investigators.

All participating institutions have agreed to abide by the terms and conditions of a Research Consortium Agreement (RCA) that provides the governance structure and decision making authority of the Network and its associated advisory committees. The RCA also provides guidance on publication, intellectual property, and data sharing and release policies. The intent of these policies is to ensure that any benefit resulting from the study is for those most in need in the developing world. Clearly delineating these issues with input from the participating institutions and investigators prior to study

initiation was very helpful; having them in place will facilitate the addition of other study sites and related projects in the future.

MAL-ED STUDY HYPOTHESES

1. Infection (and/or co-infection) with certain enteropathogens leads to malnutrition by causing intestinal inflammation and/or by altering the barrier and absorptive functions of the gut.
2. The combination of enteric infections and malnutrition results in growth and cognitive impairments in children and may lead to impaired immunity as measured by vaccine response.
3. Particularly sensitive periods exist during early childhood when environmental exposure, infection, and malnutrition lead to exacerbated and lasting effects on development.

STUDY DESIGN

The MAL-ED study employs standardized and harmonized study protocols. Use of a shared Manual of Procedures and common data collection forms by all eight field sites ensures that comparable data will be collected and that the data can be pooled for analysis. Each site has received approval of study protocols from their local Institutional Review Board (IRB) and from the IRBs of collaborating U.S. institutions.

Prior to enrolment, each site conducted an extensive census to determine the location of women of childbearing age and pregnancies that served to identify potential enrolees. Recruitment plans were developed in collaboration with science/administrative core staff to ensure that enrolees would be representative of the overall population in the community. In addition, each site conducted a pilot study in 100 households in an area representative of the study population in order to determine socio-economic status, food security, and anthropometric base line characteristics of the community.

Each field site will enrol a minimum of 200 children before they are 17 days old in a birth cohort study that will follow children from birth to two years of age. Recruitment of the cohorts will be distributed over two years in order to control for, and allow analysis of, seasonal variation in infectious disease burden, or the quantity and nature of the food supply, for example. Further details of the birth cohort study design are presented in Tables 2 and 3.

Although only a cohort study design can adequately assess sequential events that precede the onset of persistent diarrhoea and/or malnutrition or growth shortfalls, close biweekly household follow-up (as is necessary for obtaining accurate data on diarrhoeal illnesses) may have a "Hawthorne Effect" that dramatically reduces diarrhoea rates and malnutrition, as well as mortality (*Ricci* et al., 2006). Hence, in order to understand the aetiologies, pathogenesis and interventions of moderate or severe malnutrition, two of our sites (Brazil and Bangladesh) are also conducting case control studies of malnutrition with 500 cases and 500 control children (cases (HAZ >-2) and controls (HAZ <-1) identified at community clinics.

Table 2: Overview of sample collection for the MAL-ED cohort study

Objectives		Measurement	Sample type
Gut functional capacity:	Gut integrity	Lactulose-mannitol	Urine
	Gut inflammation	Quantitative lactoferrin, A-1-antitrypsin	Stool
Enteric infection assessment:	Incidence and prevalence of enteric pathogens	Microbiological assays[a]	Stool
	Diarrheal disease[b] history	Frequency, severity, and duration	Interview
Growth and development:	Anthropometry	Length, weight, head circumference	Examiner administered
	Nutrition	Breastfeeding status (exclusivity, partial/full cessation)	Interview
		Child feeding practices (e.g. introduction of solids, feeding patterns, key foods)	Interview
	Micronutrients	Iron (hemoglobin, trans-ferrin receptor, ferritin)	Blood
		Zinc (plasma)	Blood
		Vitamin A (plasma retinol)	Blood
		Plasma protein (α-1-acid glycoprotein)	Blood
		Others (e.g. iodine, lead, glutamine, arginine)	Blood
	Cognitive function	Global	Examiner administered
		Language (verbal fluency)	Interview
		Others (e.g. child temperament)	Interview
	Assessments on mother/household	Home environment SES/demographic	Examiner administered
		Maternal IQ	Examiner administered
		Other (e.g. depressive symptoms)	Interview
Vaccine response:	Vaccine immunogenicity	Antibody titers to mucosal vaccines: - Rotavirus vaccine - Oral Polio vaccine	Blood
		Antibody titers to EPI vaccines: - tetanus, measles, pertussis	Blood
Other illness surveillance (syndrome):	Incidence of respiratory and other illnesses	Frequency, severity, and duration	Case report form

[a] Microbiological assays include bacterial culture, microscopy, and PCR for identification of site-specific bacterial, viral, and parasitic pathogens.
[b] Diarrhea defined as 3 or more unformed stools in a 24 hour period; episodes separated by 2 diarrhea-free days.

Table 3: Timing of measurements conducted during the two years of obseervation in the MAL-ED cohort study

Sample	Measured	When
Blood	Haemoglobin, ferritin, zinc, vitamin A, lead, α-1 acid glycoprotein, transferrin receptor, amino acids	7, 15 months
	Immune response to pertussis, tetanus, polio, measles, rotavirus	7, 15 months
Urine	Gut integrity: Lactulose-mannitol permeability test	3, 6, 9, 15 months
	Iodine	6, 15 months
Stool	Lactoferrin, α-1-antitrypisn	Monthly 0- 24m, AND one time during each diarrhea episode
	Enteric pathogens	Monthly 0- 24m, AND one time during each diarrhea episode
General survey	Length, weight, head circumference	Monthly 0- 24 months
	Comprehensive diet	Monthly 0- 24 months
	Cognitive function	6, 15, 24 months
	Demographic/ SES/ medical history	0, 6, 15 months
	Household/ maternal assessment	0, 8, 15 months
	Incidence of diarrhea and other illness	2x per week until 2 years
	Breastfeeding, supplemental diet	2x per week until 2 years

COMPANION PROJECTS

The MAL-ED Network provides both a scientific and administrative platform from which to launch additional related projects. These projects could include hypothesis driven research and targeted interventional trials. We anticipate that such opportunities will present themselves as our analysis proceeds over the next four years and the clinical situation at each site becomes better defined. Currently, the Network collaborates with three associated companion projects which, together with the cohort and case control studies, constitute the larger MAL-ED Consortium. Companion project institutions and investigators have agreed to abide by the same Research Consortium Agreement, as have all Network investigators. The three companion projects are briefly described below:

1) Studies of the human gut microbiome and its role in nutrition, is being conducted at Washington University, St. Louis, by Dr. Jeff Gordon, and at the University of Colorado, Boulder by Dr. Rob Knight.
2) Genome wide studies aimed at identifying candidate human genes associated with undernutrition and growth impairment are being conducted at the University of Virginia by Dr. William Petri, Dr. Pat Con-

cannon and Dr. Steve Rich. The University of Virginia investigators are working with the Bangladesh site.

3) Development of a multiplex PCR assay capable of detecting all of the bacterial, viral and parasitic pathogens being studied in the MAL-ED project is headed by Dr. Eric Houpt at the University of Virginia in collaboration with Dr. Jim Nataro at the University of Maryland.

CURRENT STATUS

The MAL-ED Network and Consortium has been operational for $1^1/_2$ years. All field sites are actively recruiting subjects, the earliest having started in November, 2009. Enrolment of new subjects will proceed evenly paced over a two-year period in order to capture seasonal variation in exposure to pathogens, disease aetiology and food availability. We look forward to the comprehensive analysis of the data and applying the findings to the improvement of the public health in the participating sites and in the rest of the developing world. We envision that the Network will accommodate expansion to include additional performance sites and companion projects related to the study objectives of the Network. By thoroughly characterizing the populations under study, and improving the local infrastructure and capacity, these sites will be ready to test appropriate intervention strategies by conducting clinical trials relevant in their setting.

ACKNOWLEDGEMENTS

We are grateful to the team of MAL-ED investigators listed in Tables 1a and 1b and to the dedicated administrative, laboratory and field staff for their implementation of the common protocol at each site. Of course, the study would not be possible without the participation of the enrolled families and children; to them we are indebted. We appreciate the dedicated efforts of the scientific and administrative staffs of the Fogarty International Center, NIH, and at the Foundation for NIH. We are especially thankful for the generous support of the Bill & Melinda Gates Foundation.

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MECHANISMS OF IMMUNE ENHANCEMENT BY BENEFICIAL MICROBES AND PROBIOTICS

CARISSA M. THOMAS[1], GEOFFREY A. PREIDIS[1], and JAMES VERSALOVIC

[1]Interdepartmental Program in Cell and Molecular Biology, Baylor College of Medicine and Department of Pathology, Texas Children's Hospital;
[2] Department of Pathology & Immunology, Baylor College of Medicine, and Department of Pathology, Texas Children's Hospital, Houston, TX, USA

SUMMARY

This review explores the abilities of beneficial microbes including probiotics to stimulate mucosal and systemic immunity, so that global vaccination strategies may be enhanced. Beneficial microbes secrete microbial factors and express cell surface features that stimulate different types of immune cells to alter their gene expression programs and produce different sets of cytokines and immune mediators. Microbes can affect the B lymphocyte program, and the production of different antibody subclasses due to class switching. Immune responses to antigenic challenges as a result of vaccination may be stimulated by B lymphocyte-promoting signals that result from microbial stimulation. Effector and regulatory T lymphocyte programs may be modulated by microbial effects on different signalling pathways. Beyond adaptive immunity, beneficial microbes may stimulate signalling pathways in intestinal epithelial cells, macrophages, and dendritic cells of the innate immune system. Beneficial microbes including probiotics may "prime" the immune system and supplement nutritional approaches so that infants and young children in the developing world are vaccine-ready. If these strategies can be combined, success rates for diverse vaccines may effectively increase in resource-poor regions of the world.

INTRODUCTION – IMMUNITY IN THE ERA OF THE MICROBIOME

The human body consists of a vast ecosystem that includes more bacterial cells than human cells, by an estimated difference that exceeds an order of magnitude. Commensal microbes have co-evolved with animals, including *Homo sapiens*, for thousands of years, and functions encoded by microbial genomes may supplement the functions encoded in the human genome. The human genome contains an estimated 25,000 genes with many genes of unknown function and genes that encode multiple proteins via alternative splicing or posttranslational processing. The rich functional repertoire of the human genome is exceeded by the metagenome, which is estimated to contain a gene content that is more than 100-fold greater in terms of gene number, with

an estimated gene pool exceeding 3 million genes. This aggregate metagenome, or microbiome, encodes for diverse metabolic pathways and signals that may have a profound impact on mammalian physiology and immunity.

In this manuscript, we will describe the impact of probiotics and beneficial microbes on the mammalian immune system, with an emphasis on mechanisms of immune enhancement or stimulation by beneficial microbes. Probiotics are defined as viable microbes that, when ingested in adequate amounts, confer some benefit to the host (*FAO/WHO*, 2001). The benefits are vaguely defined, and these benefits may include stimulation or modulation of host immunity. In this review, we will use beneficial microbes as a broader term that refers to any microbe with a benefit to the host that has been published in any single study. Beneficial microbes include probiotics as a subgroup, and this subgroup includes organisms that have been vetted in published clinical trials and meta-analyses in terms of benefits to human health.

Our hope is that beneficial microbes can facilitate the development of a robust immune system that may protect animals from various pathogens, radiation, and diverse biochemical challenges. Microbes may stimulate the development and differentiation of effector T lymphocytes, thereby enhancing populations of helper and cytotoxic T cells. Regulatory T cell populations may also be expanded in number as a result of microbial stimulation, resulting in inhibitory effects on cell mediated immunity and cytokine responses. Such responses may serve to quench immune responses, enabling the host to avert immunopathology as a result of overzealous immune responses. Conversely, commensal microbes may suppress the functions of regulatory T cells, thereby promoting more robust immune responses when hosts are challenged. B lymphocytes produce pathogen-specific antibodies, and the differentiation of antibody-secreting cells can be stimulated by beneficial microbes. Innate immunity, including dendritic cells and macrophages, may be affected so that subpopulations of these cells may "tilt" immune responses towards inflammation or more effective neutralization of pathogens.

The central question of this Old Herborn University seminar is whether vaccine challenges can be more effective with respect to protection if the host is exposed to the optimal combination of beneficial microbes. This review describes multiple mechanisms of immune enhancement (Figures 1 and 2), and the final section will attempt to point the way forward regarding strategies for harvesting the power of the microbiome and antigenic diversity of these communities to stimulate immunity and efficacy of vaccination programs for global health.

B LYMPHOCYTES AND ANTIBODY RESPONSES

Probiotics and beneficial microbes may stimulate humoral immunity by stimulating the production of mucosal and systemic antibodies. Microbes may promote differentiation of B lymphocytes and class switching, and such stimulation may serve to "prime" or prepare the immune system for subsequent pathogen or vaccine challenges. Intestinal microbes may strictly promote mucosal immunity, and such immune enhancement may be sufficient

to enhance enteric or mucosal vaccination strategies.

The consumption of probiotics during pregnancy may stimulate production of antibodies in the mother and consequently serve to transfer passive immunity to the infant via breast milk. In human studies, oral consumption of either *L. rhamnosus* or *B. lactis* during pregnancy stimulated the production of IgA in human breast milk at one week and 3 months post-partum (*Prescott* et al., 2008). The consumption of these probiotic strains resulted in the elevation of cord blood interferon-gamma levels in neonates, and these results indicate that stimulation of immunity in mothers may be effectively linked to enhanced systemic immunity in newborns. The production of mucosal IgA may be enhanced by signals derived from intestinal epithelial cells such as APRIL, BAFF, or TGF-β. Human enterocytes produce APRIL (a proliferation inducing ligand) in response to microbial signals from commensal bacteria such as *Lactobacillus plantarum* or *Bacillus subtilis*. APRIL mediates class-switch recombination in B lymphocytes to IgA_2 (*He* et al., 2007), and this antibody subclass is known to promote mucosal protection. Our recent work in an outbred, new-born mouse model suggests that probiotic *Lactobacillus reuteri* stimulates pathogen-specific mucosal IgA responses (G. Preidis, unpublished data). Whereas mucosal rotavirus-specific IgA antibodies are elevated in the presence of a single probiotic strain, systemic rotavirus-specific IgA responses do not seem to be affected.

Several studies with different vaccine challenges have documented the potential of probiotics to serve as "adjuvants" or to function as enhancers of vaccination. The delivery of systemic vaccines in parallel with probiotics may be enhanced by the ability of probiotics to stimulate antigen-specific IgG responses in peripheral blood. Oral or mucosal vaccination challenges with whole organisms or recombinant subunits have also demonstrated enhancement of antigen-specific IgA or IgG responses when probiotics are co-administered. New genetically engineered vaccines that are based on commensal microbes as "delivery vectors" may contain immunostimulatory or adjuvant properties that serve to boost vaccine responses (*Van Huynegem* et al., 2009).

T LYMPHOCYTES AND CELL-MEDIATED IMMUNITY

Beneficial microbes stimulate the proliferation of effector T lymphocytes globally or in response to specific antigens. In the presence of specific antigens, probiotics can stimulate proliferation of antigen-specific T lymphocytes. Probiotic species are known to promote anti-apoptotic signalling pathways and suppression of caspases in T lymphocytes and other immune cells. Lamina propria or intra-epithelial lymphocyte populations may be enhanced *in vivo*, and these immunostimulatory effects have been documented in mouse models (*Ivanov* et al., 2009; *Mileti* et al., 2009). Probiotic strategies may stimulate antigen-presenting cell function with subsequent effects on effector T cell stimulation. Effects on signalling pathways in macrophages and dendritic cells will be described later in this review, and several studies have documented stimulation of dendritic cell function by probiotics. Dendritic cells treated by probiotics will subsequently drive effector T lymphocyte proliferation and function in response to specific antigens (*Baba* et al., 2009; *Mileti* et al., 2009).

Regulatory T lymphocytes may suppress the functions of effector T cells, and the functions of Treg populations may be enhanced by probiotics and beneficial microbes. Diverse microbes such as *B. lactis* W51, *L. acidophilus* W55, and *L. plantarum* W62 induce $FOXP3^+$ Treg cell differentiation, and $FOXP3^+$ Treg cells demonstrate a suppressive phenotype that is contact-dependent with T effector cells (*Izcue* et al., 2009). TGF-β-expressing regulatory T cells were induced by a probiotics cocktail (VSL#3), and these cell subpopulations were associated with protection against colitis (*Di Giacinto* et al., 2005). Conversely, probiotics may inhibit the functions of regulatory T cells, thereby promoting more robust immune responses to pathogen or vaccine challenges. Three of six probiotic strains, *L. acidophilus* NCFM and *B. bifidum* (2 strains), suppressed Treg activity in a contact-dependent manner by modulation of spleen-derived APCs. Splenic entero-antigen-presenting cells (APCs) were exposed to individual probiotic strains and used to stimulate $CD4^+CD25^-$ proliferative T cells in the presence of absence of Treg cells (*Schmidt* et al., 2010). The proliferation of $CD4^+CD25^-$ cells was effectively enhanced by probiotic-mediated suppression of Treg function.

IMMUNE SIGNALLING IN INTESTINAL EPITHELIAL CELLS

Some probiotics stimulate NFκB activation, and consequently these microbes promote immunity and increase cytokine secretion (Figure 1). The commensal anaerobe *Bacteroides vulgatus* activates NFκB in intestinal epithelial cells via TLR4 signalling, interleukin-1 receptor associated kinase-1 (IRAK1) degradation, and RelA phosphorylation. The end-result is enhanced transcriptional activity of NFκB secondary to increased DNA binding capacity. The presence of peripheral blood mononuclear cells counteracts the effects on intestinal epithelial cells, resulting in suppression of NFκB activation (*Haller* et al., 2002), and these results indicate that different cell types can modulate signalling pathways in response to microbial agonists. The cytokine interleukin-6 (IL-6) has important roles in the promotion of innate and adaptive immune responses. For example, *Bifidobacterium lactis* BB12 increased IL-6 secretion by transient induction of RelA. RelA is the p65 sub-unit of NFκB that is the active component responsible for transcriptional activation of multiple cytokine genes. B. *lactis* BB12 also stimulates p38 MAP kinase by phosporylation, and both RelA and p38 MAP kinase are necessary for induction of IL-6. Stimulation of IL-6 is dependent on the Toll-like receptors, specifically TLR2 (*Ruiz* et al., 2005), and TLR2 has also been implicated in suppression of IL-6 production using the porcine IPEC-J2 line (*Liu* et al., 2010).

Microbial signals may also modulate the activity of hormone receptors that may result in attenuation of intestinal inflammation. The nuclear hormone receptor, PPARγ, is one such target that may contribute to cycling of transcription factors such as RelA in and out of the nucleus (Figure 1). The commensal organism *Bacteroides thetaiotamicron* may diminish secretion of the chemokine interleukin-8 (IL-8) by promoting nuclear export of RelA through a PPARγ dependent pathway (*Kelly* et al., 2004). Other microbes,

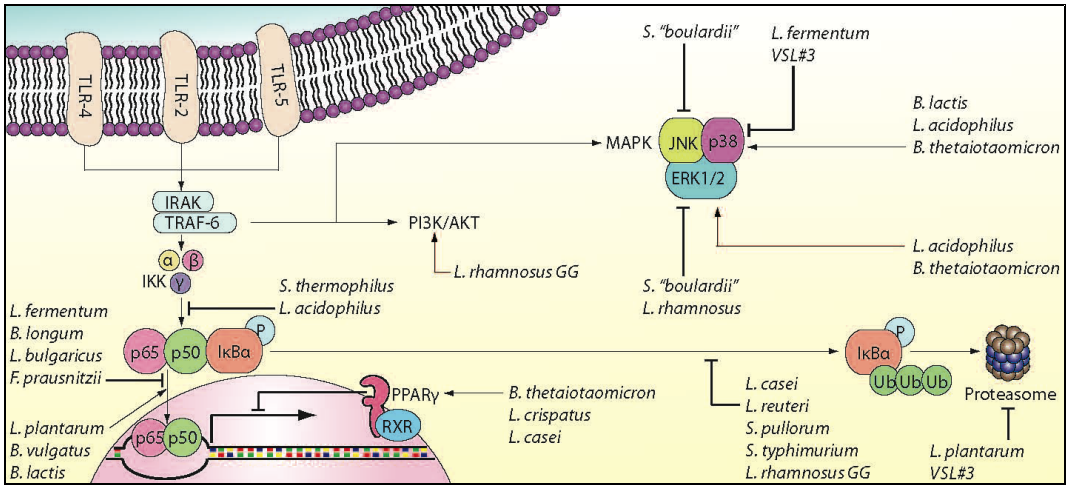

Figure 1: Probiotics modulate key signalling pathways in intestinal epithelial cells. Various probiotics prevent NFκB activation by inhibiting IκBα phosphorylation, ubiquitination, proteasomal degradation, or translocation of NFκB into the nucleus (suppression is indicated by a block sign "⊣"). Probiotics can also enhance RelA export from the nucleus via PPARγ. Other probiotics increase NFκB activation through enhanced translocation into the nucleus (activation is indicated by an arrow sign "→"). Apoptosis of intestinal epithelial cells can be prevented by probiotic modulation of the PI3K/ Akt pathway. Probiotic-induced changes in phosphorylation levels of p38, JNK, and ERK1/2 MAPKs can affect cytokine secretion and apoptosis. ERK, extracellular signal-regulated kinases; IκBα, inhibitor of NFκB α; IKK, IκB kinase; IRAK, interleukin-1 receptor-associated kinase; JNK, c-Jun N-terminal kinase; P, phosphorylation; PPARγ, peroxisome proliferator activated receptor-γ; RXR, retinoid X receptor; TLR, Toll-like receptor; Ub, ubiquitin. [Reprinted from: Thomas, C.M. and Versalovic, J.: Probiotics-host communication: Modulation of signaling pathways in the intestine. Gut Microbe 1, 1-16 (2010)].

such as the lactobacilli, *Lactobacillus crispatus* and *Lactobacillus casei*, also enhance signalling via PPAR-γ. In addition to inhibition of cytokines, modulation of PPARγ by *Enterococcus faecalis* may also stimulate production of regulatory cytokines such as interleukin-10 (IL-10) (*Are* et al., 2008). *E. faecalis* is a commensal bacterium that colonizes the human intestine early in infancy. So, microbes may stimulate immune signalling pathways that promote immune tolerance and reduce inflammation. Conversely, suppression of signalling pathways such as NFκB or MAP kinases may alleviate disease phenotypes such as colitis, depending on the genetic background of the host.

One published study examined the effects of oral ingestion of one probiotic strain on mucosal signalling pathways in the proximal small intestine. In this study, gene expression profiles were gathered from samples of duodenal mucosa in human volunteers 6 hours after oral ingestion of *Lactobacillus plantarum* strain WCFS1 (*van Baarlen* et al., 2009). This strain yielded different gene expression profiles depending on the growth phase of the organism. Stationary phase *L. plantarum* increased expression of several genes involved in NFκB signalling, whereas midlog phase *L. plantarum* modulated genes involved in the cell cycle and cell proliferation such as MYC and cyclin D1. The central lesson of this study is that the physiologic state of microbes affects the production of microbial signals that may differentially modulate mammalian signalling pathways.

In summary, probiotics and beneficial microbes may modulate different signalling pathways involved in innate or adaptive immune responses (Figure 1). Microbial signals may activate or suppress NFκB signalling, and activation of NFκB signalling may stimulate cytokine production and enhance immune "readiness." NFκB signalling may be regulated at the level of the inhibitor IκB, entry of NFκB sub-units into the nucleus, or extrusion of sub-units from the nucleus via PPARγ-dependent pathways. Microbes may also modulate MAP kinases such as JNK or p38, resulting in immune activation. Populations of microbes may produce complex combinations of counteracting signals so the net effect on mammalian physiology may depend on relative quantities and the physiologic status of complex microbial communities.

IMMUNE SIGNALLING IN MACROPHAGES AND DENDRITIC CELLS

Innate immunity may be enhanced by beneficial microbes as a consequence of the stimulation of pattern recognition receptors and various signalling pathways. Prior studies showed that microbial stimulation of Toll-like receptors was important for maintenance of homeostasis in the intestine, and microbial eradication or lack of MyD88 signalling increased the vulnerability of the host to chemical challenge (*Madara*, 2004; *Rakoff-Nahoum* et al., 2004).

Some probiotic strains can stimulate innate immunity by activating NFκB or STAT signalling pathways in macrophages (Figure 2). STATs are cytoplasmic proteins that may become activated by cytokine or antigen signals, resulting in functional transcription factors after nuclear translocation. *Lactobacillus crispatus* induced the production of pro-inflammatory TNF and interleukin-1β (IL-1β) following the activation of NFκB in the human myeloid cell line THP-1 (*Klebanoff* et al., 1999). The established probiotic strain, *Lactobacillus rhamnosus* GG (ATCC 53103), induced DNA binding by STAT1 and STAT3, resulting in enhanced immune signalling in human PBMCs (*Miettinen* et al., 2000). Different *Lactobacillus* species may counteract each other, so that the net effect on dendritic cell populations may depend on relative quantities or potencies of microbial signals. For example, *L. casei* strain CHCC3139 induces production of IL-12, IL-6, and TNF by dendritic cells, but *L. reuteri* DSM 12246 counteracts this effect and suppresses the production of these cytokines in the presence of *L. casei* CHCC3139 (*Christensen* et al., 2002).

MICROBIAL SIGNALS THAT TRIGGER IMMUNE STIMULATION

Complex microbial communities in the intestine may secrete or present diverse signals that serve to enhance immune responses in the mammalian host. Germ-free animals have poorly developed mucosal immune systems with a relative paucity of lymphoid tissue (*Brandztaeg*, 2009). Individual microbial species or microbe-derived molecules with defined immunostimulatory activities have been challenging to isolate from this complex assemblage of microorganisms, but recent studies highlight exciting new findings. From hundreds of possible microbial species, a single organism could be identified

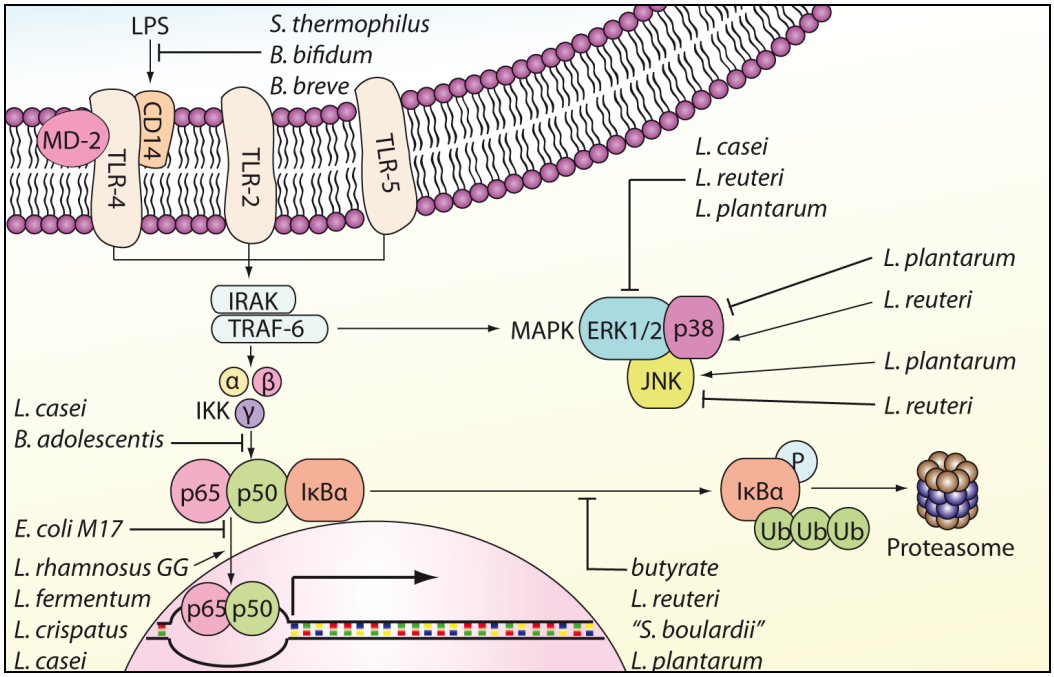

Figure 2: Probiotics modulate inflammatory signalling pathways in macrophages. Select probiotics can block binding of LPS to the CD14 receptor, interfering with LPS signal transduction. Various probiotics prevent activation of NFκB by decreasing phosphorylation or ubiquitination of IκBα or blocking NFκB translocation into the nucleus (suppression is indicated by a block sign "⊣"). NFκB activation is enhanced by other probiotics via increased nuclear translocation of transcriptionally active NFκB sub-units (activation is indicated by an arrow sign "→"). MAPK proteins p38, JNK and ERK1/2 are also targets of probiotic modulation in macrophages. ERK, extracellular signal-regulated kinases; IκBα, inhibitor of NFκBα; IKK, IκB kinase; IRAK, interleukin-1 receptor-associated kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MD-2, myeloid differentiation 2; P, phosphorylation; TLR, Toll-like receptor; Ub, ubiquitin. [Reprinted from: Thomas, C.M. and Versalovic, J.: Probiotics-host communication: Modulation of signaling pathways in the intestine. Gut Microbe 1, 1-16 (2010)].

with potent effects on mucosal immunity. A fascinating combination of studies by two research groups demonstrated the requirement of a specific commensal intestinal microbe for effective induction of Th17 effector cell populations in the mammalian intestine (*Gaboriau-Routhiau* et al., 2009; *Ivanov* et al., 2009). This uncultured organism was identified as *Candidatus Arthromitus*, or Segmented Filamentous Bacterium (SFB), by high density micro-array studies with the PhyloChip (*Ivanov* et al., 2009). Mouse colonies that were defective in this missing microbial ingredient lacked robust populations of Th17 lymphocyte populations, and these cell populations normally produce cytokines such as IL-17 or IL-22 that contribute to protection of the host from bacterial and fungal infections.

A mutant strain of *L. plantarum* NCIMB8826, defective in D-alanylation of cell surface lipoteichoic acids, induced production of the regulatory cytokine IL-10 while suppressing production of pro-inflammatory IL-12 (*Grangette* et al., 2005). These findings suggest that cell wall components such as lipoteichoic acids may play an important role in immunomodulation by

probiotics, and the alanyl component of lipoteichoic acids may serve to stimulate immunity or oppose immunosuppressive features of the microbiome. Consistent with this concept, lipoteichoic acids from *L. casei* YIT 9029 and *Lactobacillus fermentum* YIT 0-159 activated NFκB signalling and induced murine TNF production by mouse macrophages (*Matsuguchi* et al., 2003. Alanylation of lipoteichoic acids correlates with upregulation of IL-12p40 by mammalian cells when treated with *Lactobacillus plantarum* L-137 (*Hirose* et al., 2010), and conversely, IL-10 induction in mouse peritoneal macrophages by *L. plantarum* depends on ERK activation (*Kaji* et al., 2010). Presumably, differences in structures of lipoteichoic acids such as patterns of amino acid or glycosyl modifications may affect relative propensities to stimulate or suppress mammalian immune signalling pathways.

In addition to cell surface components, bacterial nucleic acids may also be released from beneficial microbes and stimulate immune responses. CpG oligodeoxynucleotides (ODN) derived from commensal bacteria upregulated immune responses via Toll-like receptor signalling pathways. CpG ODN from *Streptococcus thermophilus* induced upregulation of IL-33 (*Shimosato* et al., 2010), and CpG-rich sequences from different *Bifidobacterium* species stimulated production of MCP-1 and TNF by murine macrophages (*Menard* et al., 2010). Stimulation of murine MCP-1 and TNF by RAW 264.7 cells is mediated by enhanced TLR9 signalling. While CpG ODN have potent immunostimulatory effects in mouse models, results in human vaccination models have been disappointing. Bacterial DNA derived from *Lactobacillus rhamnosus* GG or *Bifidobacterium longum* suppressed chemokine production and NFκB signalling in polarized human intestinal epithelial cells. The addition of DNA to cultured human intestinal epithelial cells suppressed TNF-induced NFκB activation and NFκB-mediated IL-8 production via inhibition of IκBα degradation and p38 phosphorylation (*Ghadimi* et al, 2010a). Probiotic-derived DNA sequences may have different effects in human cells, versus mouse cells, despite the fact that effects in both mammalian systems depend on TLR9 signalling.

VACCINATION AUGMENTATION STRATEGIES

Probiotics may stimulate Th1 responses by enhancement of interferon-γ production from peripheral blood mononuclear cells (PBMCs) and human monocyte-derived macrophages (HMDMs), and beneficial microbes can suppress production of Th2 cytokines such as IL4 and IL13 (*Ghadimi* et al., 2010b). Co-treatment with *Mycobacterium tuberculosis* antigen with either *L. rhamnosus* LGG or *B bifidum* resulted in the stimulation of IFN-γ and NO production, resulting in greater IFN-γ / IL4 and IFN-γ / IL13 ratios. Autophagy biomarkers such as Beclin-1 and LC3-I were induced by treatment of PBMCs with *M. tuberculosis* antigen and either LGG or *B bifidum*. In this model, the presence of a vaccine-related antigen and probiotics stimulated autophagy and an associated Th1 response; these results suggest that probiotics may effectively augment vaccination strategies for *M. tuberculosis* and other microbial pathogens.

Probiotics can stimulate immune responses to pathogens and augment vaccination strategies via enhanced mucosal and systemic immunity. Probiotics enhance protection by the influenza vaccine (*Namba* et al., 2010; *Olivares* et al., 2007). The ingestion of human breast milk-derived *L. fermentum* enhanced the production of antigen-specific IgA following intramuscular influenza vaccination and reduced the incidence of influenza infection in the probiotics group (*Olivares* et al., 2007). *Lactococcus lactis* engineered to produce pneumococcal protective protein A induced effective protection against *Streptococcus pneumoniae* infection in mice via nasal vaccination (*Vintini* et al., 2010).

SUMMARY AND FUTURE DIRECTIONS

In summary, beneficial microbes including probiotics may serve as potent stimulators of mucosal and systemic immunity. As microbial communities have co-evolved with the immune systems of mammals for thousands of years, it is reasonable to suggest that microbes have played an important role in the development of immunity in the context of human individuals and entire populations. Mankind should exploit the fruits of human microbiome research and mucosal immunology to effectively couple vaccination strategies with probiotics and commensal microbiology.

New vaccine strategies may include combinations of micronutrients, recombinant vaccines, and probiotics to enhance the success rates of mucosal vaccination strategies in the developing world. In the context of undernutrition, novel approaches may be necessary to deliver efficacies comparable to the success stories in the developed world. Firstly, nutrition should be considered as part of the overall strategy for improving efficacy of vaccines in the developing world. The delivery of micronutrients and adequate nutritional support enables each child to fully develop immunity and responsiveness to vaccine challenges. Breastfeeding is a primary source of nutrition in infancy, in addition to its role in maternal:infant bonding. The quality of the breast milk is dependent on maternal nutrition so that the mother's diet becomes an important consideration for any comprehensive disease prevention strategy during infancy and early childhood. Supplementation of the maternal diet with probiotics, prebiotics, and other nutrients may maximize the production of complex saccharides in human breast milk and facilitate the establishment of a "beneficial breast milk microbiome". Bifidobacteria and other species are considered to be part of human breast milk in healthy, lactating women, and these breast milk-associated microbes may lay the foundation for the human intestinal microbiome.

The presence of complex microbial communities on mucosal surfaces early in life promotes the development and differentiation of a robust immune system. The combination of adequate nutritional support and a probiotics/prebiotics strategy will provide the "substratum" for mucosal immunity to flourish in children. Vaccine challenges with the proper mucosal adjuvant(s) will be poised to succeed if delivered on a solid foundation of nutrition and a rich microbiome. Enteric vaccines may be re-engineered to combine the best of both worlds by creating recombinant vaccines within probiotic strains as delivery vectors. Such recombinant

vaccines could merge the microbial-derived immunostimulatory signals and adjuvant-like properties of probiotics with the specific antigenic challenge. New research tools such as "humanized" mouse models, or mice with a human-derived microbiome and a human-like immune system, may enhance research in mucosal vaccinology and combination strategies with probiotics. New tools for clinical research may include micro-volume assays for different antibody subclasses and T lymphocyte function, and new protein arrays that can provide more complete assessment of immunity in the field. Our hope is that new research tools, when combined with nutritional support and microbiome-reshaping strategies for vaccine delivery, will point the way towards improved success rates with vaccine strategies for enteric and systemic infections in the developing world.

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EFFECTS OF MALNUTRITION AND MICRONUTRIENT DEFICIENCY ON HYPO-RESPONSIVENESS TO ORAL VACCINES: WHAT CAN BE DONE TO OVERCOME THIS?

FIRDAUSI QADRI

Immunology Unit, International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dhaka, Bangladesh

SUMMARY

The purpose of this review is to bring to light, important factors that may contribute to lowered immune responses to vaccines in children in developing countries. Of the over 546 million under five year children in developing countries, 50 million are severely underweight. These children are also those most at risk of death due to malnutrition and infectious diseases, both of which follow a vicious cycle. Malnutrition includes both macronutrient and micronutrient deficiencies. Deficiencies in vitamins and trace elements are present in children in developing country settings because of the lack of access to food and also because of the lack of the knowledge for accessing these from available and affordable sources. Identifiable micronutrient deficiencies seen in countries that have been studied in the developing world include vitamin A and zinc. Natural infection studies show that zinc enhances innate and adaptive immune responses to enteric bacterial and parasitic infections. Vitamin A supplementation results in improved immune responses to both mucosal and parenteral vaccines. Additive effect of zinc and vitamin A has also been seen to oral cholera vaccine in children 2-5 years of age. Ways to implement such strategies to increase immune responses in younger children have been undertaken. Infants 10-18 months of age responded with increased vaccine specific immune responses while this effect was not seen in younger children. Overall, the analyses of data from different studies show that improvement of immunogenicity of natural infections and vaccines can be made with micronutrient interventions. The use of these strategies for improvement of vaccine efficacies and health of children is a challenge that will need to be met in order to improve lives of children in developing country settings and to meet the millennium development goals using available vaccines.

REVIEW AND DISCUSSION

The need for better understanding of the barriers that cause oral vaccines to be less effective in children in developing countries has become imperative. We ask ourselves time and again the reason as to why hypo-responsiveness to oral mucosal vaccines is being seen (*Sack* et al., 2008). The potential causes of hypo-responsiveness may include frequent breast-feeding practices in de-

veloping countries, pre-existing immunity, malnutrition, high parasitic load and also genetic and environmental factors. These factors may synergistically interact to cause the detrimental effect of lowered responses to oral vaccines.

The review is based on evidence obtained from different studies where Vitamin A and/or zinc have been used to improve immune responses in natural infection or vaccination. Vitamin A supplementation is known to decrease morbidity and mortality and also enhances the immune system. Zinc is a trace element and is needed for functional and structural integrity of proteins. Deficiency results in hampering of the immune system rapidly and extensively, more than it affects other organs and tissues. Deficiency also results in high rates of infectious diseases as well as a decrease of the humoral and cell mediated immune responses of the body. Zinc given as daily doses has been studied in children with natural bacterial infection in shigellosis, cholera and enterotoxigenic *E. coli* (ETEC) diarrhoea. It has also been used to study effect on enteric parasitic infections e.g. *Giardia lamblia* infections in children, *Entamoeba histolytica* associated diarrhoea and *Ascaris lumbricoides* infections (*Long* et al., 2007). In shigellosis, 14 days of supplementation with zinc resulted in increased B cells, proliferation of lymphocytes, plasma cells, shigellacidal killing activity and reduced duration of diarrhoea (*Raqib* et al., 2003, 2004, *Roy* et al., 2008). A randomized controlled trial was carried out to determine the benefit of zinc treatment (20 mg/day for 10 days), followed by zinc supplementation (10 mg/day for 3 months) on the clinical and immunological outcome of acute watery diarrhoea in children 6-24 months of age in a low income urban setting in Dhaka city in Bangladesh (*Larson* et al., 2010, *Sheikh* et al., 2010). The children were followed for 9 months after initiation of the study. In addition a sub-group of children with diarrhoea caused by ETEC were studied to determine the effect of this intervention on the innate and adaptive immune responses. Zinc supplementation followed by zinc treatment resulted in an additional 30% reduction in diarrhoeal incidence during the period of the intervention (*Larson* et al., 2010). It also provided an additional 20% reduction in acute diarrhoea and 12% reduction of the duration of diarrhoea over 9 months period of the study. There was no impact on acute respiratory infections in the children in this study. In children with ETEC diarrhoea in the cohort, an increase of complement C3 was seen on initiation of zinc treatment in comparison to the levels seen in children who were not given the interventions (*Sheikh* et al., 2010). The levels remained elevated in both the treatment and supplementation groups over the duration of the study. Increased phagocytic activity of granulocytes and monocytes were also observed. A reduction of reactive oxygen species in these cells was observed, suggesting that zinc decreased oxidative stress. A decrease in memory T cells and an increase of the naïve to memory T cell ratio was seen. The adaptive immune response to vaccine antigens, e.g. tetanus toxoid and diphtheria toxoid remained unchanged. There was no effect on the IgA and IgG antibody response to the heat labile toxin (LT) of ETEC contrary to that seen in the immune response in toddlers given Dukoral together with supplementation of zinc (*Qadri* et al., 2004).

Elegant studies in experimental models suggest that there is a role of the gut microbiota in altering the mechanism by which the immune re-

sponse becomes tuned to produce a less effective and appropriate response to pathogens, antigens and vaccination (*Sack* et al., 2008). The purpose of this review is to see what happens in the natural setting in developing countries and the factors that are responsible for lowered immune responses and determine ways to enhance these responses. Infants and children time and again are not responding to oral vaccines in rates seen in children in industrialized settings with high GDI/GDP (gross domestic product/gross domestic income). A major factor leading to such lowered responses in the children in developing countries is the high rates of malnutrition and micronutrient deficiency. The largest number of under-five children is in the developing countries of the world. About 90% of children are the developing countries including the less developed countries. About 50.6 million children are malnourished of whom 90% are in the developing world (*Faruque* et al., 2008). Major micronutrient deficiencies that have been identified include vitamin A and zinc and other trace elements. Malnutrition includes both macronutrient and micronutrient deficiencies. Mortality in severely malnourished children can be decreased by protocolized management interventions. Deficiencies in vitamins and trace elements exist due to lack of access to costly food and the knowledge to access it from affordable sources.

The role of micronutrients on the immune response to vaccines is reviewed. Hypo-responsiveness to many vaccines have been seen which include oral polio vaccine, the oral typhoid vaccine (Ty21A), Shigella vaccine (SC602), rotavirus vaccine (Rotarix, RotaTeq), cholera vaccines (Dukoral, CVD103HgR, Peru-15). It has not been studied whether this lowered immune response can also be observed after using parenteral vaccines and we do not know how this reflects on vaccination of adults living in developing countries.

The effect of vitamin A deficiency on the yellow fever vaccine (YFV) in adults is a question that was asked in a recent analysis (*Ahmad* et al., 2008). Adults with low vitamin A store were compared with those with high vitamin A stores. A distinct difference was seen in the response to YFV specific proliferation of peripheral blood mononuclear cells and TNF-α production which also correlated with whole body vitamin A stores.

In summary, vitamin A supplementation in Bangladeshi adults resulted in increased YFV and tetanus toxoid specific proliferative responses. It also resulted in increased YFV specific IL-5, IL-10 and TNF-α responses. The available results suggest that adults may also need to be targeted for micronutrient supplementation to achieve better responses. The response to oral vaccines has not been studied in this age group or in the elderly. The reasons for vaccine failures can be better targeted when all age groups have studied and compared.

Studies in toddlers (2-5 years of age) showed that daily dosing with 20 mg/day of zinc sulphate for three weeks prior to vaccination increases the innate immune response in natural disease, diarrhoeal diseases caused by bacterial or parasitic microbes.

An additive effect of zinc and vitamin A supplementation was seen when these two micronutrients are given together to 2-5 year old children and immunization with the oral cholera vaccine was carried out (*Albert* et al., 2003). When zinc was given prior and during oral cholera vaccination to younger infants and children, this resulted in an increased vaccine specific vibriocidal antibody response (*Ahmed*

et al., 2009). This effect was seen in children 2-5 years of age and in those 10-18 months of age but not in younger children 6-9 months of age. This is an important observation and caution needs to be taken when these results are extrapolated to immunization strategies for other vaccines and even younger age groups.

CONCLUSIONS

Overall, the analyses of the available data suggest that improvement of immunogenicity of natural infections and vaccines can be made with micronutrient interventions. The use of these strategies for improvement of vaccine efficacies and health of children is a challenge that will need to be met in order to improve lives of children in developing country settings and to meet the target of the millennium development goal towards improvement of lives and decreasing childhood deaths.

ACKNOWLEDGEMENTS

The work presented is from collaborative work of F. Qadri with scientists at the ICDDR,B (researchers and colleagues), University of Gothenburg (Ann-Mari Svennerholm) and at the Johns Hopkins University (David Sack). Funding for different aspects of the work is acknowledged to Sida, Bill and Melinda Gates Foundation and funds from the ICDDR,B.

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SUBLINGUAL DELIVERY OF VACCINES

LOUISE B. LAWSON, LUCY C. FREYTAG, and JOHN D. CLEMENTS

Department of Microbiology and Immunology,
Tulane University School of Medicine, New Orleans, USA

SUMMARY

Sublingual immunization, where the vaccine is placed directly under the tongue and absorbed through the sublingual mucosa, has the advantages of ease of delivery without the complication of oral administration or the safety concerns of intranasal vaccines. Sublingual vaccine delivery has been shown to induce development of humoral and cell mediated immunity in both the systemic and mucosal compartments to a variety of bacterial, viral, and protozoan antigens. Sublingual immunization represents and exciting breakthrough in vaccine delivery with great potential to overcome the barriers to effective mucosal immunization; this strategy holds great promise specially for vaccinating infants and children in developing countries.

INTRODUCTION

A great deal of effort is being directed towards developing non-parenteral (needle-free) alternatives to traditional vaccine delivery. Non-parenteral vaccines offer a number of potential advantages over traditional vaccines including:

1) the ability to confer mucosal as well as systemic immunity,
2) increased stability,
3) increased shelf-life,
4) elimination of needles and the need for specially trained healthcare workers to administer vaccines, and
5) lower costs.

Nasal delivery of vaccines has demonstrated efficacy in numerous animal models and in humans (e.g., FluMist®) but there are safety concerns regarding some antigens and adjuvants administered intranasally (*Lewis* et al., 2009). Transcutaneous delivery (e.g., by skin patch) has shown potential but requires large amounts of antigen and is device-dependent (*Glenn* et al., 2007; *Frerichs* et al., 2008). Oral immunization is effective for some live-attenuated vaccines (e.g., poliovirus, rotavirus, typhoid fever) and killed whole-cell vaccines (e.g., cholera). However, oral immunization with non-replicating vaccines requires large antigen doses and some means to bypass or neutralize the gastric acidity; furthermore, the outcome of oral vaccination may be impacted by the age and immune status of the host, the presence of maternal antibody in infants, colonization and growth of microbial flora in the bowel, and numerous other factors.

The sublingual mucosa has been a site for administration of therapeutic drugs for over a century. This tissue is highly vascularized, which allows rapid entry of antigens into the systemic circulation and avoidance of the harsh pH

and proteases found in the gastrointestinal tract and first-pass metabolism of the liver (*Zhang* et al., 2002). As with sublingual drug delivery, the ease of administration, safety benefits and increased patient compliance also make this route attractive for vaccine delivery. Sublingual immunization (SLI) is accomplished by placing the immunizing preparation directly under the tongue where it is absorbed through the sublingual mucosa. The presence of immune sentinel cells within the oral mucosa makes it a suitable site for direct antigen uptake and initiation of an immune response. Moreover, SLI induces immune responses systemically as well as in distal mucosal compartments, making this a promising delivery route for mucosal protection.

ANTIGEN PERMEATION, UPTAKE AND PROCESSING WITHIN THE SUBLINGUAL MUCOSA

Different compartments of the oral mucosa, specifically the lining, masticatory, and lingual and sublingual mucosa, exhibit different structural, immunological and chemical properties, and consequently have different permeabilities to exogenously applied proteins and other substances. The sublingual mucosa of humans is covered with a non-keratinized stratified squamous epithelial layer. As a result, this surface is more permeable than other regions of the oral mucosa, such as the gingiva and hard palate, which are keratinized and more closely resemble the epidermis of the skin (*Squier*, 1991). However, permeation does not occur freely, since the presence of extruded amorphous intercellular lipids provide some barrier to chemical permeation in the supra-basal sublingual mucosa; and salivary mucins also inhibit permeability of some substances at the sublingual surface (*Squier*, 1991).

In marked contrast to humans, rodents have a highly keratinized sublingual mucosa; but despite this difference, mice are suitable models for preclinical evaluation of sublingual vaccines. Using this animal model, it has been observed that small volumes of sublingually applied antigens stay localized and do not "spread" to other tissues. When fluorescently-labelled ovalbumin (OVA) was administered sublingually in a small volume (5-10 µl) to mice, the antigen remained exclusively on the sublingual mucosa for up to two hours. No fluorescent OVA was detected in the buccal tissues, palate, oesophagus, or small intestine, indicating that initiation of the immune response occurs specifically within the sublingual mucosa. When larger volumes were sublingually applied, some OVA was swallowed and detected in the oesophagus and duodenum (*Çuburu* et al., 2007).

Resident and migratory antigen-presenting cells (APCs) of the sublingual mucosa are directly involved in antigen transport to the draining lymph nodes. The sublingual lamina propria of naive mice contains a wide distribution of leukocytes, including MHC-II$^+$ cells, located mainly along and beneath the basal layer of the epithelium. Almost all of these are CD11c$^+$ dendritic cells, and represent 3-4% of all cells in the sublingual compartment. Within 2 hours of sublingual administration of cholera toxin (CT), the number of MHC-II$^+$ dendritic cells in the submucosa and epithelium increased, indicating a rapid recruitment to the site of administration (*Çuburu* et al., 2007; *Song* et al., 2009); the dendritic cell

numbers then returned to basal levels 6 hours post-treatment (*Çuburu* et al., 2007).

Typically associated with antigen uptake and presentation in the skin epidermis, Langerhans cells represent a small population of cells interspersed in the human sublingual epithelia (*Allam* et al., 2008). In mice, the presence of langerin^{+} cells, a hallmark of Langerhans cells, has also been noted in naive animals (*Çuburu* et al., 2007; *Song* et al., 2009; *Hervouet* et al., 2010). Depletion of this cell population had no impact on CD4 T-cell proliferation in draining lymph nodes after SLI in the murine model, suggesting that these cells are not essential for activation of CD4 T-cells (*Song* et al., 2009. However, sublingual langerin^{+} cells did induce proliferation of CD8 T-cells, indicating that these cells are functional APCs. Their role is distinct from langerin^{-} dendritic cells, which were effective in inducing both CD4 and CD8 T-cell proliferation (*Hervouet* et al., 2010). Dendritic cells of the sublingual tissue prime B- and T-cells in the draining lymph nodes via the CCR7-CCL19/CCL21 pathway (*Song* et al., 2009). This pathway has similarly been shown to regulate skin dendritic cell entry into the dermal lymphatics (*Ohl* et al., 2004). A role of resident macrophages in shaping the immune response to sublingually applied antigens has also been reported (*Jee* et al*.,* 2010). Within the human sublingual mucosa, mast cells localizing in the gingiva along basal cells and within lobes and ducts of glands are also known to participate in the ensuing immune responses (*Allam* et al., 2008). It is likely that other cell populations of the sublingual mucosa have some impact on the immune response to antigens and adjuvants; however, the direct or indirect role of other cell populations is not yet fully understood.

IMMUNE RESPONSE TO SUBLINGUALLY-ADMINSTERED ANTIGEN

Humoral response to sublingual immunization

The ability of sublingual immunization to induce a humoral immune response systemically as well as in multiple mucosal compartments has been demonstrated with a variety of viral, bacterial, and protozoan antigens. In the murine model with OVA in the presence of CT as a mucosal adjuvant, a significant systemic antibody response was observed upon sublingual administration, as shown in Figure 1. The response to sublingually-applied OVA was similar in magnitude and isotype distribution to the response seen with intranasal immunization and both sublingual and intranasal responses were enhanced when compared with intragastric administration (*Çuburu* et al., 2007). BenMohamed and co-workers (*BenMohamed* et al., 2002) also noted a strong systemic immune response upon SLI with synthetic lipopeptides derived from *Plasmodium falciparum*; importantly these antibodies were able to recognize and bind intact parasites.

Antibody-production in the gastrointestinal tract has also been noted in response to SLI. Negri and co-workers noted detectable salivary IgA up to four months post-immunization (*Negri* et al., 2010). Likewise, Song and co-workers detected significant IgA in saliva and faecal extracts of mice (*Song* et al., 2008). This suggests the suitability of SLI for vaccines against enteric pathogens that primarily colonize the gastrointestinal epithelium.

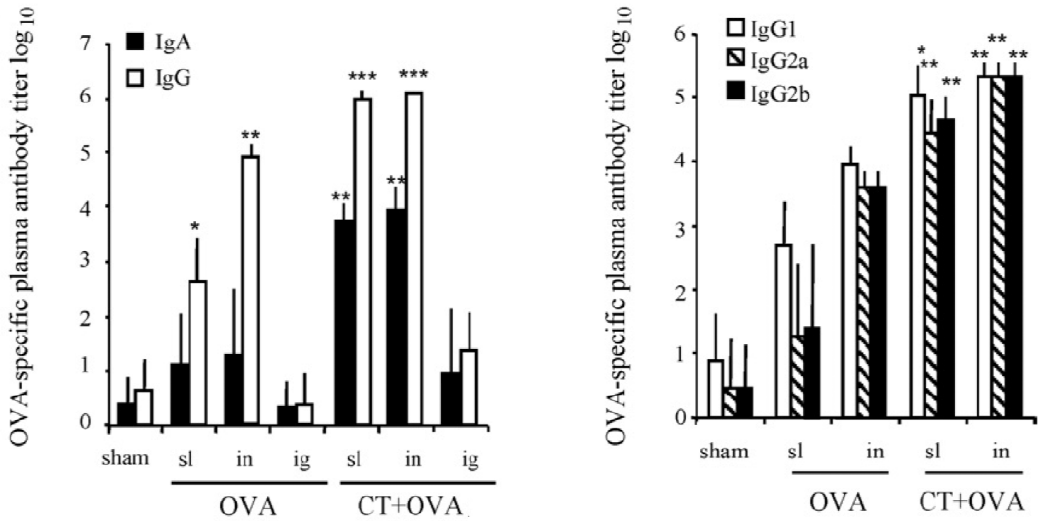

Figure 1: Systemic antibody levels upon sublingual (sl), intranasal (in), and intragastric (ig) immunization with ovalbumin and cholera toxin (CT). From *Çuburu* et al. 2007; with permission.

Generation of antibody responses in the genital tract is also an important aspect of SLI and suggests that this route of immunization could be promising for vaccines against sexually transmitted diseases, as well. Sublingual administration of a human papillomavirus antigen (HPV16L1) induced higher vaginal secretory IgA than intravaginal administration of a larger dose of the same antigen. Intranasal administration also elicited significant vaginal secretory IgA, but saliva antibody production was lower than with sublingual delivery (*Cho* et al., 2010). In a recent investigation, sublingual administration of an HIV-1 ectodomain protein was shown to induce antigen-specific IgG and IgA in genital secretions at levels comparable to levels induced by local intravaginal immunization (*Hervouet* et al., 2010).

In the respiratory tract, antigen-specific antibodies were detected following SLI with OVA. Significant OVA-specific IgG and IgA titres were detected in the nasal wash and broncho-alveolar lavage of immunized mice (*Çuburu* et al., 2007). Immunization with inactivated influenza virus also yielded significant anti-viral IgA and IgG in lung and nasal wash fluids (*Song* et al., 2008).

The origin of antibodies produced in response to SLI has been assessed to better understand the response to sublingual vaccination. Upon SLI with inactivated influenza virus administered in conjunction with a chimeric mucosal adjuvant composed of the B subunit of LT and the A subunit of CT (mCTA/LTB), IgA antibody-secreting cells were detected in various mucosal tissues, including the lung, nasal passage, submandibular glands, and small and large intestine, in addition to the spleen (Figure 2) (*Song* et al., 2008). Çuburu and co-workers detected IgG- and IgA-secreting cells in the lung, spleen and submandibular lymph nodes but not in the mesenteric lymph nodes following SLI (*Çuburu* et al., 2007). Antibody-secreting cells in the genital tract were also noted in response to SLI with an HIV antigen. While antigen-specific antibody-secreting cells were

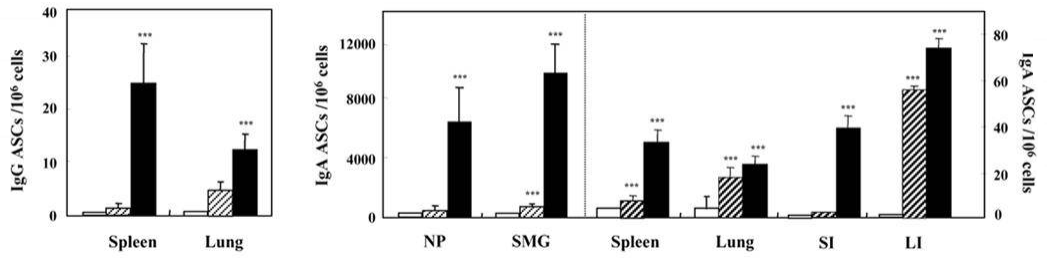

Figure 2: IgG and IgA antibody-secreting cells were detected in the spleen and mucosal compartments of mice treated sublingually with PBS (open bar), inactivated A/PR/8 influenza virus (hatched bars), or inactivated virus plus a mutant chimera of the B subunit of LT and the A subunit of CT (mCTA/LTB) (closed bars). Antibody-secreting cells were measured in the spleen, lung, nasal passage (NP), submandibular gland (SMG), small intestine (SI), and large intestine (LI). From *Song* et al., 2008; with permission.

detected in the genital tract of sublingually immunized mice, none were detected in the ileosacral lymph nodes, which drain the genital mucosa. This suggests that antibody-secreting cells migrated to the genital tract from another location, most likely the draining lymph nodes of the sublingual mucosa (*Hervouet* et al., 2010). CCL28 was shown to be an important chemokine in migration of IgA antibody-secreting cells to the genital tissue upon SLI (*Çuburu* et al., 2009). Indicating the duration of antibody production, antigen-specific antibody-secreting cells were found in the bone marrow of mice immunized with tetanus-toxoid and appropriate adjuvants up to four months post-immunization (*Negri* et al., 2010).

Cell-mediated responses to sublingual immunization

Several investigators have characterized the cell-mediated immune response to sublingually administered antigens. Although the quality of the Th1/Th2 response is influenced by both the antigen and the adjuvant, investigations of CD4 T-cell responses have noted a mixed Th1/Th2 response following SLI with various formulations. This was noted in the spleen and submandibular lymph nodes following SLI (*Çuburu* et al., 2007; *Çuburu* et al., 2009; *Zhang* et al., 2009; *Cho* et al., 2010). The development of CD8 cytotoxic lymphocytes in distal mucosal compartments has been noted as well. Hervouet et al. observed IFN-γ production and cytolytic activity of CD8 cells in the genital tract of mice immunized sublingually with an HIV reverse transcriptase peptide conjugated to the binding subunit of cholera toxin (CT-B) (*Hervouet* et al., 2010). Cytotoxic T cell activity was also detected in the lung following SLI with ovalbumin (Figure 3) (*Çuburu* et al., 2007). CD4 and CD8 T-cell expansion in response to antigen re-stimulation was noted in the spleen, submandibular (regional) lymph nodes, and distal lymph nodes draining the genital mucosa (iliac lymph nodes) four months post-immunization (*Negri* et al., 2010).

Adjuvant incorporation in sublingual vaccines

The incorporation of appropriate adjuvants into sublingual vaccines is critical and enhances the immune responses in many instances (e.g., [*Çuburu* et al., 2007, 2009; *Song* et al., 2008]). The most commonly used adjuvants in SLI are derivatives of the

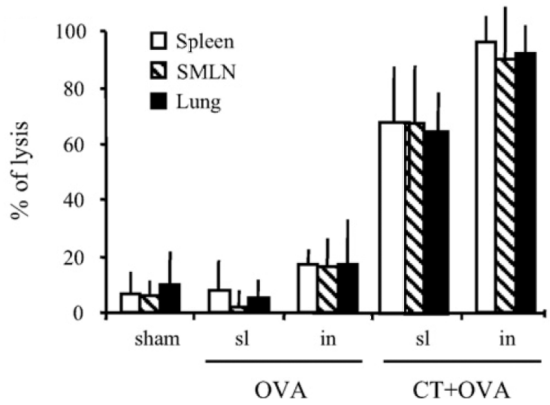

Figure 3: Sublingual (sl) and intranasal (in) immunization with ovalbumin (OVA) with or without cholera toxin (CT) induced *in vivo* cytotoxic activity in the spleen, submandibular lymph node (SMLN), and lung. From *Çubur*u et al., 2007; with permission.

bacterial ADP-ribosylating enterotoxins, CT and LT. In a direct comparison of several adjuvants co-administered with a human papillomavirus protein 16 L1, only the B subunit of CT significantly enhanced mucosal and systemic immune responses. Other adjuvants included various TLR agonists, NOD agonists, vitamin D3, and nanoparticles of a bacterial capsular exopolymer (*Cho* et al., 2010). CpG ODNs have also been successfully used in a sublingual Salmonella vaccine delivered to neonatal mice (*Huang* et al., 2008). Negri and co-workers noted the impact of LT-derived adjuvants in eliciting long-lasting systemic and mucosal immune responses (*Negri* et al., 2010).

Protective efficacy of sublingual immunization

In vivo challenge studies in mice have shown the protective efficacy of SLI against a variety of pathogens. Incorporation of mCTA/LTB with an inactivated whole influenza virus induced complete viral clearance in the

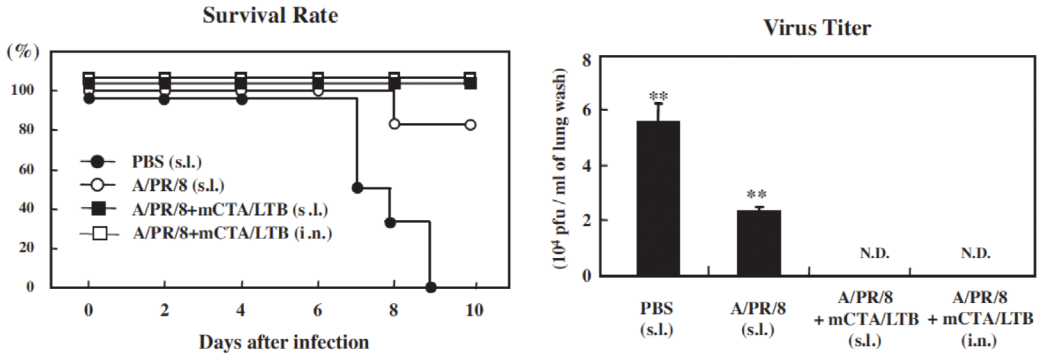

Figure 4: Sublingual immunization with adjuvant conferred complete protection against intranasal viral challenge and resulted in no detectable viral titres in the lung. Mice were immunized sublingually (sl) or intranasally (in) with inactivated A/PR/8 influenza virus with our without a mutant chimera of the B subunit of LT and the A subunit of CT (mCTA/LTB). From *Song* et al., 2008; with permission.

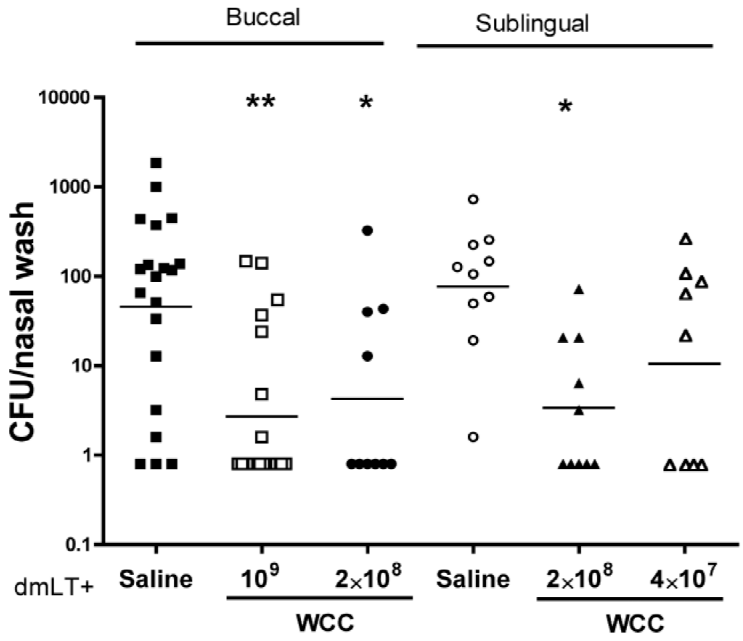

Figure 5: Dose-dependent protection was induced following sublingual and buccal immunization with killed pneumococcal whole-cell vaccine containing a detoxified double mutant of LT (dmLT). Mice were immunized three times and challenged intranasally one week following the last immunization with the indicated dose. Determination of CFU per nasal wash was performed 1 week post-challenge. From *Lu* et al., 2010; with permission.

lungs and 100 percent protection following homologous intranasal viral challenge; without adjuvant, mice had significant viral titre in the lungs 3 days post-challenge and only partial protection (80% survival) as seen in Figure 4 (*Song* et al., 2008). SLI with human papillomaviris virus-like particles resulted in complete protection against genital challenge with papillomavirus pseudovirions (*Çuburu* et al., 2009). Protection against *Porphyromonas gingivalis* was observed in mice immunized sublingually with an outer membrane protein of this organism plus a cDNA vector plasmid encoding a Flt ligand; protection was noted by reduced bone loss following oral challenge (*Zhang* et al., 2009). A detoxified mutant of LT (dmLT) developed in our own laboratory and administered as part of a whole-cell killed pneumococcal vaccine sublingually also conferred significant protection against intranasal challenge as seen in Figure 5 (*Lu* et al., 2010).

SAFETY OF SUBLINGUAL IMMUNIZATION

Several studies have addressed issues related to safety of administering vaccine formulations sublingually because of the potential risk associated with trafficking of intranasally administered antigens to the central nervous system. Live influenza virus administered sublingually in mice was undetectable in the olfactory bulb or brain tissue 24-hours post-inoculation; however, there

was detectable viral RNA and labelled virus in these compartments following intranasal administration (*Çuburu* et al., 2007; *Song* et al., 2008). Additionally, administration of CT-B sublingually resulted in no adverse events or other side effects that have been noted upon intranasal administration (*Cho* et al., 2010).

CONCLUSION

Despite the availability of antibiotics and vaccines, infectious diseases remain a leading cause of morbidity and mortality worldwide, especially in developing countries. Even when vaccines exist, they are often impractical, especially for administration to children. New strategies for vaccine discovery, formulation and delivery are desperately needed, and successful development of vaccines that are safe, well tolerated and effective will have a profound impact in improving health globally. Oral vaccines are potentially advantageous but must overcome a number of physical and physiological barriers (e.g., gastric acidity, age and immune status of the host, maternal antibody in infants and bowel microbial flora) to be effective. Sublingual immunization, where the vaccine is placed directly under the tongue and absorbed through the sublingual mucosa, has the advantages of ease of delivery without the complication of oral administration or the safety concerns of intranasal vaccines. Sublingual vaccine delivery has been shown to induce development of humoral and cell mediated immunity in both the systemic and mucosal compartments to a variety of bacterial, viral, and protozoan antigens. Sublingual immunization represents and exciting breakthrough in vaccine delivery with great potential to overcome the barriers to effective mucosal immunization; this strategy holds great promise specially for vaccinating infants and children in developing countries.

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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.

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