第 45 回日本小児感染症学会招待講演

Mucosal Microbiome and its Impact on Mucosal Immune System in Childhood

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Mammalian mucosal immune system is a complex network of functional elements of innate and adaptive immunity distributed in mucosa associated lymphoid tissue (MALT), including the gut (GALT), Broncho epithelium (BALT), nose, nasopharynx and Waldeyer's ring (NALT), conjunctiva, sublingual tissue (SLLT), mammary glands, and skin and the underlying subcutaneous regions (SALT). Exposure of a diverse spectrum of microbial agents to the organized lymphoid tissue at the inductive sites in GALT, BALT, SLLT, and possibly in SALT is followed by the activation of specific antigen-reactive B and T lymphoid cells and their migration to the lamina propria in the immunized regions as well as in other effector sites in distant mucosal sites. Such migration is highly regulated via the binding of homing receptors expressed on antigen-sensitized lymphocytes and their binding ligands in different homing sites.

Mucosal surfaces and the skin of the human neonate are essentially sterile at birth, but shortly thereafter, begin to be continually colonized by microorganisms. This life-long interaction begins at a time when the component elements of the neonatal immune system are not fully mature. The postnatal development and function of the mucosal immune system is critically influenced by the acquisition, the temporal pattern, and the qualitative nature of mucosal microflora acquired in the neonatal period and early infancy. The potential role of mucosal microbiome on the functional development of several aspects of mucosal immune system and their clinical implications are briefly discussed in this presentation.

Ⅰ. Human mucosal microbiome

The interaction between the cellular mass of the human host and the environmental microorganisms begins during and immediately after birth and continues throughout life. The acquisition of microflora by the human neonate occurs initially via the maternal genital tract after normal vaginal delivery, followed by the organisms in the maternal gastrointestinal tract, other maternal mucosal surfaces, skin, and the process of breast-feeding. Subsequently, the neonate acquires other environmental microorganisms from other humans, pets and animal species, and the organisms in the soil. Colonization with "normal physiologic" microflora is usually completed after one week of postnatal life. Breast fed infants soon begin to develop a gut microflora in which *Bifidobacterium species* predominate. Subsequently, the mucosal microflora becomes more diverse with a predominance of *Fir-*

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Table 1 Microbial load in different human body surfaces

Site	No. Organisms
Skin	$>10^{12}$ (total skin surface)
Naso-oro-pharynx	10^{8-10}/mL
Lungs	$<10^{1-2}$ mL
Stomach	$0-10^{3}$ mL
Small intestine	10^{3-5}/mL
Large intestine	10^{10-11}/g (feces)
Genital tract (vagina, lower urinary tract)	10^{8-10}/mL

(Adapted from referrence 4)

micutes and *Bacteroidetes species*. In addition to breast-feeding, the mucosal microflora is also significantly affected by diet and other aspects of mucosal microenvironment including, antibiotics, other environmental macromolecules and supplemental formula feeding. Although the qualitative and quantitative aspects of microbial diversity exhibit marked fluctuations under different environmental conditions during the first year of life, the mucosal microflora, once established remains surprisingly stable, unique and specific to each individual. As pointed out earlier, the primary source of neonatal colonization is the mother. The maternal genital tract contains over 10^{12} organisms representing many aerobic and anaerobic species with predominance of *Coliform*, *Streptococcal species*, *Gram positive anaerobes*, *Lactobacilli*, *Prevotella* and *Sneathia species*. Human intestinal lumen contains over 10^{14} bacteria representing as many as 2000 microbial species, with over 160 species per individual[1,2]. It is estimated that human gut microbiota contains more genes than the entire human genome. Recent observations have suggested that human breast milk contains over 700 microbial species which exhibit significant changes in quality and quantity after establishment of lactation. The microbiome in the early colostrum appears to be most diverse, with high numbers of *Streptococcus*, *Lactococcus*, *Leuconostoc*, and *Weisella* and *staphylococcus species*. Subsequently, milk obtained after establishment of lactation exhibits a microbiome which is somewhat limited to the organisms in *Prevotella*, *Veillonella* and *Leptotrichia species*. The microbial content of milk exhibits significant difference in mothers who undergo C-section for the delivery and in those who exhibit abnormal nutrition[3]. The surface area of the human mucosal surface and the skin is extremely large. However, the relative load of the microorganisms and the qualitative nature of microbiome is strikingly different in different human body surfaces (**Table 1**). The microbial load appears to be lowest in the lungs, stomach and small intestine[4]. It is estimated that once fully colonized, there are over 100 trillion or more microorganisms representing over 2000 bacterial species residing in and on each human being[1,2,5]. Of these, only about 150 species have been cultured and studied in vitro to date. The remaining organisms in the human mucosal microbiome await detailed characterization. On the other hand, it has also been estimated that the total cellular mass of fully developed human being is composed of approximately 10 trillion cells derived from the original fertilized egg. Assuming that these estimates of microbial and human cellular load in each individual are reasonably accurate, it is a sobering conclusion to recognize that for every single human cell there may be as many as 10-100 microbial cells representing the total cellular mass of each human being throughout its life span. As a result, the development of immune system may possibly be one of the most effective evolutionary adaptations of the human cellular mass to the acquisition of the massive and life-long load of microorganisms.

Ⅱ. Human immune system

The functional components of human immune system include diverse and complex mechanisms of innate and adaptive immunity distributed in different systemic tissues, mucosal sites, skin and the mammary glands. The structural and functional

aspects of the human immune system have been reviewed extensively in many recent publications[6~8].

Briefly, both the innate and adaptive immunity begins to develop in fetal life, prior to and independent of specific antigenic stimulation. However, subsequent activation of B and T lymphoid cells, induction of lymphoid hyperplasia and secondary follicle-germinal center development, maturation of MALT and the acquisition of mucosal tolerance are critically dependent on microbial and other antigenic stimulation during and after birth.

1. Innate immunity

Important components of the innate immunity include mucosal epithelium, microbial recognition receptors, antigen processing and presenting cells, and a number of cellular and soluble mediators of inflammation.

The mucosal epithelium, especially the follicle associated epithelium (FAE) and M cells in the gut mucosa are derived from stem cells in crypt regions following cellular differentiation into crypt villus and follicular epithelium. The FAE is better suited for antigen sampling of microbial agents, and transcytotic activity for sampling by Dendritic cells (DC) via M cells[9]. The FAE also serves as receptor sites for many organisms, including the *Commensals*, *Salmonella*, *listeria*, *Yersinia*, reoviruses and other viral agents. The induction of M cells is initially a B cell independent process. However, the active phase of M cell function is a B cell dependent event[9]. M cell induction has also been observed in villus epithelium, via receptor activation of nuclear factor Kβ ligand (RANKβ). The microbial and pathogen recognition receptors (PRR) are designed specially to recognize unique and conserved pathogen and other microbial associated molecular patterns (PAMP) integral to the survival of the microorganisms[9]. The PRR function is genetically predetermined and antigen independent. In contrast to B and T cells, the relative number of PRP and their receptor density is limited to few hundred to a thousand in number. The PRP play an important role in regulating the quality and quantity of microbial content in the mucosa, epithelial proliferation, mucosal permeability in response to epithelial injury, and induction of adaptive immune responses. The PRR also play an important role inflammation in response to pathogens secondary to signaling in the mucosal lamina propria.

Other important components of innate immunity, such as antigen presenting cells (APC : CD40+, CD88+), macrophages (CD68+), dendritic cell (CD83+) and NK cell (CD94+) begin to appear in fetal life as early as 11-16 weeks[7].

2. Adaptive immunity

B cell and T cell progeny begins to appear in the systemic and mucosal tissues early in fetal life. Surface immunoglobulin+ cells (SIg) such as, SIgM+ B cells appear in fetal life as early as 9 weeks and in the peripheral blood and spleen by 12 weeks. SIgA, SIgG and SIgD+ B cells appear in the peripheral blood and lymph nodes by 12-16 weeks, and discrete plasma cells are detectable by 20 weeks of gestation[7].

Recent observations have suggested that B cell subset B_1 (BIa : CD5+ CD19+ CD45R+, CD11b+ SIgM (high), SIgG (low) ; (B1b : CD5− CD11b+) and B_2 (CD19+ CD45R+) B cells appear in different systemic and mucosal sites between 14-17 weeks of gestational life. However, B1 cells predominate in cord blood (>90%) and during infancy (70-80%), but represent only about 25-30% of adult B cells. The B_1-B cells interact with innate immune system, and maintain a critical homeostatic interaction with mucosal microflora. These cells distribute to regional lymph nodes and differentiate into polyclonal IgM secreting cells with immediate and broad spectrum antimicrobial protection. This response is independent of B cell receptor (BCR) activation. These cells differentiate into IgA producing cells after migration to the gut. They also enhance neutralization of pathogens and promote uptake or clearance of apoptotic cells in

the mammalian host[10].

Quantitative and qualitative changes in the distribution of B_1 B cells has been observed in several disease states, including common variable immune deficiency, IgA nephropathy, multiple sclerosis and inflammatory bowel disease[11~13]. B_1 cells also appear to be selectively induced in mucosal surfaces after mucosal immunization with rotavirus and influenza vaccines[14,15]. The precise role of B_1 B cells remains to be fully elucidated. It has been suggested that these cells may have a roll in induction of memory responses, possibly via positive selection for self-reactivity and formation of a pool of long-lived self-renewing B cells. These cells have also been shown to enhance IgG mediated adaptive immune response usually associated with the adult type (B_2) B cells[10].

Many T cell phenotypes have been identified in fetal tissues as early as 9-10 weeks. CD7+ T cells begin to appear around 10 weeks of fetal development. Subsequently CD3+, CD3+ CD7+, and CD28+ T cell subsets have been detected around 14-15 weeks of gestation. CTLA4+ CD40L+ T cells are usually observed by 16th week. However CD3+ CD7− T cells observed in the adult are not seen during fetal life[6,7].

3. Mucosa associated lymphoid tissue (MALT)

Significant amounts of antigen-reactive lymphoid tissue are observed at the time of birth as evidenced by the presence of Peyer's patches and in other organized mucosal lymphoid follicles. The naïve antigen reactive cells in the lymphoid tissues of GALT, BALT, NALT and SLLT represent the primary inductive sites for development of specific mucosal immune responses. The lamina propria of all mucosal surfaces, male and the female genito-urinary tracts and mammary glands represent the sites destined for eventual expression of effector function of mucosal immunity[16,17].

The neonates exhibit over 70-150 visible Peyer's patches at birth. However, there is significant lack of expression of effector functions. Maturation of such functions, qualitative and further quantitative expansion of mucosal lymphoid tissue in the GALT as well as in NALT occurs after birth (**Table 2**). In animal models, the development of NALT occurs typically after birth and peaks later in life. On the other hand, the peak number of Peyer's patches is observed in early life with significant decline in later years of life (Table 2). Little or no IgA (the principal immunoglobulin in human external secretions) is detected at birth. However, it begins to appear rapidly in the secretions and serum during the first 12 weeks of postnatal life. Over 90% of infants exhibit detectable level of serum (7S) and secretory (11S) IgA activity after 3-4 months of age[16~19].

Ⅲ. Impact of microbiome on mucosal immunity

As pointed out earlier, the functional maturity of neonatal mucosal immune system occurs largely after exposure to the post natal environmental microflora. This period of neonatal growth is characterized by, reduced levels of innate mechanism of defense including reduced levels of complement components, lower number and function of leukocytes and macrophages ; impaired IFN-γ and IL-10 production ; reduced APC function ; and altered antibody and cell mediated immune response at systemic as well as mucosal levels. Human neonates also exhibit reduced Th_1-mediated T cell responses and enhanced Th_2-mediated T cell response. The shift to Th_2 response may be related to increased apoptosis of Th_1 cell by IL-4[20]. The neonate also exhibits delayed maturation of IL-12 producing DC, reduced CD4+ T cells responses, reduced delayed type hypersensitivity responses, but normal graft rejection ; and reduced intracellular killing of cell-associated organisms[20]. It is during this window of significant alterations of immunologic functions that the neo-

Table 2 Age related distribution of Peyer's patches（PP）

Age	Structures	Peyer's Patches
Prenatal 10-11 weeks	Rudimentary；HLA-DR＋, CD4＋	No visible patches
11-16 weeks	CD8＋T cells, IgM＋IgD＋B cells	No visible patches
16-18 weeks	CD5＋, IgA＋B cells	No visible patches
20-40 weeks	Appearance of B and T cell zones	Visible patches 60 （45-70）
Birth	Abundant lymphoid tissues, No germinal centers, no secondary follicle formation	60 （50-90）
24 hrs-6 weeks	Expression of germinal centers after antigen exposure	94 （70-150）
Adult	Peak distribution of Peyer's patches	
12-15 years	Increasing lymphoid tissue and Peak numbers of Peyer's patches	295 （185-325）
20-40 years	PP number declining	160 （100-285）
＞70 years	Further decline in PP number	100 （60-170）

（quote from refference 18）

nate is initially colonized by the microflora from the maternal genital tract, gastrointestinal tract and the products of lactation.

In earlier clinical observations, it was shown that breast-feeding was associated with reduced severity of rotavirus disease and increased replication of *Bifidobacterium*. Subsequently oral feeding of *Bifidobacterium* resulted in significant reduction in the endotoxin levels in the gut, a possible reflection of altered microflora. In other studies, it was shown that supplemental feeding with *Bifidobacterium* during rotavirus infection resulted in significant amplification of fecal（16-fold）and serum（4-fold）virus-specific IgA antibody response[21～23]. There is now sufficient evidence to suggest that mucosal microflora directly influences the outcome of mucosal immune responses. Non-pathogenic *Salmonella species* have been shown to inhibit NFKβ -induced activation of genes coding for inflammatory cytokine expression. Similarly, induction of decay-accelerating factor（DAF）has been observed after infection with some *commensals*. The DAF appears to inhibit cytotoxic damage from microbial activation of complement components, such as C-reactive protein and Ductin. Ductin is a possible receptor for a family of prolein-rich proteins（mucosal trefoil factors；sprrza）involved in mucosal barrier function, as reviewed earlier[8,24]. There is now evidence to suggest that certain *commensal* bacteria acquired during neonatal period and early infancy are critical for the development of tolerance to dietary proteins. Development of tolerance to IgE production against ovalbumin in the gut in an animal model was found to require colonization with single or polymicrobial flora in the gut mucosa. No tolerance was observed in germ-free state. Microbial colonization also appears to affect expression of host genes regulating maturation, nutrient uptake, metabolic processing of xenobiotics and, development of angiogenesis[8,24].

During the last few years, a number of elegant

studies conducted in experimental model settings have provided more direct evidence for a critical role of mucosal microbiome on the development and functional maturation of mucosal immune system. Mucosal microflora has been associated with, increased expression of toll-like receptors (TLR2, 4, 5) on mucosal tissues[25], enhanced development and proliferation of organized lymphoid tissue in the gut with certain organisms, often working in a synergistic manner, induction of proliferation and plasticity of IgA response in the gut, up-regulation of the shift of T cell response towards Th_1 type, and down regulation of NK T cell mediated inflammatory specificity at 3 weeks of age. Some microorganisms, especially segmented filamentous bacteria (SFB) are unique in their ability to induce pro-inflammatory cytokines. Recent studies have shown that the diversity and presence of certain organisms is more important in the expression of disease in animal models. Furthermore, the outcome and nature of regulatory immune responses (tolerance, pro and auto inflammatory) is often determined by the presence or absence of only certain bacteria. It has also been observed that the nature of microbial exposure in early infancy determines the development of appropriate protective or inappropriate T cell responses which determine the development or outcome of disease in later life. This information has been reviewed in several recent reports[26~31].

Ⅳ. Clinical implications

Based on the observation summarized above, it is apparent that mucosal microbiome is essential for the structural enhancement and functional development of several aspects of mucosal immune system. Remarkably however, it has also been shown that the qualitative as well as quantitative nature of the post natal mucosal microflora itself is regulated by the nature of mucosal immune response induced.

During the past four decades, there has been a substantial increase in the incidence of many immunologically mediated or autoimmune disorders in the developed world, but less so in underdeveloped economic settings. At the same time, markedly different composition of microbiome is beginning to be observed between well developed and under developed economic settings. In experimental models as well as in certain clinical situations, many bacteria, viruses or parasites have been implicated in the pathogenesis of, or protection against the development of some disorders. Some microorganisms and parasitic agents still prevalent in underdeveloped economic settings appear to be more effective in protection against autoimmunity, allergy or induction of tolerance. These observations form the basis of hygiene hypothesis, proposed initially nearly 3 decades ago[26].

As mentioned earlier in this discussion, human beings harbor for their lifetime, >100 trillion microbial organisms acquired immediately after birth. This relationship has been established since the evolution of man itself. Virtually all microbial organisms residing or producing infections in man are acquired from other life forms. It is apparent that benign colonization of human mucosal surfaces is the rule with virtually all human microbiome and the development of disease and death is an exception especially with long established microflora, otherwise mankind would have ceased to exist a long time ago.

Acquisition of new microbial colonization may result in local inflammation, which often culminates in prompt termination of infection. However, under certain circumstances the host-microbial interaction can result in chronic inflammation which can lead to chronic alterations in long term host-microbial interaction, development of illness and possibly death of the host.

It has been proposed by Rook[32] that since Paleolithic period to the modern age (**Table 3**), the human mucosal microbiome has continuously undergone significant changes. In recent times,

these changes have been associated with a clear temporal association, with increased expression of different types of allergy, inflammatory bowel diseases, multiple sclerosis, diabetes mellitus, other forms of autoimmune disorders, depression, malignancy, autism spectrum disorder and possibly other disorders. Specific qualitative and or quantitative alterations in mucosal microbiome have been observed in many such disease states[24,33]. These include, A) increased *Proteobacteria*, IL-4 and decreased *Bifidobacterium*, *Clostridia*, *Lactobacilli* and *H. pylori* in allergic disorders ; B) increased *Bifidobacterium* : *Firmicutes* ratio, *Bacteroides ovatus* ; and decreased microbial diversity, *Clostridium* and *Firmicutes* in type 2 Diabetes Mellitus ; C) increased *Firmicutes*, *Acinetobacteria*, TNF-α, and decreased *Bacteroides* and classic inflammatory responses in obesity ; D) increased antibiotic resistance gene reservoir in the gut microbiome, and decreased Bacteroides, and long term *microbial diversity* in prolonged antibiotic usage. And, E) Significant changes in mucosal microbiome in Crohn's disease, persistent *Clostridium difficile* infection, experimentally induced depression and ASD[24,33~35]. However, It must be emphasized that for all the clinical situations listed above, a clear-cut cause and effect relationship has yet to be established. Nevertheless, it is clear that trillions of microorganisms live in and on us for a lifetime. "Normal physiologic flora" represents microorganisms for which human host has served as primary reservoir for several hundred to thousands of years, They must be considered benign or commensals and only in rare or exceptional cases as pathogens. During the prolonged residence in the human host, their evolution towards attenuation of virulence and overall peaceful coexistence beneficial to both has in general established a symbiotic relationship. However, under certain exceptional circumstances, driven either by the host, external environment, or by the microorganism itself during its shift to a new host, the organism may sustain pathogenic relationship or by-pass effective host defenses to induce a disease or pathogenesis detrimental to the host[36].

Table 3 Changing microbial ancestry of man

1. Paleolithic period －>10,000 BCE Microorganisms present in early humans for as long as>100,000 years More : Helminths ; Toxoplasma *Mycobacteria-TB* ; *Helicobacter* ; *Salmonella* HAV other established *microbiome* ; *Lactobacillus*
2. Neolithic period －3,500 BCE More settled life style, novel sporadic infections from farm animals More : Helminths, Rotavirus, Calciviruses, Influenza, Measles, Smallpox, *Cholera*, *Plague*, *Typhus* increasing oral or fecal contact
3. Modern age －1,800-present Replacement of earlier flora with recent immigration "alien" arrivals : Significant alteration in prior native *microflora* Less : Helminths, Toxoplasma, *Helicobacter*, *Salmonella*, *TB*, *Commensals from mud and water*, HAV More : HIV and *other restricted "alien" animal flora*

(quote from refference 32)

Summary

Based on the observation discussed very briefly in this overview, it is suggested that human mucosal immune system and its functions continually evolve and mature after birth. Mucosal microflora derived from the maternal mucosal sites, is critical for its functional maturation and outcome. The nature of immunologic, nutritional and metabolic homeostasis is determined by the balanced host-microbial interaction. There are no good or bad microbes. The induction of protection against or pathogenesis of microbial or host immunologically mediated diseases in the host is a reflection of normal (symbiotic) or abnormal (altered) mucosal microflora established in early childhood and possibly later in life under altered environmental conditions[37].

"To regard any form of life merely as a slave or a foe will one day be Considered poor philosophy, for all living things constitute an integral part of the cosmic order." [38]

Rene Dubois, 1901-1982

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