Current Understanding of Gastrointestinal Immunoregulation and Its Relation to Food Allergy

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ABSTRACT: Tolerance to food antigens induced via the gut ("oral tolerance") appears to be a rather robust adaptive immune mechanism. However, the neonatal period is particularly critical in terms of mucosal defense, with regard to infections and priming for allergic disease. This is so because the intestinal barrier function provided by secretory antibodies, as well as the immunoregulatory network, is poorly developed for a variable period after birth. Notably, the postnatal development of mucosal immune homeostasis depends on the establishment of a normal commensal microbial flora and also on adequate timing and dose of dietary antigens when first introduced. In this context, breastfeeding apears to exert both shielding and positive regulatory effects. Altogether, the intestinal immune system normally seems rather fit for tolerance induction against innocuous antigens because most children with food allergy "outgrow" their problems, whereas airway allergy tends to persist.

KEYWORDS: B cells; breastfeeding; food allergy; gut-associated lymphoid tissue; mucosal immunoregulation; oral tolerance; secretory immunity; T cells

INTRODUCTION

The mucosae are bombarded immediately after birth by a large variety of microorganisms as well as by protein antigens from the environment, the latter particularly in formula-fed infants; and the mucosal surface to be protected is enormous, probably almost 200 times that of the skin. During evolution over millions of years, the mucosal immune system has generated two arms of adaptive defense to handle these challenges: (i) antigen exclusion per-
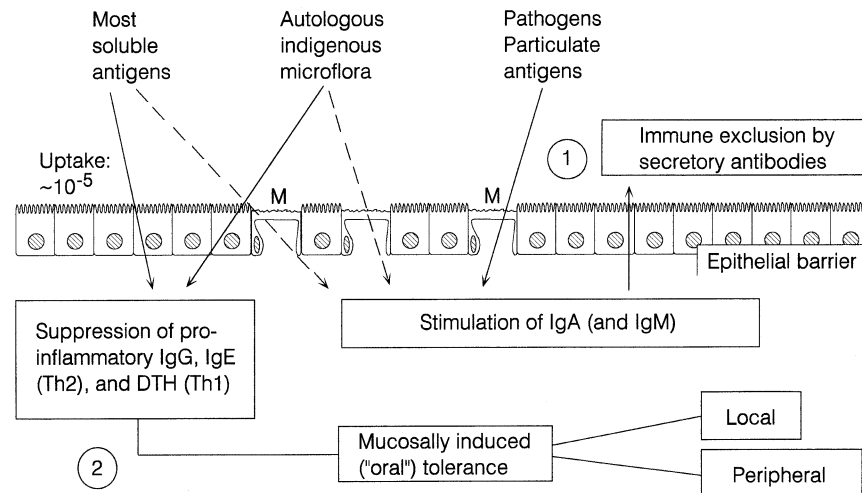
FIGURE 1. Schematic depiction of two major adaptive immune mechanisms operating at mucosal surfaces: (1) Immune exclusion limits epithelial colonization of pathogens and inhibits penetration of harmful foreign material. This first line of defense is principally mediated by secretory antibodies of the IgA (and IgM) class in cooperation with various nonspecific innate protective factors (not shown). Secretory immunity is preferentially stimulated by pathogens and other particulate antigens taken up through thin M cells (M) located in the dome epithelium covering inductive mucosa-associated lymphoid tissue (see FIG. 2). (2) Penetrating innocuous soluble environmental and dietary antigens (magnitude of uptake indicated) as well as the autologous indigenous microbial flora are less stimulatory for secretory immunity (self-limiting responses, *broken arrows*), but induce suppression of proinflammatory humoral immune responses (IgG and Th2 cytokine-dependent IgE antibodies) as well as Th1 cytokine-dependent delayed-type hypersensitivity (DTH). The homeostatic Th2/Th1 balance is regulated by a complex and poorly defined phenomenon called mucosal or "oral" tolerance (see FIG. 5), which exerts downregulatory effects both locally and in the periphery. Modified from Brandtzaeg *et al.*[5]

formed by secretory IgA (SIgA) and secretory IgM (SIgM) antibodies to modulate or inhibit colonization of microorganisms and dampen penetration of potentially dangerous soluble luminal agents and (ii) suppressive mechanisms to avoid local and peripheral overreaction (hypersensitivity) against innocuous substances bombarding the mucosal surfaces (FIG. 1). The latter arm is referred to as "oral tolerance" when induced via the gut against dietary antigens[1]; it probably explains why overt and persistent immunological hypersensitivity, or allergy, to food proteins is relatively rare.[2] Similar downregulatory mechanisms apparently operate against antigens from the commensal microbial flora.[3]

Oral tolerance generally seems to be a rather robust adaptive immune function in view of the fact that more than a ton of food may pass through the gut

of an adult every year, resulting in substantial uptake of intact antigens (some 10^{-5} of the intake) even in the healthy state. Nevertheless, the neonatal period is particularly critical in terms of mucosal defense, both with regard to infections and priming for allergic disease.[4] This is so because the mucosal barrier function and the immunoregulatory network are poorly developed for a variable period after birth.[5,6] Notably, the postnatal development of mucosal immune homeostasis appears to depend on the establishment of a normal commensal microbial flora as well as on adequate timing and dose of dietary antigens when first introduced.[3,7,8]

Interestingly, the postnatal colonization of commensal bacteria is important both to establish[9] and regulate[10] an appropriate epithelial barrier. Also, as discussed in this review, an optimal mucosal barrier function in the neonatal period unquestionably depends on an adequate supply of breast milk, particularly in relation to mucosal infections in the developing countries.[11] In the Westernized part of the world, the value of breastfeeding is clinically most apparent in preterm infants,[12] but accumulating evidence (referred to below) also suggests a significant role in the protection against hypersensitivity reactions to food.

MUCOSA-ASSOCIATED LYMPHOID TISSUE

Induction and Homing of Immune Cells

Lymphoid cells are located in three distinct compartments in the gut: organized gut-associated lymphoid tissue (GALT), the lamina propria, and the surface epithelium. GALT comprises the Peyer's patches, the appendix, and numerous solitary lymphoid follicles,[5] especially in the large bowel.[13] All these lymphoid structures are believed to represent inductive sites for intestinal immune responses. The lamina propria and epithelial compartment constitute effector sites but are nevertheless important in terms of cellular expansion and differentiation within the mucosal immune system. GALT and other mucosa-associated lymphoid tissue (MALT) structures (see below) are covered by a characteristic follicle-associated epithelium (FAE), which contains membrane (M) cells (FIGS. 1 and 2). These specialized thin epithelial cells are particularly effective in the uptake of live and dead antigens from the gut lumen, especially when particulate in nature.[14] Many enteropathogenic infectious bacterial and viral agents use the M cells as portals of entry.

The GALT structures resemble lymph nodes with B-cell follicles, intervening T-cell areas, and a variety of antigen-presenting cell (APC) subsets, but there are no afferent lymphatics supplying antigens for immunological stimulation. Therefore, the exogenous stimuli must come directly from the gut lumen, probably in the main via the M cells. Among the T cells, the CD4$^+$ helper
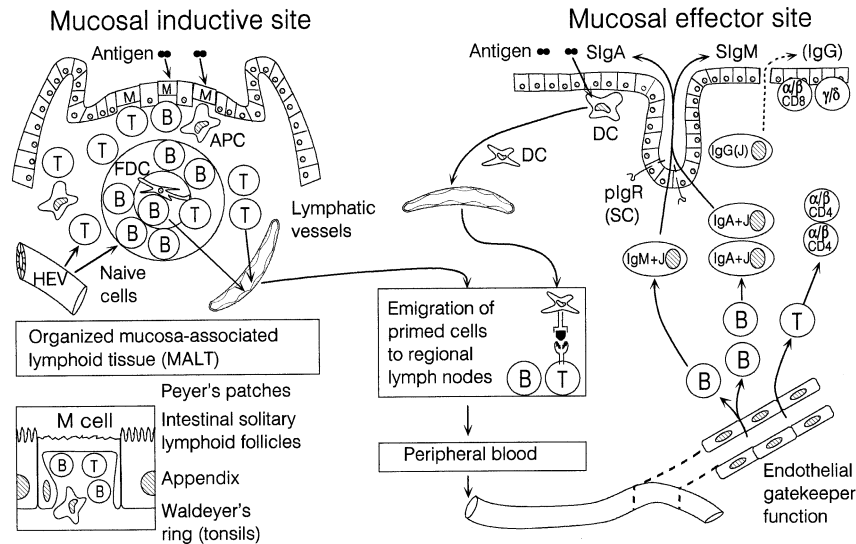
FIGURE 2. Schematic depiction of the human mucosal immune system. Inductive sites are constituted by regional mucosa-associated lymphoid tissue (MALT) with their B-cell follicles and M cell (M)-containing follicle-associated epithelium through which exogenous antigens are actively transported to reach professional antigen-presenting cells (APC), including B cells (B) and follicular dendritic cells (FDC). In addition, mucosal dendritic cells (DC) may capture antigens and migrate via draining lymph to regional lymph nodes where they become active APCs, which stimulate T cells (T) for positive or negative (down-regulatory) immune responses. Naive B and T cells enter MALT (and lymph nodes) via high endothelial venules (HEV). After being primed to become memory/effector B and T cells, they migrate from MALT and regional lymph nodes via lymph and peripheral blood for subsequent extravasation at mucosal effector sites. This process is directed by the profile of adhesion molecules and chemokines expressed on the microvasculature, the endothelial cells thus exerting a "gatekeeper" function for mucosal immunity. The intestinal lamina propria is illustrated with its various immune cells, including B lymphocytes, J chain-expressing IgA and IgM plasma cells, IgG plasma cells with a variable J-chain level (J), and CD4+ T cells. Additional features are the generation of secretory IgA (SIgA) and secretory IgM (SIgM) via pIgR (SC)-mediated epithelial transport, as well as paracellular leakage of smaller amounts (*broken arrow*) of serum-derived and locally produced IgG antibodies into the lumen. Note that IgG cannot interact with J chain to form a binding site for pIgR. The distribution of intraepithelial lymphocytes (mainly T-cell receptor α/β+CD8+ and some γ/δ+ T cells) is schematically depicted. Insert (*lower left corner*) shows details of a M cell and its "pocket" containing various cell types.

subset predominates, the ratio between CD4 and CD8 cells being similar to that of other peripheral T-cell populations.[5] In addition, B cells aggregate together with T cells in the M-cell pockets, which thus represent the first contact site between immune cells and luminal antigens.[15,16] The B cells may perform important antigen-presenting functions in this compartment, perhaps promot-
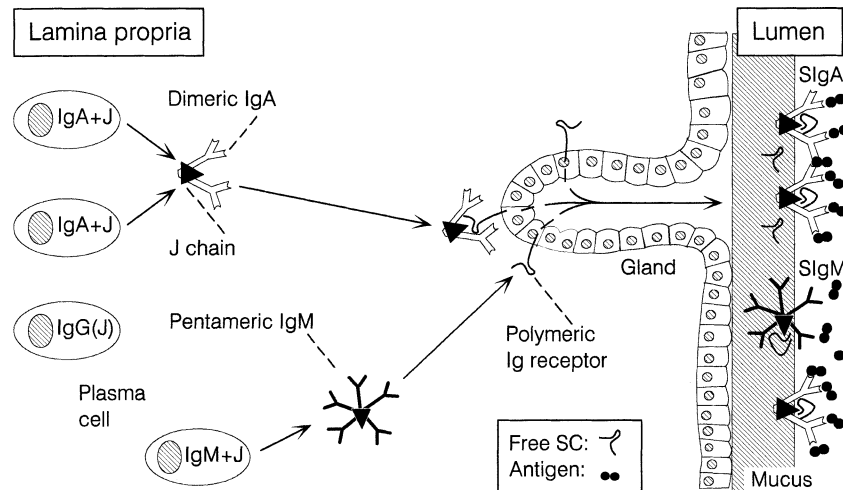
FIGURE 3. Model for epithelial transport of J chain-containing dimeric IgA (IgA+J) and pentameric IgM (IgM+J) by the polymeric Ig receptor (pIgR) expressed basolaterally on secretory epithelial cells. The resulting secretory immunoglobulins (SIgA and SIgM) act in a first line of defense by performing immune exclusion of antigens in the mucus layer at the epithelial surface (to the right). Note that although J chain (J) is often produced by mucosal IgG plasma cells, it remains free in the cytoplasm and becomes degraded. Serum-derived or locally produced IgG is not subjected to active external transport (see FIG. 2). Free secretory component (SC) is generated when unoccupied pIgR (at the top of gland) is cleaved at the apical face of the epithelial cell, in the same manner as bound SC in SIgA and SIgM.

ing antibody diversification and immunological memory or contributing to tolerance induction.[17] Other types of professional APCs, macrophages and dendritic cells (DCs), are located below the FAE and between the follicles.

Pioneer studies performed in animals almost 30 years ago demonstrated that immune cells primed in GALT are functionally linked to mucosal effector sites by an integrated migration or "homing" pathway.[1] T cells activated by microbial and other antigens in GALT preferentially differentiate to CD4+ helper cells which, aided by DCs and secretion of cytokines such as transforming growth factor (TGF)-β and interleukin (IL)-10, induce the differentiation of antigen-specific B cells to predominantly IgA-committed plasma blasts.[5,17] The germinal-center cells express small amounts of surface IgA along with less IgM or IgG.[18] Such isotype skewing reflects differentiation to precursors for IgA-producing cells. The drive for isotype switching towards IgA, together with J-chain expression in B cells, is for unclear reasons much more evident in Peyer's patches than in other MALT structures.[5,17] The combination of IgA- and J-chain production is a prerequisite for generation of SIgA antibodies (FIG. 3).

The GALT-derived B-cell blasts proliferate and differentiate further on their route through mesenteric lymph nodes and the thoracic duct into the bloodstream (FIG. 2). Thereafter, they home preferentially to the gut mucosa where they complete their terminal differentiation to IgA-producing plasma cells. As reviewed elsewhere,[5,19] this migration of lymphoid cells is facilitated by "homing receptors" interacting with ligands on the microvascular endothelium at the effector site ("addressins"), with an additional fine-tuned level of navigation conducted by local chemoattractant cytokines (chemokines). Under normal conditions, therefore, the local microvasculature exerts a "gatekeeper" function to allow selective extravasation of primed lymphoid cells belonging to the mucosal immune system (FIG. 2).

Regionalization of MALT

Although GALT constitutes the major part of MALT, induction of mucosal immune responses can also take place in the palatine tonsils and other lymphoepithelial structures of Waldeyer's pharyngeal ring, including nasal-associated lymphoid tissue (NALT) such as the adenoids in humans[17,19,20] and probably also bronchus-associated lymphoid tissue (BALT). Because BALT is lacking in normal lungs of newborns and adults,[21] Waldeyer's ring may represent a significant component of human MALT. Accumulating evidence suggests that a certain regionalization exists in the mucosal immune system, especially a dichotomy between the gut and the upper aerodigestive tract with regard to homing properties and terminal differentiation of B cells.[5,17,20] This disparity may be explained by microenvironmental differences in the antigenic repertoire as well as adhesion molecules and chemokines involved in preferential local leukocyte extravasation. It appears that primed immune cells selectively home to effector sites corresponding to the inductive sites where they initially were triggered by antigens. Such regionalization within the "common" or integrated mucosal immune system has to be taken into account in the development of local vaccines.

B-Cell Homing to Mammary Glands

Lactating mammary glands are part of the integrated mucosal immune system, and milk antibodies reflect antigenic stimulation of MALT in the gut as well as in the airways. This fact has been documented by showing that SIgA from breast milk exhibits antibody specificities for an array of both intestinal and respiratory common pathogens.[22] The secretory antibodies are thus highly targeted against infectious agents in the mother's environment, which are those likely to be encountered by the infant during its first weeks of life. Therefore, breastfeeding represents an ingenious immunological integration of mother and child (FIG. 4). Although the protection provided by this humor-
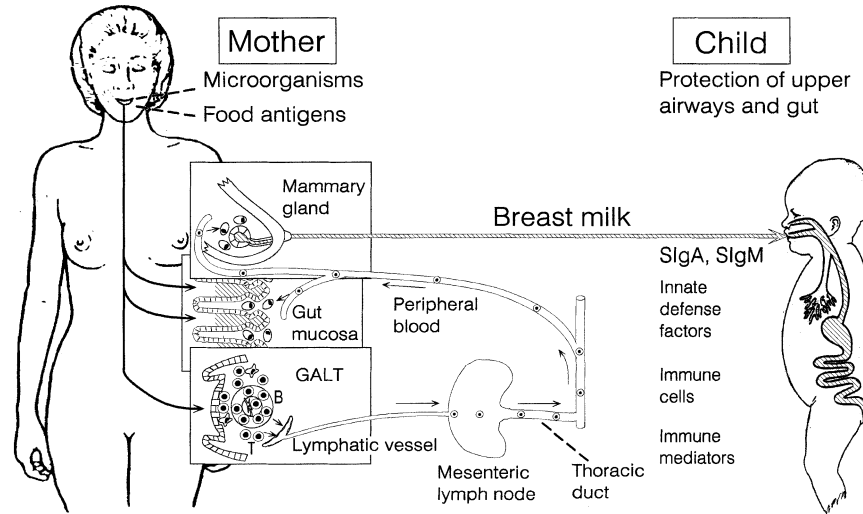
FIGURE 4. Integration of mucosal immunity between mother and the newborn, with emphasis on migration (*arrows*) of primed B (and probably T) cells from gut-associated lymphoid tissue (GALT) such as Peyer's patch, via lymph and peripheral blood to lactating mammary gland. This distribution of precursors for IgA plasma cells beyond the gut mucosa is crucial for glandular production and subsequent occurrence in breast milk of secretory antibodies (SIgA and SIgM) specific for enteric antigens (microorganisms and food proteins). By this mechanism, the breast-fed infant will receive relevant secretory antibodies directed against the microflora initially colonizing its mucosae (reflecting the mother's microflora) and hence be better protected both in the gut and in the upper airways by SIgA and SIgM (*hatched areas*) in the same way as the mother's gut mucosa is protected by similar antibodies (*hatched areas*).

al defense mechanism is most readily demonstrable in populations living in poor sanitary conditions,[11,23] a beneficial clinical effect is also apparent in the industrialized world,[24] even in relation to relatively common diseases such as otitis media and acute lower respiratory tract infections.[25–27]

Antibodies to various dietary antigens, such as cow's milk proteins[28] and gluten,[29] are also present in breast milk. Nevertheless, little is known about the preferential site where soluble luminal antigens exert immune priming. Thus, dietary proteins may be taken up mainly through the extensive epithelial surfaces covering the diffuse immunological effector tissue of the intestinal mucosa rather than by M cells and may therefore largely be transported to the mesenteric lymph nodes. As discussed below, their fate and possible immune-inductive or tolerogenic effects will depend on how they are handled locally and whether they reach lymph or portal blood.[1,30,31]

POSTNATAL DEVELOPMENT OF MUCOSAL IMMUNITY

Effects of Antigen Exposure and Nutrition

In most mucosal tissues, the surface epithelium is monolayered and therefore quite vulnerable, so the defense of this large area is a formidable task. Nevertheless, most babies growing up under privileged conditions show remarkably good resistance to infections if their innate nonspecific mucosal defense mechanisms are normally developed. This can be explained by the fact that immune protection of their mucosae is additionally provided by maternal IgG antibodies, which are distributed in interstitial tissue fluid at a concentration 50–60% of the intravascular level. In the first postnatal period, only occasional traces of SIgA and SIgM normally occur in the intestinal juice, whereas some IgG is more often detectable; this reflects "leakage" from the highly vascularized lamina propria, which contains maternal IgG, particularly after 34 weeks of gestation.[32] On the other hand, the inductive sites of the secretory immune system, such as the Peyer's patches, depend on exogenous stimuli for maturation. Thus, although GALT structures generally develop in fetal life, hyperplasia with secondary follicles containing germinal centers signifying B-cell activation does not occur until shortly after birth.[33–37]

Antigenic constituents of food clearly exert a stimulatory effect on the intestinal B-cell system, as suggested by the occurrence of fewer lamina propria IgA immunocytes both in mice fed on hydrolyzed milk proteins[38] and in parenterally fed babies.[39] Likewise, mice given total parenteral (intravenous) nutrition have reduced numbers of B and T cells in the gut, as well as decreased SIgA levels,[40–42] and they show impaired SIgA-dependent influenza-specific immunity.[43] The effect of food in the gut lumen could be direct immune stimulation or stimulation mediated via release of gastrointestinal neuropeptides. The indigenous microbial flora is also extremely important for mucosal immunity as shown by the fact that the intestinal IgA system of germ-free or specific pathogen-free mice is normalized after about 4 weeks of conventionalization.[44,45] *Bacteroides* and *Escherichia coli* strains seem to be particularly stimulatory for the development of intestinal IgA immunocytes.[46,47] The large dietary and bacterial antigen load in the gut lumen therefore explains that the greatest density of IgA immunocytes is seen in the intestinal lamina propria, amounting to some 10^{10} cells per meter of adult gut.[5]

In keeping with an important stimulatory effect of luminal antigens, defunctioning colostomies in children showed a 50% numeric reduction of mucosal IgA and IgM immunocytes after 2–11 months.[48] Prolonged studies of defunctioned ileal segments in lambs revealed even more strikingly a scarcity of mucosal immunocytes; this was caused by decreased local proliferation and differentiation of B-cell blasts and perhaps reduced homing from

GALT.[49] Accordingly, the postnatal establishment of the mucosal IgA system is usually much faster in developing countries than in the industrialized part of the world, a difference that seems to hold true even in undernurished children.[50] However, severe vitamin A deficiency has been reported to have an adverse effect on mucosal IgA antibody responses in rodents,[51] but with no consistent downregulation of epithelial IgA transport.[52]

The possibility exists that suboptimal stimulatory reinforcement of the SIgA-dependent mucosal barrier function might contribute to the increased frequency of certain diseases in industrialized countries, particularly allergies and other inflammatory mucosal disorders. This "hygiene hypothesis" has been tested in several experimental and clinical studies by evaluating the beneficial effect of probiotic bacterial preparations. Especially viable strains of the commensal intestinal microflora, such as lactobacilli and bifidobacteria, have been reported to enhance IgA responses, both in humans and experimental animals, apparently in a T-cell-dependent manner.[53–58] Interestingly, early colonization of infants with a nonenteropathogenic strain of *E. coli* has been reported to have a long-term beneficial effect by reducing both infections and allergies.[59] Likewise, a recent double-blind study of infants with a family history of atopic (IgE-mediated) allergy reported the prevalence of atopic eczema to be reduced by 50% at the age of two years in those receiving the probiotic *Lactobacillus* GG strain daily for 6 months compared with those receiving placebo.[60] It remains to be shown whether this striking beneficial effect was mediated via SIgA enhancement or by promotion of oral tolerance as discussed below.

Importance of Breastfeeding and Secretory Immunity

When much of the transferred maternal IgG has been catabolized by around 2 months of age, the infant becomes still more dependent on antibodies from breast milk for specific humoral immunity. Notably, IgA-producing immunocytes are normally undetectable in human intestinal mucosa before 10 days of age, but thereafter a rapid increase takes place, although IgM immunocytes usually remain predominant up to 1 month.[7,8,32] Adult salivary IgA levels are reached quite late in childhood, but only a small increase of IgA-producing cells has been reported to take place in the intestinal mucosa after 1 year.

At least 90% of all pathogens attacking humans use the mucosae as portals of entry; mucosal infections are in fact a major killer of children below the age of 5 years, being responsible for more than 14 million deaths of children annually in the developing countries. Diarrheal disease alone claims a toll of 5 million children per year. These sad figures document the importance of breastfeeding. Convincing epidemiological data suggest that the risk of dying from diarrhea is reduced 14–24 times in nursed children.[11,23] Indeed, exclusively breastfed infants are better protected against a variety of

infections[24–26,61] and apparently also against atopy, asthma,[62–64] and celiac disease.[65] Interestingly, experiments in neonatal rabbits strongly suggest that SIgA is a crucial antimicrobial component of breast milk.[66] The role of secretory antibodies for mucosal homeostasis is furthermore supported by the fact that knockout mice lacking SIgA and SIgM show increased mucosal leakiness.[67]

The survival of the infant will to an increasing extent depend on its own adaptive immune responses. When the mucosal immune system is adequately developed, exocrine glands and secretory mucosae contain most of the body's activated B cells, which are terminally differentiated to Ig-producing blasts and plasma cells (collectively called immunocytes).[5] These cells produce mainly J chain–containing dimers and some larger polymers of IgA (collectively called pIgA) which, along with pentameric IgM, can be actively transported through serous-type secretory epithelia,[68–72] including lactating mammary glands,[73] to act in a first-line mucosal defense (FIG. 3). This transport depends on the epithelial polymeric Ig receptor (pIgR), which consists of a transmembrane glycoprotein also known as the secretory component (SC). The generated SIgA and SIgM antibodies reinforce the epithelial barrier function by performing immune exclusion of live and dead antigens.

Developmental Variations and Food Allergy

The postnatal mucosal B-cell development shows large individual variations, even within the same population.[32] This disparity could partly reflect a genetically determined effect on the establishment of the mucosal barrier function. Thus, it has been proposed on the basis of serum IgA levels that a hereditary risk of atopy is related to a retarded postnatal development of the IgA system.[74] This notion was later supported by a report showing significantly reduced IgA immunocyte numbers (with no compensatory IgM enhancement) in jejunal mucosa of atopic children.[75] Also, an inverse relationship was found between the serum IgE level and the jejunal IgA cell population in children with food-induced atopic eczema.[76] It was subsequently reported that infants born to atopic parents showed a significantly higher prevalence of salivary IgA deficiency than age-matched control infants.[77] Interestingly, Kilian *et al.*[78] more recently found that the throats of 18-month-old infants with presumably IgE-mediated allergic problems contained significantly higher proportions of IgA1 protease-producing bacteria than age-matched, healthy controls, thus supporting a previous report showing much less intact IgA in nasopharyngeal secretions from children with a history of atopic allergy than from controls with episodes of acute otitis.[79] In this context it is important to note that it takes up to 3 months after birth before the IgA2 to IgA1 immunocyte ratio in salivary glands has increased to the adult value, with approximately 33% IgA2-producing cells.[80]

Altogether, a poorly developed or enzymatically reduced SIgA-dependent mucosal barrier function, combined with a hereditary and/or cytokine-driven hyper-IgE responsiveness (see below) could contribute to the pathogenesis of allergy. This notion accords with the increased frequency not only of infections, but also of atopic allergy and celiac disease seen in subjects with permanent selective IgA deficiency,[81] although compensatory overproduction of SIgM apparently may counteract the adverse consequences of their absent mucosal IgA responses, particularly in the gut.[32,82]

MUCOSAL INDUCTION OF IMMUNOLOGICAL TOLERANCE

Oral Tolerance Appears to Exist in Humans

The concept of oral tolerance has a long history, mainly based on feeding experiments in rodents.[1] An overwhelming mechanistic complexity has hampered the understanding of this mucosally induced down-regulatory or suppressive phenomenon. Identifiable experimental variables include genetics; age, dose, and timing of postnatal feeding; antigenic structure and composition of fed protein; epithelial barrier integrity; and the degree of concurrent local immune activation as reflected by microenvironmental cytokine profiles and the expression of costimulatory molecules on mucosal APCs.[1,83,84] Also, rodent studies suggest that the commensal microflora is important both for induction of oral tolerance and for reconstitution of this mechanism after its experimental abrogation.[3] This effect is probably mediated mainly through immune stimulation of GALT as discussed above.

It seems justified to believe that that oral tolerance also operates in humans. Indirect evidence of this is provided by the fact that the vulnerable intestinal mucosa, which is separated only by a monolayered epithelium from the enormous luminal load of live and dead antigenic material, in the normal state exhibits no substantial IgG response[5,30] and contains very few T cells with markers of hyperactivation such as CD25 or the IL-2 receptor.[85] Moreover, the systemic IgG response to dietary antigens tends to decrease in humans with increasing age,[86,87] and a hyporesponsive state to bovine serum albumin has been documented by intradermal testing in adults.[88]

Interestingly, experimental feeding in healthy adults with a protein to which humans normally are not exposed, keyhole limpet hemocyanin (KLH), did result in downregulation of the peripheral T-cell response, although stimulation of local as well as systemic humoral immunity was observed.[89] Conversely, intranasal application of KLH tended to suppress both cell-mediated and humoral peripheral immunity to this antigen.[90] The mechanisms remain unclear, however, and sequestration of specific immune cells into the antigen-exposed mucosae or regional lymph nodes is one possible pitfall that is diffi-

cult to refute because local immunity was enhanced in both studies. Such a mechanism has been suggested in untreated celiac disease patients whose circulating T cells show decreased response to gluten compared to treated patients on a gluten-free diet.[91] Nevertheless, human feeding with KLH was recently repeated with parallel systemically immunized controls, and mucosally induced T-cell tolerance was indeed confirmed in peripheral blood (Mayer, L. *et al.*, unpublished observations). [84] Also notably, feeding low doses of myelin basic protein to patients with multiple sclerosis resulted in a higher frequency of circulating T cells with a potency for production of the down-regulatory cytokine TGF-β compared with T cells from placebo-fed patients.[92]

Putative Lympho–Epithelial Interactions

A central role of the gut epithelium in oral tolerance is suggested by the observation that its experimental induction depends on preserved integrity of the mucosal barrier.[93,94] Suppressive effects resulting from interactions between the dominating T-cell receptor (TCR)α/β^+CD8$^+$ intraepithelial lymphocyte (IEL) subset and a normal epithelium represent one intriguing possibility, and there is some supporting evidence to this effect[95]; it is possible that luminal antigenic peptides are presented by resting enterocytes with inadequate costimulation to IELs or subepithelial CD4$^+$ T cells.[96] Experiments in CD8-knockout mice have suggested that CD8$^+$ T cells are crucial for the downregulation of enterically elicited mucosal immunity but not for mucosally induced suppression of systemic antibody responses.[97] Moreover, the chief effect obtained when enterocytes have been used as unconventional APCs in various test systems has been stimulation of CD8$^+$ T cells with suppressor function.[84,98] Human enterocytes express a ligand (gp180) that, by interaction with the α chain of CD8, may rapidly activate the tyrosine kinase p56^{1ck} and thereby preferentially trigger CD8$^+$ T cells.[99] Antigen presentation by major histocompatibility complex (MHC) or CD1d molecules on enterocytes in this context could theoretically leave cognate IELs, and even CD4$^+$ lamina propria T helper (Th) cells, in an unresponsive state or induce an active down-regulatory potential by a deviated cytokine profile (FIG. 5). Moreover, basolateral exosomes with MHC class II–dependent antigen-presenting capacity may be released from the gut epithelium and act as "tolerosomes,"[100] either locally or at distant sites such as mesenteric lymph nodes or the liver (FIG. 5).

The additional involvement of TCRγ/δ^+ IELs in oral tolerance is also an intriguing possibility (FIG. 5) in view of the suggestion that this subset in the mouse may act as "contrasuppressor cells," thereby being able to release intestinal IgA responses from T-cell-mediated suppression.[101] Subsequent studies have shown that this effect probably can be ascribed to IL-10 secreted
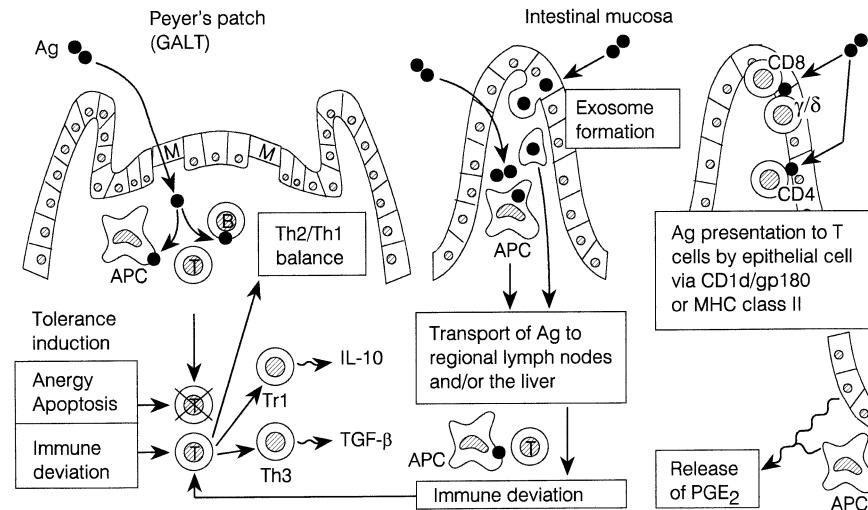
FIGURE 5. Schematic depiction of putative mechanisms suggested for induction of tolerance via the gut ("oral tolerance"). Hyporesponsiveness to innocuous antigens (Ag) gaining access to immune cells through M cells (M) in gut-associated lymphoid tissue (GALT) or through the intestinal surface epithelium, may be explained by T-cell anergy, clonal deletion by apoptosis, and cytokine-mediated active suppression (immune deviation), either locally or at distant sites after dissemination of absorbed Ag or transport of Ag in antigen-presenting cells (APC) or epithelial exosomes. In the normal state, when only low-grade activation takes place, subepithelial APCs migrate quickly to regional lymph nodes with acquired Ag, thus prohibiting mucosal hyperactivation of T cells locally. Special regulatory T cells (Tr1 and Th3) producing the suppressive cytokines IL-10 and TGF-β appear to be important for the development of a balanced Th2/Th1 profile. A down-regulatory tone in the gut may also be ascribed to unconventional Ag presentation by epithelial cells (to the right) and the effect of prostaglandin E_2 (PGE_2) released from the epithelium or APCs. Details are discussed in the text.

by $CD4^+$ T cells that are controlled by γ/δ T cells operating through this down-regulatory cytokine in low-dose tolerance.[102] If this mechanism operates also in humans, the preferential expansion of intraepithelial γ/δ T cells in the celiac lesion might contribute to the striking increase in Ig-producing immunocytes and activated lamina propria $CD4^+$ T cells seen in untreated patients.[103] However, the increase of TCRγ/δ$^+$ IELs in celiac disease could rather reflect that they are cytotoxic cells involved in the clearance of microorganisms or damaged epithelium to preserve the surface barrier.[1,98,104]
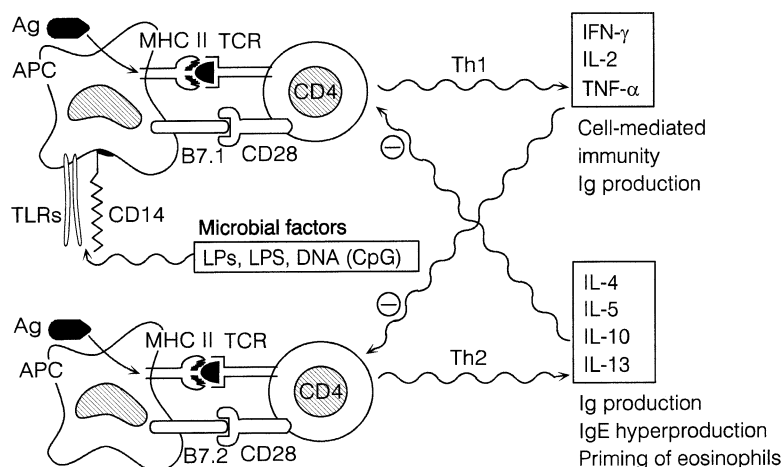
FIGURE 6. Schematic representation of polarized patterns of cytokines produced by activated T helper (Th) cells. When naive CD4$^+$ Th cells are primed by a professional antigen-presenting cell (APC) providing adequate costimulatory signals, they differentiate into Th1 or Th2 cells. Such skewing of the immune response depends on the presence of microenvironmental factors, including cytokines and danger signals from microbial products interacting with APC receptors, thereby determining the expression level of various costimulatory signals. For simplicity, only the lipopolysaccharide (LPS) receptor CD14 and Toll-like receptors (TLRs) are indicated, together with the costimulatory molecules. B7.1 and B7.2. Th1 cells produce predominantly IFN-γ, IL-2 and TNF-α, while Th2 cells are mainly capable of IL-4, IL-5, IL-10, and IL-13 secretion. Distinct Th1 and Th2 profiles are further promoted by inhibitory feedback loops as indicated. Abbreviations: Ag, antigen; MHC II, major histocompatibility complex class II molecules; TCR, T-cell receptor; LPs, lipoproteins; CpG, unmethylated nucleotide motif.

Role of Costimulation by Antigen-Presenting Cells

Productive T-cell activation with appropriate proliferation and cytokine secretion requires two signaling events, one through the TCR and another through a receptor for some costimulatory molecule (FIG. 6). Without the latter signal, the T cells mount only a partial response and, more importantly, may be subjected to active tolerance induction[83] or anergy with no capacity for production of their own growth factor IL-2 upon restimulation.[105] The required costimulation for productive immunity is provided by soluble mediators such as IL-1 and through cellular interactions, especially ligation of B7

(CD80/CD86) on professional APCs with CD28 on the T cells.[106] There is particularly great interest in the role of DCs in shaping the phenotypes of naive T cells during such initial priming. Also, because DCs have migratory properties, they largely determine the tissue site in which primary immune responses will take place.[107,108]

Immature DC subsets are found both in the circulation and in most peripheral tissues from which they, after endocytosis of antigen, generally migrate via draining lymphatics into regional lymph nodes to perform antigen presentation. The actual expression level of various costimulatory molecules on the matured and activated DCs during the priming process influences the differentiation of naive T cells in terms of cytokine production—that is, a Th1 (IFN-γ, IL-2, and tumor necrosis factor (TNF)-α) versus a Th2 (IL-4, IL-5, IL-10, and IL-13) profile (FIG. 6). Interaction of the T-cell CD28 receptor with B7.1 (CD80) appears to favor the former, and with B7.2 (CD86) the latter cytokine profile.[109] This Th1/Th2 paradigm is important in relation to atopic allergy, because IgE production as a basis for type I hypersensitivity (atopy) is highly dependent on IL-4 and IL-13.[110] Also, homeostatic cross-regulation should ideally take place between the Th1 and Th2 responses.[111]

Considerable information exists about putative aberrant immunoregulatory functions of nonprofessional APCs such as keratinocytes, because they lack appropriate costimulatory molecules necessary for productive immunity.[112] As alluded to above, this also applies to enterocytes (FIG. 5). Thus, both B7 and intercellular adhesion molecule-1 (CD54) are virtually absent on normal human enterocytes.[113] Low levels of B7 might actually engage the high-affinity costimulatory molecule CTLA-4 on Th cells,[114] which could result in a down-regulatory response contributing to oral tolerance.[115]

In the normal state, even the subepithelial professional APCs in human gut mucosa, which have both macrophage and DC properties, show an extremely low level of B7 expression[15,116] and might therefore ligate CTLA-4 rather than CD28 on T cells. Also, only B7.2 (CD86) is normally detectable, and this molecule has been shown in animal experiments to be important for low-dose oral tolerance.[117] Functional characteristics of normal human lamina propria CD4$^+$ T cells actually do suggest that they are tightly controlled by suppression. First, they are remarkably unresponsive to signaling via the classical TCR/CD3 pathway alone, whereas anti-CD2 (particularly together with engagement of CD28) induces proliferation and cytokine secretion.[118,119] Second, they appear to be particularly susceptible to Fas (CD95)-mediated apoptosis, which might contribute to the limitation of clonal proliferation in the normal gut.[120] Third, they may be kept in check by prostaglandin E$_2$ released by the gut epithelium or lamina propria macrophages.[121]

The fact that resident APCs from normal human gut mucosa are quite inert in terms of immune-productive stimulatory properties[122] supports the notion that they play a central role in induction of oral tolerance. One possibility is that, in the normal state (i.e., when subjected only to low-grade activation),

they carry penetrating dietary and innocuous microbial antigens away from the mucosa, thereby avoiding local hyperactivation of immune cells (FIG. 5). Indeed, normal human intestinal mucosa shows only very low expression levels of mRNA for IFN-γ, the key cytokine of activated Th1 cells.[123] The same is true for Th2 cytokines such as IL-4 and IL-5. Moreover, animal experiments have demonstrated that intestinal APCs can be triggered by proinflammatory factors to become mobilized[124] and even constitutively migrate rapidly with acquired epithelial elements and antigens away from the intestinal mucosa.[125,126] Such successful "silent" antigen clearance probably depends on relatively low doses of absorbed antigen and may result in systemic T-cell-dependent tolerance induction (FIG. 5). Interestingly, *in vivo* expansion of the intestinal APC population enhanced the induction of oral tolerance in mice,[127] whereas concurrent APC activation by immunization with cholera toxin or treatment of the animals with IL-1 resulted in productive immunity against the fed antigen.[128]

Animal studies have suggested differential effects of antigen dose and feeding frequency on the mechanisms of tolerance induction.[8] At very high doses, both Th1 and Th2 cells were shown to be deleted following initial activation, an event apparently depending on apoptosis in Peyer's patches.[129] Anergy and clonal deletion would be antigen-specific events, in contrast to active suppression resulting from deviation of cytokine profiles induced by T-cell stimulation locally or in regional lymph nodes or the liver[130] after distant transport of antigen in APCs or epithelial exosomes (FIG. 5). Experiments performed to induce therapeutic tolerance via the gut in various autoimmune disease models have relied on a bystander effect of stimulated T cells which, through immune deviation, preferentially have secreted down-regulatory cytokines, particularly TGF-β.[131] The gut has been suggested to harbor T cells with a propensity for secretion of TGF-β (so-called Th3 cells), which appear to be particularly resistant to apoptosis,[129] but this subset has not been clearly identified in humans. Another regulatory T-cell subset (Tr1) with a remarkable propensity for IL-10 production has been identified both in the murine and human gut.[132,133]

Altogether, a complex scenario may be proposed for oral tolerance, depending on apoptosis when intestinal antigen exposure is excessive and on anergy due to lack of costimulatory APC molecules, antigen clearance from the mucosa, and induction of immune deviation (skewing of T-cell cytokine profile) at lower antigen doses (FIG. 5). This scenario is further complicated by the fact that several cytokines contributing to the local profile are produced not only by T cells, but also by APCs and epithelial cells, for instance the down-regulatory cytokines TGF-β and IL-10. Furthermore, it remains unclear whether the most important immunoregulatory events for oral tolerance against dietary antigens takes place in the Peyer's patches, in the lamina propria, or in systemic lymphoid organs.[134–136]
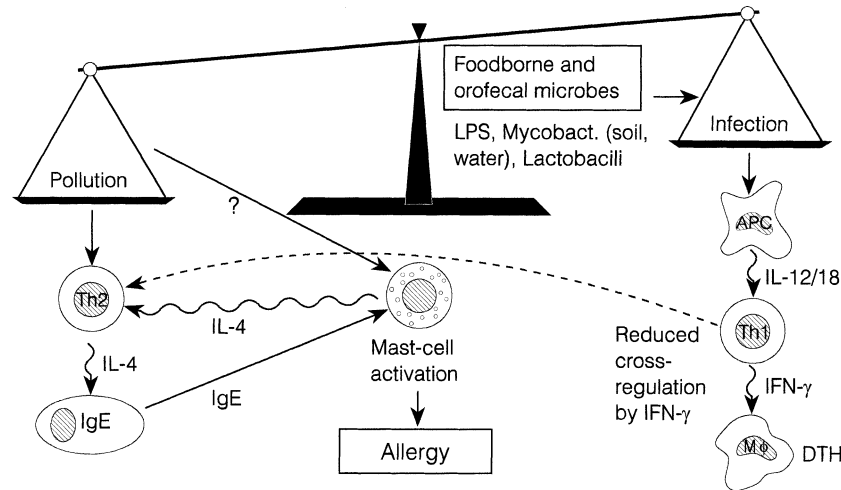
FIGURE 7. Shift of the balance between microbial exposure and pollution as a possible cause of the increase in IgE-mediated allergy over the last decades. Infections due to foodborne and orofecal microbes are dramatically decreased in the Westernized part of the world, and so are other Th1-driving stimuli such as lipopolysaccharide (LPS) from gram-negative bacteria, saprophytic mycobacteria from soil and water, and lactic acid-producing bacteria. Reduced secretion of IL-12 and IL-18 by antigen-presenting cells (APC) is one explanation for downregulated Th1-cell responses, which are the basis for delayed type hypersensitivity (DTH)-dependent immune defense. Consequently, there is also less negative crossregulation of Th2 cells by IFN-γ, allowing enhanced IgE responses to protein antigens (allergens). Pollution may also directly (or indirectly via mast-cell activation) stimulate IgE production. Modified from Brandtzaeg.[138]

Mucosal Homeostasis versus Allergy

It may seem paradoxical that mucosal disorders such as inflammatory bowel disease (IBD) and celiac disease appear to depend, at least initially, on putative Th1-cell-driven pathogenic mechanisms,[103,137] while atopic (IgE-mediated) allergy originates from Th2-cell responses[110,138] which generate the essential cytokines IL-4 and IL-13 (early phase) as well as IL-3, IL-5, and GM-CSF (late phase). According to the hygiene hypothesis, the increasing incidence of allergy in Westernized societies over the last decades[139,140] may to some extent be explained by a reduced microbial load early in infancy,[140–142] resulting in too little Th1-cell activity and therefore an insufficient level of IFN-γ to cross-regulate optimally Th2-cell responses (FIG. 7). In this context an appropriate composition of the commensal bacterial flora[144] and exposure

to foodborne and orofecal microbes[145,146] most likely exert an important homeostatic impact, both by enhancing the SIgA-mediated barrier function (see above) and by promoting oral tolerance through a shift from a predominant Th2-cell activity in the newborn period to a more balanced cytokine profile later on.[147] Thus, the intestinal microflora of young children in Sweden was found to contain a relatively large number of *Clostridium* sp., whereas high levels of *Lactobacillus* sp. and *Eubacterium* sp. were detected in an age-matched population from Estonia.[148] Perhaps this difference could explain the lower incidence of allergy in the Baltic countries compared with Scandinavia. Interestingly, the intestinal microflora of children in Estonia were deemed to be somewhat similar to that of Swedish children in the 1960s. Also, the intestinal microflora of Estonian children with allergy appeared to differ from that of their healthy counterparts, particularly by containing less lactobacilli.[149] A recent Finnish study likewise reported that atopic infants had more clostridia and tended to have fewer bifidobacteria in their stools than nonatopic controls.[150]

Such observations make a good case for studying the potential clinical benefits of prebiobics and probiotic bacterial strains from the indigenous gut flora.[143,151,152] Similarly, there is some hope that immunization with mycobacterial antigens might skew the cytokine profile towards Th1 and thereby, through cross-regulation, dampen Th2-dependent allergic (atopic) symptoms.[153,154] Newborns are in fact able to mount a Th1-type immune response when appropriately stimulated.[155] Also notably, the bacterial endotoxin or LPS receptor CD14 together with the Toll-like receptor (TLR4) on APCs, as well as other TLRs that recognize microbial products (e.g., lipoproteins and peptidoglycans) as danger signals or PAMPs (pathogen-associated molecular patterns) are in this respect an important link between innate and specific immunity (FIGS. 6 and 7). This link operates via the NF-κB activation pathway to release proinflammatory cytokines,[156,157] including the Th1-inducing IL-12 and IL-18.[158,159] Even certain CpG motifs of bacterial DNA have been shown to promote Th1-cell activity through interaction with TLR9.[160–162] Subepithelial intestinal APCs most likely express TLRs, although this has not yet been studied properly in the human gut.[163] However, low levels of CD14 are normally present on these cells, and its expression is enhanced together with that of B7.1 and B7.2 by proinflammatory factors.[15,116]

Altogether, it appears that the human intestinal immune system preferentially responds with a dominating Th1 profile,[123] even against various food antigens in the seemingly normal state.[164] This appears to be true for T cells also in the duodenal mucosa of children with cow's milk hypersensitivity[165] and might to some extent reflect a high expression level of the Th1-promoting cytokine IL-12 observed for putative APCs situated below the FAE of Peyer's patches in children.[166] The strong bias towards Th1-cell responses in the human gut could thus contribute to the fact that the majority of food-allergic

children outgrow their problems.[2] This is in contrast to respiratory atopic allergy, which tends to persist and increase in severity.[167,168] Most likely, danger signals from an established intestinal bacterial flora, as well as the environmental microbial exposure, exert an important drive towards an adequate Th1 skewing in the gut, thus counterbalancing excessive Th2 responses (FIGS. 6 and 7). Nevertheless, allergen-specific mucosal Th2 cells have been detected in patients with presumably cow's milk–induced gastroenteritis.[169]

Although the immune system in the airways also responds to antigen stimulation in the presence of danger signals (infection or inflammation) with a Th1 profile,[107] an increasingly prominent Th2 profile generally develops as the basis for IgE-mediated (type I) respiratory allergy[167,170] in individuals with hereditary atopic predisposition.[171,172] This skewing towards Th2-cell responses may be influenced by the so-called "lymphoid" DC type, recently named plasmacytoid DCs (P-DCs), which can be identified by their high level of IL-3-receptor (CD123) in allergic nasal mucosa.[173] *In vitro*, P-DCs have been shown to drive naive T cells towards a Th2 response with IL-4 and IL-5 production.[174] Interestingly, we have been unable to detect P-DCs in the intestinal lamina propria, even in IBD and celiac disease (Jahnsen *et al.*, unpublished observations). Therefore, the apparent inability of this DC subset to home to intestinal effector sites might contribute to the Th1 dominance of immune responses in the human gut as a result of little cross-regulation from local Th2 responses. The paucity of human intestinal Th2 responsiveness[166] is emphasized by the fact that there is usually no detectable IgE production at this mucosal effector site, even in adult food-allergic persons with overt atopy.[175] Hence, there may be several mechanisms other than a local mucosal Th2 response to explain gastrointestinal allergy against dietary antigens,[2,176] including recruitment of mast cells armed with IgE from mesenteric lymph nodes, type III (immune complex)-mediated reactions and type IV (delayed-type) hypersensitivity.[2,138]

The feeding and treatment regimens (e.g. antibiotics) to which the newborn is subjected, and even the nutritional state, have a significant impact on its establishing indigenous microbiota as well as on gut integrity and hence may disturb the balance of its developing mucosal immune system.[144,177,178] The role of commensal bacteria for mucosal tolerance induction in humans was highlighted in a recent clinical trial with postnatal colonization (for 6 months) of a probiotic lactobacillus strain[60]; after 2 years, a 50% reduction of atopic eczema was observed in these children compared with placebo controls. Intestinal colonization of lactobacilli and bifidobacteria is promoted by breast milk because of its large amounts of oligosaccharides, which have prebiotic properties[178]; these microorganisms may directly enhance the Th1 profile in the gut (FIG. 7) by inducing IL-12, IL-18, and IFN-γ.[179,180] Also notably, *E. coli* is a strong inducer of IL-10 secretion, apparently derived from APCs.[181,182] This has directly been shown to be an important suppressive cy-

tokine in the gut.[102] Thus, the indigenous microbiota may have an impact on mucosal homeostasis beyond that of enchancing the SIgA system or promoting a Th1-cytokine profile that counterbalances Th2-cell responsiveness.[184]

Effect of Breastfeeding on Oral Tolerance

Through avoidance of too-early local immune activation, for instance limiting the intestinal upregulation of the costimulatory B7 molecules,[8,134] the shielding effect exerted by SIgA from breast milk on the suckling's GALT may contribute to the establishment of oral tolerance not only against the indigenous microflora, but also against dietary antigens such as gluten. Antibodies to gluten peptides are present in breast milk,[29] and breastfeeding has in fact been shown to protect significantly against the development of celiac disease in children unrelated to the time of solid food introduction.[65] Early exposure to cow's milk has been suggested to be associated with predisposition to type 1 (insulin-dependent) diabetes, and investigations have particularly focused on immune stimulation by bovine serum albumin,[185] β-lactoglobulin,[186] and insulin.[187] In a recent study, short-term breastfeeding and early introduction of cow's milk were found to be associated with progressive signs of type 1 diabetes-related autoimmunity.[188]

On the basis of such observations, it may be tentatively concluded that mixed feeding, rather than abrupt weaning, appears to promote tolerance to food proteins and thereby also avoidance of potentially harmful cross-reactive autoantibodies. This notion is further supported by reports suggesting that cow's milk allergy is more likely to develop in infants whose mothers have relatively low levels of milk IgA antibodies to bovine proteins.[28,189] It is also noteworthy in this context that allergic mothers appear to have decreased levels of ovalbumin-specific IgA[190] and elevated levels of Th2-promoting IL-4[191] in their breast milk.

On the other hand, the presence of TGF-β and IL-10 in breast milk might contribute to its tolerogenic properties, because these cytokines exert pronounced immunosuppressive effects in the gut.[183,192] The balance between IL-10 and the Th2-promoting IL-4 might be of particular significance. Moreover, TGF-β enhances the epithelial barrier function.[193] Interestingly, TGF-β has been reported to be present at a higher level in maternal colostrum provided for infants that did not develop atopic eczema during exclusive breastfeeding compared to those with early-onset symptoms.[194]

Many recent epidemiological studies do support the view that breastfeeding protects against atopic allergy and asthma,[62–64] although this is still a somewhat controversial issue.[177] Food antigens appear in breast milk, but dietary restriction during pregnancy and breastfeeding has shown no conclusive effect on the development of atopic diseases in the child.[177,178] It remains an open question whether early exposure to small amounts of food antigens may

actually have a positive effect on tolerance induction, especially when occurring in its natural context in the gut lumen of a suckling.[195]

CONCLUSIONS

Several more or less well-defined variables influence the development of productive mucosal immunity and oral tolerance, therefore constituting a complex and rather enigmatic mechanistic basis for adaptive immune defense and adverse immunological reactions to foods. An inadequate epithelial barrier against luminal antigens is an important primary or secondary event in the pathogenesis of several mucosal diseases—being influenced by the individual's age (e.g., preterm versus term infant), activation of the epithelium and subepithelial elements such as APCs and mast cells (e.g., by infection, cytokines, or neuropeptides), and the shielding effect of SIgA provided by breast milk or produced by adaptive B-cell responses in the infant's gut. The consequences will depend on how fast mucosal homeostasis can be attained or re-established after abrogation.

SIgA is the best defined effector component of the mucosal immune system, and it operates by immune exclusion against infectious agents and other harmful substances. Breastfeeding provides the infant with this important first-line specific defense. Breast milk also contains an array of important immunoregulatory factors and promotes colonization of lactic acid-producing bacteria. These members of the indigenous microbiota are powerful in combating pathogenic intruders that may break oral tolerance,[151,152] and they also appear to exert a beneficial effect on the cytokine balance of the host and thereby on the developing immunological responder phenotype. Animal experiments have indeed documented that the commensal bacterial flora are crucial both for induction of oral tolerance and for its re-establishment after abrogation.[3] This effect not only might be mediated through immune modulation, but could also be partly explained by enzymatic activity of the indigenous flora that degrades food proteins to tolerated peptides.[196]

ACKNOWLEDGMENTS

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