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Review article

Postnatal development of intestinal immune system in piglets: implications for the process of weaning

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Abstract – European-wide directives are in place to establish a sustainable production of pigs without using production enhancers and chemotherapeutics. Thus, an economically-viable pig production is now only possible when the physiological mechanisms of defense against pathogens and tolerance against nutrients and commensal bacteria in the intestinal immune system are taken into account. During the postnatal period the piglet is facing first the time large amounts of new antigens and at weaning a second wave of nutritional antigens is entering the intestinal tract. The appropriate development of humoral and cellular functions of the intestinal immune system is essential for optimum growth and performance of the piglets. The integrity of the intestinal surfaces is a prerequisite of intestinal immunity and tolerance. Secretory IgA serves to exclude harmful antigens from uptake. The induction of intestinal immune reactions starts with antigen presentation by professional antigen presenting cells of Peyer's patches and mesenteric lymph nodes. In addition, the intestinal lamina propria serves as a mucosal compartment for regulation of immune responses. Here especially T regulatory cells ($CD4^+$ $CD25^+$) have their function for maintaining intestinal homeostasis. The network of mucosal T and B cells develops after birth in a programmed sequence; it is almost completed at week 7 after birth. Weaning is associated with changes in the regulation of the lymphoid cells in the mucosa. In small and large intestine increases in pro- and anti-inflammatory cytokines were observed after weaning in lymphocytes. Epithelial cells were studied both in intestinal samples and in vitro. Here the cytokine patterns provide evidence that weaning is inducing a transient inflammation of the mucosa. Piglets weaned under conventional conditions have a thicker mucosa than pigs weaned from isolators. Cells of isolator-reared pigs show slightly higher levels of activation markers – probably

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reflecting the interaction of the foreign protein derived from bovine milk. The results presented in this overview demonstrate that further effort is necessary to elucidate the function of the porcine intestinal immune system in the postnatal period and at the time of weaning to provide criteria for porcine intestinal health.

inflammation / intestinal immunity / mucosa / nutrition / pig / postnatal / weaning

Résumé – Développement postnatal de l'immunité intestinale chez le porcelet : implications pour le processus de sevrage. Des directives européennes ont été adoptées, en vue de mettre en place une production durable de porcs sans facteurs de croissance ni antibiotiques. Dans ce contexte, un élevage porcin ne sera économiquement viable que si sont pris en compte les mécanismes physiologiques de défense contre les agents pathogènes, et de tolérance vis-à-vis des aliments et des bactéries commensales, mécanismes engageant le système immunitaire intestinal des animaux. A la naissance, le porcelet fait face, une première fois, à de grandes quantités d'antigènes ; puis, au sevrage, une seconde vague de nouveaux antigènes alimentaires entre dans le tube digestif. Il est donc essentiel pour la croissance et les performances optimales des porcelets d'avoir un développement approprié des fonctions humorales et cellulaires du système immunitaire intestinal. L'intégrité des surfaces intestinales est un préalable indispensable à l'immunité intestinale et à la tolérance. Les IgA sécrétoires contribuent à prévenir l'absorption des antigènes dangereux. L'induction de la réponse immunitaire intestinale débute avec la présentation des antigènes par des cellules spécialisées des plaques de Peyer et des ganglions lymphatiques mésentériques. De plus, la lamina propria intestinale représente le compartiment muqueux de régulation des réponses immunitaires, en particulier, avec les cellules T régulatrices ($CD4^+$ $CD25^+$) intervenant dans le maintien de l'homéostasie. Le réseau de cellules muqueuses T et B se développe après la naissance selon une séquence programmée, et est pratiquement achevé 7 semaines après la naissance. Le sevrage est associé à des changements dans la régulation des cellules lymphoïdes dans la muqueuse. Ainsi, des augmentations dans l'expression des cytokines pro- et anti-inflammatoires dans les lymphocytes intestinaux ont été observées après le sevrage. Les cellules épithéliales ont également été étudiées dans les échantillons intestinaux et in vitro. Ici, les profils de cytokines montrent que le sevrage induit une inflammation transitoire de la muqueuse. Des porcelets sevrés dans des conditions conventionnelles présentent une muqueuse plus épaisse que des porcelets élevés et sevrés en isolateurs. Les cellules de ces derniers montrent des niveaux de marqueurs d'activation légèrement supérieurs, reflétant probablement l'interaction des protéines étrangères dérivées du lait de bovin. Les résultats présentés dans cette synthèse démontrent que des efforts renouvelés sont nécessaires pour élucider les fonctions du système immunitaire intestinal chez le porc durant la période postnatale et au moment du sevrage, afin de fournir des critères de santé intestinale appropriés.

Immunologie / intestin / porc / sevrage

1. INTRODUCTION

Pig production represents an important source of revenue for the agricultural industry of the European Union. However, as a direct result of environmental and animal welfare concerns, European-wide directives are now in place which severely restrict the use of both growth promoters and antimicrobials. These legislative events have had a major impact on pig production within Europe, rendering current production systems wholly inappropriate for sustainable and economically-viable production of pigs. New and novel systems for animal production are clearly required if the pig industry is to maintain, or improve, its economic competitiveness within Europe and world markets. Development of new systems for pig production that deliver immunocompetent, healthy and fast growing animals is a significant challenge. This objective will be met only when the interactions between neonatal biology and maternal factors, rearing environment and post-weaning nutrition are fully understood.

2. GUT FUNCTION AND IMMUNITY

The piglet is profoundly immunodeficient at birth and is highly dependent upon a supply of both specific and non-specific immune factors present in maternal colostrum and milk for immune protection, development and survival. Directly after birth the piglet takes up macromolecules from the intestinal lumen in a non-selective way. IgG from the sow's colostrum is absorbed by the newborn via enterocytes [22, 23]. A rapid "closure" of the gut for the macromolecular uptake occurs within 24 and 48 h after birth [25]. Colostral intake is essential for efficient immune reactions, and the germ-free colostrum deprived "virgin" piglet can serve as model for the assessment of development of defence against of pathogens [24]. Lymphocytes migrating into the lactating mammary gland obviously provide the immunological information necessary for the production of secretory IgA that is released into the sow's milk for maintenance of humoral immunity in the offspring [35].

The functional immaturity of the neonatal cellular and secretory immune systems is such that newborn pigs are only able to generate limited T and B cell responses when challenged with pathogens, thus contributing to their immuno-compromised state [13]. Clearly, development of immunocompetence is an absolute requirement for optimum growth and performance. However, in the context of exposure to a wide range of antigens associated with pathogens and with commensal bacteria and food, a definition of immunocompetence must consider the ability to mount appropriate responses to antigens. This will include the ability to generate tolerance to food and commensal bacterial antigens as well active immune responses to pathogens [5]. There is a considerable body of evidence to indicate that early nutrition and environment impacts on immune development and function (the hygiene hypothesis) [11, 21]. There is also a view that the immune system requires specific stimulatory inputs during very defined periods of early postnatal development in order to promote optimum immune function and that failure to provide these inputs can markedly impair immune function in early and later life [10, 20]. While these viewpoints are conceptually important there is little mechanistic data to either support or refute them.

In pigs as in other species, significant quantities of fed protein are absorbed immunologically intact across the intestinal mucosa [42, 47]. Immune responses to harmless dietary components must be regulated to prevent tissue damage and impaired absorption of macromolecules, and systemic tolerance to fed proteins ("oral tolerance") has been demonstrated in the pig [3, 40]. Depending on the food composition and on the presence of oral food components the morphology of both the intestinal mucosa and the intestinal immune cells changes [15, 30, 36, 37]. Thus, the immunological structures associated with gastrointestinal tract have evolved to generate different types of response to pathogens and to harmless environmental antigens. The sites at which such "value judgements" are made are unclear in any species, but models of the function of mucosa-associated lymphoid tissues must account for both of these outcomes.

In order to control infections from with the gut the local mucosal immune system has adopted a remarkably different strategy to that which operates systemically. Antigens that gain entry into tissue via cuts or the mouth parts of biting insect have to be actively eliminated, a process which involves inflammation and some degree of tissue damage. In contrast within the gastrointestinal tract, micro-organisms which remain within are unlikely to cause harm. Only once the organism (or its toxin) has adhered to the epithelial cells and gained entry across the epithelium will it cause disease. In this way maintaining the integrity of the epithelial surface is a critical process in preventing infections. It is then not surprising that mucosal immune defence mechanisms

tend not to be inflammatory and primarily directed toward keeping potentially harmful antigens within the lumen of the intestine, where peristalsis and the constant flow of digesta will effectively remove them. IgA antibodies, which are unable to activate complement, are clearly well able to fulfil this role. In health the physical integrity of the mucosal barrier (epithelial cells and mucus) together with IgA form an effective barrier to the entry of potentially harmful antigens. Only once the barrier is breached do other defensive processes play a role. It is then not surprising that both innate and acquired immune defences play an important role in intestinal defence.

In pigs the structure of the Peyer's patches, under various antigenic stimuli, has been described [7, 26, 29], and the appearance of lamina propria and intraepithelial lymphocyte subpopulations have been analysed [32, 34, 43]. To analyse these various methods to isolate lymphocytes and dendritic cells from the intestine have been developed [2, 18, 19, 33, 39].

3. INNATE IMMUNE DEVELOPMENT

Gut epithelial cells are now recognised to play a major role in innate immunity, forming a highly specialised physical and functional barrier to dietary and microbial antigens. These cells respond directly to colonising bacteria using specific cell-surface pattern recognition receptors to detect and respond to the presence of bacteria and specific bacterial moeities. A number of diverse receptor systems are expressed on epithelial cell surfaces that recognise bacteria and communicate signals to underlying lymphoid cell populations. These receptor systems comprise glycan receptors, which recognise fimbrial lectins found on many commensal and pathogenic strains of bacteria and viruses, and toll-like receptors that recognise microbial molecular patterns.

Toll-like receptors (TLRs) are emerging as a functionally important class of membrane receptors with key roles in bacterial recognition and immune-modulation. TLRs recognise conserved molecular patterns (pathogen-associated molecular patterns, PAMPs and commensal-associated molecular patterns, CAMPs) shared by large groups of bacteria and other gut micro-organisms. Currently, in humans there are ten known TLRs; of these, the ligands for TLR 2, 3, 4, 5 and 9 have been identified. In addition to their important function in bacterial ligand recognition, TLR also activate signal transduction pathways (e.g. NFkB and MAPkinases) that trigger gene expression. By these mechanisms TLR ligation triggers the release of many cytokines and chemokines that can exert a profound influence on cells of the innate and adaptive immune systems.

4. PROTECTIVE IMMUNITY AND TOLERANCE IN THE MATURE INTESTINE

Induction of adaptive immune responses begins with processing and presentation of antigen by professional APCs, probably in the organised tissues of the Peyers patches or mesenteric lymph nodes [16]. Dendritic cells (DC) represent a key antigen-presenting cell and are important for development of innate and adaptive immunity. The subsequent response to mucosal antigens either active cellular or IgA antibody immune responses or immunological tolerance depends on previous exposure of the dendritic cell to TLR-binding molecules, or it may involve the subsequent recognition of antigen by primed T-cells in cytokine microenvironments such as the intestinal lamina propria. The outcome to antigen is a critical step in the maintenance of "enteric health" and requires the ability to discriminate between, and respond appropriately to, pathogen and harmless antigens. Studies over the last decade have identified a number of mechanisms by which immune responses in the intestine are regulated. These include clonal deletion, clonal anergy and active control by regulatory cells. Clonal anergy is a feature

of very high doses of food antigen while the induction of regulatory T-cells (T_{reg}) appears to be important at physiological levels of exposure [38]. In the mouse, regulatory T-cells have been identified by their expression of cell surface molecules, which include CD4, CD25, CTLA4 and lack of expression of high molecular weight isoforms of CD45, and by their secretion of specific cytokines, primarily interleukin-10 (IL-10) and transforming growth factor β (TGFβ) [8, 17, 45]. Although some studies have clearly demonstrated a role for centrally-generated (thymic) T_{reg} in control of autoimmunity, regulation of responses to novel, foreign antigens at mucosal surfaces is likely to involve antigen-driven peripheral differentiation of regulatory cells from naïve cells in a local cytokine/hormone micro-environment [5, 8]. The intestinal mucosal represents a likely environment for generation of T_{reg} as these cells show selective expression of TLR-4, 5, 7 and 8 and are activated by bacterial ligands such as LPS. Unfortunately, $CD4^+CD25^+$ T_{reg} from mucosal sites have been difficult to study in murine systems due to the difficulty of isolation of sufficient numbers. This has not been a problem with pig intestinal T-cells. Isolation techniques recover large numbers of cells (3×10^8 in a routine isolation), which remain viable in culture or after flow cytometric sorting [2, 19]. Previous studies in mature pigs have demonstrated unusual surface phenotype and cytokine profiles of these cells (including high levels of IL-4 and IL-10, Bailey and Stokes, unpublished data), consistent with the constitutive presence of mucosal regulatory T-cells controlling responses to normal microbial flora and food antigens [4, 18]. Two recent studies further suggest unusual functions for the intestinal mucosal environment. Firstly, it has been shown that pig lamina propria T-cells, like human mucosal T-cells and regulatory cells [9, 41], are extremely susceptible to activation-induced cell death (AICD) in vitro. Consistent with the idea that mucosal T-cell function is controlled by apoptosis, we have shown, in vivo, that very few of the $CD45RC^+$ cells which are the majority of the $CD4^+$ T-cells in the intestine ever migrate away from the intestine (Bailey and Rothkötter, unpublished observation). Secondly, addition of recombinant pig IL-2 into the cultures activated to cell death results in full rescue of $CD8^+$ T cells, but has no effect on the survival of $CD4^+$ T cells. In contrast, addition of TGF-β rescued LPL $CD4^+$ T cells whilst having no effect on $CD8^+$ T cells (Plunkett and Bailey, unpublished observations).

Thus, our data strongly suggests that the intestinal lamina propria is a specialised microenvironment, which in homeostasis, contains and promotes differentiation of regulatory and regulated T-cells. During challenge by potential pathogens, epithelial and T-cell cytokines may recruit or promote transient or permanent differentiation into effector T-lymphocytes.

5. DEVELOPMENT OF MUCOSAL IMMUNOLOGICAL ARCHITECTURE

Unlike the mature pig [43, 47], where there is a high degree of organisation, it has been shown that the cells and structures involved in mucosal immune responses are initially absent at birth and populate the intestine of the young pig in a highly programmed sequence [26, 32, 44]. To date four phases to this process have been identified:

1. The newborn piglet has very few lymphocytes in its intestinal epithelium or lamina propria. Clusters of lymphocytes are present in the mucosa, in the areas that will subsequently develop into Peyer's patches [7, 28], but these clusters have no clear immunological structure.

2. In the first two weeks of life the intestine rapidly becomes colonised with lymphoid cells. These cells express the CD2 surface marker but do not coexpress CD4 or CD8. The Peyer's patches begin to organise

during this period, reaching a relatively "adult architecture" by 10–15 days.

3. In piglets 2–4 weeks old the intestinal mucosa becomes colonised by $CD4^+$ T cells, primarily in the lamina propria. $CD8^+$ cells are still largely absent. Small numbers of B cells appear, preferentially expressing IgM.

4. From the age of five weeks onwards, $CD8^+$ cells begin to appear in the intestinal epithelium and around the epithelial basement membrane. In the crypt areas many IgA^+ B cells are appearing. By 7 weeks the architecture of the intestine is comparable to that of the mature animal.

These phenotypic studies strongly suggest that the mucosal immune system remains relatively immature throughout the "normal commercial weaning" period. If, as we have postulated, the intestinal lamina propria is critically involved in determining active immune responses and tolerance in mature pigs, then it is of importance to determine the mechanisms by which this microenvironment is established and maintained in neonatal pigs and the impact of weaning and rearing environment on immunological homeostasis.

The young piglet is capable of active immune responses to live virus and to dietary components by three weeks old, but quantitatively and qualitatively these responses differ markedly from that in older animals. For example, whereas injecting 9-week-old piglets with soya results in a vigorous IgG1 and IgG2 response, injection at 3 weeks stimulates only a small IgG1 response [6]. Importantly, piglets weaned at three weeks old onto soya or egg-based diets do develop very strong antibody responses to the fed protein, entirely comparable in magnitude to the response to antigen injected with adjuvant, suggesting that they are unable to make the critical distinction between "dangerous" and harmless antigens and to develop tolerance to the latter [3]. Consistent with the hypothesis, tolerance to continuously fed proteins appears not to be fully achieved until 8 weeks of age [3, 27]. Further, weaning is associated with a transient reduction in the ability of both intraepithelial lymphocytes to respond to mitogens [46] and splenic T cells to secrete IL-2 [1].

6. WEANING AGE AND ENVIRONMENT: IMPACT ON MICROBIAL CLONISATION AND DEVELOPMENT OF IMMUNITY

Immediately following birth, the neonatal pig is dependent upon the ingestion of maternal colostrum and milk for protection and survival. Factors derived from colostrum, and indeed secreted in milk throughout the lacation period, provide important back-up for the neonatal innate host defences particularly as the adaptive immune system is clearly functionally immature [2] (Bailey et al., personal communication). In addition, to this passive protection, colostrum and milk provide a rich source of biologically active factors that actively promote the development of immune competence. We have hypothesized that, in addition to active immunity, certain of these factors may promote the development of tolerance mechanisms and the expansion of T_{reg} cells and that early weaning may dramatically impair these processes.

In addition to maternal factors and weaning age, much emphasis has been placed on the role of the microflora on development of gut function and immunity. Studies in germ-free piglets have highlighted the importance of an intestinal microflora on the phased development of the mucosal immune system [12, 29, 32]. Although it has long been considered that the gut microflora participates in health maintenance by forming a barrier, preventing gut invasion by pathogenic bacteria, a phenomenon known as colonisation resistance, other mechanisms of action are clearly important. Furthermore, although much emphasis has been placed on the role of bifidobacteria and lactobacilli (less than 1% the normal colonic flora) in promoting intestinal barrier

integrity and immune ontogeny, other prevalent bacterial species are emerging as potentially important candidates (e.g. gram-negative Cytophaga-Flavobacteria-Bacteroides cluster and gram-positive Clostridial clusters XIVa and IV). Valuable information on the relationship between immune development and microbial colonisation by such organisms can therefore be derived from molecular profiling studies which factor in, important environmental (microbial) and dietary variables, correlated with life-time performance data.

In recent experiments we have demonstrated that four to six week old piglets (after weaning) express mRNA for the pro-inflammatory cytokines IL-2, IFN-γ and IL-12p40 and the anti-inflammatory cytokines IL-4, IL-5 and IL-10 in all parts of the gastrointestinal tract. In the large intestine cells containing IL-2-, IL-12p40-, IFN-γ-, IL-4- and IL-5-mRNA transcripts were located in the lamina propria close to the crypts. No significant effect on the expression of IL-2 mRNA was observed by weaning on to conventional, compared with fermentable carbohydrate enriched diet (shown by in situ hybridisation and RT-PCR) were observed (Sowa, Rothkötter et al., in preparation). To date no effect of fasting on the expression of this cytokine has been observed and it seems that non-fermentable carbohydrates in the food had only a moderate effect on the expression of IL-1β. Further analyses of other cytokines showed that there is a late decrease (day 10) of IL-6 and IL-12p40 mRNA in the colon in all conditions tested. For TNF-α and IL-18, no variation in the expression of these 2 cytokines were observed post-weaning [31].

The weaning period in young pig is frequently associated with morphological and biochemical changes of the gut epithelium. In term of immunological changes, some authors have recently suggested that weaning time is also correlated with an inflammation of the gut. As pro-inflammatory cytokines play a key role in the inflammatory process in the intestine, pro-inflammatory cytokines were studied at different time post-weaning in piglets feed with different diets. For this study an RT-PCR followed by a dot blot hybridisation was used to detect six different cytokines: TNFα, IL-1β, IL-6, IL-12p40, IL-8 and IL-18. Weaning was associated with a transient increase of pro-inflammatory cytokines in the small intestine and the colon. A more detailed analysis revealed that at early time points after weaning (day 0 to day 2) there is an increase of TNFα, IL-1β and IL-6 mRNA level. At later time points (day 2 to day 8) there is a decrease of IL-12p40 in all parts of the gut except in the jejunum. TNFα and IL-8 mRNA also decrease in the proximal part of the gut but increase in the distal part of the intestine (Oswald et al., personal communication)

The role of enterocytes in cytokine production after weaning was studied in vitro using Caco-2 cells infected with ETEC. The gene expression of pro- and anti-inflammatory cytokines were analyzed by RT-PCR on cultured human epithelial cells (Caco-2) and co-cultures of Caco-2 cells with PBMC (peripheral blood mononuclear cells), with and without infection with ETEC. The expression of pro-inflammatory TNFα, IL-8, and GROα were drammatically increased after ETEC infection of Caco-2 cells either cultured alone or with lymphocytes. IFNγ was also increased but to a lesser extent. In contrast, TGFβ was markedly reduced. A similar cytokine pattern was observed on the lymphocytes cultured with Caco-2 cells, although the differences were less marked than on Caco-2 cells (Mengheri et al., in press).

Previous studies have examined the local immune response to challenge with *E. coli* at weaning by the quantitation of total IgA and IgA specific anti-k88 antibodies [14]. A similar approach to determine the effect of diet and heavy is currently under examination (Bosi et al., in press).

Initial examination of the intestines from isolator and farm reared showed that those from the farm-reared animals were thicker.

It was surprising therefore that significantly more cells were isolated from piglets reared in the SPF isolator. Overall the T cell populations were very similar in the farm reared and isolator reared piglets. There was however a tendency for a greater level of expression of activation markers in the mesenteric lymph node cells prepared from farm reared pigs and in the first few days of life in the lamina propria cells prepared from isolator reared animals. These results would suggest that there was an early influx of activated cells into the lamina propria and that this was pronounced in the isolator reared piglets which had been removed from there sow and transferred on to a bovine milk based diet (Stokes, Miller, Bailey, Haverson et al., recent observations).

7. CONCLUSION

Currently, our understanding of the mechanisms by which microbial colonisation potentiates immune development and modulates the adaptive immune response is poor. As discussed above, the interactions between colonising micro-organisms and epithelial/immune cells and the consequent gene/protein events in relation to gut barrier function and immunity is physiologically very significant. It is likely that even within the complex ecosystem of the gut, dominant bacterial antigens direct and determine the immune response. Unravelling their mechanism of action and establishing the impact of such antigens on immune homeostasis, particularly in the context of a background colonising flora, is clearly important.

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