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Research paper

Practical immunoregulation: Neonatal immune response variation and prophylaxis of experimental food allergy in pigs

Bruce N. Wilkie*, Prithy Rupa, Julie Schmied

Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada N1G2W1

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ABSTRACT

The importance of environment in immune response is identified and the increase in prevalence of allergic, autoimmune and chronic inflammatory diseases reviewed. In particular, altered opportunity to acquire evolutionarily anticipated commensal microbiota is associated through the “hygiene hypothesis” with defective developmental and response signals to the innate and adaptive immune systems. Evidence of the detrimental effects of such environments is reviewed as is evidence for remediation using controlled exposure to bacteria or their active components such as LPS or peptidoglycan ligands for TLR and NOD-like receptors. Occurrence of major environmentally associated changes in porcine immune response phenotype are described. The prophylactic effects of heat-killed *Escherichia coli* given intramuscularly or of oral *Lactococcus lactis* on experimental ovomucoid-induced allergy in piglets are described in the context of altered immune response bias favouring reduced type-2 phenotypes. The high frequency of clinical tolerance to developing allergic signs even in the face of classical sensitization indicates possible function in this pig model of regulatory effectors such as Treg cells.

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1. Introduction

Since innate and acquired immune response (IR) are integrated in mediating much of host adaptation to adverse environments they are a logical target in pursuit of alternative health-management methods for animals (Wilkie and Mallard, 1999). Interventions based on genetic approaches to improving general rather than agent and disease-specific IR and indirectly, health and productivity, require objectively and reproducibly measurable quantitative trait phenotypes as correlates. Such approaches have allowed selection for high and low IR as unweighted, combined antibody and cell-mediated IR estimated breeding values, in which heritability of IR is approximately 20% (Mallard et al., 1992, 1998). It is apparent that the

larger source of phenotypic variation is the environment such that profound negative or positive responses may arise from agent and disease non-specific environmental changes (Blaser and Falkow, 2009; Djuardi et al., 2009; Inman et al., 2010; Mulder et al., 2009). Amongst the large number of possible modern environmental variables that may influence IR and health, considerable attention has been given to early ontogeny of gastrointestinal commensal microbiota that under principles of “Darwinian medicine” (Rook, 2008) have evolved as necessary mediators of host–environmental interactions. Alteration or loss of these anticipated environmental stimuli predisposes to human diseases reflecting dysregulation of the IR (allergy, asthma, autoimmunity) and others (obesity, type 2 diabetes, esophageal adenocarcinoma, inflammatory bowel disease) (Rook, 2008; Blaser and Falkow, 2009). It has been suggested that man is unique amongst animals in ability to both change the environment to which the species has adapted in evolution and to adapt to adverse consequences of this change using cultural and technological means such

* Corresponding author. Tel.: +1 519 824 4120x54760; fax: +1 519 824 5930.

E-mail address: bwilkie@uoguelph.ca (B.N. Wilkie).

that genetic evolution has not been the principal adaptive mechanism since the societal change from hunter gatherer to agriculture and pastoralism (Rook, 2008).

2. Hygiene and immune-mediated diseases

In modern developed society there has been a “post industrial revolution epidemic” of immunoregulatory diseases such as allergy and asthma, first addressed in an epidemiological study that associated diminished risk of developing these diseases with birth into larger families and having older siblings (Strachan, 1989). This led to the “hygiene hypothesis” that in modern society, reduced exposure to previously ubiquitous, co-evolved infections or “old friends”, has omitted or altered expected signals, mainly from microbial or helminth pathogen-associated molecular patterns (PAMPs) to pattern recognition receptors (PRR) on antigen presenting cells (APC). In this circumstance, in response to ubiquitous, innocuous antigens such as allergens, type-1 IR bias was reduced and type-2 bias was sustained leading to induction of IgE-related antibody and allergic sensitization (Rook, 2008; Palomares et al., 2010). This “missing immune deviation” interpretation was rendered insufficient by the simultaneous increase in prevalence of autoimmune diseases (type-1 diabetes, multiple sclerosis and inflammatory bowel disease) that associated with type-1 IR. A major confounding observation was that persistent helminth infection, itself biasing to type-2 IR to helminth antigens and allergens, was not associated with allergy and infection of mice with *Schistosoma mansoni* prevented type-1 diabetes while curing the infection led to exacerbation (Rook, 2008). Such observations suggested failure of mechanisms that controlled chronic inflammation (“reduced immune regulation”) (Palomares et al., 2010) as being the main defect in modern societies associated with reduced presence of “old friends” or diverse mucosal, especially gastrointestinal, commensals. It is probable that in both human and animal health care practice, adoption of a “Darwinian Medicine” philosophy to investigating possible deletion of evolutionarily expected environmental signals for adaptive responses might suggest practical interventions that reduce predisposition to disease rather than agent and disease-specific approaches. Such treatments are likely to be prophylactic and relevant to very young neonates.

3. Neonatal immune response bias and disease

It was proposed by Wegmann et al. (1993) based on studies of inbred mice, that pregnancy success was in part due to type-2 bias of maternal and fetal IR to prevent rejection of the fetal allograft. This was thought to predispose to induction of allergy in neonates as well as contributing to susceptibility to pathogenic infections given deficient ability to mount protective type-1 IR (Morein et al., 2002). However, in a cohort of some 400 trios of mother, infant and father, blood mononuclear cell (BMC) cytokines induced by mitogen or allergen indicated individual variation as well as strong intrafamilial correlation relating mother to child and to father. This suggests strong environmental effects in familial cytokine patterns with balanced type-

1/type-2 ratios rather than consistent type-2 cytokine bias in mothers or in infants (Halonen et al., 2009). Similarly, in a study of 146 Indonesian mother–infant pairs, village of residence and intestinal protozoan parasite infection accounted for most of the correlation between maternal and infant mitogen (PHA) or lipopolysaccharide (LPS)-induced BMC cytokines without evidence of type-2 bias (Djuardi et al., 2009).

De Groot et al. (2005) investigated BMC mitogen (PHA)-induced IL-2, IFN- γ , IL-4 and IL-10 production at 2, 5 and 8 weeks of age in 32 piglets from 8 litters by 5 sires. While results varied by test method (mRNA vs culture supernatant cytokine antigenic protein), variation was high (coefficient of variation 40–200%) at all times with significant effects of litter for IL-2, IL-4 and IL-10 and individual accounting for most variation in IFN- γ . Increasing ratio of IFN/IL-10 with age, but not of equivalent mRNA, and of IL-2/IL-4 as mRNA but not as protein, may suggest early postnatal type-2 bias which balances with age, however confirmation would be desirable. Nevertheless, as the authors conclude, consistency of litters as high or low in type-1/type-2 cytokine ratios across all ages from 2 to 8 weeks suggests early acquisition of IR phenotype with implications for early predisposition to infectious or immunologically mediated diseases. Classically such variation is attributed to gene by environment interactions, however maternal and neonatal piglet adaptation to the environment is likely to be the largest contributor to observed variation.

Piglets are born with detectable serum IL-12 and TGF- β , which are likely constitutively produced since they occur in hysterectomy-derived, colostrum-deprived piglets (Nguyen et al., 2007). Interleukin-4, IL-6, IFN- γ and IL-10, in decreasing order of frequency in piglet serum, are absorbed from colostrum until gut closure on day 2 (Nguyen et al., 2007). Both TGF- β and TNF- α are produced in the mammary gland, however TNF- α was not detected in piglet serum. Piglets born naturally rather than by hysterectomy had higher serum IL-12 and TGF- β . In gnotobiotic pig cells in vitro, low concentrations of TGF- β induced IgM and IgA from LPS-stimulated B-cells while high concentrations reduced frequency of Ig secreting cells. Interleukin-4 induced dose inverse switch to IgA and IgG. It is proposed that IgA switching facilitates acquisition of gut commensal bacteria and promotes immunoregulation at the intestinal mucosa (Nguyen et al., 2007) possibly modulating weaning-associated upregulation of gut mucosal inflammatory cytokines (Pié et al., 2004). Hence piglet cytokines derived from endogenous and maternal sources would be expected to regulate and reflect IR phenotype as implied by variable effects in conventional pig cells on Ig isotype switching to IgG₁ or IgG₂ (Crawley et al., 2003) or T-cell cytokine response to in-contact monocyte-derived dendritic cells (DCs) pretreated with type-1 or type-2 antigens (Raymond and Wilkie, 2004).

4. Gut commensals, environment and immune response

There is abundant evidence of beneficial effects of normal colonization with gut commensal bacteria on

development of ability to maintain noninflammatory innate and adaptive IR to commensals themselves as well as to ubiquitous, innocuous allergens and self-antigens while maintaining ability to mount protective, regulated, inflammatory IR to virulent pathogens (Kelly et al., 2005; Blaser and Falkow, 2009). The mechanisms involve the resident microflora and high frequency food and environmental antigens being systematically sampled transepithelially at non-Peyer's patch, non-inductive sites and inducing a "tolerant" state mediated by lamina propria downregulatory DCs, monocytes and Treg cells with induction of secreted, noninflammatory IgA-related antibody. The IgA antibody may be induced by direct PAMP-PRR-activated, type 1 interferon-producing DCs interacting with B-cells directly without T help thus limiting immunoglobulin switching (MacPherson and Lamarre, 2002). Participation of toll-like receptors (TLR) and ligands derived from commensal bacteria, particularly LPS, in induction of mucosal and systemic homeostasis is well established. Mice lacking functional TLR4, the signalling component of the LPS receptor complex, have heightened predisposition to experimental oral sensitization with peanut allergen Ara h1 in the adjuvant cholera toxin and wild-type mice expressing TLR4 have similar susceptibility after antibiotic depletion and alteration of their gut commensal bacteria, a status that is reversed by commensal bacterial re-colonization (Bashir et al., 2004). Susceptibility is associated with several type-2 IR correlates and can be prevented by the TLR9, type-1-biasing ligand, hypomethylated CpG nucleotide (Bashir et al., 2004). Similarly, the TLR2 ligand peptidoglycan (Pgn) reduces allergy in a mouse model (Velasco et al., 2005) thus the well known protective effect on allergy of prenatal and neonatal exposure of infants to animals in a farming environment (Debarry et al., 2007; Schaub et al., 2009) may be mediated by both LPS and Pgn as PAMPs for the PRRs TLR4 and TLR2 inducing both deviation in anti-allergen response away from type-2 and induction of Treg cells to enhance regulation of both induction and effector functions in response to allergen (Schaub et al., 2009; Palomares et al., 2010).

Both Gram negative (LPS source) and Gram positive (Pgn source) bacteria *Acinetobacter lwoffii* and *Lactococcus lactis* isolated from anti-allergic cowshed environments reduced allergy in mice with increased type-1 IR polarization. *L. lactis* bound PRRs nucleotide binding and oligomerization domain (NOD)-2 and TLR2 while *A. lwoffii* bound TLR2, TLR4, NOD1 and NOD2 (Debarry et al., 2007). Recently *L. lactis*-derived Pgn particles (GEM) with viral antigen passively adsorbed using as intermediary a fusion protein of vaccine antigen replacing the active site of an *L. lactis* cell wall-cleaving hydrolase which binds avidly to *L. lactis* Pgn, was shown to induce neonatal DC maturation and TLR2-dependent protective type-1 murine immune response. Significant antigen non-specific protection was recognized (Ramirez et al., 2010). Taken together, evidence suggests that Gram negative or Gram positive bacteria or their components, LPS, Pgn and CpG are candidates for modulation of neonatal IR by substitution for missing "old friends" and hence providing prophylaxis of diverse type-1 and type-2 IR-mediated diseases (Schaub et al., 2009; Palomares et al., 2010).

5. Pigs and the hygiene hypothesis

The relevance of testing hygiene and immune response-related hypotheses in pigs in their own right and as large outbred animals similar to humans has recently been confirmed by a report of rearing environment affecting ileal microbiota quantity and bacterial 16sRNA gene sequence profiles of overall microbiota diversity (Inman et al., 2010). Piglets housed conventionally with the dam were compared over 28 days with littermates placed in isolators at 24 h. Early (days 2–5) similarity was followed by diversification which was greatest in the farm piglets. Isolator pigs had more lamina propria DC and T-cell-derived IL-4 at day 20 only but did not differ from farm piglets in frequency of any other cell types. Another report (Mulder et al., 2009) revealed marked differences in ileal epithelial resident microbiota of outdoor, indoor and indoor isolator-antibiotic treated pigs to age 56 days such that the phylum Firmicutes and particularly members of the beneficial Lactobacillaceae family, were abundant in the outdoor (77.2% of sequences) vs indoor (12.8%) and isolator-antibiotic (3.58%) groups. Similar differences were seen in corresponding sows indicating stability of neonatally acquired microbiota. Potential pathogens were more frequent in indoor groups and were not detected in outdoor sows. Ileal gene expression detected by Affymetrix porcine microarray indicated increased expression of several potentially inflammation-related genes in the isolator group suggesting disrupted mucosal homeostasis. Overall diversity of microbiota was least in the outdoor group and there was no evidence of altered type-1 vs type-2 IR bias by treatment groups. It remains to be seen if these dramatic environmentally induced differences are reflected in health and IR phenotypes, such as response to induction of allergy or to test immunizations.

6. Experimental allergy in pigs and spontaneous clinical tolerance

Natural and experimental allergy in pigs has recently been reviewed (Rupa et al., 2009a). To test hypotheses regarding the conditions for expression or suppression of allergy and of correlates of clinical signs as well as of clinical tolerance, conditions for systemic induction of oral allergy to the major egg allergen, ovomucoid (Ovm) were described (Rupa et al., 2008a). The functional role of anti-allergen antibody associated with IgE was studied by heat labile passive cutaneous anaphylaxis reactivity of sensitized pig sera and later swine IgE was purified and used to produce rabbit anti-swine IgE (Rupa et al., 2008b) to measure anti-Ovm associated with this isotype. Additional immune response bias criteria include ratios of antibody association with IgE, IgG₁ (type-2 IR) and IgG₂ (type-1 IR) (Crawley et al., 2003; Crawley and Wilkie, 2003) and cytokine production by mitogen-stimulated BMC. The model is able to reveal gastrointestinal, cutaneous and rarely respiratory clinical signs commencing immediately or up to 2 h after challenge, with variable severity, usually resolving spontaneously and occasionally requiring epinephrine treatment.

Variability both in individual and litter in frequency and severity of signs is a hallmark of the model. While positive cutaneous immediate hypersensitivity (skin test [ST] positive), is induced in virtually all sensitized controls (not treated with putative allergy modulators) and similarly, nearly all have serum Ovm-specific IgG and IgE antibody, these criteria are not sufficient to predict clinical signs. However, clinical allergy has never been observed in the model in individuals that are ST negative. Hence ST and IgE antibody positivity are necessary but not sufficient conditions for clinical allergy, suggesting frequent occurrence of litters and/or individuals within litters otherwise having responsive individuals, that are immunologically sensitized on the afferent limb of IR but are clinically tolerant in that they apparently control the efferent, effector limb. One of the three litters described in the original report had nearly 100% (7/8) of individuals in this category with 1/8 and 2/8 in the remaining 2 litters (Rupa et al., 2008a). This and subsequent experience with some 176 individual control animals from several litters confirms that neither serum IgE nor IgG anti-Ovm or ST positivity are good correlates of predisposition to develop allergic signs after oral challenge. Amongst unrelated litters over the period June 2006–August 2010 a total of 176 individuals were sensitized as positive allergy controls. Between June 2006 and August 2009 of 78 sensitized pigs 91.73% were ST+ with 51.81% positive and 39.92% negative (clinically tolerant) for allergic signs. In the subsequent period January 2009–August 2010, of 98 sensitized pigs 100% were ST+ with 3.03% positive and 96.97% negative (clinically tolerant) for allergic signs. The 3.03% positive in the second period were in two litters at the end of the period. These observations suggest strong but unidentified environmental effects that render essentially all sensitized individuals of all litters clinically tolerant, the objective of anti-allergic immunotherapy (Rupa et al., 2009b; Ozdemir et al., 2009). It may be that transient changes in background infection or alteration in the population of commensal microbiota contribute to this condition, knowledge of which could indicate direction for investigating etiology of a potentially useful treatment effect. Our preliminary and ongoing studies test the hypothesis that blood Treg cell frequency and function are correlated with the clinically tolerant state.

7. Prophylaxis of porcine experimental allergy

7.1. Heat-killed bacteria given to newborns

To test the hypothesis that modulation of the IR in newborn pigs prior to initiation of sensitization to Ovm newborn pigs of two litters were pretreated with killed bacterial cells with or without the type-1 cytokine IFN- γ . Sensitization was by intraperitoneal injection of Ovm with cholera toxin adjuvant on days 14, 21 and 35 of age (Rupa et al., 2008a). Heat-killed *Escherichia coli* (10^9 cfu) (EC) or *E. coli* expressing recombinant pig IFN- γ (ECI) or phosphate buffered saline (PBS, control group, C) were injected intramuscular (im) into the neck of piglets on days 1–7 of life. All piglets were orally challenged with egg white in yoghurt on day 46 after withholding food overnight. The experiment has been described in detail

(Rupa et al., 2009b). Clinical signs of allergy were detected in 7/8 (87.5%), 3/7 (42.8%) and 4/8 (50%) of the C, EC and ECI groups, respectively with the treatment and control groups differing significantly without difference between the treated EC and ECI groups. Similarly mean clinical scores for allergy were significantly higher in the C group than in either treated group which however did not differ from each other. Skin tests were positive in all but one piglet of each litter and there was no significant difference in IgG (H+L), IgG₁, IgG₂ or IgE-related antibody between any of the groups. Graded passive cutaneous anaphylaxis responses by individual pig sera indicated positive correlation with clinical scores. These results indicate a strong prophylactic effect of daily im injections of *E. coli* on expression of clinical allergy. However this advantage appears to be independent of induction of skin sensitization and serum anti-Ovm antibody, including antibody associated with allergy-mediating IgE, or potentially type-1 and type-2 IR-related IgG₂ and IgG₁. While mechanistic studies were not conducted here, it seems likely that effects were driven by bacterial PAMPs (e.g. LPS, CpG) acting on piglet DCs via TLRs to induce downregulation of efferent mediators of clinical signs or a state of clinical tolerance. Furthermore, the bacterial cells may have simulated signals normally derived from the intestinal commensal population that ensure a healthy IR to allergens and other ubiquitous, innocuous antigens

7.2. *L. lactis* given orally before and during sensitization

L. lactis is a generally regarded as safe (GRAS) lactic acid bacterium that is a vehicle for intestinal delivery of biologically active molecules such as cytokines or vaccine antigens, can prevent allergic signs when given intranasally and its derived Pgn potently biases to type-1 IR (Debarry et al., 2007; Rupa et al., 2008; Ramirez et al., 2010). Biological containment and the non-colonizing nature of *L. lactis* have been confirmed in pigs (Steidler et al., 2003). Given the inherent anti-allergic, type-1 IR biasing potential of *L. lactis* and the ability of IFN- γ to bias IR away from type-2, it was decided to test *L. lactis* alone (LL) or as a vehicle for porcine recombinant IFN- γ (LL+IFN) vs controls (C) for prophylaxis of allergy in the pig model of Ovm allergy (Rupa et al., 2008a, 2011). In this protocol 10^9 live LL or LL+IFN were given orally to piglets on days of age 1–7, 10, 12, 14, 21, 28 and 35 prior to and during ip sensitization with Ovm and cholera toxin (days 14, 21 and 35). Oral challenge with egg white was on day 46. Three litters of conventional Yorkshire pigs were used for each treatment without vaccination or antibiotics. The LL, LL+IFN and C groups had 30, 33 and 32 piglets, respectively. Response to treatment was assessed by direct skin test with Ovm (days 14, 21, 35 and 45), day 45 serum anti-Ovm antibody as IgG (H+L), IgG₁, IgG₂, and IgE as well as ratios of these. Mitogen (PHA)-induced BMC cytokine production was measured from cells collected on the day preceding challenge (day 45). Overall the LL group had significant and dramatic reduction in clinical signs with corresponding evidence of enhanced type-1 IR bias while in the LL+IFN group, although clinical signs were also significantly less than in C, the difference was less. Correlated data suggests that paradoxically the

LL – IFN group had less evidence of type-1 bias than did the group receiving LL only. Results are as follows: clinical signs were expressed by 3%, 15% and 40% of LL, LL + IFN and C pigs, respectively and positive skin tests occurred in 50%, 94% and 94% of each group. On day 45, anti-Ovm antibody associated with the type-1 isotype IgG₂ was greater in LL than in LL + IFN or in C group piglets. Antibody of the type-2 IR isotype IgG₁ did not differ between LL and C but was more in LL than in LL + IFN. The allergy-mediating IgE isotype was greater in LL than C and in LL + IFN it was less than in C but did not differ from LL. Ratios of type-1 to type-2 Ig isotype-related anti-Ovm indicate significantly more IgG₂ relative to IgG₁ and to IgE in LL vs C groups consistent with enhanced type-1 bias in the LL treated group. In the LL + IFN group the relative amount of IgG₂-associated anti-Ovm was less than in the LL group. While BMC production of the type-2 cytokines IL-4 and IL-10 was very significantly reduced in LL vs C, in LL + IFN piglets mean IL-4 exceeded that of C and for IL-10 it equalled the C value and exceeded LL. Interleukin 13 was detected only in the five piglets of the LL + IFN group that had clinical signs. This apparent type-2 biasing effect of an acknowledged type-1 cytokine may reflect its reported enhancing effect on allergic conjunctivitis (Magnan et al., 2000) and the complexity of actual regulatory interactions involved in the Th1:Th2 paradigm (Gor et al., 2003). Dose of IFN- γ could not be defined in the present experiments and the bell-shaped dose response in the bioassay used here (Rupa et al., 2008) may allow for expression of opposing pleiotropic effects. In a previous study IFN- γ had no additive effects over heat-killed *E. coli* alone in preventing allergy in piglets (Rupa et al., 2009b).

Hence LL was a potent down-regulator of allergy to Ovm with evidence of altered bias of IR favouring increased type-1 and decreased type-2 phenotypes but with clinical signs not expressed in some individuals positive for skin test reaction to the allergen and in serum IgE-related anti-Ovm antibody suggesting regulatory mediators of clinical tolerance apart from altered type-1: type-2 IR bias (Rupa et al., 2011).

8. Conclusions

Immunology of host resistance to infectious and immunological diseases is benefiting from conceptual advances that identify the evolutionarily ancient relationship between host and the commensal microbiota as critical for ontogeny and function of a balanced immune system. In this perspective appropriate responses are obviated under modern environmental conditions which are fundamentally removed from those to which species are genetically adapted. Recent studies of pigs indicate important detrimental effects of indoor housing and use of antibiotics on the profile of intestinal commensals and potential pathogens. Using allergy as a model of a modern disease predisposed by high hygiene conditions, it has been shown that unidentified environmental effects can drastically alter prevalent immune response phenotype. Simulating healthy environments by treating neonatal pigs with killed *E. coli* or with living *L. lactis* significantly prevents allergic signs, an effect that is correlated with reduced type-2 bias in immune response and also induces clinical

tolerance even in the face of classical evidence of allergic sensitization. Opportunities exist for functional studies of these positive immunoregulatory approaches.

Conflict of interest statement

None of the authors are in conflict of interest.

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