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A LINK BETWEEN MUCOSAL REGULATORY LYMPHOCYTES AND CHILDHOOD FOOD ALLERGY

SIMON H. MURCH

Royal Free and University College School of Medicine, London, UK

INTRODUCTION

There has been important recent advance in understanding basic concepts of intestinal food allergy, and the role of infectious challenge in the prevention of allergy. There has also been increasing appreciation of the role of non-IgE-mediated pathology, and the basic concepts of food allergy have broadened as the mechanisms of oral tolerance have been unravelled. It has been suggested that the traditional emphasis on IgE-mediated allergy has become less appropriate, as evidence mounts that the role of IgE may be one of modulation of the response to sensitising antigen, rather than as a prime mediator of sensitisation itself (*Walker-Smith* and *Murch*, 1999).

IgE responses to dietary antigens do occur in children in the tropics, but without consequent disease in most children. In allergic children of the developed world, children may manifest immediate and obvious reactions or a complex of delayed symptoms including diet-responsive eczema and a marked disturbance of intestinal motility (*Murch*, 2000).

THE INCREASE IN THE INCIDENCE OF FOOD ALLERGIES

Food allergies are not alone in showing increased incidence. It is well-recognised that there has been substantial increase in incidence of all types of childhood allergy. It is not simply a matter of incidence. Previously rare allergies, such as to peanuts, have become common (*Ewan*, 1996; *Hourihane*, 1997). Thus, in addition to advance in the scientific basis of allergic sensitisation, there has been recognition of novel patterns of food allergic disease in children. Marked increase in the incidence of food allergies of all kinds has occurred. In addition, multiple food allergies and sensitisation of exclusively breast-fed infants to maternal dietary antigens have become commonplace (*Walker-Smith* and *Murch*, 1999; *Murch*, 2000).

DOES IgE OR IgA DETERMINE SENSITISATION?

Genetic predisposition is clearly important in allergy, and candidate genes for allergy susceptibility have been identified, most concerned with IgE generation (*Cookson*, 1999). A population study from Iceland however demonstrated that an IgA concentration in the lower quartile of the normal range was more strongly predictive of allergic disease than elevated IgE (*Ludviksson*, 1993). This concords with Soothill's early report (*Soothill*, 1976) of transient IgA deficiency of infancy in the pathogenesis of allergic sensitisation. It is

probably not coincidental that the cytokine most centrally involved in isotype shift to IgA is transforming growth factor-β (TGF-β), a molecule now recognised as central in oral tolerance mechanisms.

The role of infectious exposures in the generation of TGF-β responses will be discussed later. However it is striking that early-life IgA concentrations, including in cord blood, are elevated in infants born in the developing world compared to developed world infants (*El Seed* and *Dafallah*, 1983). There is as yet little study of specific placental mechanisms regulating cord blood IgA, but breast milk cytokine concentrations may differ in atopic and non-atopic mothers (*Jones* and *Warner*, 2000). It will be intriguing to compare breast milk cytokines in developing and developed-world mothers.

GENETIC PREDISPOSITION TO FOOD ALLERGY

While a history of other atopic diseases is common in food allergic individuals, the major genetic studies have so far been only been carried out in classic atopy, and thus may not be a true representation of susceptibility for food allergy itself. There are regions on chromosomes 2q, 5q, 6q, 12q and 13q that are consistently linked with atopic disorders, with candidate genes including the IL-1 cluster, the IL-4/IL-9/IL-13 cluster, the Major Histocompatibility Complex and the interferon-γ gene (*Rosenwasser*, 1997; *Cookson*, 1999). Several susceptibility regions are shared with chronic inflammatory bowel disease, where tolerance is lost to the enteric flora rather than dietary antigen.

Epidemiological studies suggest a role for T cell responses and MHC type in food allergy, with regional variation in patterns of sensitisation despite broadly similar antigen exposures (*Hill* et al., 1999). Although peanut hypersensitivity is common in Indonesia, it is uncommon in Malaysia, Japan and the Philippines.

REGULATION OF IgE RESPONSES

While non-IgE-mediated food allergy may be the most frequent cause of chronic symptoms, IgE-mediated mechanisms account for the majority of immediate hypersensitive reactions to foods. Transient IgE responses to foods are found in many normal children and these may thus not be clinically relevant (*Sigurs* et al., 1994). However exaggerated IgE responses are clearly important in severe food allergies and anaphylaxis.

Isotype shift to IgE is regulated by products of Th1 and Th2 T cells (reviewed by *Corry* and *Kheradmand*, 1999). Th1 cytokines (particularly IFN-γ and IL-2) limit IgE production, as do Th1-associated cytokines such as IL-12 and Il-18. By contrast, Th2 cytokines, particularly IL-4 and IL-13, directly promote IgE synthesis. This Th1 effect may partly explain the protection against allergy provided by childhood within the developing world, but do not encompass the role of Th2 responses against helminths (*Yazdanbakhsh* et al., 2002).

Class-switching to IgE in response to IL-4 and IL-13, whose receptors share a common α chain (IL-4Rα), is mediated by a signal cascade involving Stat-6 (signal transducer and activator of

transcription-6), and gain of function mutations in this pathway are associated with both murine and human allergic sensitisation (*Shimoda* et al., 1996; *Hershey* et al., 1997). There is evidence of compartmentalised IgE responses within both the intestine and lung, with transportation of mucosally-produced IgE into the gut lumen or airway by a IL-4-dependent mechanism distinct from the poly-immunoglobulin transporter that mediates IgA secretion (*Ramaswamy* et al., 1994).

MULTIPLE FOOD INTOLERANCE, FOOD-ALLERGIC DYSMOTILITY AND THE EOSINOPHIL RESPONSE

In addition to clear increase in IgE-mediated and non-IgE-mediated food allergies, a remarkable alteration in disease presentation has been noted in several countries, where increasing numbers of infants are now sensitising to multiple antigens despite exclusive breastfeeding, often within the first weeks of life (*Hill* et al., 1999; *Walker-Smith* and *Murch*, 1999; *Murch*, 2000). This was rare a generation ago and is still almost unknown in the developing world. These children represent a major clinical challenge, and extensive dietary exclusions are often required. There is a clear association with eczema, food-allergic colitis or enteropathy, and these infants demonstrate a prominent disruption of intestinal motility. This pattern of disease suggests a primary failure to establish basic oral tolerance mechanisms, rather than the loss of previously acquired tolerance of classic food allergy.

There is now increasing evidence that both gastro-oesophageal reflux and constipation may be features of the food-allergic dysmotility syndrome, which is characterised by local eosinophilic infiltration. Epithelial expression of the eosinophil chemokine eotaxin appears to distinguish allergy-associated gastro-oesophageal reflux from primary mechanical reflux in infants (*Butt*, 2002). Eotaxin-deficient mice are protected from the dysmotility associated with mucosal allergy, suggesting that this is an important local response (*Hogan* et al., 2001).

DEMOGRAPHICS OF ALLERGIC SENSITISATION: THE ROLE OF ENTERIC CHALLENGES

Improvement in social conditions in an individual country appears to cause rapid increase in childhood allergies. Thus former East Germany, Estonia and Singapore have seen an increased incidence of allergic diseases of all kinds (*Goh* et al., 1996; *von Mutius* et al., 1998). The particular exposures that reduce risk of sensitisation appear to be gastro-enterological rather than respiratory. Serology performed in Italian military recruits demonstrated that past exposure to food-borne and oro-faecal pathogens in childhood was associated with a reduced risk of allergic sensitisation (*Matricardi* et al., 2000). Both rural upbringing and exposure to animals offers protection against later allergy in both developed-world and developing-world children (*Braun-Farländer* et al., 1999; *Lewis*, 2000).

There may thus be an important role for early environmental exposures in the determination of immune tolerance,

modulating the effects of genetic predisposition. Interest now centres on the specific links between the innate immune system and bacterial exposures in early life (Table 1). There is increasing evidence to suggest an obligatory role for the gut flora and probably also a maturational role for bacterial pathogens in the establishment of immune tolerance (*Fearon* and *Locksley*, 1996; *Sudo* et al., 1997; *Rook* and *Stanford*, 1998; *Sebra*, 1999).

MUCOSAL CHALLENGES IN CHILDREN BORN IN DEVELOPING COUNTRIES

Most children born within the tropics have evidence of enteropathy, and mucosal biopsies would usually be considered abnormal by UK standards. Our study of regulatory lymphocyte responses in the mucosa of developing world children has been based on biopsies obtained at the MRC Unit at Keneba in the Gambia. Gambian children show a pattern of growth faltering typical of deprived areas of the developing world, with UK normal growth velocity for the first 4 months, prior to weaning, followed by decline against UK centiles. At age 2 the mean weight-for-age lies 2SD below UK standards (z-score –2). Previous studies from Keneba confirmed biochemical and dietary deficiencies in these infants. Despite massive dietary supplementation (twice recommended values for energy, $2^1/_2$ times for protein), there was some short-term catch-up growth in malnourished children following gastroenteritis, which reversed as soon as the child was discharged (*Rowland* et al., 1981; *Sullivan* et al., 1992). Because of the failure of dietary intervention to restore growth, other factors have been studied. The most important is small bowel enteropathy, with particular evidence of a role for excess paracellular permeability on lactulose:mannitol (L:M) dual sugar permeability testing (*Lunn* et al., 1991). Over a 1-year period, increased L:M ratio accounted for 40% of growth faltering in Gambian children. Infection alone accounts for a minority of cases: Bacterial pathogens were isolated in <12% in one Keneba study and viruses detected more frequently in non-diarrhoeal controls (*Rowland* et al., 1978). Small bowel bacterial overgrowth and *Giardia lamblia* infection are found in >80% of rural Gambian infants, but neither correlate with growth or gut permeability (*Lunn* et al., 1999).

It is noteworthy that infant mortality rates in Gambia 2002 (c. 100/1000) are similar to those that were seen in London, Paris or New York 1902, where gastro-enteritis and wasting was also the major cause of infant death in underprivileged children. There is clear evidence that polymorphisms in cytokine response genes may give survival advantage in tropical children, and that these vary from country to country (e.g. high TNF producers do better against intracellular pathogens but have higher mortality from cerebral malaria). It is likely that similar selection pressures will have existed a century ago in European children, and that these may play a role in the development of atopy as infectious challenge decreases.

Table 1: Some potential interactions between innate immunity and the gut flora (After: *Murch*, 2001)

Recognition element	Distribution	Bacterial component	Effect transduced
Mannose receptor	Dendritic cells, macrophages, B cells	High-mannose carbohydrates	Increased efficiency of antigen presentation
Natural Antibody	Secreted by peritoneal and intestinal B-1 cells	Surface glycans	Modulation of T cell activation
Complement	Synergy with natural Antibody	O- and N-linked glycans	Opsonisation. Also regulates T cell activation and B cell tolerance
Toll-like Receptors TLR's 1-10 identified, most with as yet undetermined ligands	Dendritic cells, macrophages, T cells, Enterocytes	TLR2 - peptidoglycans TLR4 -LPS TLR9 - Unmethylated CpG repeats in bacterial DNA	NF-κB signaling pathway Increased surface expression of Class II MHC and co-stimulatory ligands
Mannan-binding lectin	Serum-derived. Binds to macrophages, monocytes and B cells	Carbohydrates on Gram-negative and Gram positive bacteria	Activates complement directly via serine proteases MASP-1 and MASP-2
Nod receptors	Intracellular recognition molecules in innate immune cells	Bacterial LPS	NF-κB signaling pathway
Vα24 NK T cells, Vδ1 γδ T cells	Epithelial lymphocyte subsets. Invariant T cell receptor chains	Conserved glycolipid sequences, presented by non-classical MHC (CD1d)	Modulate enterocyte responses, polarise towards Th1 and mucosal IgA production

CONTRASTING CHANGES IN EARLY-LIFE GUT FLORA IN THE DEVELOPED-WORLD CHILD

Lack of appropriate early infectious exposure has been postulated for many years as a cause of the overall increase in allergies (*Rook* and *Stanford*, 1999). These changes may be occurring from very early in life. There is evidence that the initial intestinal colonisation of the developed-world neonate has altered dramatically compared to infants born in the developing world, with reduced colonisation by previously dominant species such as *Bifidobacteria* and frequent discordance between the flora of the mother and her child (*Grutte* and

Muller-Beuthow, 1979; *Simhon* et al., 1982). These changes were not found in infants born in Nigeria, when compared to London-born infants (*Simhon* et al., 1982). Infants born by caesarean section show prolonged abnormality in composition of the intestinal flora and distinct alterations in immune function (*Gronlund* et al., 1999a,b).

We examined the hypothesis that changes in infant handling practices at the time of initial gut colonisation may be important, by study of the 1970 UK national birth cohort, where every infant born in one week in April 1970 has been followed long-term. Those infants who spent the first night away from their mother in the communal nursery had a significantly increased incidence of hayfever at age 26, suggesting that increased exposures to non-familial microorganisms or reduced colonisation by family microorganisms, may be associated with later allergic disease (*Montgomery* et al., 2000). In addition, early analysis suggests that this is a risk factor for inflammatory bowel disease, but not diabetes mellitus (unpublished data). Further support that early-life colonisation is a determinant of later sensitisation has been provided by studies of gut flora in Estonian and Swedish children, and allergic children from either country showed reduced lactobacilli and anaerobes but higher numbers of coliforms and *Staphylococcus aureus* (*Björksten* et al., 1999, 2001). These changes are detectable very early in life, before the development of clinical allergies.

Neonatal administration of probiotics to infants at risk of later allergies induced a remarkable reduction in later eczema (*Kalliomaki* et al., 2001). However it was notable that systemic IgE responses were unaffected, arguing for compartmentalisation of mucosal and cutaneous responses (*Murch*, 2001).

THE DEVELOPMENT OF ENTERIC TOLERANCE

Food allergy requires breakdown of oral tolerance, in which systemic immunological tolerance to an antigen is induced by its ingestion. The molecular mechanisms of oral tolerance to dietary antigens have recently been partially elucidated. The dose of ingested antigen is important in determining the means by which tolerance is maintained (*Weiner*, 1997; *Strobel* and *Mowat*, 1998; *Strober* and *Kelsall*, 1998). Tolerance for high doses of antigen occurs through anergy of potentially responsive T cells. This may reflect antigen presentation by gut epithelium in the absence of co-stimulatory ligands, or active suppression of the lymphocytes by suppressor cells or regulatory cytokines (*Mayer*, 2000). These pathways may be abrogated by breakdown of the epithelial barrier and presentation of dietary antigen by activated antigen presenting cells, as seen in infant sensitisation to cow's milk formula following gastro-enteritis. High dose dietary antigen induces apoptosis of antigen-specific lymphocytes within Peyer's patches in mice, but this has not yet been demonstrated in man (*Chen* et al., 1995). By contrast, tolerance to low-dose antigen is an activation-dependent process, where antigen-specific regulatory lymphocytes producing transforming growth factor-β (TGF-β) are generated (T_H3 cells). This activation-dependent response to low doses may be more difficult to establish in infancy than high dose tolerance, which is mediated by T cell anergy, as neonatal lymphocytes are relatively difficult to activate. Indeed, oral administration of low-dose myelin basic protein sensitised

neonatal rats, while similar amounts induced protective tolerance in adults (*Miller* et al., 1994).

Both T_H3 cells and IL-10 producing lymphocytes (Tr1 cells) suppress immune reactivity within the intestine by a process termed "bystander tolerance", in which they home to the intestinal mucosa and release TGF-β or IL-10 upon encountering antigen, thus suppress potential reactivity of all surrounding lymphocytes (*Groux* and *Powrie*, 1999). If this process breaks down, immunological tolerance may be lost and allergy or gut inflammation the consequence.

MECHANISMS OF ORAL TOLERANCE: A CENTRAL ROLE FOR NF-κB

The final pathway in the generation of tolorogenic lymphocytes appears to be shared with Th1 responses, with the nuclear transcription factor NF-κB in a pivotal role. Impaired NF-κB responses are clearly linked to mucosal sensitisation, to both the intestinal flora and to dietary antigen in experimental models, and indeed human IBD.

Spontaneous IBD develops in response to the normal flora in CE3Hej mice, who are genetically deficient in the LPD-sensing Toll-like receptor Tlr4 (*Poltorak* et al., 1998). Targeted deletion of NF-κB sub-units in murine knockouts also induces intestinal inflammation. In man, the primary genetic association in Crohn's disease is a loss-of-function mutation in Nod2, an intracellular LPS sensor which induces a NF-κB response to bacterial responses (*Ogura* et al., 2001). More profound multi-system inflammation occurs in children with mutations in the NEMO molecule (IKKγ), which is an important regulator of NF-κB function (*Courtois* et al., 2001). Thus the paradox is seen that a sub-optimal inflammatory response to the normal flora leads to an exaggerated pro-inflammatory response.

Similar mechanisms appear to apply in the generation of tolerance to dietary antigen. Again, blockade of a sufficiently pro-inflammatory response prevents the normal establishment and maintenance of tolerance to dietary antigen. In study of transgenic mice, whose only T cells recognised a class II MHC-restricted peptide in hen egg lysozyme, *Newberry* and colleagues (1999) demonstrated an obligatory role for physiological inflammation in the establishment of oral tolerance. These mice could only respond to their luminal flora through innate immune cells, but were fully tolerant to dietary hen egg lysozyme in normal conditions. When mucosal production of prostaglandin E2 (PgE2) was prevented by cyclo-oxygenase 2 (COX-2) antagonists, tolerance was abrogated and enteropathy developed in response to antigen ingestion. Thus PgE2 appears to play a fundamental role prevention of immune reactivity to dietary antigens. Constitutive production of PgE2 occurs in lamina propria macrophages in response to the enteric flora, and it functions as a potent inducer of IL-10 production in lymphocytes (*Newberry* et al., 1999). In turn, IL-10 is critical in the generation of regulatory lymphocytes, probably through facilitating generation of TGF-β producing cells (*Groux* and *Powrie*, 1999). There is also clear evidence that mucosal inflammation induces a compensatory TGF-β response (*Xian* et al., 1999).

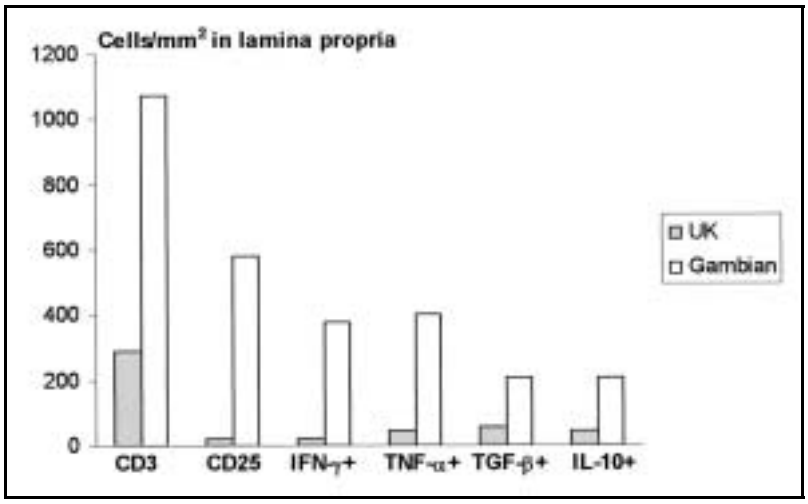

Figure. Mean density of lymphocyte populations in the duodenal lamina propria of well-grown UK and Gambian infants. (Data from *Campbell* et al., 2003).

IS FOOD ALLERGY RELATED TO DEFECTIVE GENERATION OF REGULATORY LYMPHOCYTES?

The data presented above argue for a specific role of early infectious exposures in the generation of enteric tolerance, mediated specifically through regulatory lymphocytes. They would suggest that children brought up in underdeveloped countries with low allergy would have higher numbers of regulatory lymphocytes. In addition, those children within privileged countries who develop allergies may be less efficient in generating regulatory lymphocytes than those who do not. Our data suggest that this might be so.

In our immunohistochemical analysis of Gambian children, the mucosal density of CD25+ lymphocytes was 50-200 times that seen in UK normal controls (*Campbell* et al., 2002). The density of IFN-γ and TNF-α expressing mononuclear cells was approximately 10 times that see in UK infants (Figure 1). Importantly, the density of IL-10+ and TGF-β+ lymphocytes in the Gambian children was also some 10 fold higher than in the UK controls. We noted progressive reduction of TGF-β producing lymphocytes with worsening nutritional status in these children, while Th1 responses were relatively maintained.

In studies of UK children with food allergies, using flow cytometry, immunohistochemistry and *in situ* hybridisation, we found the dominant abnormality to be failure to generate mucosa TGF-β producing lymphocytes, rather than simple deviation of Th1/Th2 responses (*Pérez-Machado* et al., 2000).

These data thus support early contention (*Murch*, 1996) that impaired generation of regulatory lymphocytes might underlie the increase in food allergies within the developed world. All the available evidence points toward a critical role for the gut flora in this process.

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