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MUTUALISM BETWEEN THE MUCOSAL IMMUNE SYSTEM AND COMMENSAL INTESTINAL BACTERIA (PART II)

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SUMMARY

Mammals normally coexist with a large load of commensal bacteria in the lower intestine that are not normally pathogenic. Commensal bacteria and their products shape mucosal and systemic immunity, epithelial differentiation and gene expression, and probably influence many other body systems. Although mammals are highly adapted to the presence of commensal intestinal bacteria, relatively little is known about the mechanisms of these adaptations or their functional significance.

We used *Enterobacter cloacae*, originally isolated from the intestinal flora of pathogen-free mice, as a model organism to investigate the mechanisms of immune handling of commensal bacteria. Intestinal challenge doses of *E. cloacae* resulted in penetration of live bacteria into dendritic cells within the Peyer's patches and these bacterial-loaded DC traffic to the mesenteric lymph nodes, but not into systemic secondary lymphoid tissues. Although live intestinal bacteria persist in DC, they are very rapidly killed by macrophages. IgA^+ B cells are induced in *ex vivo* cultures in the presence of intestinal DC loaded *in vivo* with live, but not heat-killed, bacteria. Repeated intestinal challenge *in vivo* with live intestinal commensal bacteria induces intestinal and serum IgA, but the response is absent if heat-killed organisms are used. Experiments with mice following mesenteric adenectomy show that mesenteric lymph nodes are important to avoid live bacteria reaching the spleen and priming of serum IgG responses to the organism. Thus under normal conditions, in the presence of mesenteric lymph nodes, priming by challenge doses of live bacteria is largely confined to the mucosal immune system, so mucosal immune adaptation can be achieved in the face of systemic immune ignorance of intestinal bacteria.

The function of intestinal IgA secretion has been investigated by challenging SPF mice with different doses of *E. cloacae*, and by parallel recolonisation of germ-free wild-type and ($J_H^{-/-}$) antibody-deficient mice using the same SPF sentinel. In each case antibody secretion did not affect the luminal densities of intestinal bacteria but reduced the levels of live bacterial translocation through the epithelial layer.

ADAPTATION OF THE IMMUNE SYSTEM TO COMMENSAL INTESTINAL BACTERIA

Interest in the mutualism between commensal intestinal bacteria and the mammalian body has a long history. Over a century and a quarter ago, micro-

scopic studies were published showing that the meconium of new-born babies was without visible microorganisms, but these appeared when the first yellow stools were passed (*Billroth*, 1874). Escherich confirmed these observations with microbiological cultures, showing that initially the postnatal intestinal discharges were sterile, but started to contain culturable bacteria within a few days (*Escherich*, 1885). The high densities of intestinal bacteria were also recognised to be non-pathogenic as long as they were contained within the lower intestinal lumen. Harvey Cushing and his collaborators showed with both clinical observations and a series of animal experiments, that accidental or belligerent perforation of the lower small intestine or the colon were likely to lead to peritonitis and septicaemia (*Cushing* and *Livingood*, 1900).

Study of the biology of mutual relationships between mammals and their intestinal bacteria also has a long history. Classical observations showed that the nutrition of humans and experimental animals influences the composition of the intestinal flora, especially when breast-feeding is substituted by formula feeding in human infants. Pasteur considered that microbes would be essential to the long-term viability of plants and animals. This provoked the challenge of raising animals in an aseptic environment, which was initially accomplished over several weeks, and later over a full life span (*Cohendy*, 1912, 1914; *Glimstedt*, 1936). A program that was able to successfully maintain experimental animals germ-free long term was started at Notre Dame University in 1928 (*Reyniers*, 1959).

The benefits of the lower intestine as a habitat for microorganisms are fairly obvious. The temperature is a steady 34-40 °C and there is a reliable supply of carbon sources, vitamins, minerals and water. The availability of germ-free animals in the early axenic and gnotobiotic programs allowed the impact of the microflora on their host to be investigated. For example, early studies showed that animals kept in sterile conditions suffered nutritional (especially vitamin K) deficiencies and had immature lymphoid structures and low levels of serum gammaglobulins (*Gustafsson*, 1959). Subsequently it has been shown that there are many changes, both immune and non-immune, within the mucosa (short range interactions) and elsewhere in the body (long range interactions) that differentiate germ-free animals from those colonised with intestinal bacteria (reviewed in *Macpherson* et al., 2005)[1]. These changes can be recapitulated in a germ-free animal within days or weeks of bacterial colonisation, and this can be done very simply, merely by placing a sentinel with an intestinal flora in the same cage as the germ-free animals. For example, there is only a sparse content of lamina propria IgA secreting cells in the mucosa of germ-free animals, and the mucosal lymphoid structures (Peyer's patches), are hypoplastic with few B cell follicles or germinal centres. The content of some intestinal lymphocyte subsets, particularly intestinal epithelial cells bearing the CD8αβ heterodimer and double positive $CD4^+$ $CD8^+$ subsets are reduced in germ-free circumstances. $CD4^+$ lamina propria lymphocytes are also deficient in germ-free animals. Immunity in the rest of the body, away from the mucosa is also shaped by the presence of commensal bacteria: systemic secondary lymphoid structure, high endothelial morphology, polyclonal responses to immunisation and serum isotype-switched immunoglobulins are all deficient or abnormal in animals living in germ-free conditions.

[1]Detailed references to the primary literature are given in this review.

We can refer to these short and long range changes of the immune system when germ-free animals are colonised as being adaptive to the commensal flora. However, there is very little evidence that the adaptation of the immune system has functional benefits. The low pathogenicity of commensal bacteria and the fact that innate immunity is sufficient to protect the animal from these organisms complicates assessment of functionality. The role of innate immunity is demonstrated by the viability of profoundly immunodeficient mice in specific pathogen-free (SPF) animal houses, where they are colonised by a simple (modified Schaedler) intestinal flora, but are screened to be free of pathogens[2]. For example SPF *scid/scid* and $RAG^{-/-}$ mice which both lack the majority of both B and T cells, are perfectly viable. Only if phagocytes are profoundly deficient in microbiocidal activity by introduction of two genetic targeted lesions of $gp^{91}phox^{-/-}$ and inducible nitric oxide $(NOS_2)^{-/-}$, which jointly abolish generation of oxygen and nitric oxide radicals, do the mice start to experience systemic sepsis with commensal organisms after they have been weaned (*Shiloh* et al., 1999). Conversely, consider the mechanisms used by endocytosed bacterial pathogens to survive in the host. The relatively small pathogenicity islands which distinguish their genome from commensals, predominantly encode proteins that subvert microbiocidal killing by phagocytes (*Sansonetti*, 2001).

MUCOSAL IMMUNITY COMPARED WITH SYSTEMIC IMMUNITY TO COMMENSALS

Our experiments have mainly used the model commensal aerobe *Enterobacter cloacae* present in the flora of our specific pathogen-free mice in Zürich. Western blots of *E. cloacae* cell wall proteins probed with purified secretory IgA from unmanipulated mice showed a poly-specific binding pattern of intestinal IgA, whether or not the strain of mice had T cells (*Macpherson* et al., 2000). In contrast, there was no binding of serum IgG even in wild type C57BL/6 mice. This was shown to be a result of immunological ignorance rather than tolerance, because an experimental injection of 10^4 to 10^6 organisms into the tail vein induced a strong, reproducible and specific immune response to a narrow range of cell wall proteins 14 days later (*Macpherson* et al., 2000). This suggests that specific pathogen-free mice are systemically ignorant rather than tolerant of their flora. We also found that there was evidence of spontaneous serum IgG priming against *E. cloacae* proteins, without prior deliberate infection, where the strains were deficient in intestinal IgA production, for example in the alymphoblastic mouse that has no secondary lymphoid structure outside the spleen and lacks IgA, or the $IgA^{-/-}$ mouse, which is deficient in IgA as a result of a targeted genetic lesion (*Macpherson* et al., 2000). This spontaneous (low level) systemic IgG priming was assumed to be the result of translocation of bacteria across the epithelial cell layer to reach deeper systemic tissues as a result of deficient intestinal secretory antibody, although the evidence was entirely indirect.

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[2]It is important to note that specific pathogen-free (SPF) mice have a simple limited bacterial flora unlike germ-free mice which contain no intestinal bacteria. SPF mice are screened to be free of murine pathogens.

SAMPLING OF BACTERIA AT THE INDUCTIVE SITES OF MUCOSAL IMMUNITY

In order to try and resolve the question of how there could be induction of the mucosal immune system by commensal bacteria independently of the mucosal immune system, allowing a significant IgA response in the former but leaving the latter ignorant of these organisms, we attempted to carry out functional experiments to measure the levels of culturable bacteria in macrophages. The intention was to determine whether translocated bacterial loads differed as a function of whether or not the mouse strain concerned was capable of secreting IgA. Following an oral dose of 10^9 *E. cloacae* given into the intestine by gavage, we found culturable organisms in washed mesenteric leukocytes between 6 and 60 hours after the dose was given. To our initial surprise, we were unable to recover any live bacteria in the sorted $CD11b^+$ $CD11c^-$ macrophage fractions. However, the dendritic cell fraction ($CD11b^+$ $CD11c^+$) contained a remarkable enrichment of live bacteria (*Macpherson* and *Uhr*, 2004). By carrying out macrophage killing assays, we could show that the explanation for being unable to culture live organisms in the macrophage fraction, was that our experimental protocols for flow cytometric sorting lasted approximately 5-6 hours, and in that time macrophages are able to kill almost all phagocytosed commensal organisms (*Macpherson* and *Uhr*, 2004). In contrast, dendritic cells are rather deficient in microbiocidal activity (*Delamarre* et al., 2005), so live organisms persist for several days (*Macpherson* and *Uhr*, 2004).

Although we had studied mesenteric leukocytes, as these are sterile by bacterial culture unless the intestine receives a challenge dose of commensal bacteria, the question arose whether dendritic cells in the mesenteric lymph nodes had taken up free *Enterobacter cloacae*, or whether *E. cloacae* were carried within dendritic cells as they travelled to the mesenteric lymph nodes. To resolve this question *in vivo*, we made two strains of *E. cloacae* that were antibiotic resistant either to nalidixic acid (Nal^R) or to rifampicin (Rif^R), both as a result of a chromosomal mutation. We also constructed isolated small intestinal loops by disconnecting segments of the small intestine and anastomosing both ends to the skin as mucous stomas. The vascular and lymphatic connections of these isolated loops of small intestine were not disturbed. Our experiment was to challenge one loop with *E. cloacae* carrying the Nal^R and the other loop with *E. cloacae* carrying the Rif^R and to isolate mesenteric leukocytes 18 hours later. The leukocytes were plated out at low density on bacterial culture media, so that any bacteria growing had arisen from a single cell. We established that approximately 30% of cells contained more than one bacteria, by lysing the cell suspension with deoxycholate prior to plating. We could now see whether each bacterial colony from a single unlysed cell growing on unselective media contained one or both antibiotic resistances by a replica plating technique. We found that if the Nal^R and Rif^R *E. cloacae* organisms were given into different intestinal loops, each cell from the mesenteric lymph nodes would always contain just one antibiotic resistance. As a control, if both antibiotic resistances were given into a single loop, a proportion of master colonies for mesenteric leukocytes had both antibiotic resistances. This result demonstrates that, *in vivo*, intestinal dendritic cells are capable of sampling bacteria at the intestinal surface and carrying them to the mesenteric lymph nodes, otherwise if *E. cloacae* were freely penetrating *either* antibiotic resistance would be just as likely to be taken up by

those dendritic cells within the mesenteric lymph nodes containing more than one organism.

Further direct evidence that intestinal dendritic cells can sample bacteria in the Peyer's patches themselves, was obtained by deriving a fluorescent form of *Enterobacter cloacae*, with green fluorescent protein (GFP) expression driven by the constitutive ribosomal rpsM promoter. Following intestinal challenge by gavage with GFP^+ *E. cloacae*, we were able to demonstrate a proportion of $CD86^+$ activated dendritic cells from the Peyer's patches that were positive for green fluorescence, this positive dendritic cell fraction was absent in mice treated with an identically transformed *E. cloacae*, but lacking the coding sequence for GFP. Thus low numbers of intestinal bacteria can be sampled after penetrating the intestinal epithelium, and persist as live organisms within dendritic cells, whereas they are very rapidly killed if phagocytosed by macrophages.

In these experiments where challenge doses of commensals are given into the intestine, the live organisms are seen only in the local mesenteric lymph nodes, and not in other systemic secondary lymphoid structures including central lymph nodes and the spleen. The presence of the mesenteric lymph nodes is critical to avoid this, because in wild type C57BL/6 mice where the lymph nodes have been removed by mesenteric adenectomy along the superior mesenteric artery, and allowed to recover with re-anastomosis of the lymphatic vessels, an intestinal challenge with *Enterobacter cloacae* subsequently results in culturable organisms detectable in the spleen. The presence of the mesenteric lymph nodes to protect the systemic secondary lymphoid system from exposure to commensal bacteria is also vital to preserve systemic ignorance, as specific priming of serum IgG, splenic enlargement and the hypertrophy of the splenic marginal zones all occur following repeated intestinal bacterial challenge of mice following mesenteric adenectomy.

Flow cytometry experiments with Peyer's patch and mesenteric lymph nodes leukocytes from animals treated with GFP^+ *E. cloacae* suggest that only a small proportion of the bacteria that are taken up across the intestinal epithelium are actually delivered to the mesenteric lymph nodes by dendritic cell trafficking.

The mechanism by which bacteria penetrate the M cell epithelial layer overlying the Peyer's patches to reach the underlying dendritic cells in the dome layer is still uncertain. In cell culture dendritic cells have been shown to be able to protrude processes between epithelial cells and sample the apical environment, and this is a pathway for the penetration of pathogenic bacteria including *Salmonella*. It has also been shown that the particular subepithelial dendritic cell subset that expresses the chemokine receptor CX_3CR1, is also able to sample intestinal organisms in the lower small intestine (ileum). In our experiments translocation across the Peyer's patches was a quantitatively far greater pathway than translocation through the villous intestinal epithelium. However, we were carrying out experiments with luminally delivered challenge doses of *E. cloacae* so these bacteria may have failed to penetrate the mucus layer overlying the villous epithelial cells as readily as they were able to penetrate the M cells overlying Peyer's patches. It is possible that subepithelial DCs away from the Peyer's patches are principally responsible for sampling bacteria that form in biofilms of the mucous layer overlying the epithelium, whereas the Peyer's patches with a reduced surface mucous layer and thin epithelial glycocalyx are principally responsible for sampling planktonic bacteria in the luminal contents.

INDUCTION OF IgA

Since the landmark papers of Cebra, Gowans, and their colleagues it has been known that IgA is induced in the Peyer's patches, and that IgA plasmablasts recirculate through the lymphatic and blood vascular systems to home back to the mucosa as they differentiate into IgA secreting plasma cells (*Craig* and *Cebra*, 1971; *Husband* and *Gowans*, 1978; *Pierce* and *Gowans*, 1975). Brandtzaeg and his colleagues showed that large amounts of secretory IgA are continuously secreted across the epithelial cell layer into the intestinal lumen coupled to the specific polymeric immunoglobulin receptor transport protein (*Brandtzaeg*, 1978; *Brandtzaeg* and *Prydz*, 1984). *Ex vivo* experiments have previously shown that dendritic cells are critical for class switch recombination to IgA. Although the factors responsible for signalling alpha specific class switch recombination are incompletely understood, TGF-β, IL-4, IL-5, IL-2 and IL-10 are know to be able to promote the IgA class switch *in vitro* or *in vivo* (reviewed by *Macpherson* et al., 2001) and *Johansen* and *Brandtzaeg*, 2004), and specific interaction between dendritic cells and B cells or T cells (*Cebra* et al., 1991; *Fayette* et al., 1997) leading to IgA induction might be promoted by the TNF family members B cell activating factor (BAFF) and APRIL (*Litinskiy* et al., 2002). *In vivo*, APRIL deficient mice have decreased spontaneous levels of IgA and reduced specific switching with T dependent and T independent immunisation protocols (*Castigli* et al., 2005).

To evaluate the importance of DC loaded with commensal bacteria in IgA induction, we set up cell cultures of B ± T cells isolated from mesenteric lymph nodes of unmanipulated mice with dendritic cells purified from Peyer's patches of mice that have been pulsed *in vivo* with 10^9 *E. cloacae*. Control experiments were carried out with dendritic cells purified from the Peyer's patches of mice that had received *E. cloacae* that were heat-killed prior to administration. Measurement of IgA^+ B cells after 3 days of culture, or levels of supernatant IgA after 6 days, both showed induction of IgA only in the presence of DC loaded *in vivo* with commensal bacteria. IgA induction was always greatest in the presence of T cells, although B cells and DC loaded with live commensals were sufficient for the response: In contrast no IgA induction occurred in cultures where DC were isolated from mice challenged *in vivo* with heat-killed *E. cloacae* (*Macpherson* and *Uhr*, 2004).

To see whether IgA was also induced *in vivo* by challenge with commensal bacteria, we carried out a protocol of repeatedly dosing mice by gavage with 10^8 *E. cloacae* every third day for a month. Control mice were gavaged with the same preparation of *E. cloacae* that had been split, and heat-killed, prior to administration. Immunohistochemistry and quantitative ELISPOT assessments of the small intestinal mucosa after this conditioning protocol showed that intestinal IgA levels were increased over 4 fold as a result of treatment with live, but not heat-killed, *E. cloacae* (*Macpherson* and *Uhr*, 2004). We have not yet determined whether the reason that heat killed *E. cloacae* does not work in this induction protocol, is that the heat killed organism is less effectively translocated epithelium into the dendritic cells, or whether it is important that live organisms per say are taken up by dendritic cells for induction of IgA by the adjacent B cells.

We did evaluate the IgA response to repeated treatments with *E. cloacae* in C57BL/6 mice that had been treated over a month previously by mesenteric adenectomy. The levels of IgA following *E. cloacae* conditioning were essentially identical in C57BL/6 mice whether or

not they had mesenteric lymph nodes, suggesting that the critical phase of the IgA induction occurred in the Peyer's patches, or that in the absence of mesenteric lymph nodes other secondary lymphoid organs are able to take over any maturation or amplification process that normally occurs within the mesenteric lymph nodes (*Macpherson* and *Uhr*, 2004). However, as described in the previous section, in the absence of the mesenteric lymph nodes it is possible for bacterially loaded dendritic cells to traffic to systemic secondary lymphoid organs, with the result that there is dramatic lymphoid hyperplasia in both the spleen and lymph nodes, and specific serum IgG responses against commensals are induced.

Our interpretation of these experiments was that normally the inductive response for IgA is focused on the Peyer's patches because of the local exposure to live commensal bacteria at that level. Mesenteric lymph nodes are not vital for *in vivo* induction of IgA per se, but are required to prevent trafficking of bacterial-loaded dendritic cells to central lymphoid sites where they provoke superfluous, and possibly harmful, immunologic reactions.

FUNCTIONAL CONSEQUENCES OF INTESTINAL IgA SECRETION

To examine the functional consequences of IgA induction we have carried out two different sorts of experiments. In the first, we used bacterial recolonisation of previously germ-free mice to examine the way in which production of secretory antibodies effects the penetration of intestinal bacteria. Experiments like this have been carried out previously, for example by Cebra and his colleagues who inoculated *Morganella morganii* into germ-free mice and followed the kinetics of bacterial translocation and IgA induction (*Shroff* et al., 1995). In order to extend these observations to the functional consequences of intestinal antibody secretion *in vivo*, we first rederived the $J_H^{-/-}$ antibody deficient strain [on the C57BL/6 background (*Chen* et al., 1993)] germ-free by aseptic embryo transfer. We then housed germ-free wild type C57BL/6 and $J_N^{-/-}$ mice together in a single cage and introduced a single C57BL/6 mouse carrying an SPF flora. We used an SPF sentinel to introduce a balanced bacteria flora, to avoid the possibility that a single species would behave unphysiologically without any microbial competition during recolonisation. We found that there was a short period of overgrowth of both aerobes and anaerobes at day 2, after which luminal bacterial densities settled down to steady state levels, that were not significantly difference whether or not the animals secreted antibodies. However, in the antibody-deficient germ-free animals undergoing recolonisation, we did measure increased aerobic bacterial translocation to the mesenteric lymph nodes peaking at day 6 and lasting until day 60 (*Macpherson* and *Uhr*, 2004). This shows that the natural IgA (produced prior to the inductive effect of commensal bacteria colonising the intestine) as well as induced IgA is effective in limiting the penetration of commensal bacteria beneath the epithelial cell layer. It also shows that although antibodies are reduce the levels of bacterial penetration during the recolonisation process, the tissues (at the level of the mesenteric lymph nodes) become sterile by day 70, indicating that antibody secretion is only one of a number of redundant mechanisms capable of limiting the ingress of bacteria below the epithelial layer.

A further experimental setup to look at the effect of antibodies in limiting

penetration of commensal organisms was to challenge either wild type C57BL/6 mice or antibody-deficient SPF $J_H^{-/-}$ mice with different doses of *E. cloacae* given into the intestine. Because in this second experiment all mice were SPF they already contained a limited flora of intestinal bacteria prior to the challenge dose of *E. cloacae* being given. The results were that with each of the graded challenge doses of *E. cloacae*, a smaller proportion of organisms penetrated as far as the mesenteric lymph nodes in mice that were secreting intestinal antibodies (*Macpherson* and *Uhr*, 2004). Moreover, if the experiment was done with C57BL/6 mice where the IgA levels had been increased by the repeated conditioning protocol referred to above, the numbers of organisms reaching the mesenteric lymph nodes was consistently lower at all doses than unconditioned C57BL/6 mice. This also indicates that antibody secretion is protective against overall penetration of commensal bacteria. We did not find evidence in our experiments for alterations in luminal densities of commensal organisms, either spontaneously or after challenge. However, these experiments are measuring overall levels of penetration, and do not exclude an effect of IgA in promoting uptake of bacteria through small areas of specialised epithelial such as the M cells.

CONCLUSIONS

Our data suggest that dendritic cells in the inductive lymphoid structures of the intestine sample live commensal bacteria, and that these loaded dendritic cells are then responsible for induction of mucosal immune responses. The experiments have been carried out with challenge doses of intestinal bacteria, and it is currently an assumption that the lower levels of spontaneous translocation of intestinal bacteria induce the mucosal immune system by a similar mechanism. Intestinal delivery of live bacteria is required to induce IgA although this may be because of better translocation rather than a requirement for endocytosis of live organisms per se. Even with high recurrent challenge doses of commensals, live organisms within dendritic cells do not penetrate further than the mesenteric lymph nodes, so the inductive effect is focused primarily on the mucosal immune system, rather than in systemic lymphoid structures. IgA has an overall protective effect against commensal bacterial exposure, but this is just a part of a multilayered system that adapts the mucosa to the presence of the microflora.

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