Communicative & Integrative Biology 3:6, 569-571; November/December 2010; ©2010 Landes Bioscience

In remembrance of commensal intestinal microbes

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Mammals contain an enormous load of commensal microbes in the lower intestine, which induce adaptive responses in the host immune system that ensure mutual coexistence of the host and its microbial passengers. The main way of studying how the host responds to commensal colonization has been to compare animals kept in entirely germ-free conditions and their colonized counterparts. We present an overview of our development of a reversible colonization system, whereby germ free animals can be treated with live commensal bacteria that do not persist in the host, so it becomes germ free again. We describe how this system has been used to demonstrate that there is little or no immune memory for specific IgA induction in the intestinal mucosal immune system by commensal intestinal bacteria.

Key words: IgA commensal bacteria, germ free, mucosal immunity

Submitted: 07/13/10

Accepted: 07/13/10

Previously published online: www.landesbioscience.com/journals/cib/article/13011

DOI: 10.4161/cib.3.6.13011

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Addendum to: Hapfelmeier S, Lawson MA, Slack E, Kirundi JK, Stoel M, Heikenwalder M, et al. Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science 2010; 328:1705–9; PMID: 20576892; DOI: 10.1126/science.1188454.

Commensal Microbes of the Intestine

The lower intestine of mammals contains an amazingly dense microbiota. The micro-organisms are approximately 10-fold more numerous than the cells of the host and, taking host and microbes together as a single super-organism, there are about 100-fold more prokaryotic than eukaryotic genes.[1] It has been thought for over 100 years that, although these commensal microbes are normally not pathogenic in a healthy host, there are profound mutual interactions between the prokaryotes and their eukaryotic home.[2,3] To show this experimentally, different species of animals have been derived germ-free.[4,5] Initially this requires aseptic Caesarian section, followed by housing and feeding the young in germ-free conditions. For small animals such as mice and rats, it is then technically possible to interbreed the germ-free offspring, using aseptic conditions in flexible film isolators.[6]

Experiments that compare germ-free and colonized animals show that many different host systems are shaped by the presence of the microbiota, both in terms of histological structure and metabolic pathways. In turn, the 'benign' microbiota fills up a microbiological niche which limits access to pathogens and helps the host by providing vitamins and salvaging energy from foods that would otherwise be indigestible.

Studying How Commensal Intestinal Microbes Shape the Mucosal Immune System

Our interest has focused on the immune system. Mucosal immunity is a minority activity in the world community of immunologists. Actually, most antibody production and a high proportion of lymphocytes are to be found in the intestine of colonized animals.[7] The presence of B- and T-lymphocytes in the lymphoid follicles (Peyer's patches) and lamina propria, and the high levels of IgA production from plasma cells in the lamina propria depends on the presence of the commensal intestinal microbiota.[8] In germ free animals these components of the adaptive immune system are absent and the intestinal immune system is relatively hypocellular.[9] This hypoplasia of the mucosal immune system in germ free animals may be reversed by colonising the intestine with commensal bacteria.

Germ free (axenic) animals are an incredibly good culture medium. Normally, we are born germ free and

colonized after birth with successive waves of microbes until a stable adult microbiota is established. The proximity of the birth canal to the anus is convenient for seeding the neonatal intestine. Studies of neonatal immunity depend both on autonomous maturation of the immune system, and on the effects of the commensal microbiota. In adult germ-free animals one can very easily achieve colonization, simply by placing a sentinel colonized animal in the germ free cage, followed by transmission of the intestinal commensal microbiota to the germ free animals. Serial experiments show that the hypoplastic mucosal immune system of the former germ free animals becomes normal in approximately 2–5 weeks.[6]

Many experiments have therefore assessed the impact of the intestinal commensal microbiota in three ways. First, there have been straightforward comparisons of the immunity (or wider biology) between animals that are germ-free and the same strain colonized with a commensal intestinal microbiota. Secondly, changes in germ free animals have been sequentially followed after intestinal microbial colonization. Thirdly the animals have been studied with different compositions of intestinal 'commensal' bacteria.

Once an animal has been colonized with commensal intestinal microbes there has been no way back to the germ free state. Antibiotics reduce, but do not eliminate, commensal microbes and although the relative composition of commensal microbes can be influenced by dietary manipulation, the animal remains colonized. This has meant that investigations of immune memory to commensal microbes and the functional effects of priming the immune system have been very difficult to investigate, because bacterial colonization could not be uncoupled from mucosal immune priming.

Development of Reversible Colonization of Germ Free Mice

We set out to solve the problem of being able to deliver live bacteria into the intestine of germ free mice, yet so ensure these bacteria would not persist and the mice would become germ free again. Our approach was to make auxotrophic mutants that would require preformed growth supplements in culture, choosing chemicals that would not be available in a mammalian host.[10] Our initial experiments were carried out with *asd* deletion mutant of *Escherichia coli* K-12 deficient for aspartate semialdehyde dehydrogenase, an enzyme central in the biosynthetic pathway of diaminopimelic acid (DAP), a component of the bacterial peptidoglycan cell wall. Although some germ-free animals gavaged with this mutant recovered their germ-free status, others were colonized by the organism. When the bacteria that had colonized were grown ex vivo, they had not only lost the requirement for DAP, but also displayed changed, mucoid colony morphology, most likely the result of stress-induced colanic acid capsule production, even when grown on DAP-containing agar medium.[11] We reasoned that sometimes the strain found a way around using DAP in the peptidoglycan crosslink under the intense selective pressure of the germ free intestine. Since the approach was partially successful, we followed up by introducing further mutations to block the biosynthesis of D-alanine, also required within the crosslink, which is also unavailable in the host which uses the L-stereoisomer of the amino acid. Under these conditions, we obtained a bacterial strain (HA107) which could be grown in culture and inoculated live into germ free animals, but after about 72 hours all animals became germ free again, even if multiple doses of HA107 were given.

Uncoupling of Commensal Mucosal Immune Induction from Intestinal Bacterial Colonization

Given that we had developed a system for transient colonization of the intestine with live bacteria, the question was whether this would be sufficient to induce an immune response? We found that a specific IgA response was induced two weeks after the administration of six doses of HA107 at 2 or 3 day intervals. The specificity of the response was judged using a flow cytometry assay that we have previously reported, which shows specific binding of the intestinal secretory IgA to the capsular surface of the HA107 strain, but not to other Enterobacteriaceae such as *Salmonella typhimurium*. This confirms, in a very clean system, that the intestinal IgA response to commensal bacteria can be highly specific, and not simply a polyclonal amplification of the mucosal B cell compartment in response to endogenous bacterial mitogens.

These results show that the induction of the mucosal immune system can be uncoupled from the persistent presence of live commensal bacteria in the intestine. Since HA107 does not proliferate in vivo, we could show that live bacteria are a far better stimulus for mucosal immune induction than equivalent numbers of killed bacteria given into the intestine.

Priming of Intestinal Immunity by Intestinal Bacterial Sampling at a very High Threshold

We, and others, have shown that IgA to commensal organisms is induced by dendritic cells that sample commensals at the intestinal epithelial surface.[12,13] In previous experiments with colonized mice, only a very tiny proportion of a test dose of live commensal bacteria given into the stomach are sampled by the intestinal dendritic cells as the bacteria pass through the intestine, however because of the effect of competition of the endogenous commensal bacteria, one could not make conclusions about the threshold of sampling. With the HA107 system we titrated the dose of oral bacteria, and found that a specific IgA resonse[14] requires extremely high doses (>10^9 c.f.u.) of commensals to be effective, in other words the threshold for sampling is extremely high. Presumably a high threshold minimizes the number of live bacteria that are allowed across the epithelial barrier, and thus minimizes the risks of infection with these organisms.

Mucosal Immunity has Limited Immune Memory but Rather Integrates Exposure to Mount an IgA Immune Response

Uncoupling of priming of the mucosal immune system by commensals from bacterial colonization of the intestine allowed

us to measure the immune memory for commensal bacterial immune response induction. We found that the specific IgA immune response was a function of the total exposure of the mucosal immune system to HA107, and the intervals of priming were not critical. Even when the same dose of bacteria was given either within 24 hours or spaced out over three doses at 7 day intervals, the extent of priming was very similar. This suggests that the mucosal immune system mounts sequential responses to commensal bacteria by integration of the effects of exposure over time, rather than showing a prime-boost effect that characterizes central immune responses and allow vaccination to be effective.

We also found that the response to HA107 was extremely persistent in germ free mice, with no loss of response even after 16 weeks. In contrast, if other intestinal bacteria were given after HA107, the specific response became attenuated as other IgA specificities appeared in the intestinal mucosa. This again suggests that the IgA response is matched to the current exposure of commensals in the intestine, with persistence of the response only as long as other organisms are not priming different IgA specificities.

Teleologically speaking, the absence of exaggerated immune responses to commensal microbes in the intestine makes considerable sense, because presumably the system needs to match the protective effect of IgA to the organisms that are currently dominant in the intestinal microbiota, rather than mount an excessive response to a minor organism that temporarily disappears from the microbiota and then reappears at a low abundance.

In *A Study in Scarlet*,[15] Mr. Sherlock Holmes tells Dr. Watson, '*I consider that a man's brain originally is like a little empty attic, and you have to stock it with such furniture as you choose. A fool takes in all the lumber of every sort that he comes across, so that the knowledge which might be useful to him gets crowded out…. (but)… the skillful workman is very careful indeed as to what he takes into his brain-attic. He will have nothing but the tools which may help him in doing his work….. It is a mistake to think that that little room has elastic walls and can distend to any extent.*' We see the mucosal IgA response to commensal bacteria in a similar way, except that 'crowding out' is used effectively to make recent mucosal immune induction relevant to the bacteria present in the intestine.

Conclusions

We have described how the development of a reversible system of colonization of germ free animals with bacteria that cannot proliferate or persist in vivo allows a different approach to understanding intestinal immunity. We have investigated the induction of specific IgA responses, which are presumed to form part of the immune barrier that ensures mutualism of the intestinal microbiota and the mammalian host. These responses are mounted best in response to live bacteria, probably because these organisms can be sampled by intestinal dendritic cells with the right molecular profile to stimulate IgA induction. Only a tiny proportion of the intestinal bacteria are sampled, and sequential exposures do not generate a prime-boost effect seen in systemic vaccination, but rather an integrative response that can be eroded by induction of new responses to different intestinal bacteria, functioning to match immunity and the repertoire of the intestinal microbes present.

There are many other important adaptive and innate layers of intestinal mucosal immunity relevant to host microbial mutualism. We still need to explore these how these reponses fare when uncoupled from the continuous presence of commensal intestinal microbes.

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