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Addressing the experimental variability associated with the microbiota

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The use of isogenic mice is now part of our research culture, not least because major histocompatibility complex polymorphism is a major roadblock for many experiments in immunology. But mouse genetics is not the only source of variation. We have come to realize that the large symbiotic microbiota is not ignored by its host, but is in fact involved in many aspects of development and physiology, and shapes the reactivity of the immune system. Microbiota potentially cause, or at least contribute to, numerous mucosal and systemic pathologies, and can even affect processes in the central nervous system. Therefore, experimental variability caused by the microbiota can no longer be ignored, and must be addressed when experiments are conducted *in vivo*.

The use of germfree and gnotobiotic (from the greek *gnostos* 'known' and *bios* 'life) mice, which carry a microbiota of known and controlled composition, have made it possible to investigate the impact of the symbiotic microbiota on its host. The technology to generate and maintain germfree mice is now available in many institutions around the globe, and such mice can be shared using adapted shipping devices. In addition, microbiotas can be engineered to reduce the natural complexity of the symbiotic microbiota and yet retain functions and metabolic pathways that are deemed essential for the host and the model under study. Such microbiotas may be stored, shared, and re-implanted into germfree mice. In short, it is now possible to perform experiments using both isogenic and isobiotic mice.

Several issues nevertheless arise when considering the use of isobiotic mice. Does the restricted microbiota provide the necessary functions to the mouse model under investigation? Does it model the functions of a 'natural' complex microbiota? Is it stable and reproducible across institutions, and can it therefore be used for experimental standardization? What is the loss of information associated with the use of restricted microbiotas? Does the decreased experimental variability associated with the use of isobiotic mice offset its costs? Or would it be enough just to report information on the microbiota when publishing *in vivo* data that may be affected by it?

In the position paper in this issue of Mucosal Immunology entitled *Standardised animal models of host microbial mutualism*, Andrew Macpherson and Kathy McCoy examine these issues and propose guidelines to develop standard microbiotas to generate isobiotic mice and offset experimental variability associated with the microbiota. We have also asked leaders in the field, to comment on important questions on how to manage the experimental variability associated with microbiota. We hope that this paper and the comments below will lead to the development of solutions acceptable to the wider community of researchers, as well as to sustainable publishing guidelines when the symbiotic microbiota is believed to affect the reported phenotypes.

QUESTIONS AND ANSWERS ADDRESSING EXPERIMENTAL VARIABILITY AND THE MICROBIOTA

Experts in the field were asked to react to the position paper of McCoy and Macpherson by discussing important questions on how to deal with microbiota and the variability associated with it in mouse experiments. The following colleagues have participated: David Artis (DA; Weill Cornell Medical College, New York), Marion Bérard (MB; Chief Veterinarian at Institut Pasteur, Paris), Nadine Cerf-Bensussan and Valérie Gaboriau-Routhiau (NC/VG; Institut Imagine INSERM, Paris), Lora Hooper (LH; University of Texas Southwestern Medical Center), Trevor Lawley (TL; Welcome Trust Sanger Institute, Hinxton, UK), Cathryn Nagler (CN; University of Chicago), Philippe Sansonetti (PS; Institut Pasteur, Paris), Balfour Sartor and Ajay Gulati (BS/AG; University of North Carolina). A person's initials in parentheses following a statement indicate that he or she has has made that comment, but the absence of initials does not mean that this person disagrees. A few remarks have been added by Gérard Eberl (GE; Institut Pasteur, Paris), who compiled these answers.

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1. Should the composition of the microbiota of experimental animals be reported for publication?

Yes, if there is evidence or reasons to think that the reported results depend on the microbiota, its composition should be reported (NC/VG, LH, PS, BS/AG). It should also be reported when contradicting phenotypes are observed by colleagues in other institutions (DA, CN). A number of questions arise, however, on how to report on the microbiota. To what degree of phylogenetic detail should the composition of the microbiota be reported (NC/VG, BS/AG)? The required detail of this analysis depends on the questions asked (NC/VG), as for example, the relevant metabolic pathway may be encoded by individual species, or rather by a group of bacteria (GE). Also, how should the microbiota be sampled? In principle, a cage effect guarantees a relatively homogeneous microbiota among littermates,[1] but microbiotas may change in long-term experiments (NC/VG, BS/AG). If mice undergo a treatment that may influence the microbiota, then microbiota should be sampled before and after this treatment (CN). The microbiota may be sampled in the feces, colon, caecum or ileum, or in niches distinct from the digestive tract, depending on the model under investigation (GE).

2. How should the composition of the microbiota be determined?

The method used to determine the composition of the microbiota is an important variable that needs to be addressed (NC/VG). Sequencing of 16 S rDNA is a popular approach, but the cost of sequencing the microbiota of a significant number of mice in an experiment may limit its general accessibility (DA, NC/VG, LH, PS, BS/AG). However, as sequencing costs fall, standards for reporting the microbiota through sequencing may be reviewed periodically (LH, PS). Furthermore, 16 S sequencing data has limitations for the identification of lower bacterial taxa, limitations that can be overcome by whole-DNA (metagenome) sequencing, an approach that is, however, too expensive to be used in routine for most of us (NC/VG, GE). Another much cheaper but less comprehensive approach to determine the composition of microbiota is quantitative PCR (qPCR). Primer pairs are reported that amplify regions of the 16 S rDNA specific for different groups or species of bacteria,[2,3] which are now commercially available (GE). A list of bacteria, known for their immunomodulatory properties, such as Segmented Filamentous Bacteria, could be established and updated periodically (NC/VG, TL), and standard qPCR assays developed for each. Reviewers may then ask to use such assays to quantify the listed bacteria, or particular bacteria, depending on the mouse model under investigation (MB). In that case, standard operational procedures would have to be established so that the site of sampling, the extraction of bacterial DNA, and the conditions of qPCR or sequencing do not become new sources of noise (NC/VG).

3. What are the important parameters of the microbiota that should be reported?

Different taxonomic levels in the composition of the microbiota can be targeted by the choice of primers to amplify 16 S rDNA for qPCR or sequencing. A more comprehensive description of the microbiota is achieved by metagenome sequencing, the accuracy of which can be increased by higher coverage (the number of bases sequenced divided by the estimated size of the metagenome). This type of description would provide a precious base of comparison when discordant results are reported, and potentially lead to discoveries on the role of particular microbes (LH). It might also be requested when the microbiota is claimed to transfer a phenotype from mouse-to-mouse (LH). Furthermore, adding to the complexity, parasites, fungi, viruses, and bacteriophages may also have a significant impact on host phenotype, and required to be analysed (MB).

However, the gene content of the microbiota does not reveal its gene expression or metabolic output. Thus, RNA sequencing, proteome analysis and metabolome analysis may be more informative in some experimental settings (MB), but cannot realistically be required on a general basis (DA, BS/AG). Alternatively, select biomarkers may be useful proxies of the microbiota, and be used as structural or functional readouts (MB, NC/VG). For example, the amount of short chain fatty acid produced by several groups of bacteria can be determined by liquid chromatography and is predictive of the induction of regulatory T-cells through the activation of G-coupled protein receptors and epigenetic modifications.

4. Should microbiotas be standardized?

First of all, authors should report on the caging methods when mutant and wild-type mice are compared (LH, CN). It has been shown that the microbiota in littermates is relatively homogeneous, but undergoes stochastic variations in different cages, even in mice of identical genotypes.[1] Therefore, the use of age-matched littermates should be the rule, unless particular reasons prevent this rule to be applied (DA, NC/VG, LH, CN, BS/AG). Furthermore, a description of the specific pathogen-free (SPF) status, that is, the microbiological quality control, should be reported as a first level assessment of the microbiological conditions in which the experiment has been performed (MB, CN, BS/AG). The quality of the drinking water, that is, the use of chlorine or acid additives, and the composition of the diet (DA, MB, CN, BS/AG), all potential modifiers of the microbiota, should also be reported. The development of microbiological and dietary standards for the SPF status would be relatively easy steps towards the standardization of the microbiota (MB).

Next level is to establish microbiotas of standardized composition. The Altered Schaedler Flora (ASF) has been created precisely to alleviate experimental noise owing to variations in microbiota, but new "synthetic" microbiotas have to be generated that more closely mimic the functions of complex mouse or human microbiotas (TL). As one standard may not fit all models and mouse genetic backgrounds, several microbiotas may be

created that can be stored, shared, and implanted into germfree mice (MB, PS, BS/AG). Construction of such microbiotas would be based on our knowledge of the metabolic pathways required in a particular model (NC/VG). It will also require a good understanding of microbial ecology, as particular bacterial species might not tolerate the presence of others, depend on the presence of others, or require a specific dietary input (NC/VG). Thus, still many "known–unknowns" need to be explored before we can impose standardized microbiotas, and standards should be re-examined periodically by a dedicated forum (PS, BS/AG). Nevertheless, in a foreseeable future, the use of a standardized microbiota may be required to support a particular claim, as now the use of mice of defined genetic backgrounds are required to model human pathologies (DA, LH). In the mean time, logic should prevail, and a reviewer should know the boundary of what can be asked, and the author should know the boundary of what can be claimed (LH, PS).

5. What are the benefits and risks of such standardization?

As discussed in the position paper by McCoy and Macpherson, standardization of the microbiota would bring many benefits. Comparison of the phenotypes within and between institutions should suffer less from variability and thus, lead to less confusion and more robust conclusions (MB, NC/VG, LH, TL, CN). It would also serve as a solid basis to investigate host-microbe interactions (DA) and the effect on the host of specific microbes (LH). Importantly, it would reduce the costs of animal experimentation, as lower numbers of animals would be required per experimental group (GE). All these benefits are replicating those we now enjoy from the use of standardized inbred mouse lines, without which major concepts in immunology, for example, would have been difficult to demonstrate (GE).

However, inbred mouse lines have their limitations, as they may lack genes or gene variants that influence a particular phenotype, and therefore limit discovery (GE). They also distance themselves from the human situation (DA, BS/AG). In the same vein, the use of standardized microbiotas may limit the discovery of microbes that have important biological properties and effects on the host (NC/VG, LH, TL), whereas at the same time exaggerating particular phenotypes (DA). Therefore, knowledge has to be accumulated on the microecology and roles of the microbiota in the host in order to limit these risks (TL). Furthermore, this reductive approach has to be complemented by a gradual return to complexity, much in the same way mouse outbreeding programs are now under way to discover new useful alleles (MB).

The standardization of microbiotas also faces technical hurdles, as discussed by McCoy and Macpherson. Genetic and population instability may plague the long-term viability of a "synthetic" microbiota, and knowledge may yet be short to build physiologically relevant consortia (LH). For example, population dynamics in complex microbiotas may drive certain phenotypes in the host, whereas more restricted microbiotas may be more static and lose such effects (LH). Furthermore, a significant investment in infrastructure is required to maintain standardized microbiotas, which may be a barrier to their use in many institutions, and also limit mouse production (LH, PS, BS/AG). However, as discussed by McCoy and Macpherson, such costs may be compensated by the reduction in the number of mice required to establish significance in experimental settings, a claim that needs to be more thoroughly documented (MB, NC/VG).

6. How could we share microbiotas between institutions?

Mouse lines with defined microbiotas can be shipped between institutions using special containers, provided that the necessary infrastructure, including germfree isolators, is available at the recipient institution (NC/VG, TL, CN, BS/AG). More convenient would be the shipping of feces or microbiotas, used at the recipient institution to inoculate germfree mice. If the interest in such practices grows to significant levels, the production, storage, and maintenance of microbiotas, or mice carrying defined microbiotas, could be centralized, or even carried out by industry (MB, BS/AG). In a perfect world, shipping would follow standardized procedures, and handling by the recipient institution would benefit from a broad program of technological transfer (MB). To make this happen, the EU and the NIH should join forces and organize an international symposium involving representatives from diverse academic institutions, industries and animal facilities (PS).

However, the production, storage, maintenance, and shipping of standardized microbiotas may face a number of technical roadblocks that must be addressed. Production may suffer from an obvious bias towards culturable microbes (NC/VG), and freeze/thaw or lyophilisation protocols may select for resistant species (NC/VG, CN). Furthermore, implantation of the microbiota into germfree mice may be complex and require a sequence of implantation rather than an all-at-once implantation procedure (GE).

7. Should we develop microbiota informatics?

A Murine Microbiome Project should be developed to store information on, and compare, the microbiotas of mice throughout the world (TL). Such a platform would be key to create standardized microbiotas based on knowledge, and could be developed following current standards and procedures existing for the investigation of the human microbiota (DA, TL). It would also include tools to mine the database and perform meta-analyses, and guide the researchers in the exploration of microbe-host interactions (DA, TL). It has to be noted that servers already exist that store metagenomes and provide tools for metagenome analysis (see MG-RAST, MOTHUR, QIIME) (CN, BS/AG). Therefore, our community needs to work more closely with groups and institutions that have already become experts in metagenome bioinformatics (CN).

8. The bonus question: what is the minimal set of experimental standards required to demonstrate the causal effect of microbiota on host phenotypes?

The minimal experiment to demonstrate that microbiota is causative of the host phenotype requires the use of germfree mice or antibiotic-treated mice (NC/VG, LH, TL, CN, BS/AG). However, both approaches have pitfalls. Germfree mice develop numerous defects,[4] and antibiotic-treatment will lead to species selection that can have dominant effects on the host phenotype (NC/VG). The field would therefore benefit from a standardization of the antibiotic cocktails and length of treatments to be applied (NC/VG). Co-housing and transfer of microbiota from one group of mice to the other is the next step in the demonstration of causality of the microbiota (DA, CN, BS/AG). Ultimately, to demonstrate that a phenotype is caused by an individual microbe or a group of microbes, one has to fulfill the Koch's postulates (DA, LH): (i) the phenotype is present in mice that carry the microbe(s), and is absent in mice that do not, (ii) the microbe(s) need(s) to be isolated and grown, (iii) the phenotype has to be recapitulated in mice that are re-colonized with the microbe(s), and (iv) the microbe(s) have to be re-isolated from that mouse.

DISCLOSURE

The author declares no conflict of interest.

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