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Microscale Gut Microbiota Spatial Heterogeneity Correlates with neuroinflammation in an Alzheimer's Disease Model

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Abstract: Rising evidence implicates composition of the gut microbiota in neurodegenerative processes, yet little is known about correlations between microscale spatial organization of microbial communities along mucosa of the intestine and brain inflammation in Alzheimer's disease (AD). In our investigation, we applied a validated transgenic mouse model of AD and age-matched wild-type controls to evaluate whether microscale heterogeneities in gut microbiota — i.e., small-scaled, spatially-resolved variations in microbial community composition over adjacent mucosal microenvironments in the intestine — are correlated with markers of neuroinflammation. We applied high-resolution spatial sampling of mucosal surfaces in small intestine and colon coupled with 16S rRNA gene amplicon sequencing and spatial in situ fluorescent hybridization to map community structure at sub-millimeter scale. Concurrent analyses of central nervous system inflammation involved immunohistochemical quantification of microglia and astrocytic activation and measurement of pro-inflammatory cytokine levels in hippocampal tissue. Behaviorally, animals received a battery of cognitive tests in order to place molecular results in context. We found our AD-model mice demonstrated enhanced microscale patchiness and beta-diversity of mucosa-associated microbiotas compared to controls, specifically in proximal colon. Regions of high local heterogeneity correlated with enhanced hippocampal microglia activation and greater levels of inflammatory cytokines. Further, variability of spatial patterns in microbiotas explained greater variability in neuroinflammatory markers compared to bulk measures of fecal community composition. These results implicate that microscale spatial structure of microbes in the gut — not only compositional content — could play a role as a causal factor in, or reflector of, neuroinflammatory states in AD, providing a new direction for mechanistic research as well as spatially-directed microbiome interventions.

Keywords: Gut–brain Axis; Microbiota Spatial Heterogeneity; Alzheimer's Disease; Neuroinflammation; Mucosa-Associated Microbiota; Spatial Microbiome Sampling.

1. Introduction

The gut–brain axis has been implicated in Alzheimer's disease (AD) pathogenesis and progression, and compositional alterations in the gut microbiome have been correlated with cognitive impairment and neuropathology in several studies. The majority of research conducted so far has employed bulk collections of feces as a proxy for mucosa-associated communities, which capture an averaged community signature, obliterating potential subtlety of spatial structure that is biologically meaningful. In the gut, microbial communities are spatially structured along gradients of mucus layer thickness, oxygen tension, substrate availability, and immune effectors, forming microscale environments that harbor unique taxa in distances of millimeters or less. Microscale heterogeneities have in other models of disease been shown to control local interactions between microbes and host, whereas its relevance in neurodegenerative disease has not been systematically investigated.

Neuroinflammation, defined by activation of microglia and astrocytes and elevations of cytokines, is a characteristic of AD and a potential mediator of microbiome–brain interactions. The pathways by which gut microbes might interact with immune responses in the brain are mechanistic pathways including microbial metabolites (short-chain fatty acids, metabolites of tryptophan), microbial-associated molecular patterns as systemic regulators of immunity, and vagal afferent signaling. If local mucosa-associated small-scale spatial heterogeneity of the microbiota alters local signaling in epithelia or priming of immunity, then it might realistically alter systemic inflammatory tone and thus neuroinflammation.

Here, we addressed the hypothesis that microscale spatial heterogeneity of mucosa-associated gut microbiota is associated with neuroinflammatory markers in a rodent model of Alzheimer's disease. We used spatially-resolved sampling and molecular characterization of mucosal communities, together with histological and biochemical markers of brain inflammation and behavioral tests, to (1) describe microscale microbial spatial distributions in AD-model and control mice, (2) establish associations between local microbial heterogeneity and central neuroinflammatory status, and (3) compare spatially-resolved microbiota measures against conventional bulk fecal analyses in terms of explanatory power.

2. Methods

2.1. Animals

Female and male transgenic mice expressing familial AD mutations in the APP/PS1 background and age-matched wild-type controls were used. The mice were housed in identical conditions under ad libitum chow and ad libitum water. These protocols have been approved by the institutional animal care committee and performed in accordance with applicable guidelines[1].

2.2. Experimental Design

AD-model and control mouse cohorts were analyzed at an age when early-to-moderate amyloid disease and neuroinflammation are generally observable. Animals were behaviorally tested during the two weeks prior to tissue harvest[2]. At sacrifice, fresh fecal pellets were obtained just before euthanasia. Distal ileum, proximal colon, and distal colon intestinal segments were obtained and prepared for spatial sampling, andbrains were obtained for histology and cytokine measurement.

2.3. Spatial Sampling of Mucosa-Associated Microbiota

For capturing microscale heterogenity, we adopted a spatial sampling grid imposed upon gently opened pieces of intestine oriented mucosal side upwards. With a sterilized stamping tool and microedissection instruments, we obtained discrete mucosal biopsies in sub-millimeter to millimeter intervals along transects 1–2 cm in length[3]. Each biopsy was picked up in individual sterilized tubes in ice and processed individually. Spatial neighbors were kept as such in adjacent grid positions for later analysis. We took care not to contaminate samples from luminal contents; parallel washes prior to sampling eliminated loosely-adhered luminal microbes[4].

2.4. Molecular Profiling

We extracted DNA from each spatial biopsy in a bead-beating optimized for low-biomass mucosal samples. The 16S rRNA gene V4 region was amplified and sequenced in a high-throughput platform in order to produce amplicon libraries for every spatial location. The sequence data were analyzed in standard pipelines in order to produce operational taxonomic units (OTUs) or amplicon sequence variants (ASVs), and then assigned taxonomically.

For spatial pattern confirmation and visualization of microbial localization, in situ hybridization by fluorescence (FISH) using universal and group-specific probes was performed in parafixed sections of mucosa adjacent to sampled sections. For qualitative validation of patchy distributions and to localize particular taxa in reference to mucus layers and epithelial surfaces, FISH photographs were used.

2.5. Brain Histology and Cytokine Assays

brains were sliced for immunohistochemistry. Microglial activation was quantified by Iba1 staining and morphological scores, and astrocytic reactivity was quantified by GFAP staining. The cortex and hippocampus were studied with stereological counting techniques to determine density and activation state of glial cells. Concurrent hippocampal tissue homogenates were analyzed in multiplex immunoassays for a variety of pro- and anti-inflammatory cytokines.

2.6. Behavioral Testing

For context for inflammation and microbial results, mice completed a novel object recognition test and a spatial memory task (a spatial version of the Morris water maze scaled for cohort size). Performance measures were obtained and applied as covariates in correlation analyses.

2.7. Data Analysis

Spatial diversity indexes were computed at each sampled location and across local neighborhoods: alpha diversity (Shannon, richness), local beta-diversity between neighboring units, and spatial autocorrelation indexes (Moran's I) to evaluate patchiness. We defined microscale heterogeneity as a measure of similarity between samples adjacent along a transect. Bulk fecal community estimates were derived from aggregated fecal sequencing.

Linkages between neuroinflammatory markers as well as spatial measures of microbiota were estimated using mixed-effects linear models, in which animal ID was a random effect in a bid to control for repeated measurement, and in which genotype as well as intestinal region were fixed effects. Community clustering was depicted through principal coordinate analysis. We compared proportion of neuroinflammatory outcomes in variation explained by spatial microbiota measures compared to bulk fecal composition using variance partitioning approaches. Statistical analyses were performed in standard software; significance was interpreted in terms of biological effect sizes.

3. Results

Spatially-resolved sequencing revealed significant differences in microscale mucosa-associated microbial community structure between AD-model and wild-type mice. The majority of immediately adjacent mucosal biopsies in control animals exhibited low-to-moderate discordance, in which compositional gradients along transects were smooth. By contrast, AD-model mice exhibited strong patchiness: extreme transitions in community composition occurred over very short distances, producing high local beta-diversity and reduced spatial autocorrelation. The strongest such effect was in the proximal colon, where transects from AD-model animals contained strongly adjacent domains in which different taxonomic groups predominated.

Individual biopsy site alpha diversity was modestly different between genotypes and was region-dependent, but local heterogeneity measurements (neighbor-to-neighbor dissimilarity) were always greater in AD-model mice, regardless of region sampled. Sequencing results were supported by FISH imaging, which visibly confirmed clustering of disparate bacterial groups to create microscale patches in mucosal layers of animals from the AD-model, in contrast to control samples, where distributions were appreciably more uniform.

Quantitative analysis revealed significant correlations between local measures of microscale heterogeneity in the gut and central neuroinflammatory markers. Higher local beta-diversity in proximal colonic transects was correlated significantly with higher scores of hippocampal microglial activation and greater numbers of reactive astrocytes. Similarly, patchier transects were correlated withgreater pro-inflammatory cytokine levels in hippocampal homogenates. These correlations persisted after correction for genotype, sex, and behavioral performance, such that spatial attributes of the microbiota yield explanatory power independent of carrier status for genotypes of AD-pathology alone.

Notably, variance partitioning showed that spatial heterogeneities explained a larger proportion of neuroinflammatory outcomes' variance compared to bulk indicators of fecal composition from pellets from the very same animals. While there was overlap between bulk and spatial measures, spatial measures contained new information, in particular, local immune activation.

Linkages between spatial heterogeneity and neuroinflammation were region-dependent. The strongest

correlations were observed in the proximal colon, after which correlations weakened in distal colon transects; ileal transects had weaker and less reliable associations. Taxonomic profiling implied that patches enriched in specific anaerobic taxa and in organisms known to have associational relationships with the mucosal surface were over-represented in transects associated with increased neuroinflammation. In contrast, transects in which taxa associated with mucus consumption and prodiction of anti-inflammatory metabolites predominated correlated inversely with neuroinflammatory measures. These are associative, not causal, findings, however, and they implicate particular configurations of microbes that could have mechanistic importance.

Behavioral testing found that animals that had higher hippocampal inflammation and that had higher gut microscale heterogeneity performed poorly in spatial memory tests. Multivariate models' incorporation of behavioral measurements modestly enhanced prediction of neuroinflammatory results, in keeping with a concept that spatial structure of microbiota, neuroinflammation, and cognitive performance are linked.

4. Discussion

This research demonstrates that mucosa-associated spatial heterogeneity at a microscale is correlated with neuroinflammatory profiles in a mouse Alzheimer's disease model. The spatially resolved method reveals patterns that are overlooked under bulk sampling: patchiness at a fine scale, most prominently in the proximal colon, was better correlated with hippocampal microglia activation and cytokine increase than were conventional fecal indicators. These findings point to a potential role for spatial structure — not only taxonomic composition — of interactions between gut and brain in neurodegeneration.

Several mechanisms may account for associations that have been seen. Micro-scale patches focused in taxa that adhere strongly to the epithelium or metabolize substrate from the host may alter local properties of the epithelial barrier, propel translocation of microbial products, or prepare mucosal immune cells; these local immune perturbations may then diffuse systemically in order to impact brain-resident immune cells. Or patchiness may be a product of disease-associated alterations in gut function (e.g., changes in mucus, changes in motility) that simultaneously redefine spatial structure of microbes and independently fuel system inflammation. Causality determination will require intervention experiments that modify spatial structure—either by introducing focussed microbial consortia, localized substrate provision, or mucus property manipulation—and assess downstream neuroimmune outcomes.

The regional selectivity of our results, in that the strongest associations were found in the proximal colon, is consistent with the colon being a predominant site of microbial intermediary metabolite synthesis and immunological interaction. The moderate dearth of association in the ileum implies that context matters at mucosa: properties of ecosystems that create patchiness along these lines (substrate heterogeneity, mucus thickness, etc.) are variable along the gut and could dictate the immunological outcomes of spatial patterns[5].

Methodologically, our work demonstrates the feasibility and usefulness of high-resolution mucosal sampling for work in microbiome–neuroscience. Spatial sampling was problematic from a technical standpoint, such as in terms of low biomass as well as risk of cross-contamination, however, thoughtful design of protocols and orthogonal validation through FISH confirmed our observation's robustness. Notably, spatial measurements yielded interpretable biological signal beyond bulk analysis of feces, in favor of integration of spatially-resolved methods in subsequent work along the gut–brain axis[6].

Limitations are that it is a observational analysis that prevents causal inference, controlled for genotype as well as behavioral covariates in analysis, though possible unmeasured confounders in housing microenvironment or micro-heterogeneities in diet may account for microbial spatial structure as well as neuroinflammation. The research adopted a single mouse model of AD and a singular timepoint; longitudinal sampling spanning disease course and replication in other models would enhance generalizability. Lastly, 16S amplicon sequencing and FISH yield interesting compositional as well as localization data, though functional activity is not directly quantified; incorporation of metatranscriptomics or targeted metabolomics at spatial resolution would elucidate mechanistic pathways better.

Despite such qualifications, our results gesture toward several tractable lines of research. Intervention experiments reducing mucosal patchiness (e.g., by inducing mucus-stabilizing taxa or spatially-localized prebiotic treatment) may evaluate if neuroinflammatory mitigation is achievable by manipulating spatial organization[7]. Experimenting in introducing patchiness by localized perturbations, conversely, may clarify causal links. Translationally, clinical studies of human AD patients based on the microbiome could employ mucosal or spatially-informed sampling protocols, keeping in mind that stool profiles have a chance of missing clinically relevant micro-scale patterns.

5. Conclusion

Microscale spatial heterogeneity of mucosa-associated gut microbes is greater in a mouse Alzheimer's disease model and is associated with hippocampal neuroinflammation and cognitive impairment. Spatially-resolved measurements of microbes predict variation in neuroinflammatory markers better than bulk measurements of stool, implicating in fine-scale community structure a dominant feature of the gut–brain axis. Future mechanistic, therapeutic work targeting spatial structure of microbes may yield new methods of manipulating neuroinflammation in neurodegenerative disease.

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