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Heterogeneity in tissue culture infection models: a source of novel host–pathogen interactions?

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Abstract

Tissue cultures have been successfully exploited to dissect cellular and molecular mechanisms of microbial infections. Most of the methods used in this model conclude with data describing host and pathogen 'average' responses. Microscopy, however, reveals that such interplay is very diverse and that both partners are composed of phenotypically heterogeneous populations. Thus, upon co-incubation in the plate assay, neither all cultured host cells are infected nor all pathogen cells inflict alterations in host physiology. Despite its obvious impact in data interpretation, the basis of this heterogeneity remains in most cases unknown. Addressing this issue is encouraging since may contribute to uncover novel interactions in the host–pathogen scenario.

Keywords: Host; Pathogen; Heterogeneity; Single-cell

Most processes occurring in Nature, as the fate of cells during their development and differentiation, are intrinsically deterministic. In contrast, many other cases exist in which decisions are taken in a stochastic manner [1]. Stochasticity leads to the generation of two stable and heterogeneous co-existing populations displaying distinct phenotypes and, therefore, having distinct capabilities for responding to a specific stress or stimulus. Such situation has been termed 'bistability' and examples are known in all forms of life, from prokaryotes to metazoans [1–4]. Some cases in which co-existence of phenotypically distinct populations occurs include the states of competence and sporulation in *Bacillus subtilis* [2,5], the phenotypic 'tolerance' or 'non-inherited resistance' to antibiotics [2], the lactose utilization in *Escherichia coli*, lambda phage lysogeny, or the synthesis of photopigment by photoreceptor cells of the *Drosophila melanogaster* eye [1,3]. Heterogeneity is also known to exist at the molecular level. Thus, single-particle electron microscopy easily detects conformational 'flexibility' in macromolecules [6]. Besides the heterogeneity generated as a consequence of random decisions, other phenomena contribute to increase such population diversity. A striking example is the heterogeneity observed in microbial communities growing in biofilms. Distinct populations exhibiting varied genotypes and phenotypes have been reported to co-exist in biofilms [7]. This heterogeneity has been associated not only to stochastic gene expression, but also to mutation and selection linked to microscale chemical gradients occurring within the biofilm [7]. Another relevant process that generates heterogeneity and mechanistically distinct to bistability is 'phase-variation', which involves regulated and reversible genome alterations occurring in only part of the population [8]. Equally relevant for the concept of natural heterogeneity is that shown by the panel of differentiated immune cells present in higher organisms. Many subpopulations of dendritic cells, T cells, and B cells have been identified in peripheral and lymphoid tissues [9–11] and this diversity is essential for mounting the most efficient response according to the type of external aggression received by the organism.

1. Microbial infections on a heterogeneous tissue culture

Although natural heterogeneity is common in the living world, very few studies, if any, have addressed how this aspect

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may shape pathogen–host interactions. A tremendous amount of work has unravelled specific, ordered and sophisticated mechanisms used by pathogens to engage and/or mimic many eukaryotic molecules to invade, proliferate, persist and disseminate in susceptible hosts [12–14]. Most of this information has been collected using in vitro infection models with cultured eukaryotic cells. However, we are still far from understanding why these sophisticated pathogen-driven mechanisms apparently operate in only a population of the host cells exposed to the pathogen. A subpopulation of cultured cells 'refractory' to the infecting pathogen is seen in virtually any assay, even when the most optimal conditions for expression of adhesion and invasion functions or high multiplicities of infection are used.

Which is the key factor(s) missing or expressed in those cultured eukaryotic cells that are resistant to the pathogen? This is an interesting question that has not been yet addressed. One could speculate on the existence of host function(s) produced in a cell cycle-dependent manner, which either modulate expression of molecules engaged by the pathogen or are targets themselves. The eukaryotic cell cycle has defined phases, G0/G1/S/G2/M, differentiated by a unique set of processes and a cohort of proteins, as the cyclins, dedicated to precisely transit among phases of cell growth, replication of genome content and cell division [15]. Progress in this field has advanced tremendously in the recent years and technologies allowing to visualizing cell cycle transitions in live cells are now available. An example is the recent technology named 'Fucci', for *f*luorescence-*u*biquitination-based *c*ell *c*ycle *i*ndicator [16]. This technology is based on fluorescent probes fused to fragments of Cdt1 and Geminin, two sensors that undergo cell cycle-dependent suicide by ubiquitination. Using 'Fucci', it is possible to distinguish which cells in a culture are in either G1 or S/G_2/M phases simply by visualizing the colour of their nuclei [16]. Such analysis can also be made in tri-dimensional settings, as animal tissues. Undoubtedly, this is a great advance that could be easily applied as a powerful tool in the field of microbial pathogenesis. Thus, using 'Fucci' one can assess whether pathogens adhere, invade, survive or proliferate within host cells in a uniform basis or, instead, interact selectively with host cells staying in a concrete phase of the cell cycle. This hypothesis could also be addressed by monitoring virulence traits in synchronized tissue culture cells. Interestingly, despite the lack of information on how the host cell cycle may influence the interaction with pathogens, it is known the ability of some pathogens to alter the host cell cycle by secreting toxins, known as cyclomodulins, which interfere with the progression of the eukaryotic cell cycle [17,18]. Future studies aimed to dissect infection traits at different stages of the host cell cycle are clearly worthy.

2. Heterogeneity inherent to the pathogen

When using tissue cultures, the experimenter adjusts growth conditions to ensure the most optimal expression of virulence determinants by the pathogen. Unfortunately, there are cases in which protocols have not yet been unified concerning the way that the pathogen should be grown for the infection assay. One of the most frequent sources of controversy is the manipulation of the intracellular bacterial pathogen *Salmonella enterica*. This bacterial pathogen has been used for infection in drastically different conditions such as logarithmic, late-log, or stationary growth phases as well as in shaking or non-shaking settings. Needless to say, these different conditions alter the outcome of the infection assay at variable extents. As an example, the *S. enterica* invasion rate is extremely sensitive to the way bacteria are grown, with differences in the number of internalized bacteria reaching more than one-log [19,20].

Besides this source of diversity in the data generated by the experimenter, we should always be aware of the natural heterogeneity of microbes. Due to their facility to adapt and rapidly proliferate in multiple niches, stable variants generated by mutation continuously arise in all microbial communities and habitats. It is also known that many virulence traits are subjected to fine regulation [21–24], but other cases exist of virulence traits expressed non-uniformly by the population. Thus, in some pathogens phase variation ensures that only part of the population expresses certain determinants as pili, lipopolysaccharide, or outer membrane proteins in a concrete niche and at a specific time [8,25]. The interactions of microbes with eukaryotic cells in culture may also be controlled by other factors contributing to heterogeneous responses. Rather low numbers of the infecting pathogen, in some cases $<1\%$ of the inoculum even using low multiplicities of infection, are able to inflict changes in the host cell physiology. Why these numbers are so low in certain models is unknown, but it is possible than a combination of multiple factors (from both the host cell and the microbe) may be required to provide the unique 'bar-code' allowing the fruitful interaction. Thus, apart from the required 'permissiveness' of the eukaryotic cell, the pathogen must express in a highly precise temporal and spatial manner adhesion, invasion or survival determinants as pili, fimbriae, flagella, adhesins or secretion systems that deliver toxins and effector proteins inside the host cell [14]. Why and how only a low proportion of the pathogen population apparently become ultimately competent to adhere, invade or survive within the host cells remains to be defined. Several recent reports demonstrate that the expression of virulence proteins is not homogeneous in the population. A representative case is the work of Schlumberger et al., which describes type III secretion in real time and only a fraction (ca. 25%) of the *S. enterica* serovar Typhimurium bacteria expressing detectable amounts of the invasion protein SipA in a certain growth condition [26]. These authors also claimed that the amount and kinetics of the delivery might vary when considering individual bacteria. These observations agree with previous studies based on *gfp*$^+$ fusions integrated in the chromosome. Using this approach, the expression of genes required for host cell invasion or intracellular survival was detected in 20–50% of the individual bacteria [27]. Is the production of invasion determinants by *S. enterica* a decision taken stochastically once a stage of 'competence' has previously been reached? This hypothesis awaits further studies