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Biofilm vs. planktonic bacterial mode of growth: Which do human macrophages prefer?

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ABSTRACT

Although the natural mode of bacterial growth in nature is as biofilm, almost all antimicrobial and immunological tests are routinely developed using planktonic inoculums. Bacterial biofilms protect the microbial community from external damage and promote the persistence of chronic infections. In this study, interactions between human macrophages and bacterial inoculums of planktonic and biofilm modes of growth have been explored using *Escherichia coli* (*E. coli*) K12. Human macrophages phagocytize planktonic *E. coli* more efficiently than bacteria grown in a biofilm. Moreover, they prefer to phagocytize planktonic bacteria. In this context, CD64 expression is involved. Our data indicate that bacteria with "a biofilm background" avoid phagocytosis by naïve macrophages, which could create a favorable environment for chronic infection. Our findings were corroborated in a clinical O25b-ST131 ESBL-producer *E. coli* isolate, which caused urinary tract infections.

1. Introduction

Bacterial biofilms are microbial communities adhered to an inert surface or to a tissue, and are encased within a complex matrix. Their formation involves several stages: planktonic bacteria attach themselves reversibly to a surface, form a microcolony and produce a polymer matrix around the biofilm [1]. Once established, the biofilm can liberate individual bacterial cells to colonize other niches where new biofilms can be formed [2]. In humans, this structure allows for the spread of bacteria over several body surfaces (e.g., heart, lung, epithelium). It has been found in the lung tissue of patients with cystic fibrosis [3], in the middle ear of patients with otitis [4], in cases of chronic rhinosinusitis [5], in chronic wounds [6,7], on medical devices such as urinary catheters and prosthetic heart valves [8], among others.

Biofilm is the typical mode of bacterial growth in nature and confers many advantages, such as its capacity to resist adverse environmental conditions and increase its resistance against antibiotics and biocides [9]. This also leads to complications in the management of human infections and thereby limits therapeutic options [10–12]. In addition, bacterial biofilms protect the bacterial community from external damage, and they pose a challenge to the host immune system [13] by promoting the persistence of chronic infections [14] that are characterized by persistent inflammation and tissue damage [3].

There are important differences between the external appearance of bacteria in a biofilm and that of planktonic bacteria [15]. Microorganisms look different in biofilm infections because they are located close to each other and are surrounded by a self-produced matrix containing exopolysaccharides, proteins, nucleic acids and other bacterial detritus [15,16]. In addition, transcriptomic studies indicate that biofilms exhibit low metabolic activity together with an upregulation of genes needed for anaerobic growth, all under the strict control of the quorum sensing guidelines [17]. Whereas planktonic bacteria is designed to colonize new niches, but with a lower chance of survival, bacteria in a biofilm provides a more secure way for bacteria to reproduce and survive. This explains, in part, biofilm's increased tolerance against antibiotics, disinfectants and the immune system [15,18].

In turn, macrophages are essential in the host defense against infection and contribute to the initial inflammatory response [19–22]. These cells orchestrate several functions such as phagocytosis, antigen processing and cytokine production. Classically, immunological analyses on bacteria-macrophage interactions have

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been performed using planktonic inoculums as the most suitable way to reproduce microorganisms. Recent scientific contributions to microbial biofilm structures make it necessary for us to deconstruct some early dogmas. Concomitant analyses of macrophage interaction with both bacterial biofilm and planktonic inoculums are scarce. The difference between how each interacts with macrophages could be due to their dissimilar external structures: whereas planktonic bacteria are individual microorganisms, biofilm is a well-connected organization of millions of them.

We studied the interaction of bacteria with human macrophages, comparing those that were grown as biofilm and disrupted later, with bacteria that was grown as planktonic. Our objective was to analyze the phagocytic ability of macrophages when these cells interact with a suspension of bacteria with a biofilm background. Note that this event takes place in several clinical situations; once established, the biofilm can liberate individual bacterial cells to colonize other spaces where new biofilms can be formed [2].

2. Materials and methods

2.1. Reagents and bacterial strains

The anti-CD14-FITC and blocking anti-CD64 were from Miltenyi Biotec (Auburn, CA, USA), and the anti-CD1a-FITC, anti-CD16b-FITC and anti-CD89-FITC were from Serotec. The Green Fluorescence Protein (GFP) was located in a plasmid that was introduced by transformation into the *Escherichia coli* K12 lineage and selected in agar plates supplemented with ampicillin (100 μg/ml). An O25b-ST131 producing-CTX-M-15 *E. coli* isolate causing a human urinary tract infection was also included. This isolate belonged to the RyC collection. The Ethics Committee of La Paz Hospital approved this study.

2.2. Biofilm formation

The biofilms were initially established over 24 h at 37 °C in a static model based in a nitrocellulose filter disk (25 mm diameter; Millipore), deposited onto 7% of sheep blood agar commercial plates (Oxoid Ltd., Hampshire, England) and inoculated with 100 μl of an overnight BHI broth culture. Finally, the filter containing the biofilm-grown bacteria was suspended onto saline solution, homogenized with a vortex mixer and passed 15 times through a 30-G needle to prepare adequate inoculums [23]. Next, samples were analyzed using immunofluorescence microscopy, the bacteria sizes were determined by LAS-AF version 2.6.0 software and the concentration was adjusted by densitometry.

2.3. Planktonic inoculums

Planktonic inoculums were obtained from the mature biofilms and cultured onto BHI broth with agitation during the 24 h prior to the experiments. The cells were further centrifuged, suspended onto saline solution, homogenized with a vortex mixer and passed through a 30-G needle to prepare adequate inoculums at the same concentration as the biofilm. Next, samples were analyzed using immunofluorescence microscopy and the bacteria size were determined by LAS-AF version 2.6.0 software.

2.4. PBMC isolation and cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats by centrifugation on Ficoll-Hypaque Plus (Amersham Biosciences) as described previously [24–27]. The composition of this adherent population of cells was analyzed by FACS, and the cultures were differentiated into macrophages for 7–9 days. All the reagents used for the cell culture were endotoxin-free, as assayed with the *Limulus amebocyte* lysate test (Cambrex). The buffy coats were obtained from healthy volunteers. Written informed consent was obtained from all subjects enrolled. The La Paz Hospital Ethics Committee approved this study.

2.5. Phagocytosis assay

The cells were exposed to bacteria (Planktonic or Biofilm) for the indicated time (usually 2 h). The cells were then washed and kept with 300 μg/ml of gentamicin (Normon SA, Spain) for 30 min or the indicated period of time. Phagocytosis was analyzed by two methods: flow cytometry analysis of GFP-positive-cells and counting the Colony Forming Units (CFU) generated when the cytosol of these cells were spread on blood agar plates [28].

2.6. Flow cytometry analysis

For surface marker staining, cells were labeled with the indicated antibodies and analyzed by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences, USA). Data was analyzed with FlowJo software (Tristar, USA). GFP-positive cells were analyzed by the technique as described above [29].

2.7. Immunohistochemistry

The cells were fixed in 2% buffered paraformaldehyde at room temperature for 15 min. The samples were blocked by incubation for 60 min at room temperature with PBS containing 3% bovine serum albumin and were then incubated overnight at 4 °C with anti-CD14 (Immunostep, Spain), followed by three 5-min washes with PBS. The bound primary antibody was detected by incubation for 45 min with the goat secondary antibody conjugated to Alexa 594 (dil. 1/1000, Life Technologies) for 30 min at room temperature. The slides were washed again three times in PBS and mounted under a coverslip with VectaShield mounting medium with DAPI (Vector Laboratories, Inc.). The samples were visualized by confocal microscopy, which was performed on a Leica SP5 using a 40× objective lens. An emission/excitation laser for DAPI and Alexa 594 was used. A sample with no primary antibody was always included to control for background generated by the secondary Abs. Image processing was carried out using PHOTOSHOP CS software (Adobe Systems, San Jose, CA).

2.8. Statistical Analysis

The number of experiments analyzed is indicated in each figure. Data were collected from a minimum of three experiments and expressed as Mean ± SD. The statistical significance was calculated using *t* test. Differences were considered significant at p-values < 0.05 using Prism 5.0 software (GraphPad, USA).

3. Results

3.1. Human macrophages phagocytize planktonic E. coli more efficiently than bacteria grown in a biofilm

In order to analyze whether the recognition of bacteria by macrophages depends on how they have been grown, we first studied the phagocytic ability of these human cells in presence of planktonic (P) and biofilm (BF) *E. coli*. For this purpose, the GFP-*E. coli* K12 was grown as both planktonic and biofilm following a reported protocol (see Section 2). The BF was suspended onto saline solution, homogenized with a vortex mixer and passed 15 times

through a 30-G needle to prepare adequate inoculums. Note that after this process, the sizes of bacteria obtained from BF were similar to the Planktonic bacteria (Supplementary Fig. 1). Viability and concentration were checked in all bacteria "solutions" by microscopy and densitometry, respectively (data not shown).

Next, macrophages isolated from healthy volunteers (Fig. 1A) were exposed to GFP-*E. coli* K12 grown as P and BF for 2 h following the experimental design shown in (Fig. 1B). Afterward, the Colony Forming Units (CFU) obtained from macrophage lysis were counted (Fig. 1C) and the GFP-positive cells were quantified by flow cytometry (Fig. 1D). Both assays indicated that human macrophages more efficiently phagocytized P bacteria than BF bacteria. Similar results were obtained when a time course assay was done (Fig. 1E and F). These findings were verified by confocal microscopy (Fig. 1G). Note that similar size and identical number of planktonic and biofilm bacteria were used in our assays.

3.2. Human macrophages preferentially phagocytize planktonic bacteria

To determine whether the phagocytosis of planktonic bacteria is a favored event, we exposed cultures of human macrophages to GFP-*E. coli* K12 planktonic and biofilm bacteria. This was done in the presence of increasing concentrations of unlabeled biofilm and planktonic bacteria, respectively (see the schematic design in Fig. 2A). The GFP-positive cells were then quantified by flow cytometry. As (Fig. 2B) shows, while the phagocytosis of GFP-planktonic bacteria was not affected when unlabeled biofilm was added, the presence of unlabeled *E. coli* K12 planktonic bacteria significantly reduced the GFP-biofilm's phagocytosis.

Additionally, internalized *E. coli* cells were killed at least as efficiently in the case of P bacteria as in BF (Supplementary Fig. 2).

3.3. Differential CD64 expression on human macrophages challenged with planktonic and biofilm bacteria

The cell surface protein CD64 had been previously involved in phagocytosis [29], therefore, we studied the expression of CD64 on human macrophages exposed to P and BF bacteria. Curiously, when cells were exposed to P bacteria they fast up-regulated CD64 levels; however, the presence of BF failed to increase CD64 expression in macrophages (Fig. 3A). Moreover, a significant CFU reduction was observed when CD64 was blocked in the macrophages exposed to P bacteria (Fig. 3B). Note that in all experiments the biofilm was deeply disrupted and the bacteria showed similar

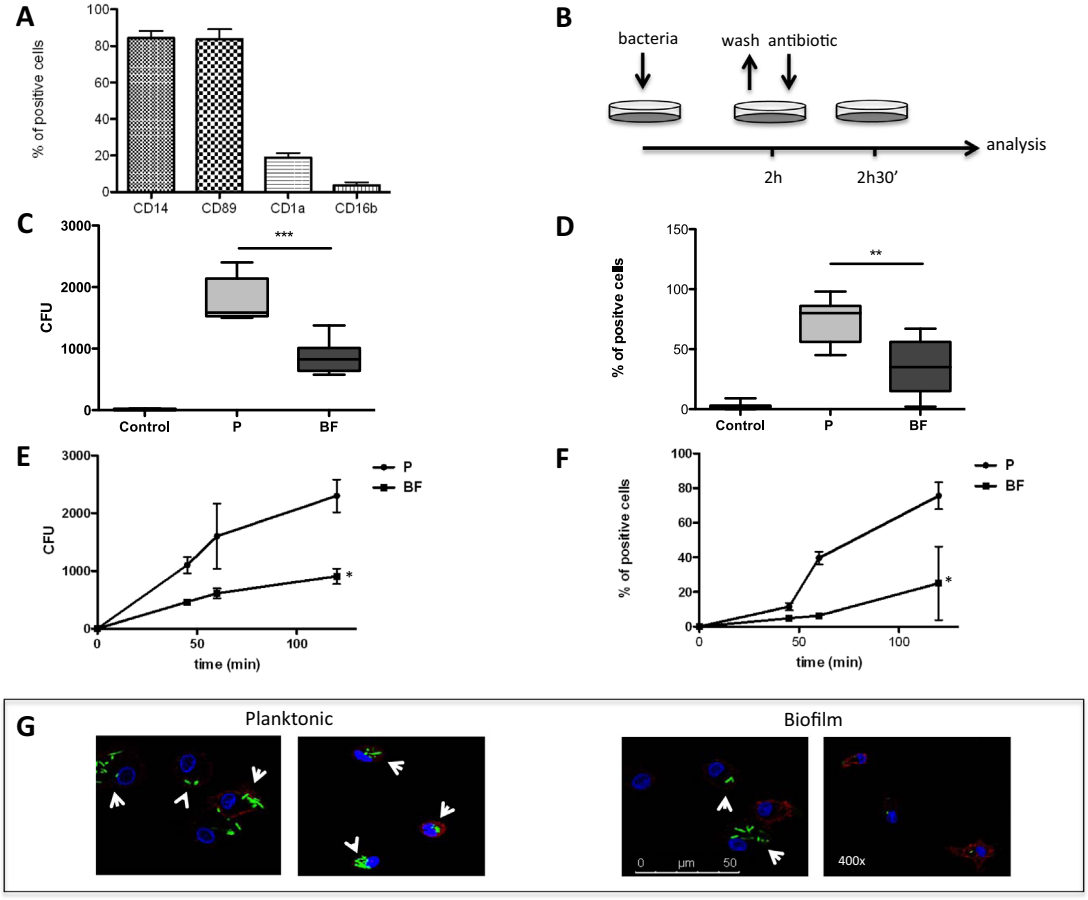

Fig. 1. Human macrophages phagocytize planktonic bacteria more efficiently than biofilm. Human monocytes were isolated from healthy volunteers and were cultured as described in the Section 2. (A) The cells were stained with indicated antibodies and analyzed by flow cytometry; the percentages of positive cells are shown. (B) The schematic design followed in phagocytosis assays. The macrophages were exposed to planktonic (P), biofilm (BF) bacteria or saline (Control) as indicated in the B for 2 h. (C) The cells were lysed and their cytosols were spreading in agar dishes overnight. The CFU were counted, and the number of CFU is shown ($n = 7$, $^{***}p < 0.01$). (D) The GFP-macrophages were analyzed by flow cytometry and the percentage of GFP-positive cells is shown ($n = 7$, $^{**}p < 0.03$). (E and F) The macrophages were exposed to P and BF for the indicated time. The CFU (E) and positive GFP-cells (F) are indicated ($n = 4$, $^{*}p < 0.05$). (G) The macrophages were exposed to planktonic (P) and biofilm (BF) bacteria for 2 h as indicated in B. The cells were stained with DAPI and CD14-antibody, and mounted as described in the Section 2. Representative pictures from confocal microscopy of five independently performed experiments are shown. Note the color legend: blue for DAPI, red for CD14 and green for GFP-bacteria. White arrows indicate ingested GFP-bacteria (400×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

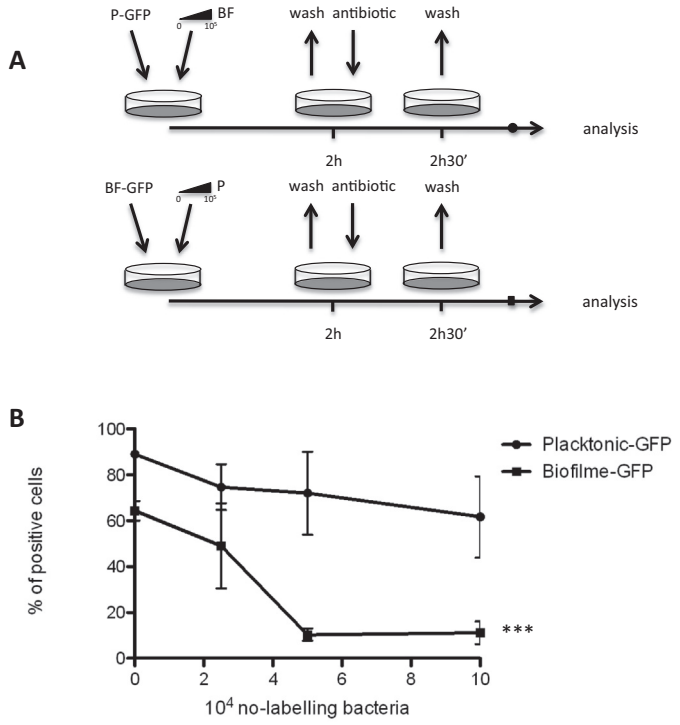

Fig. 2. Human macrophages prefer planktonic bacteria. Macrophages were exposed for 2 h to GFP-planktonic or GFP-biofilm bacteria in the presence of increasing amounts of unlabeled planktonic or biofilm bacteria, respectively. The cultures were then washed and left with an antibiotic for another 30 min. After washing, the cells were analyzed by flow cytometry. (A) Schematic design. (B) Percentage of GFP-positive cells by flow cytometry ($n = 3$, $^{***}p < 0.01$).

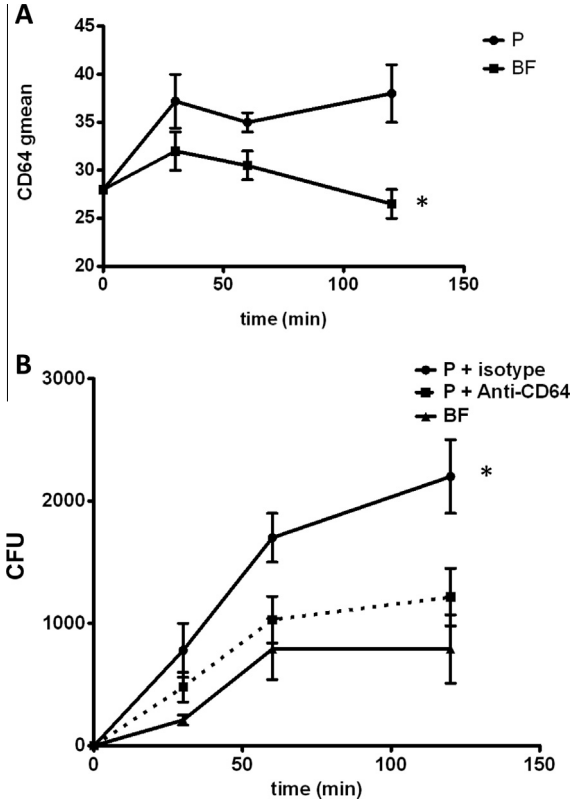

Fig. 3. Human macrophages exposed to planktonic bacteria express CD64. (A) The cells were exposed to P or BF bacteria for the indicated time. They were then washed, stained with antiCD64 and analyzed by flow cytometry; CD64 mean is shown ($n = 5$, $^{*}p < 0.05$). (B) The macrophages were exposed to BF and P bacteria in the presence of Anti-CD64 (2 μg/ml) or an isotype for the indicated time. The cells were lysed and their cytosols were spread in agar dishes overnight. The CFU were counted, and the number of CFU is shown ($n = 3$, $^{*}p < 0.05$).

sizes than in the case of planktonic. In addition, the same density of planktonic and BF was used.

3.4. Macrophage interaction with O25b-ST131ESBL-producer E. coli causing urinary tract infection

Our findings reveal there are differences between the interactions of human macrophages with P and BF *E. coli*. However, all these data were obtained with the standard bacterium *E. coli* K12, which is commonly used in research laboratories. In order to explore the pathophysiological relevance of our data, we repeated our experiments, using a clinical ESBL-producer *E. coli* isolate previously typed as O25b-ST131. Planktonic and biofilm bacteria were reproduced in the same conditions as previously described for the *E. coli* K12 isolate. We then analyzed the phagocytic ability of human macrophages following the experimental protocol

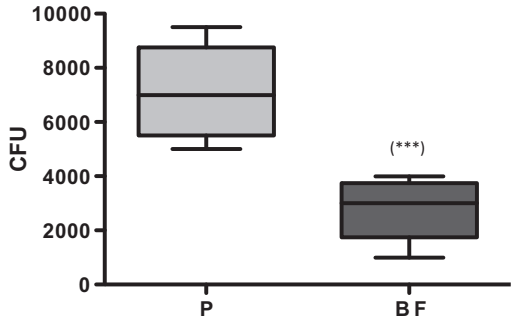

Fig. 4. Human macrophages phagocytize ST131 planktonic bacteria more efficiently than biofilm. The macrophages were exposed to ST131 planktonic (P) or biofilm (BF) bacteria as indicated in Fig. 1B for 2 h. The cells were lysed and their cytosols were spread in agar dishes overnight. The CFU were counted, and the number of CFU is shown ($n = 7$, $^{**}p < 0.03$).

shown in (Fig. 2B). As in the previous case, this assay indicated that human macrophages more efficiently phagocytized P bacteria than BF bacteria (Fig. 4).

4. Discussion

Clinical infections associated with biofilm formation, either on natural body surfaces or on foreign devices, often lead to significant morbidity due to their chronicity, their resistance to antimicrobial therapies and their immunological evasion [30,31]. In fact, these heterogeneous bacterial communities represent a serious clinical problem in modern medicine.

Most human microbiota colonizing the human body are organized under biofilm structures that are continuously being remodeled through the formation of new bacterial generations and their effective interaction with our immune system and tissues [9]. The first stage of a pathogenic infection is typically caused by planktonic bacteria that are detached from a mature biofilm. Bacterial adherence is crucial to both colonize the niche and develop a new biofilm structure, which usually represents a challenge to the immune system, but a considerably lesser challenge than in the planktonic mode.

A limited number of studies have compared the innate response to planktonic bacteria versus biofilm bacteria, and contradictory data have been obtained. Findings appear to strongly depend on the model used in each study [15,32–35].

Until now, most of microbiological and immunological studies have been developed with planktonic inoculums and have obtained results that might not be reproduced using biofilm formations. Several authors have suggested that biofilm-associated macrophages are polarized toward an alternative activated phenotype, which limits the proper immune response against infection [34]. However, our purpose was to study the differences between them when biofilms are disrupted and individual bacteria can be sensed by innate immune cells such as macrophages. It has been demonstrated that, *in vivo*, biofilm "exports" individual bacteria to colonize other niches as a mechanism of expansion, and this fact increases the clinical relevance of the results we present here.

Our data demonstrated that bacteria grown as biofilm retain some distinctive features that provide them with protection against phagocytosis. A significant difference in the phagocytosis of planktonic bacteria versus biofilm bacteria by human macrophages was observed using several techniques (e.g., flow cytometry, CFU count and confocal microscopy). Moreover, our findings indicated that human macrophages prefer planktonic bacteria to biofilm. A competition assay showed that the presence of increasing numbers of planktonic bacteria affected biofilm phagocytosis by macrophages. The mechanism by which biofilm avoids phagocytosis might involve the expression of CD64. This receptor has been previously implicated in phagocytic activity [29]. According to our data, while planktonic bacteria induced an increased expression of CD64, the presence of biofilm did not affect its baseline level. In addition, in a CD64-blocking assay, macrophages significantly reduced their ability to phagocytize planktonic bacteria. Further research must be done to clarify the potential role of this receptor in the observed phenomenon.

Finally, we corroborate our primary results using a worldwide disseminate and well-recognize clinical pathogen [36]. Human macrophages also phagocytize the urinary infection linked ST131 lineage more efficiently when bacteria were grown as planktonic rather than as biofilm. Altogether, our results suggest that new antimicrobial therapies, including DNAses, must take into account several biofilm features and, in particular, its interaction with innate immune cells. In the specific case of those pathogens that do not need internalization for their life cycle, such as *E. coli*, clinicians must evaluate the opportunity the strategies involving biofilm disruption have to offer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.012.

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