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J Immunol 2005; 175:7512-7518; ;
doi: 10.4049/jimmunol.175.11.7512
http://www.jimmunol.org/content/175/11/7512

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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The Exopolysaccharide Alginate Protects *Pseudomonas aeruginosa* Biofilm Bacteria from IFN-γ-Mediated Macrophage Killing[1]

Jeff G. Leid,[2]* Carey J. Willson,* Mark E. Shirtliff,† Daniel J. Hassett,‡ Matthew R. Parsek,§ and Alyssa K. Jeffers*

The ability of *Pseudomonas aeruginosa* to form biofilms and cause chronic infections in the lungs of cystic fibrosis patients is well documented. Numerous studies have revealed that *P. aeruginosa* biofilms are highly refractory to antibiotics. However, dramatically fewer studies have addressed *P. aeruginosa* biofilm resistance to the host's immune system. In planktonic, unattached (nonbiofilm) *P. aeruginosa*, the exopolysaccharide alginate provides protection against a variety of host factors yet the role of alginate in protection of biofilm bacteria is unclear. To address this issue, we tested wild-type strains PAO1, PA14, the mucoid cystic fibrosis isolate, FRD1 (*mucA22*$^+$), and the respective isogenic mutants which lacked the ability to produce alginate, for their susceptibility to human leukocytes in the presence and absence of IFN-γ. Human leukocytes, in the presence of recombinant human IFN-γ, killed biofilm bacteria lacking alginate after a 4-h challenge at 37°C. Bacterial killing was dependent on the presence of IFN-γ. Killing of the alginate-negative biofilm bacteria was mediated through mononuclear cell phagocytosis since treatment with cytochalasin B, which prevents actin polymerization, inhibited leukocyte-specific bacterial killing. By direct microscopic observation, phagocytosis of alginate-negative biofilm bacteria was significantly increased in the presence of IFN-γ vs all other treatments. Addition of exogenous, purified alginate to the alginate-negative biofilms restored resistance to human leukocyte killing. Our results suggest that although alginate may not play a significant role in bacterial attachment, biofilm development, and formation, it may play an important role in protecting mucoid *P. aeruginosa* biofilm bacteria from the human immune system. ***The Journal of Immunology,*** **2005, 175: 7512–7518.**

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Bacterial biofilms are increasingly being recognized as significant pathogenic mechanisms underlying the development of certain chronic infections in humans. When bacteria transition from planktonic, free-living organisms to attached, matrix enclosed communities with three-dimensional structure, they are inherently less susceptible to antibiotics and to the innate and adaptive killing components of the host (1–8). For years, studies have attempted to address the mechanisms behind reduced biofilm susceptibility to antibiotics (6, 9–13). Recently, genes involved in antibiotic resistance in the important opportunistic pathogen *Pseudomonas aeruginosa* were correlated with the biofilm phenotype (14). To our knowledge, this was the first article that connected biofilm-specific gene expression with antibiotic susceptibility (14). Biofilm resistance mechanisms to human leukocyte killing and clearance have not been well characterized and may consist of 1) limited penetration of leukocytes and their antimicrobial products into the biofilm; 2) inactivation of or suppression of leukocyte-specific processes by the biofilm matrix or bacterial components; 3) decreased ability of leukocytes to phagocytize biofilm bacteria; 4) the presence of global response regulators and quorum sensing that increase resistance to leukocytes in biofilms; and/or 5) genetic switches that lead to increased resistance to components of the human immune system.

One of the most classical examples of *P. aeruginosa* biofilm in human disease is that associated with the chronic lung infection of cystic fibrosis (CF)[3] patients (15–21). In the clinical setting, ~80–90% of the lungs of CF patients are colonized by this organism between the ages of 16 and 20 (21, 22). The chronic nature of CF lung infections correlates with increased morbidity and mortality (23–25). Over two decades ago, Costerton and others (15, 16, 26) demonstrated that lung sputum from CF patients contained *P. aeruginosa*, likely in the form of biofilms. More recently, Singh et al. (18) showed that biofilms of *P. aeruginosa* exist in the CF lung. These important works underscore the significance of biofilm formation in vivo and document the inability of the host's immune system to clear *P. aeruginosa* biofilm infections.

One of the most intensely studied virulence determinants of *P. aeruginosa*, both in the context of planktonic and biofilm growth, is the viscous exopolysaccharide alginate. Alginate is an important extracellular component of mucoid *P. aeruginosa* and has been shown to scavenge hypochlorite, reduce polymorphonuclear chemotaxis, inhibit activation of complement, and decrease phagocytosis of planktonic mucoid bacteria by neutrophils and macrophages (27–32). Overproduction of alginate protects against certain antibiotics as well as against protozoan grazing on

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Received for publication May 19, 2005. Accepted for publication September 13, 2005.

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[1] This work was supported by funds from the Northern Arizona University Intramural Grants Program and funds from the State of Arizona Proposition 301.

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[3] Abbreviations used in this paper: CF, cystic fibrosis; LB, Luria-Bertani; algD$^-$, alginate-negative strains of *P. aeruginosa*; PVC, polyvinyl chloride.

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P. aeruginosa biofilms, a process not dissimilar to leukocyte phagocytosis (33, 34). A previous study demonstrated that rabbit Abs against alginate led to killing of *P. aeruginosa* biofilm bacteria but not Abs generated by CF patients (35). The same laboratory demonstrated that a new generation of humanized anti-alginate Abs protected against planktonic, mucoid *P. aeruginosa* bacteria (36). Despite these recent findings, a direct examination of the role of alginate in *P. aeruginosa* biofilm resistance to host killing mechanisms has not been reported.

Currently, it is unclear which genetic mechanism(s), including the capacity to produce the exopolysaccharide alginate, are responsible for the lowered susceptibility of *P. aeruginosa* biofilms to leukocyte killing. It is also unclear which specific mechanism(s) host leukocytes use that render *P. aeruginosa* biofilms susceptible to killing. This study addresses these issues and we have demonstrated that alginate protects *P. aeruginosa* biofilm bacteria from leukocyte phagocytosis. In the presence of the potent leukocyte activator IFN-γ, human phagocytes killed *P. aeruginosa* biofilm bacteria lacking the ability to produce the exopolysaccharide alginate. Wild-type (PA01, PA14, and the mucoid, CF isolate, FRD1) and an isogenic PA01 mutant that over expresses alginate (PA01 *mucA22*, PDO300) were unaffected by this treatment regimen. Cytochalasin B, with blocks actin polymerization and therefore blocks phagocytosis, inhibited killing of alginate-negative (algD$^-$) strains of *P. aeruginosa* biofilm bacteria. Phagocytosis of algD$^-$ biofilm bacteria was markedly increased compared with the wild-type controls. Complementation of the algD$^-$ biofilm bacteria with exogenous alginate protected the bacteria against killing. These data are the first to directly demonstrate that alginate protects against biofilm bacterial phagocytosis and killing by human leukocytes.

Materials and Methods

Bacterial strains

P. aeruginosa wild-type strains PA01 (Iglewski variant), PA14 (37), and the mucoid, CF isolate FRD1 (38), isogenic algD$^-$ mutants of *PA01*, *PA14*, and *FRD1* and strain PDO300, a mucoid *mucA22*$^+$ mutant of strain PA01 that overproduces alginate, were used in this study. All bacteria were grown on Luria-Bertani (LB) agar and in LB broth for the experiments and the number of viable biofilm bacterial enumerated by CFU on LB agar.

Reagents

Recombinant human IFN-γ was purchased from BioSource International at a stock concentration of 10^6 U/ml. Cytochalasin B (50 μg/ml) and paraformaldehyde were purchased from Sigma-Aldrich. Purified, sterile alginate (1 mg) extracted from *P. aeru*ginosa was kindly supplied as lyophilized crystals by Dr. G. Pier (Harvard University, Boston, MA).

Growth of biofilms in microtiter plates

Biofilms were grown in microtiter plates as previously described (39, 40). Briefly, bacteria were inoculated into LB broth (Fisher) and grown overnight at 37°C with vigorous aeration. The suspension was diluted 1/100 into fresh LB broth and incubated aerobically for 3 h at 37°C, allowing the bacteria to reenter the exponential growth phase. After 3 h, the cultures were further diluted 1/50 in fresh LB and 100 μl of bacterial suspension added to sterile 96-well polyvinyl chloride (PVC) plates (Falcon; BD Biosciences) in triplicate. The plates were covered in parafilm (Fisher) and biofilms were allowed to form for 24 h. The biofilms were then used in the leukocyte killing assays described below.

Isolation of human peripheral blood leukocytes

Healthy human donors were used as sources of peripheral blood leukocytes subsequent to reading and signing donor consent forms. The protocol was approved by the appropriate Northern Arizona University Institutional Review Board. Peripheral blood leukocytes were obtained from healthy human donors as previously described with modifications (41, 42). Briefly, peripheral blood was collected into acid citrate Vacutainer tubes, centrifuged for 15 min at 400 $\times$ *g*, the peripheral blood buffy coat and autologous plasma were collected, the buffy coat was washed, and erythrocytes were lysed by hypotonic treatment for 10 s followed by rapid dilution in HBSS (Sigma-Aldrich). The purified leukocytes were counted and resuspended at a final concentration of 5×10^7/ml containing 50% autologous plasma for the killing assays described below. Silverstein and colleagues (43) reported that a critical concentration of neutrophils is required for effective planktonic bacterial killing. We designed our studies so that the concentration of total peripheral blood leukocytes was 5-fold greater than their reported critical concentration of neutrophils since we used a mixed population of white blood cells, and biofilms are inherently more resistant to killing. This protocol constantly yielded >99% pure populations of human leukocytes that were >99% viable by trypan blue exclusion.

To determine which leukocyte subpopulation was primarily responsible for killing of the *P. aeruginosa* biofilm bacteria, leukocyte subsets were fractionated as previously described with modifications (42, 44). Briefly, ~10 ml of peripheral blood was diluted 1/1 with warmed HBSS and underlayed with 10 ml of Histopaque 1.077 (Sigma-Aldrich) and underlayed again with 10 ml of Histopaque 1.119 (Sigma-Aldrich) for a total volume of 40 ml in a sterile 50-ml conical tube (Fisher). The tubes were centrifuged at 700 $\times$ *g* for 30 min and the 1.077 and 1.119 buffy coats containing mononuclear cells and neutrophils, respectively, collected. If erythrocytes were present after collection of the buffy coats, they were hypotonically lysed for 10 s in nanopure water followed by rapid dilution to a 50-ml final volume in HBSS. The separated leukocyte populations were counted and resuspended at 1×10^7/ml in HBSS containing pooled human serum (Mediatech) for introduction into the biofilm killing assay. For all killing assays, 10 mM HEPES (Mediatech) buffer was added to maintain constant pH throughout the duration of the assays.

Biofilm killing assay

To determine whether or not human leukocytes kill *P. aeruginosa* biofilm bacteria lacking the exopolysaccharide alginate, the biofilms described above were incubated with the respective leukocyte populations for 4 h at 37°C under static conditions. In preparation for the addition of the human leukocytes, the PVC plates containing the biofilms were quickly inverted and nonadherent cells washed away. Subsequently, 100 μl of either the total leukocyte population or purified subpopulations was added in triplicate to the wells of the microtiter plate containing biofilms. Control treatments included incubation of the triplicate wells with LB alone, HBSS plus 50% autologous plasma or pooled human serum alone, LB containing 10% bleach, and with 4% paraformaldehyde-fixed leukocytes in HBSS for the duration of the assays. These different assay permutations served as a positive control for growth (LB), a leukocyte medium and plasma/serum control for potential nonspecific leukocyte killing (HBSS plus 50% plasma/serum), a positive control for biofilm killing (10% bleach), and a control for the requirement of viable leukocytes in the killing assays (paraformaldehyde-fixed cells). By this protocol, no viable bacteria were recovered with the bleach treatment and no killing of *P. aeruginosa* biofilm bacteria was observed by incubation with paraformaldehyde-fixed leukocytes. Additionally, leukocytes were incubated in the presence of exogenous recombinant human IFN-γ (50 U/ml; BioSource International, for all figures presented here) in triplicate. After 4 h, the PVC-attached biofilms were washed, sonicated in a Microhorn sonicator (Misonix) and serially diluted in sterile nanopure water. Sonication by this protocol did not affect bacterial viability as measured by untreated controls and by fluorescent staining. Ten-fold serial dilutions were plated on LB agar in triplicate spots per dilution and incubated overnight. CFU were counted after a 24-h incubation at 37°C and the data are reported as CFU/ml. Percent survival was calculated by normalizing the LB treatment to 100% survival. All data were obtained from at least three separate experiments from three different human donors. The average CFU $\pm$ SEM are reported for each experiment and where appropriate, the percent survival is listed in parentheses. For these experiments, the ratio of biofilm bacteria to leukocytes was ~100:1.

Cytospin assay

To determine whether the presence or absence of alginate affected the ability of leukocytes to engulf biofilm bacteria, the biofilm killing assays were preformed as described above but instead of serial dilution after sonication, 300 μl of biofilm and leukocyte suspension was added to the bottom of the cone of a Cytofunnel with attached filter card (Shandon Lipshaw) linked to microscope slides (Fisher) by a slide clip (Shandon Lipshaw). The slides were centrifuged for 10 min at 90 $\times$ *g* in a Cytospin 2 (Shandon Lipshaw), allowed to air dry for 10 min, fixed in 100% methanol, and stained with DiffQuick. After drying, the slides were fitted with coverslips, and the number of engulfed rods enumerated from 50 phagocytes counted blindly from three separate individuals. The average number of engulfed bacteria per leukocyte from the three different individuals was calculated for wild-type and algD$^-$ biofilms in the presence and absence of

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exogenous IFN-γ. The average number of engulfed bacteria per 50 cells ± SEM are reported.

Phagocytosis inhibition assay

To establish that phagocytosis was the main killing mechanism being used by human leukocytes to kill biofilm bacteria, similar assays were used as described above in the presence of cytochalasin B (50 μg/ml; Sigma-Aldrich), a potent inhibitor of actin polymerization. The number of viable bacteria were enumerated by serial dilution plating and data are reported as CFU/ml.

Complementation of algD$^-$ biofilms with purified alginate

*FRD1*algD$^-$ or *PA01*algD$^-$ biofilms were prepared as described above, except that 250 μg/ml purified alginate was added to the wells of the 96-well plate in triplicate. The algD$^-$ bacteria were allowed to form biofilms in the presence of exogenous alginate for 24 h at 37°C. Controls included wild-type strains and the algD$^-$ biofilms without the exogenous addition of alginate. After 24 h, the biofilm bacteria were challenged with human leukocytes for 4 h at 37°C, sonicated, serially diluted, and CFU plated on LB agar and incubated overnight. CFU were counted the next day and viable biofilm bacteria were enumerated.

Statistical analysis

To determine whether there were significant differences between the average numbers of engulfed bacteria in the microtiter and cytospin assays, the leukocyte treatment of algD$^-$ biofilms in the presence of exogenous IFN-γ was compared by Student's *t* test to all other treatments. The CFU/ml were compared with respect to the treatment administered and $p < 0.05$ was considered to be significant.

Results

P. aeruginosa *biofilm bacteria that lack the exopolysaccharide alginate are killed by human leukocytes in the presence of IFN-γ*

Alginate has previously been reported to inhibit phagocytosis, thereby decreasing susceptibility of planktonic *P. aeruginosa* to human leukocytes (27–30). However, little mechanistic evidence exists that correlates alginate production and bacterial biofilm resistance to human leukocytes other than the reports identifying alginate as an important component of mucoid biofilms in the lungs of chronically infected CF patients (21). We hypothesized that *P. aeruginosa* alginate may play an important role in biofilm defense against the host's immune system. To test this hypothesis, three different strains of *P. aeruginosa* and their isogenic algD$^-$ mutants were tested in a novel, static biofilm leukocyte killing assay developed in our laboratory.

In the presence of exogenous, recombinant human IFN-γ, human leukocytes killed *P. aeruginosa* biofilm bacteria that lacked the ability to produce alginate (Fig. 1). Following a 4-h challenge with leukocytes and IFN-γ at 37°C, a 10-fold reduction in viable biofilm bacteria was observed compared with other control and leukocyte treatments (Fig. 1). The killing of PA01algD$^-$ biofilm bacteria was specific because 4% paraformaldehyde-fixed leukocytes did not reduce biofilm bacterial viability vs medium control (data not shown). There was modest killing observed when algD$^-$ biofilms were incubated with HBSS and 50% autologous plasma alone, but this effect was not statistically different from LB medium alone (Fig. 1). The reduction in bacterial viability in the presence of HBSS and autologous human plasma was likely a result of reduced nutritional substrate and the presence of antibacterial human plasma components compared with the rich nutrient environment of the LB medium. Incubation with IFN-γ alone had no effect on bacterial viability under assay conditions (data not shown). To determine the percent survival of biofilm bacteria after challenge with human leukocytes, the LB treatment was normalized to 100% survival. In this context, >90% of the biofilm bacteria were killed (Fig. 1, parentheses noting percent survival).

Human leukocyte killing in these assays was dependent on appropriate concentrations of IFN-γ. Titration studies demonstrated

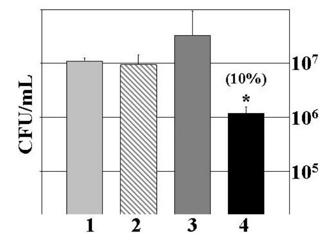

FIGURE 1. In the presence of IFN-γ, biofilm bacteria of *P. aeruginosa* that lack the exopolysaccharide alginate are killed by human leukocytes. Graphic representation of CFU/ml PA01algD$^-$ biofilm bacteria after treatment with medium control (LB, *column 1*), HBSS plus 50% autologous human plasma (*column 2*), human leukocytes in HBSS containing 50% autologous plasma (*column 3*), and leukocytes in HBSS containing 50% autologous plasma plus IFN-γ (50 U/ml; *column 4*). Percent survival was calculated by normalizing the LB medium control to equal 100% survival and is listed in parentheses above the appropriate treatment. *, $p < 0.001$ for the leukocytes plus IFN-γ treatment when compared with all other treatments. Data are representative of three separate experiments with various blood donors.

that in the presence of concentrations of IFN-γ below 8 U/ml, no biofilm bacterial killing was observed (data not shown). Another cytokine, TNF-α, had no effect on biofilm bacterial killing in our experiments over a wide range of concentrations (data not shown). Finally, under these assay conditions, we found no role for nitrogen and oxygen radicals in bacterial biofilm killing (data not shown).

Bacteria from wild-type biofilms are not killed by human leukocytes, even in the presence of exogenous IFN-γ

P. aeruginosa strain PA01 is widely used as a model biofilm organism in a variety of studies relevant to human disease (1, 3, 4, 6–11, 14, 21). It is clear from these studies that strain PA01 growing as a biofilm is less susceptible to antibiotics and to killing by host factors. When PA01 biofilms were grown in a microtiter plate for 24 h at 37°C and challenged with human leukocytes in the presence and absence of exogenous IFN-γ, no appreciable killing of biofilm bacteria was observed (Fig. 2). This was in stark contrast to the 10-fold reduction seen under identical conditions with the PA01algD$^-$ biofilms (Fig. 1). Although up to 25% of the bacteria were rendered nonviable in some treatments, these results were not statistically significant (Fig. 2). Another commonly used *P. aeruginosa* strain that efficiently forms biofilms, PA14, demonstrated similar resistance to killing by human leukocytes (data not shown).

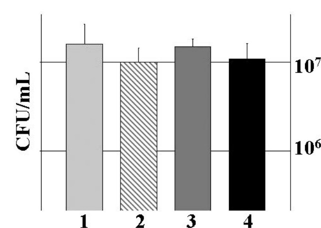

FIGURE 2. Wild-type PA01 biofilm bacteria are not killed by human leukocytes, even in the presence of IFN-γ. Graphic representation of CFU/ml wild-type PA01 biofilm bacteria after treatment with medium control (LB, *column 1*), HBSS plus 50% autologous plasma (*column 2*), leukocytes in HBSS containing 50% autologous plasma (*column 3*), and leukocytes in HBSS containing 50% autologous plasma plus IFN-γ (*column 4*). The differences in CFU/ml were not significant when compared with the LB medium control. Data are representative of three separate experiments with various blood donors.

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To determine whether alginate overproduction protected biofilm bacteria against killing by leukocytes as previously shown for antibiotics and protozoal grazing (33, 34, 45), the isogenic PA01 mutant, PDO300 (*mucA22*$^{+}$), which overproduces alginate, was tested in our leukocyte killing assay. Again, no significant killing was observed by any treatment administered (data not shown). Interestingly, percent survival of PDO300 biofilm bacteria was similar to that seen with wild-type biofilms (PA01, PA14, and FRD1, see below), suggesting that the overproduction of alginate did not increase *P. aeruginosa* biofilm bacterial resistance to leukocyte killing. However, a recent work demonstrated that after 48 h of infection with this mutant strain, there was marked inflammation and high planktonic bacterial load in a variety of mouse organs vs the parent strain PA01 (46). Thus, the ability to overproduce alginate seems to have an early protective effect in acute infection models.

An isogenic algD^{-} *mutant of the mucoid, clinical* P. aeruginosa *isolate FRD1 is susceptible to human leukocyte killing*

Since alginate is associated with the mucoid phenotype in many clinical isolates from CF patients, we tested whether the best-characterized clinical isolate, strain FRD1, and its isogenic algD^{-} biofilm bacteria were susceptible to human leukocyte-specific killing. As expected, FRD1 biofilm bacteria were not susceptible to human leukocyte killing (Fig. 3*B*). In contrast, the FRD1algD^{-} biofilm bacteria were highly susceptible to leukocyte killing in the presence of IFN-γ (Fig. 3*A*). Interestingly, greater bacterial killing was observed with the algD^{-} mutant from the FRD1 background than was observed for the other strains (PA01 and PA14) above. In the mucoid background, >98% reduction in viable bacteria was observed (Fig. 3).

Complementation of the algD^{-} *biofilm bacteria with purified, exogenous alginate restored the resistance to human leukocyte killing*

A previous study by Hassett (47) demonstrated that FRD1 produced between 200 and 500 μg/mg alginate per cell dry weight when growing in a biofilm. To determine whether exogenous alginate protected algD^{-} bacteria growing as biofilms, 250 μg/ml purified alginate was added to the wells of the 96-well microtiter dishes and the biofilms were allowed to form for 24 h. Upon challenge by human leukocytes, FRD1algD^{-} biofilm bacteria were protected from killing (Fig. 4). Similar results were observed when the PA01algD^{-} strain was complemented with exogenous alginate (data not shown). As Fig. 4 shows, the exogenous alginate protected the biofilm bacteria from human leukocyte killing as well as the native alginate produced from the FRD1 wild-type strain (Fig. 4 vs Fig. 3*B*). In further studies, the amount of complemented alginate was titrated and as expected, 500 μg protected the FRD1algD^{-} biofilm bacteria from killing whereas 5 μg did not (data not shown).

Mononuclear cells are the main subset of human leukocytes that kill algD^{-} *biofilm bacteria*

To determine which human leukocyte subpopulation, if any, was primarily responsible for the killing observed with the algD^{-} biofilm bacteria, peripheral blood leukocytes were separated into neutrophil and mononuclear (lymphocytes and monocytes) cell fractions. The subpopulations were then incubated with wild-type and algD^{-} biofilms for 4 h at 37°C as described above and the number of viable cells was determined. Under these conditions, wild-type PA01 (and PA14 and FRD1, data not shown) biofilm bacteria were not killed by either purified population of leukocytes. However, when algD^{-} biofilm bacteria were challenged with the mononuclear cell population containing lymphocytes and monocytes in the presence of IFN-γ, a 10-fold reduction in viable bacteria was observed after 4 h (Fig. 5). This result was similar to that seen with the whole peripheral blood leukocytes in Fig. 1. Since monocytes/macrophages are known to be potently activated by IFN-γ, these data suggested that monocytes were the main leukocyte subpopulation responsible for killing. Further studies using mouse (RAW264) and human (SC) monocyte cell lines treated with IFN-γ demonstrated similar killing, supporting the notion that monocytes were the main leukocyte subset responsible for biofilm bacterial killing (data not shown). Still, some killing of the algD^{-} biofilm bacteria was observed in the presence of purified neutrophils, although not to the extent as the IFN-γ-treated mononuclear cells (Fig. 5). The mononuclear cell killing of the algD^{-} biofilm bacteria resulted in 12% survival of bacteria after 4 h (Fig. 5) vs ~70% in the presence of neutrophils alone (Fig. 5). No killing was observed if 4% paraformaldehyde-fixed mononuclear or neutrophil subpopulations were used (data not shown).

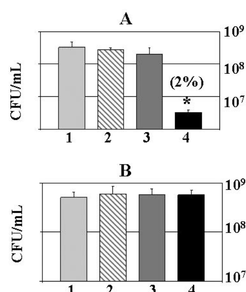

FIGURE 3. The lack of alginate in a mucoid, clinical isolate (FRD1algD^{-}) of *P. aeruginosa* renders biofilm bacteria susceptible to human leukocyte killing. *A*, Graphic representation of CFU/ml FRD1algD^{-} biofilm bacteria after treatment with medium control (LB, *column 1*), HBSS plus 50% autologous plasma (*column 2*), leukocytes in HBSS containing 50% autologous plasma (*column 3*), and leukocytes in HBSS containing 50% autologous plasma plus IFN-γ (*column 4*). *B,* The same experiment performed with the wild-type parental strain FRD1. Percent survival is listed in parentheses above the appropriate treatment. $*, p < 0.01$ for *column 4* vs LB treatment in *A*. Data are representative of three separate experiments with various blood donors.

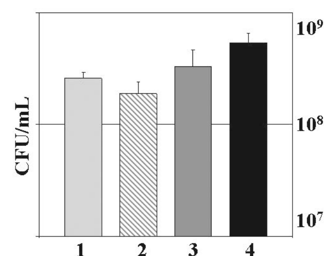

FIGURE 4. Complementation of FRD1algD^{-} bacteria with exogenous alginate restores the resistance of biofilm bacteria to killing by human leukocytes. Graphic representation of CFU/ml FRD1algD^{-} biofilm bacteria complemented with exogenous alginate (250 μg/ml) after treatment with medium control (LB, *column 1*), HBSS plus 50% autologous plasma (*column 2*), leukocytes in HBSS containing 50% autologous plasma (*column 3*), and leukocytes in HBSS containing 50% autologous plasma plus IFN-γ (*column 4*). There was no statistically significant difference between the treatments. Data are representative of three separate experiments with various blood donors.

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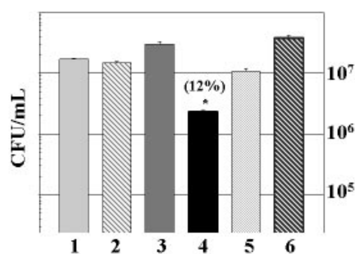


FIGURE 5. Human mononuclear cells are the primary leukocyte subset responsible for killing of the algD$^-$ biofilm bacteria. Graphic representation of CFU/ml algD$^-$ biofilm bacteria after treatment with medium control (LB, *column 1*), HBSS plus 50% pooled human serum (*column 2*), mononuclear cell fraction (lymphocytes and monocytes, *column 3,* without IFN-γ; *column 4,* with IFN-γ) and neutrophils (*column 5,* without IFN-γ; *column 6,* with IFN-γ). Percent survival (in parentheses) with the mononuclear cell fraction, in the presence of IFN-γ, was ~12% after 4 h. *, $p < 0.01$ for *column 4* vs control LB treatment (*column 1*). Data are representative of three separate experiments with various blood donors.

Cytochalasin B, a potent inhibitor of actin polymerization and phagocytosis, prevents killing of algD$^-$ biofilm bacteria, even in the presence of IFN-γ

Since the main subpopulation of leukocytes primarily responsible for the biofilm bacteria killing was mononuclear cells and the fact that killing was observed when exogenous IFN-γ was present, we hypothesized that phagocytosis was the main leukocyte killing mechanism. To test this, we treated leukocytes with cytochalasin B, a potent inhibitor of actin polymerization. When whole leukocyte populations or specific subpopulations were incubated with the algD$^-$ biofilms in the presence of cytochalasin B and IFN-γ, no reduction in viable bacteria was observed (Fig. 6). Indeed, in the presence of cytochalasin B, leukocytes, and IFN-γ, there was an increase in the number of viable algD$^-$ biofilm bacteria recovered after 4 h (Fig. 6) that correlated to ~130% survival of the normalized control. Treatments with cytochalasin B, IFN-γ, and leukocytes were statistically significant compared with the same treatments without cytochalasin B (Fig. 6). Cytochalasin B alone, in the concentrations used in this study, had little or no effect on bacterial viability under our assay conditions (data not shown).

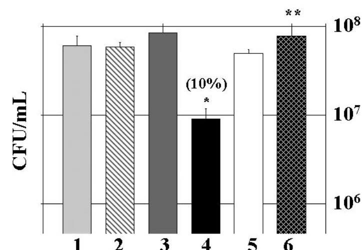


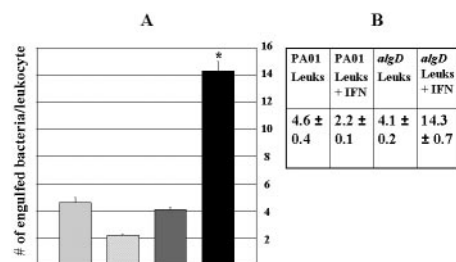
FIGURE 6. Cytochalasin B inhibits phagocyte killing of algD$^-$ biofilm bacteria. Graphic representation of CFU/ml algD$^-$ biofilm bacteria after treatment with medium control (LB, *column 1*), HBSS plus 50% autologous plasma (*column 2*), leukocytes in HBSS containing 50% autologous plasma (*column 3*), leukocytes in HBSS containing 50% autologous plasma plus IFN-γ (*column 4*), leukocytes in HBSS containing 50% autologous plasma plus cytochalasin B (*column 5*), and leukocytes in HBSS containing 50% autologous plasma plus IFN-γ plus cytochalasin B (*column 6*). Percent survival is listed in parentheses above the appropriate treatment. *, $p < 0.001$ vs medium control (*column 1*); **, $p < 0.001$ vs *column 4*. Data are representative of three separate experiments with various blood donors.

Phagocytosis of algD$^-$ biofilm bacteria is significantly enhanced relative to that of wild-type strains

To enumerate the extent of phagocytosis of algD$^-$ biofilm bacteria, the samples were centrifuged in a Cytospin 2 to quantify the number of engulfed bacteria by light microscopy. When blind counts were performed on the wild-type and algD$^-$ biofilms, a markedly higher number of bacteria were engulfed by phagocytes in the presence of IFN-γ with the algD$^-$ bacteria (Fig. 7). There was a statistically significant 5-fold average increase in the number of engulfed bacteria in this treatment vs all of the other treatments (Fig. 7*B*). These results directly demonstrate that phagocytosis was markedly enhanced with *P. aeruginosa* biofilms lacking alginate in the presence of exogenous IFN-γ. Experiments performed with strain FRD1 and its isogenic algD$^-$ strain gave similar results (data not shown).

Discussion

In this report, we demonstrate a correlation between the exopolysaccharide alginate in *P. aeruginosa* and biofilm susceptibility to human leukocyte phagocytosis in the absence of opsonizing Ab. These data show the importance of alginate in biofilm bacterial protection against leukocyte killing and more specifically against leukocyte phagocytosis in the biofilm mode of growth. Three different isogenic algD$^-$ strains, including a clinical, mucoid isolate (FRD1), were susceptible to human leukocyte killing in the presence of IFN-γ. Complementation of the algD$^-$ biofilm bacteria with exogenous alginate restored resistance to human leukocyte killing. Blood monocytes were the main leukocyte subset responsible for the biofilm bacterial killing. Cytochalasin B, a fungal toxin that prevents actin polymerization, inhibited the killing of the algD$^-$ biofilm bacteria, implicating phagocytosis as the main molecular killing mechanism of *P. aeruginosa* biofilm bacteria. By direct microscopic observation, we determined that engulfment of algD$^-$ biofilm bacteria was dramatically enhanced in the presence of IFN-γ. Collectively, these data demonstrate the important role alginate plays in protection against human leukocyte phagocytosis and killing and represents an important link in establishing chronic infections in the lungs of CF patients.



PA01 Leuks	PA01 Leuks + IFN	*algD* Leuks	*algD* Leuks + IFN
4.6 ± 0.4	2.2 ± 0.1	4.1 ± 0.2	14.3 ± 0.7

FIGURE 7. Phagocytosis of algD$^-$ biofilm bacteria was markedly enhanced vs the wild-type PA01 strain. *A,* Graphic representation of the number of engulfed biofilm bacteria per treatment ± SEM from three separate blind counts. *Column 1,* PA01 plus leukocytes in HBSS containing 50% autologous plasma; *column 2,* PA01 plus leukocytes in HBSS containing 50% autologous plasma plus IFN-γ; *column 3,* PA01algD$^-$ plus leukocytes in HBSS containing 50% autologous plasma; and *column 4,* PA01algD$^-$ plus leukocytes in HBSS containing 50% autologous plasma plus IFN-γ. The numerical values are listed to the *right* of the graph (*B*) and represent the average number of engulfed bacteria ± SEM. When compared with the rest of the treatments, there was a statistically significant increase in phagocytosis of algD$^-$ bacteria in the presence of leukocytes plus IFN-γ ($p < 0.001$). Data are representative of three separate experiments with various blood donors.

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The inflammatory cytokine IFN-γ is a potent activator of the phagocytic mechanisms of macrophages. This Th1 cytokine is often one of the first soluble immunomodulators secreted early during inflammatory events. Our previous report on *Staphylococcus aureus* demonstrated that secretion of this cytokine by human leukocytes was induced within 2 h of exposure to *S. aureus* biofilms (7). Interestingly, Moser et al. (48, 49) have demonstrated that *P. aeruginosa* induces a Th2 type response in mouse models of CF yet to clear the infections, a Th1 response is required. IFN-γ, a Th1-type cytokine, was vital to *P. aeruginosa* algD^{-} biofilm bacterial killing in this study. Together, these data demonstrate that for biofilm infections of medically common bacteria such as *P. aeruginosa* and *S. aureus*, a Th1 response may be vital for biofilm bacterial clearance. If true, this may represent one fundamental difference in immune recognition and clearance of planktonic vs biofilm bacteria where opsonization by Th2 Abs and killing with polymorphs are likely more active against planktonic bacteria. Further studies are needed to address this important question.

A previous paper demonstrated that *P. aeruginosa* biofilm bacteria were killed if the appropriate Th2-mediated opsonic Abs against exopolysaccharides, namely alginate, were present (35). Unfortunately, Abs against *P. aeruginosa* isolated from CF patients were unable to act as effective opsonins and did not lead to killing and clearance of the biofilm bacteria in vitro (35). Recently, Pier et al. (36) demonstrated that a new generation of Abs against alginate promoted clearance of planktonic *P. aeruginosa* in an acute lung infection model in mice. The opsonic Abs were required for killing and clearance of these organisms. These studies, combined with our studies, suggest that if appropriate Abs from a Th2 response are not present, a Th1 response must be present to protect patients early on. It is intriguing to think that in the CF lung, where appropriate Abs are not produced, alginate protects the biofilm bacteria from IFN-mediated clearance and may key the transition from acute to chronic infection. Collectively though, these studies may help guide more successful treatment of both acute and chronic *P. aeruginosa* lung infections.

Wozniak et al. (50) demonstrated that the commonly used laboratory *P. aeruginosa* strains, PA01 and PA14, do not express significant amounts of alginate when they grow as biofilms under specific in vitro conditions. On the surface, our data would seem to contradict this work because isogenic algD^{-} biofilms of both of these strains were susceptible to human leukocyte killing, whereas the parent, wild-type strains were not. However, two recent articles demonstrated, at least for the PA01 strain, that alginate production is heightened in vivo within 1 h of injection into a mouse (36, 51). These data correlate better with the presumed transition from nonmucoid to the mucoid phenotype in the lungs of CF patients likely mediated by reactive oxygen species (52). Still, the survival of the wild-type PA01 and PA14 biofilm bacteria is not easily explained. One possible explanation is that yet undefined alginate-independent mechanisms of protection against host factors exist. Conversely, in the presence of human leukocytes and their products, these strains may produce alginate in vitro. Studies are ongoing in our laboratory to determine whether alginate production is up-regulated in the presence of specific white blood cell populations. Other factors, such as quorum sensing and toxin production, likely play a role and need further study in the biofilm/host setting to elucidate other important resistance mechanisms. Clearly, though, our data demonstrate that there is a central role for alginate in protection of *P. aeruginosa* biofilm bacteria from host phagocytosis.

In general, as Jesaitis et al. (53) have reported for PAO1/human neutrophil interactions, the killing mechanisms of the human immune system are diminished when they encounter bacteria living in the biofilm mode of growth. Our data suggest that a target to fight chronic *P. aeruginosa* infections in humans may be biofilm-derived alginate combined with augmentation of the Th1/Th2 response. This point is underscored by the fact that many clinical isolates from chronically infected patients express the mucoid phenotype that correlates with alginate expression (21). For example, it may be therapeutically feasible to decrease alginate expression by administration of alginate lyase and exogenous, recombinant human IFN-γ to enhance the Th1 response and clearance of chronic *P. aeruginosa* biofilm bacteria in the CF lung. IFN therapy is already commonplace in patients with hepatitis C and has also shown potential in treatment of some cancer patients (54). Modulation of other cytokines that shift the Th1/Th2 balance may also provide therapeutic efficacy. It is presently unclear how the human immune system recognizes and responds to alginate but preliminary studies by our laboratory suggest alginate induces specific cytokines from human and mouse leukocytes. These types of alternative treatments will likely become even more important in the current era of increasing antibiotic resistance.

Importantly, our novel leukocyte bacterial biofilm killing assay described here represents a tool to begin to address the molecular aspects of human white blood cell interactions with medically significant biofilm-forming bacteria. The results of this study provide insight into the relationship between *P. aeruginosa* alginate produced during biofilm growth and susceptibility to human host factors, notably human phagocytes. Specifically, these data have defined alginate as a vital link in bacterial biofilm protection against host phagocytosis and the eventual transition to a chronic lung infection. Clearly, more studies are needed and animal models used to determine toxicity and efficacy of potential therapeutic treatments as described above. Nonetheless, this report provides promise for treating the devastating, chronic biofilm-mediated lung infections in CF patients caused by *P. aeruginosa*.

Acknowledgments

We thank Dr. Gerald Pier of Harvard University for his gift of purified *P. aeruginosa* alginate.

Disclosures

The authors have no financial conflict of interest.

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