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Biological Trojan Horse: Antigen 43 Provides Specific Bacterial Uptake and Survival in Human Neutrophils▽†

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***Escherichia coli* is a versatile pathogen causing millions of infections in humans every year. This bacterium can form multicellular aggregates when it expresses a self-associating protein, antigen 43 (Ag43), on its surface. We have discovered that Ag43-expressing *E. coli* cells are efficiently taken up by human defense cells, polymorphonuclear neutrophils (PMNs), in an opsonin-independent manner. Surprisingly, the phagocytosed bacteria were not immediately killed but resided as tight aggregates within the PMNs. Our observations indicate that Ag43-mediated uptake and survival in PMNs constitute a mechanism to subvert one of the primary defense mechanisms of the human body.**

Escherichia coli is a member of the normal intestinal flora of humans. However, it is also one of the most prominent human pathogens and accounts for millions of infections every year. This versatile pathogen can infect a variety of tissues and causes a broad spectrum of diseases, such as urinary tract infections, diarrhea, pneumonia, bacteremia, and meningitis (4, 15, 24). Microorganisms that embark on infecting human hosts face formidable obstacles. Thus, human tissues are superbly equipped to deal with hostile intruders. Apart from a range of specific and nonspecific antimicrobial mechanisms, the invader must face phagocytic killer cells in the form of polymorphonuclear neutrophils (PMNs). PMNs usually form the first line of defense against invading microorganisms and are a critical component of the human innate host response against bacterial infections (13). PMNs readily accumulate at sites of acute inflammation, and during bacterial infections, such as those of *E. coli*, the PMNs are the first phagocytes to arrive. Invading bacteria may be opsonized by complement proteins or antibodies and subsequently phagocytized and killed by PMNs.

Antigen 43 (Ag43) is a surface protein of *E. coli* that confers bacterial cell-cell aggregation via an intercellular Ag43-to-Ag43 handshake mechanism (8, 12, 17). Ag43-mediated aggregation can be visualized macroscopically as flocculation of cells from static liquid suspensions; hence, the name *flu* was originally coined for the corresponding genetic locus by Diderichsen (6). Ag43 is a member of the autotransporter protein family, which is defined by the fact that the proteins contain all information required for traverse of the bacterial membrane system and final routing to the bacterial cell surface (9). Ag43 is found in most *E. coli* strains, including many pathogenic strains (12, 17, 19). Ag43 is a multifunctional protein and promotes bacterial binding to some human cells as well as biofilm formation on various surfaces (12, 22).

We have previously demonstrated that Ag43-mediated aggregation enhances bacterial tolerance to bactericidal agents (21). Human blood contains multiple antibacterial agents, including PMNs. With a view to see if Ag43-mediated bacterial aggregation provided enhanced resistance toward antibacterial agents, we probed the ability of Ag43-expressing *E. coli* K-12 to survive exposure to human PMNs.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All strains and plasmids are described in Table 1. All *E. coli* strains used in this investigation were Δ*flu* derivatives of the *E. coli* reference strain MG1655, except for experiments where the urinary tract infectious *E. coli* strain 83972 was used. MG1655 and 83972 strains used for fluorescence microscopy investigations were fluorescently tagged with *gfp* and *yfp*, respectively, OS56 (MG1655 Δ*flu attB*::*bla*-$P_{A1/04/03}$-*gfp**-T_0), and VR48 (83972 *attB*::*bla-rrnB*P1-*yfp*-T_0) as described previously (7), and MS528 (MG1655 Δ*flu* Δ*fim*) was used in experiments not requiring fluorescence tagging. Transformants were selected on ampicillin-containing plates and checked for their ability to fluoresce. Proper insertion of the fragment into the λ attachment site was confirmed by PCR. Bacteria were grown at 37°C on solid or in liquid LB supplemented with appropriate antibiotics.

Cloning and DNA manipulation. All plasmid constructs were derivatives of the pACYC184 cloning vector with the tetracycline resistance gene promoter for consecutive expression. The *flu* gene encoding Ag43 was amplified by PCR and cloned into the BamHI and SalI sites of pACYC184, resulting in plasmid pPKL330. pPKL330 was transformed into MS528 (MG1655 Δ*flu* Δ*fim*), OS56, and VR48. All strains carrying the Ag43-expressing plasmid showed heavy aggregation, and the whole batch was used in the experiments. The RGD motif at position 208 in Ag43 was mutated using the QuikChange site-directed mutagenesis kit (Stratagene), such that RGD was changed to KGE using primers 655 (5′-CCGGGCAGTTTGTTAAAGGGGAAGCCGTACGCACAACCATC-3′) and 656 (5′-GATGGTTGTGCGTACGGCTTCCCCTTTAACAAACTGCCCGG-3′). The resulting plasmid, pSF2, with mutant *flu* was introduced into *E. coli* strains MS528 and OS56.

Isolation of human PMNs. Human blood samples were obtained from healthy volunteers by venous puncture and collected in BD Vacutainers with 0.129 M sodium citrate (388335; Becton Dickinson). The blood was mixed with dextran (T-500) 1:5, and the erythrocytes were left to precipitate for 40 min. The supernatant was applied to Lymphoprep (Axis-Shield PoC) and centrifuged at 850 × *g* for 15 min at 5°C. The pellet was treated with 2 ml 0.2% NaCl to lyse remaining erythrocytes. Lysis was terminated by addition of 2 ml 1.6% NaCl and 6 ml RPMI

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TABLE 1. *Escherichia coli* strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference
Strains		
MG1655	K-12 reference strain	3
MS528	MG1655 Δ*flu* Δ*fim*	11
OS56	MG1655 Δ*flu attB*::*bla*-$P_{A1/04/03}$-*gfp*mut3b*-T_0 GFP	23
83972	Asymptomatic bacteriuria *E. coli* isolate	2
VR48	83972 *attB*::*bla-rrnB*P1-*yfp*-T_0 YFP	This study
Plasmids		
pLH44	*aah-aidA* genes from *E. coli* O126:H27 strain 2787 in pACYC184	23
pPKL330	*flu* gene from *E. coli* in pACYC184	This study
pSF2	*flu* gene from pPKL330 with RGD motif at position 208 changed to KGE	This study

1640. The cells were centrifuged at 350 × *g* for 10 min at 5°C, the supernatant was discarded, and the PMNs were resuspended in RPMI 1640 buffer at 5×10^6 PMNs/ml.

Blood sensitivity. Bacteria were tested for sensitivity to human whole blood by incubating the individual *E. coli* strains with blood to a final blood concentration of 75%. Overnight cultures of bacteria grown in LB were standardized and suspended in phosphate-buffered saline (PBS) (to a density of 4×10^9 CFU/ml). Aliquots of cells (triplicates) were kept in microtiter plates at room temperature for 2 h to allow aggregation, followed by addition of fresh human blood or RMPI 1640 buffer (control). The mixtures were shaken at 200 rpm and 37°C for 15 min, and serial dilutions were plated.

Bacterial internalization by PMNs. Internalization of bacteria was examined by confocal scanning laser microscopy (CSLM). Overnight cultures of fluorescent bacteria (green fluorescent protein [GFP] or yellow fluorescent protein [YFP]) were standardized and suspended in PBS to a density of 4×10^9 CFU/ml. Aliquots of cells (50 μl) were kept in microtiter plates at room temperature for 2 h to allow aggregation, followed by the addition of 150 μl freshly purified PMNs or RPMI 1640 buffer for control. Bacterium-PMN interaction was visualized after incubation for 45 min at 37°C and 200 rpm. All CSLM images were taken using a Zeiss LSM 510 system (Carl Zeiss) equipped with an argon laser and a helium-neon laser for excitation of the fluorophores. Simulated fluorescence projections were generated using the IMARIS software package (Bitplane AG). Images were further processed for display using the PhotoShop software (Adobe).

Internalization of *E. coli* in PMNs was assessed by 45-min incubation of bacteria and PMNs prepared as above, followed by the addition of gentamicin (18 μg/ml) and another 30 min of incubation. Bacterial survival in PMNs was tested by preparing mixtures of PMNs and bacteria as described above, followed by incubation for 0, 30, 60, and 120 min at 37°C and 200 rpm. Bacterial viability was determined by plating serial dilutions. To block bacterial uptake by phagocytosis, the isolated PMNs were treated with 3 μM cytochalasin D (C8273; Sigma-Aldrich) for 1 h at 37°C before incubation together with bacteria followed by gentamicin treatment as described above.

Fluorescence-activated cell sorter analysis of phagocytosis. Phagocytosis of bacteria by PMNs was analyzed using FACSort (Becton Dickinson) equipped with a 15-mW argon ion laser turned to 488 nm for excitation. Light scatter and exponentially amplified fluorescence parameters from at least 10,000 events were recorded in list mode after gating on light scatter to avoid debris, cell aggregates, and bacteria on its own. The instrument was calibrated using Calibrite (Becton Dickinson). PMNs were identified according to their morphology and their content of DNA. GFP-expressing *E. coli* cells, both nonexpressing control cells and Ag43-expressing cells, were mixed with PMNs as above. External *E. coli* cells were removed by washing in cold PBS. The remaining PMNs were fixed in fluorescence-activated cell sorter lysing solution (Becton Dickinson) containing 150 μg/ml propidium iodide (P-4170; Sigma) for discrimination of eukaryotic cells according to their DNA content.

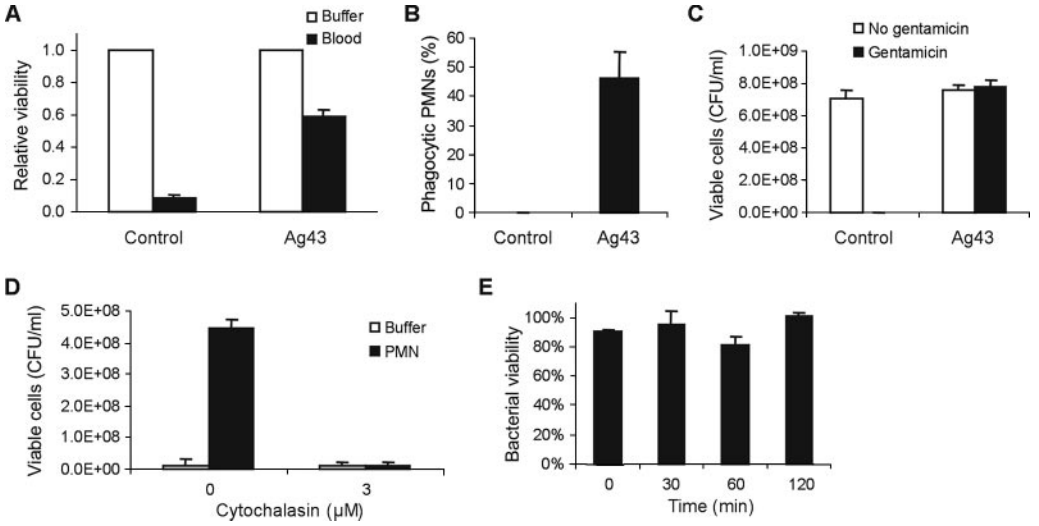

FIG. 1. *E. coli* exposed to human blood and internalization of Ag43-expressing *E. coli* by PMNs. (A) *E. coli* strain MG1655 Δ*flu*, harboring control plasmid (pACYC184) or Ag43-encoding plasmid (pPKL330), was incubated with blood or buffer for 15 min, and bacterial viability was determined. Results are presented as survival in blood compared with buffer and means from three independent experiments; error bars indicate standard deviations (values were 4.4×10^8 and 6.0×10^8 CFU/ml in buffer for control plasmid and Ag43, respectively). (B) Bacterial uptake by human PMNs monitored by flow cytometry to determine the percentage of phagocytic PMNs after bacterial incubation for 45 min at 37°C. Phagocytic green fluorescent PMNs were detected after incubation with GFP-expressing *E. coli* control (pACYC184) and Ag43-expressing (pPKL330) cells followed by a wash of external cells. Values are means of results from 10 samples, and error bars indicate standard deviations (unpaired *t* test, $P < 0.0001$). The experiment was performed in duplicate with separate cultures and blood donors. (C) Internalization of bacteria in PMNs was assessed by addition of gentamicin (18 μg/ml) to mixtures of PMNs with control or Ag43 *E. coli*; bacterial viability was determined after 30 min of incubation. Error bars indicate standard deviations of results from three independent experiments. (D) Internalization of Ag43-expressing bacteria in PMNs was blocked by treatment of PMNs with cytochalasin D, leading to lack of protection against gentamicin. Error bars indicate standard deviations of results from two separate experiments. (E) Survival of phagocytosed Ag43-expressing *E. coli* inside PMNs. Coincubation of bacteria and PMNs for 0 to 120 min at 37°C was followed by serial dilutions and plating. Bacterial viabilities are presented relative to survival in buffer and are means of results from three independent experiments, and error bars indicate standard deviations.

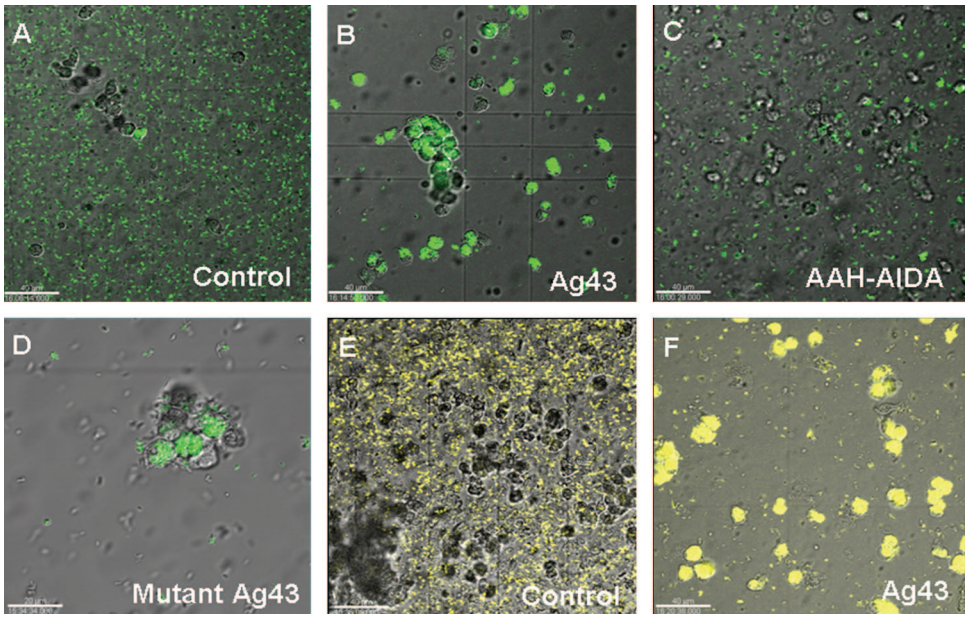

FIG. 2. Bacterial adherence to human PMNs. Fluorescent *E. coli* cells, nonexpressing control (pACYC184), Ag43-expressing (pPKL330), and AIDA-expressing (pLH44) cells, were mixed with PMNs. (A to C) Representative fluorescence micrographs of *E. coli* MG1655 Δ*flu* (GFP), demonstrating that the majority of Ag43-expressing cells were taken up by PMNs in an opsonin-independent manner while neither control cells nor AIDA-expressing cells were taken up by PMNs. (D) *E. coli* MG1655 Δ*flu* expressing the mutant Ag43 (pSF2; with RGD motif changed to KGE) was also taken up by PMNs. (E and F) Fluorescence micrographs of *E. coli* 83972 (YFP), nonexpressing control and Ag43-expressing cells, illustrating Ag43 adherence and uptake to PMNs. All images were obtained after coincubation of *E. coli* cells and PMNs for 60 min at 37°C. Bars, 40 μm (A, B, C, E, and F), 20 μm (D).

RESULTS AND DISCUSSION

Ag43-expressing *E. coli* survives exposure to PMNs. As a consequence of our previous observations concerning the Ag43-mediated aggregation-enhanced bacterial tolerance to bactericidal agents (21), we wanted to investigate if Ag43-mediated aggregation also protected the bacteria against human blood. Human blood contains multiple antibacterial agents; for example, the complement system and PMNs. It turned out that Ag43-expressing *E. coli* survived sixfold better than nonexpressing control cells when exposed to human blood (Fig. 1A). We suspected that bacterial aggregates were difficult to ingest for the PMNs. To investigate this phenomenon further, a combination of *E. coli*-expressing GFP, washed PMNs, and CSLM was employed. This technique permits spatial imaging of PMNs and bacteria. When bacteria and PMNs were mixed and examined by fluorescence microscopy, we discovered that Ag43-mediated bacterial aggregation did not protect bacteria against uptake by PMNs, but it actually enhanced uptake, i.e., the large majority of Ag43-expressing *E. coli* was taken up by PMNs in an opsonin-independent manner, whereas nonexpressing controls were not (Fig. 2; see movie in the supplemental material). This result was further corroborated by flow cytometry, which demonstrated that up to 50% of PMNs were phagocytic, i.e., they contained internalized Ag43-expressing bacteria tagged with GFP (Fig. 1B). To discriminate between bacteria that were internalized and those that were firmly bound to the PMN surface, we added gentamicin to a mixture of bacteria and PMNs (Fig. 1C). For the control, the number of viable bacteria decreased significantly in the presence of gentamicin. In contrast, the number of Ag43-expressing bacteria was not reduced by the treatment. Since gentamicin is unable to penetrate the PMN plasma membrane and kill intracellular bacteria (27), this experiment demonstrated that Ag43-expressing bacteria were protected by PMN internalization. To bolster the notion that enhanced resistance to gentamicin of Ag43-expressing *E. coli* was due to PMN phagocytosis, the PMNs were treated with cytochalasin D that blocks phagocytic bacterial ingestion by disruption of the actin microfilaments (16, 26). Gentamicin efficiently killed Ag43-expressing *E. coli* bacteria that were incubated with cytochalasin D-treated PMNs, while untreated PMNs protected the bacteria from being killed (Fig. 1D). Taken together, the results indicate that Ag43-expressing *E. coli* bacteria are specifically internalized by PMNs via phagocytosis.

To test if the bacterial host background played a role, we also investigated PMN uptake of a wild-type *E. coli* strain, i.e., the urinary tract infectious *E. coli* strain 83972. Fluorescence-tagged *E. coli* 83972 cells expressing Ag43 were presented to PMNs and subsequently inspected by fluorescence microscopy (Fig. 2). Ag43-expressing *E. coli* 83972 cells were efficiently taken up by the PMNs, whereas *E. coli* 83972 controls were not, suggesting that the uptake was not restricted to a particular host.

Ag43-assisted bacterial uptake by PMNs does not require the RGD motif. Proteins containing an RGD motif have been implicated in binding and internalization of microorganisms by human integrin receptors (10). Ag43 contains an RGD motif at position 208, and it seemed conceivable that this motif played a role in PMN uptake of Ag43-expressing bacteria. To test this, we made a version of Ag43 with an RGD-to-KGE change by

PCR-assisted site-directed mutagenesis of the corresponding gene. However, bacteria expressing the KGE version of Ag43 were taken up by PMNs with similar efficiency as bacteria that expressed the wild-type version of Ag43 (Fig. 2D). The RGD-to-KGE change does not affect the aggregation properties of Ag43.

The bacterial uptake is Ag43 specific rather than dependent on aggregation. The lack of RGD motif-dependent uptake led us to speculate that aggregation per se could provoke PMN uptake. To investigate this, we used another autotransporter protein, AIDA, which shares ~25% sequence identity with Ag43 and also causes bacterial aggregation (23). Meanwhile, aggregating bacteria expressing AIDA were not taken up by PMNs any better than the controls (Fig. 2). Taken together, the results indicate that Ag43-assisted bacterial uptake by PMNs does not require RGD motifs nor does bacterial aggregation by itself suffice to provide uptake.

Ag43-expressing internalized cells survive inside PMNs. PMN phagocytosed bacteria are usually killed rapidly (14, 18), and we therefore set out to investigate the fate of phagocytosed Ag43-expressing bacteria. Accordingly, the survival rate of such bacteria was monitored for 120 min (Fig. 1E). It transpired that, during this period of time, no reduction in bacterial viability was observed, indicating that the tight aggregates of Ag43-expressing bacteria tolerated antimicrobial mechanisms inside the PMNs for extended periods of time. In contrast, bacteria devoid of Ag43 were not internalized at all and did not survive as well in whole blood compared to Ag43-expressing bacteria (Fig. 1A and 2). Ag43-mediated cell aggregation significantly protects bacteria against exposure to hydrogen peroxide (21). Since PMNs use peroxides to eliminate bacteria, similar protective mechanisms could be at play inside the PMNs. It therefore seems that Ag43 not only confers efficient bacterial uptake in PMNs but also seems to provide a survival mechanism for the bacteria after phagocytosis.

In this study, we have described a possible bacterial evasion technique, "hiding-in-the worst-place," i.e., inside the killer cells of the host defense. The bacterial strategy for the uptake and survival of Ag43-expressing *E. coli* might be evasion, survival, and possibly transport. Since the PMNs play a central role in the host defense, it should follow that the ability to foil PMN-mediated killing should be a major advantage for an infecting microorganism. *E. coli* is the primary cause of urinary tract infections in humans (24). The antibacterial defense of the urinary tract is highly dependent on innate immunity where PMNs are crucial players (25). Arguably, Ag43-mediated uptake and survival in PMNs could play an important role in this type of infections. The sessile state of bacteria (biofilms, microcolonies, and aggregates) shows an increased tolerance to a variety of antimicrobial measures compared to its planktonic counterparts (5, 20). Ag43 has been shown to be expressed by *E. coli* in the urinary tract in the mouse model (1). By expressing Ag43, the bacteria become readily internalized, and we speculate that they resist elimination internally due to aggregation. Firmly inside the PMN, the bacteria are protected from the remaining host defenses, as evidenced by the improved survival observed in whole blood. However, it needs to be clarified whether it is the internalization or the clumping which protects the cells. Even killing by antibiotics in general may be evaded within the PMNs. It is interesting to speculate that, once internalized, the intruder may be favored even further by being transported around the body by macrophage scavengers, which in turn phagocytose the dead PMNs. In this way, the PMNs become Trojan horses, which might liberate their content of bacteria at times and places that will increase the probability of successful infection.

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