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EXPERIMENTAL ARTICLES

Effect of Inherent Immunity Factors on Development of Antibiotic Tolerance and Survival of Bacterial Populations under Antibiotic Attack

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Abstract—Effect of human inherent immunity factors, a gene-encoded antibacterial peptide indolicidin (Ind) and a cytokine interleukin 1 (IL-1), on formation of antibiotic-tolerant persister cells surviving in the presence of ciprofloxacin (Cpf, 100 μg/mL) and ampicillin (Amp, 100 μg/mL) in submerged bacterial cultures (*Staphylococcus aureus* FGA 209P, *Escherichia coli* K12, and *Pseudomonas aeruginosa* PAO1) was studied. While Ind in physiological concentrations (0.3 and 3.0 μg/mL) introduced to the lag- or exponential-phase cultures of test organisms exhibited no reliable effect on population growth, the number of persisters increased at 3.0 μg/mL. Bactericidal Ind concentrations (9 μg/mL) suppressed *S. aureus* growth (~0.1% of surviving cells) with subsequent recovery due to development of the more antibiotic-tolerant white variant. Treatment with Cpf after Ind addition resulted in mutual potentiation of their antimicrobial activity, with the number of *S. aureus* persisters 2 to 3 orders of magnitude lower than in the case of the antibiotic alone. IL-1, another immunity factor, when introduced (0.1–1 ng/mL) to the exponentially growing *S. aureus* culture (but not to the lag phase culture) had a temporary growth-static effect, with the number of persisters surviving Cpf treatment (100 μg/mL) increasing by 1 to 2 orders of magnitude. Electron microscopy revealed significant alterations in the outer cell wall layer of surviving *S. aureus* cells, which should be associated with their changed antigenic properties. Thus, the factors of human inherent immunity have a dose-dependent effect on the growth of bacterial populations. In combination with antibiotics, they exhibit synergism of antimicrobial action (indolicidin) and minimize (indolicidin) or increase (interleukin 1) the frequency of formation of persister cells responsible for survival of a population subjected to an antibiotic attack.

Keywords: antibiotic tolerance, persister cells, inherent immunity factors, indolicidin, interleukin 1, synergism of antimicrobial action

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A large body of evidence is presently available indicating that genetically monoclonal bacterial populations are phenotypically heterogeneous, which provides for survival of small subpopulations (~0.001–1%) when the overwhelming majority of the cells die as a result of treatment with bactericidal doses of antibiotics (Lewis, 2007; Gefen and Balaban, 2009; Balaban et al., 2013). This phenomenon was first described by Hobby et al. in 1942 in their observations of *Staphylococcus* cultures treated with penicillin, and it is based on the ability of a small number of cells genotypically sensitive to antibiotics to acquire the surviving (persister) phenotype in their presence (Gefen and Balaban, 2009; Lewis, 2010). According to the conventional point of view, such cells, termed persisters (Bigger, 1944), are indifferent to the presence of any antibiotics in the environment, i.e., they exhibit multidrug tolerance (Balaban et al., 2004; Lewis, 2010). In contrast to the cells with antibiotic-resistant genotype that possess the required genes, surviving persister cells cannot divide (Guilfoile, 2007). Another feature of the antibiotic-tolerant (AT) phenotype is that persister cells inoculated into fresh medium will fully reproduce the original antibiotic-sensitive population, which, in turn, will contain a very small persister subpopulation. When antibiotic-resistant (AR) cells are transferred into fresh medium, all cells of the resulting population

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retain the AR phenotype, i.e., they are able to grow in the presence of a certain antibiotic (Antonopoulos, 2009). At the same time, the population of genetically resistant AR cells will contain an AT subpopulation of persisters, which will make it possible for the population to survive when subjected to other stressors (e.g., antibiotics of a different type).

Thus, the principal functional difference between AT persisters and AR cells is that the former enable survival of the population when subjected to various stress factors, including antibiotics and medications of different chemical nature, while the latter ensure its survival in the particular circumstances of treatment with a certain antibiotic.

Not surprisingly, microbiologists, as well as biologists specializing in other areas, investigate the conditions that give rise to persisters and the properties of these cells. According to one of the current viewpoints, there exist two types of persisters: type I persister cells arise in response to stress (starvation) in the stationary phase, while type II persisters are constitutively and with increasing frequency formed during the whole growth phase of a bacterial culture (Balaban et al., 2004). Although the mechanism underlying the switching to an AT phenotype is largely unclear, it was proposed that it is a cell differentiation program induced by an imbalance in the expression of certain toxin–antotoxin modules and associated with stress response (Gerdes and Maisonneuve, 2012).

Taking into account that the problem of bacterial persistence is extremely significant for medical practice, researchers have so far been studying mainly the effects of stress factors that exist in the host organism during infection. It was shown that the intensity of persister formation is affected by such factors as low pH, limited nutrient supply, or elevated concentration of reactive oxygen species (Levin and Rosen, 2006; Wayne and Lin, 1982). However, no information is available about how innate immunity factors affect the formation of persisters. The scarce data concern only their effects on the growth of bacterial cultures.

At the same time, genetically encoded antimicrobial peptides produced by a variety of organisms (from microorganisms to humans), including those that are part of innate immunity in humans, are currently widely studied as promising new-generation antibiotic agents. Antimicrobial effects of indolicidin (Ind) and its artificial derivatives, but not their effects on persister formation, have been investigated in considerable detail (Smirnova et al., 2004). There are also no data on how the formation of surviving subpopulations can be affected by another group of immunity factors, interleukins (ILs), although there is some information on their effects on growth of microbial populations (Romanova and Gintsburg, 2000).

The goal of the present work was to investigate the effects of innate immunity factors, indolicidin (an antibacterial peptide) and interleukin 1 (a cytokine), on the development of antibiotic tolerance and on survival of bacterial populations treated with antibiotics.

This work showed how survival efficacy and persister formation rate depends on the concentration of immunity factors added to the cultures of different physiological ages prior to antibiotic attack selecting the persisters.

MATERIALS AND METHODS

Subjects of research. The study was performed with *Staphylococcus aureus* FDA 209P and *Escherichia coli* K12 strains obtained from the collection of the Gause Institute of New Antibiotics, Russian Academy of Medical Sciences, as well as with *Pseudomonas aeruginosa* PAO1 obtained from the Molecular Genetics Institute, Russian Academy of Sciences. Bacteria were stored on Luria–Bertani (LB) agar slants at 4°C.

Cultivation. Bacterial cultures from slant agar were inoculated into 50 mL LB medium in 250-mL flasks and grown at 28°C on an orbital shaker at 120 rpm to the stationary phase, then used to inoculate fresh medium (1%, vol/vol) and grown for 12–16 h under the same conditions. The obtained overnight cultures were used to inoculate fresh LB medium (1%, vol/vol), and 1-mL aliquots were distributed into 2-mL Eppendorf tubes and incubated at 28°C on a TS100 thermal shaker (BIOSAN, Latvia) with constant agitation (250, 500, or 700 rpm). Depending on the purpose of the particular experiment, test compounds were added to growing cultures either in the lag phase or in the exponential phase.

Persister cells were detected by adding bactericidal concentrations of ciprofloxacin (Cpf, a quinolone antibiotic) or ampicillin (Amp, a beta-lactam antibiotic) to the test cultures in the exponential or decelerated growth phase. The numbers of viable persister cells (CFU/mL) were determined in 3–240 h of incubation.

Effects of innate immunity factors on the formation of persisters. The factors in question—indolicidin (Ind) in the concentrations of 0.3, 3, and 9 μM or interleukin 1 (IL-1) in the concentrations of 0.1 and 1 ng/mL—were added to Eppendorf tubes containing 1 mL of test cultures either in the lag phase (together with the inoculum) or in the exponential phase. Antibiotics (Cpf or Amp, 100 μg/mL) were added after 4–6 h of cultivation if the test factors were added in the lag phase or 60 min after adding them in the exponential phase. In the control variants (no peptide added) antibiotics were introduced at the same time points as in the test variants. In several experiments, antibiotics were added during the decelerating growth phase of *S. aureus* cultures (24 h).

Throughout the experiments, the numbers of viable cells in the experimental and control cultures were determined using three "sacrifice samples" for each evaluation (three Eppendorf tubes for each analysis).

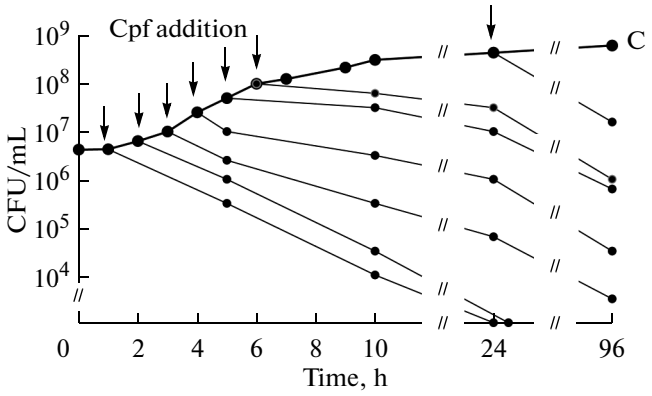

Fig. 1. Numbers of *S. aureus* 209P cells (CFU/mL) that survived antibiotic treatment depending on the physiological age of the cultures treated with ciprofloxacin (Cpf, 100 μg/mL). Arrows indicate the moments of Cpf addition to the culture.

Viable cell counts (CFU/mL) were carried out using a modified Koch method. For each of the three tubes, a series of tenfold dilutions was prepared from 0.1 mL of test culture to plate on LB agar in petri dishes; for each dilution, ten 5-μL aliquots were applied onto agar, and the dishes were incubated in a thermostat at 28°C. The resulting colony numbers (CFU/mL) were counted on day 3–30 after plating. Platings were repeated three times in three replicates and three parallel counts in each. Typical results of these experiments are shown on the figures.

Microscopic investigations were performed using Zetopan (Reichert, Austria) or Axioplan (Carl Zeiss, Germany) microscopes. In some experiments, bacterial cells were stained with propidium iodide (PI) or Live/Dead Baclight Kit® L-13152 dye (Molecular Probes) to identify live or dead cells. Low-density cell suspensions were concentrated 10- or 100-fold.

Electron microscopy of the cells surviving for 19 days after Cpf addition was performed on ultrathin sections prepared using conventional techniques. The sections were examined under a JEM-1400 electron microscope (JEOL, Japan) at accelerating voltage of 80 kV.

Statistical analysis was performed using conventional methods: the root-mean-square deviation test, the Student's *t*-test, and correlation analysis. The differences were considered significant at $P < 0.05$.

RESULTS

Detection of Surviving Type II Persister Cells

The relationship between the physiological age of a bacterial population and the persister formation rate described previously (Balaban et al., 2004; Lewis, 2010) was confirmed for the growing cultures of studied strains. The number of surviving cells (persister fraction) in staphylococcal cultures treated with antibiotics (Cpf, 100 μg/mL) increased from 0.001% to 0.1–1.0% with culture aging (Fig. 1). Similar results were obtained for submerged cultures of other strains. In all bacterial strains studied, the fractions surviving after antibiotic treatment were heterogeneous: the cultures plated on solid medium with antibiotics (1–5 μg/mL Cpf or, for *E. coli*, 5–10 μg/mL Amp) gave rise both to AR colonies and to AT slowly growing small-colony variants of the SCV type (Levin and Rosen, 2006). These 0.5- to 1.0-mm colonies developed later than AR colonies, on day 5–10. For all test cultures, the number of colonies growing on antibiotic-containing media was by 2 or 3 orders of magnitude lower than the CFU numbers obtained on media without antibiotics; therefore, the surviving population was mostly composed of AT cells, type II persisters. The cells of these subpopulations were, in turn, also heterogeneous in their ability to form colonies for different periods of time: this ability decreased when the surviving fraction was stored for more than 10 days, up to lack of CFU in some variants that had been stored for 1–3 months. However, microscopic investigations using PI or Live/Dead staining showed that these specimens (stored for 1–3 months) did contain intact cells, which meant that they had switched to a nonculturable state. Therefore, the lack of growth on standard media cannot be considered as a definite sign of the absence of viable cells in the specimen.

Effects of Physiological Concentrations of Indolicidin on Persister Formation

Taking into account that detectable (μM) concentrations of genetically encoded antimicrobial peptides are present in the inflammation foci both in the beginning and throughout the inflammation process, we added Ind to the model submerged cultures of tester bacteria both in the lag phase and in the exponential phase.

Effect of Ind on growth of *S. aureus* 209P cultures and formation of type II persisters. Ind in the concentration of 0.3 or 3 μM was added to *S. aureus* cultures growing in microaerophilic conditions (thermal shaker, 250 rpm), which was followed by addition of a bactericidal Cpf dose (100 μg/mL) (Fig. 2). When the peptide was added together with the bacterial inoculum, it did not affect significantly the culture growth and type II persister formation (Fig. 2a), whereas Ind addition (in both concentrations) to exponentially growing cultures temporarily decreased their growth rate (bacteriostatic effect) and slightly increased the number of type II persisters (Fig. 2b). Since the available data suggest that microaerophilic or anaerobic growth conditions have a stimulating effect on antibiotic tolerance of bacterial populations (Hamsley et al., 2014), subsequent series of experiments were designed to exclude this factor, and tester bacteria were cultured

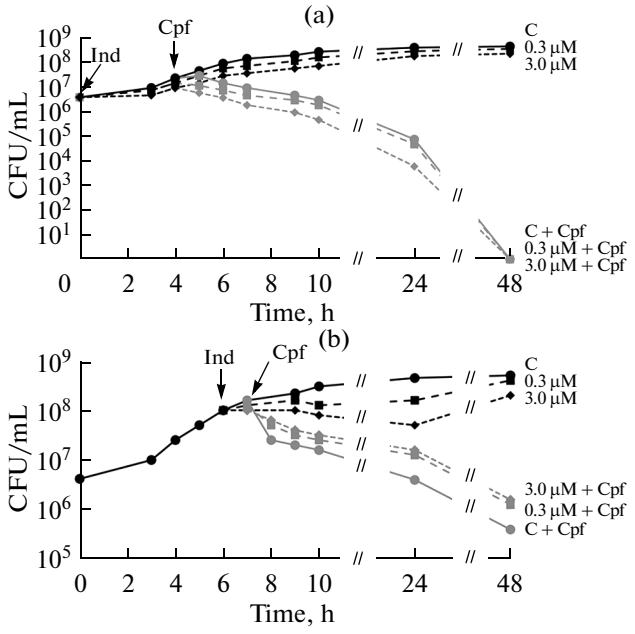

Fig. 2. Effect of indolicidin (Ind, 0.3 and 3 μM) on the number of surviving persister cells (CFU/mL) when added to *S. aureus* 209P cultures in the lag phase (a) or exponential growth phase (b) prior to Cpf treatment (100 μg/mL) revealing persister cells. Arrows indicate the moments of Ind and Cpf addition to the cultures.

with more intense aeration (thermal shaker, 500–700 rpm).

Effect of indolicidin on growth and formation of type II persisters in the cultures of gram-negative bacteria. Ind (0.3 or 3 μg/mL) added to an *E. coli* culture (thermal shaker, 700 rpm) together with the bacterial inoculum did not affect its growth (Fig. 3a). Subsequent selective exposure to antibiotics (Cpf, 100 μg/mL, or Amp, 100 μg/mL, added to 4-h cultures with 5×10^8 cells/mL) showed that, firstly, there was no difference in persister formation rates between the experimental (with Ind) and control (without Ind) variants, and, secondly, the number of surviving cells was significantly higher in 168-h test cultures treated with Cpf than in similar cultures treated with Amp (5×10^4 vs. 10–100 cells/mL, Fig. 3a). Similar results were obtained in experiments with submerged *P. aeruginosa* cultures: Ind added at the lag phase together with the inoculum did not affect the culture growth or persister numbers in 10- to 32-h cultures (Fig. 3b).

When Ind (0.3; 2; 3 μg/mL) was added to exponentially growing 4-h *E. coli* cultures, subsequent exposure to Cpf revealed a significant increase in the number of surviving persister cells in 240-h test cultures (by 2–3 orders of magnitude, Fig. 3c). When physiological Ind concentrations were added to 6-h *P. aeruginosa* cultures, the number of surviving persister cells also increased (by 1.5 orders of magnitude in 10-h cultures, Fig. 3d).

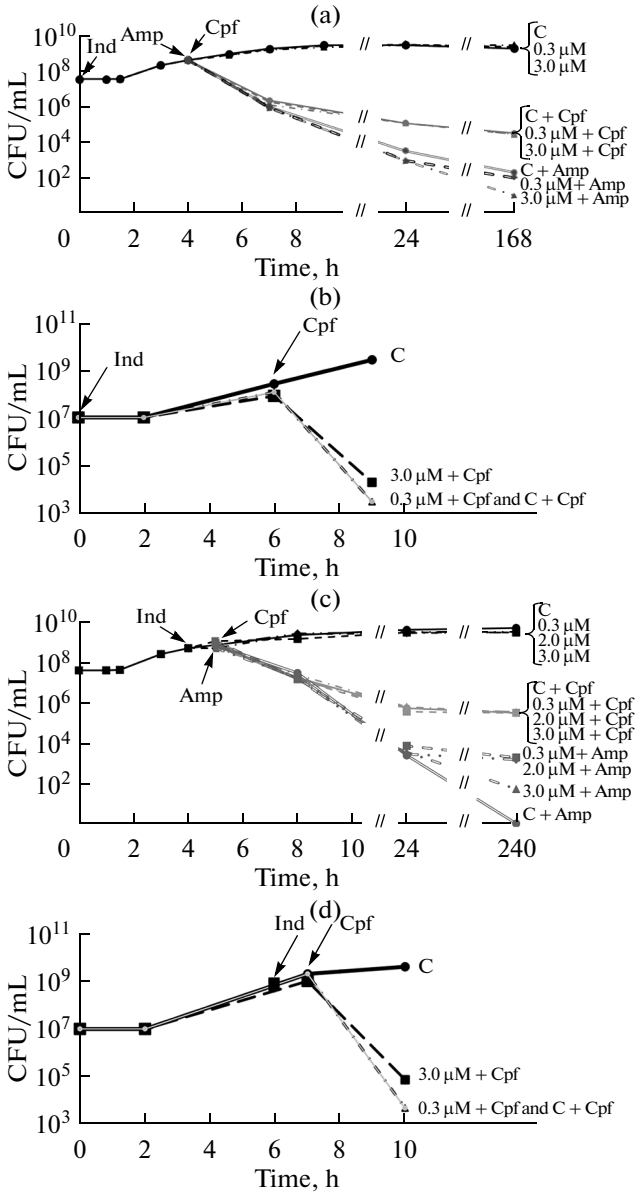

Fig. 3. Effect of indolicidin (Ind, 0.3 and 3 μM) on the number of surviving persister cells (CFU/mL) when added to *E. coli* K12 (a, c) and *P. aeruginosa* PAO1 (b, d) cultures in the lag phase (a, b) or exponential growth phase (c, d). Persister-detecting antibiotics: Cpf (100 μg/mL) or Amp (100 μg/mL).

Effects of Bactericidal Concentrations of Indolicidin on S. aureus 209P Culture Growth and Persister Formation

Effect of indolicidin on growth and death of *S. aureus* populations. Antimicrobial Ind amounts were added to *S. aureus* cultures (thermal shaker, 500 rpm) in the lag phase or the exponential phase (Fig. 4). In both cases, it caused death of bacterial cells, as expected. The younger the culture at the moment of Ind addition and the higher the Ind dose,

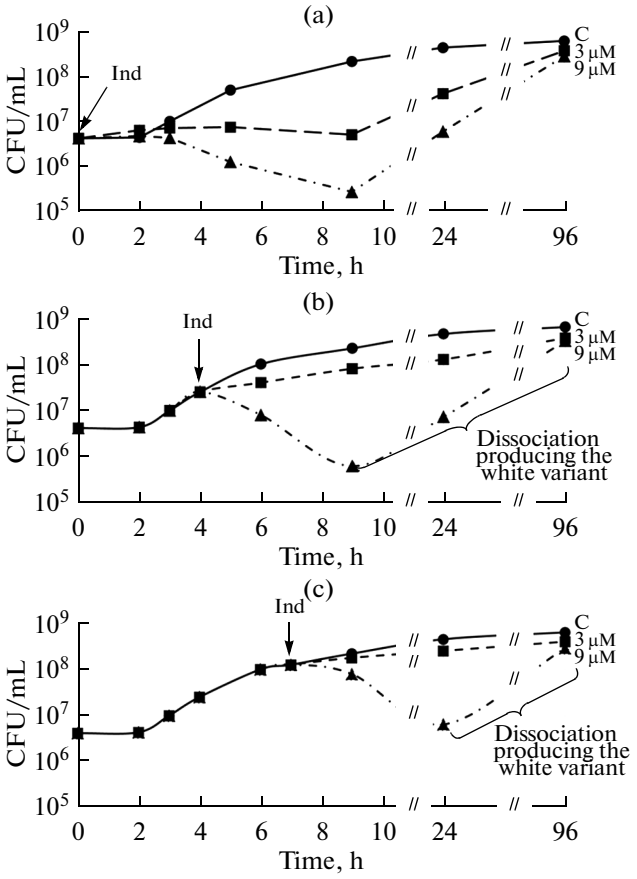

Fig. 4. Effect of indolicidin as an antimicrobial peptide (Ind, 3 and 9 μM) on growth, death, and survival of *S. aureus* 209P cultures (CFU/mL) when added in the lag phase (a), in a 4-h (b), and in a 7-h (c) culture.

the more pronounced was its antimicrobial effect. When the peptide was added at inoculation, the 3 μM dose caused lag phase extension, while the 9 μM dose killed most cells (90%) of the population; however, by day 4, the culture growth regained the level of the Ind-free control (Fig. 4a). Similar effects were observed after Ind addition to exponentially growing 4-h or 7-h cultures (Figs. 4b and 4c, respectively); they were more pronounced in the first variant. Growth was restored due to a phenotypic switch of viable AT persisters into normal dividing cells; in the two latter variants, these cells were mainly of the more tolerant "white" phenotype, which replaced the dominant one and on which the Ind doses used did not have a bactericidal effect. (In the stationary control culture, the portion of the minor white variant was approximately 1%.) This phenotype reversal from persisters to normally dividing cells, which is typical of AT bacteria, was described previously by Gefen and Balaban (2009). Novel is the fact that effects of Ind (and probably other defensins) on growing bacterial cultures involve, in particular, changes in their phase variation spectrum. Although phase variation activity of bacterial populations is a subject of special investigations, the described phenomenon should be taken into account in further research of antibiotic resistance in bacteria and antibiotic efficacy, since intrapopulational variation of opportunistic pathogenic bacteria is associated with their virulence and pathogenicity.

Effect of indolicidin on the formation of AT persister cells. Further experiments determined the effect of 9 μM Ind on the formation of type II persisters (Fig. 5a): Ind was added to exponentially growing 4-h cultures, which were 3 h later exposed to an antibiotic (Cpf, 100 μg/mL). In both the experimental and control cultures, bacteria were killed by Cpf; however, the number of surviving type II persisters in 96-h test cultures was lower by 1.5 orders of magnitude (10^4 cells/mL vs. 3×10^5 cells/mL in the control cultures). It should be noted that, in the experimental variant, Cpf acted on the cultures already in the course of Ind-induced lysis, i.e., it was potentiating the effect of the antimicrobial peptide, thus preventing the culture from regenerating due to growth of "white" bacteria, as observed in previous experiments in the Ind only variants (Figs. 4b, 4c). The number of type II persisters detected in the experimental variant corresponded to their number in a 4-h culture. On the other hand, in the control variant, it was a 7-h growing culture that was exposed to Cpf, and it contained a higher portion of type II persisters than a 4-h culture (Fig. 1), which agrees with the difference in the numbers of type II persisters between experiment and control (no Ind) 96-h cultures.

The same trends were observed in the next series of experiments, where Ind was also added to exponentially growing cultures (4-h), but the selecting agent (Cpf) was added to 24-h cultures to account for all type II persisters that appeared in the course of population growth (Fig. 5b). At this point of time, control cultures were reaching the stationary phase, while the experimental cultures were exponentially growing, with the white variant with higher antibiotic tolerance being the predominant form substituting the original dominant phenotype (Fig. 4b). In these experiments, the number of persister cells in the experimental and control 96-h cultures differed by 4–5 orders of magnitude, and the number of type II persisters in experimental cultures reached 7×10^3 cells/mL, or 0.1%. The large difference between the control and experimental variants can be explained by the fact that the cells in the decelerating or stationary growth phase (24-h) are considerably more resistant to the Cpf dose used than the cells of exponentially growing cultures, and probably by a developing metabolic adaptation, which was previously described as a form of antibiotic tolerance (Corona and Martinez, 2013). A similar effect was observed in our previous work on *E. coli* exposed to heat shock prior to Amp treatment (Loiko et al., 2015).

Thus, exposure of exponentially growing cultures to bactericidal concentrations of Ind prior to antibi-

otic treatment with Cpf produced a synergistic killing effect, affected the phase variation spectrum of the population, and significantly decreased the number of type II persisters.

In the third experimental series, Ind was added to experimental cultures in the lag phase, and Cpf was added to 24-h cultures (both experimental and control ones). It was found that the numbers of surviving cells after 96 h incubation differed by 3.5 orders of magnitude (Fig. 5c). It should be noted that, in experimental cultures, Cpf acted in fact on the 4-h population of the variant that replaced the dominant phenotype. In 96-h test cultures, the number of surviving persister cells was 8×10^4 cells/mL, i.e., 0.1% of the cell count in the 24-h culture that had been exposed to Cpf. Moreover, Cpf had a weaker bactericidal effect on growing populations of the white variant (Figs. 5b, 5c) than on the cells of the dominant phenotype, whose death rate was higher (Fig. 1).

Thus, the cumulative effect of exposure to an antimicrobial peptide (Ind) and then to an antibiotic (Cpf) on the formation of surviving persister cells in growing submerged bacterial cultures depended on the Ind concentration used and on the physiological state of bacterial target cells determined by the age of culture.

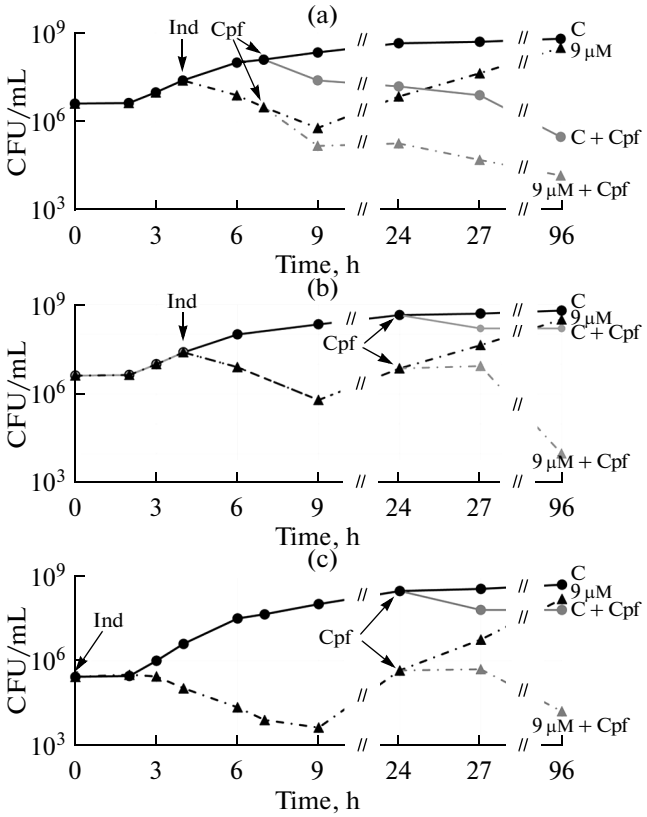

Fig. 5. Effect of indolicidin as an antimicrobial peptide (Ind, 9 μM) in combination with ciprofloxacin (Cpf, 100 μg/mL) on the number of surviving persister cells (CFU/mL). Ind was added to *S. aureus* 209P cultures in the exponential phase (4 h), (a, b) or in the lag phase (c), and Cpf was added to 7-h (a) or 24-h (b, c) cultures.

Effect of Interleukin 1 (IL-1) on Formation of Persister Cells

The final series of experiments analyzed how the formation of type II persisters was affected by another immunity factor, interleukin 1 (IL-1). When added at inoculation, IL-1 had no effect on the development of *S. aureus* 209P cultures and the number of persisters following the subsequent Cpf treatment: in contrast to experiments with the antimicrobial peptide, the differences between the control and experimental variants were not significant (Fig. 6a). However, when IL-1 was added to the exponential-phase culture, the number of surviving cells increased significantly (by nearly two orders of magnitude) in comparison to the control (Fig. 6b). At the same time, IL-1 temporarily but significantly inhibited the growth of submerged *S. aureus* cultures, in agreement with the published data suggesting that some interleukins affect the growth of bacterial populations (Wilson et al., 1998). This growth-arresting stress effect of IL-1 stimulated the phenotypic transformation of normal cells into persisters.

In a gram-negative *E. coli* K12 culture, exposure to IL-1 also changed the number of cells surviving after an antibiotic attack. However, in contrast to observations on *S. aureus*, the effect was observed when IL-1 was added at the lag phase (Fig. 6c). Subsequent Amp exposure revealed that the number of viable cells in 168-h cultures was significantly increased (by 2 orders of magnitude). In experiments with *P. aeruginosa* PAO1 cultures, a significant change in the number of type II persisters was observed only when IL-1 was added during the exponential phase (Fig. 6d).

Thus, IL-1 effects in tester bacterial populations were specific and depended on the growth phase of the bacterial culture exposed to this factor.

These results demonstrate that innate immunity factors (Ind and IL-1) have a significant effect on antibiotic tolerance and phase variation activity (Ind) of growing bacterial cultures. The most important are the changes in the number of AT persisters appearing in bacterial populations, since they ensure survival of the population and are responsible for recurrence of chronic infections.

Ultrastructural Organization of Persisting S. aureus 209P Cells

As discussed above, cell fractions surviving after antibiotic treatment were heterogeneous in their colony-forming ability retained for different periods of time. Visualization of surviving *S. aureus* cells also demonstrated heterogeneity of their ultrastructural organization (Fig. 7). In autolyzing *S. aureus* cultures

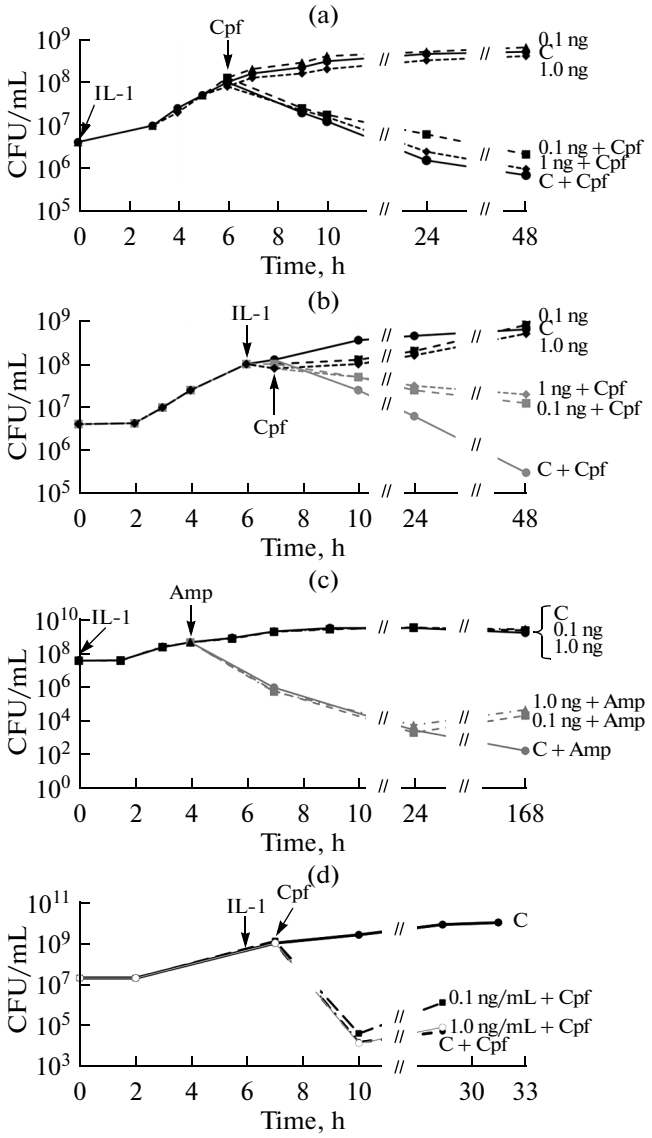

Fig. 6. Effect of interleukin 1 (IL-1, 0.1 and 1 ng/mL) on the number of surviving persister cells (CFU/mL) added to *S. aureus* 209P (a, b), *E. coli* K12 (c), and *P. aeruginosa* PAO1 (d) cultures in the lag phase (a, c) or exponential growth phase (b, d). Persister-detecting antibiotics: Cpf (100 μg/mL) or Amp (100 μg/mL).

(12 days after exposing a 24-h culture to Cpf (100 μg/mL)) against the background of debris of autolyzed cells (A) (Fig. 7a), we identified intact cocci typical for this species, as described by different authors (Wilmes et al., 2014), and varying in size and electron density of the cytoplasm (Figs. 7a–7e). The cocci were commonly surrounded by a microcapsule, a layer composed of short fibrils (FL) (Figs. 7a, 7b, 7e, 7f). Similar cells with radial fibrils were revealed on the cell surface in ultrathin sections of staphylococci growing in a biofilm (Archer et al., 2011). Cells of the surviving fraction had cell walls lying close to the plasma membrane and composed of peptidoglycan and teichoic acids, electron-dense cytoplasm, and a centrally located compacted nucleoid (CN) (Figs. 7c, 7h). In some cells, a septum (S) was visible, possibly supporting the notion that persister cells may be capable of very slow division (Figs. 7c–7e). The same specimen also contained cocci where peptidoglycan layer (PG) of the cell wall had grown into the cytoplasm (Figs. 7b, 7f), while the cytoplasm was compacted and small in volume. It is unclear whether such cells are capable of growing. The specimen also contained cells with loosened and shedding external layer of the cell wall (EPG) (Figs. 7a, 7c, 7h). The internal cell wall (ICW) was composed of a thin layer similar in density to peptidoglycan (Figs. 7a, 7d, 7f). The cells were filled with granular and fibrous matter (Figs. 7b, 7d, 7f–7h). There were forms with cell wall defects, protoplasts, and lysed cells. In cells with cell wall defects, EPG was thinned and partially destroyed, while a more or less developed FL was retained.

Thus, the distinguishing features of persister cells were the following: (1) shedding of the external peptidoglycan layer of the cell wall; (2) formation of numerous fibrils that give an appearance of a "woolly" envelope characterizing only intact cells; (3) uniformly disperse cytoplasm texture; and (4) compacted nucleoid. The further fate of persister cells stored for a long time will be discussed in a separate article.

DISCUSSION

The purpose of this study was to investigate the effects of Ind and IL-1 on the formation of persister cells and on survival of bacterial populations under antibiotic attack, which involved two aspects. On the one hand, we planned to analyze the effects of Ind and IL-1 as innate immunity factors in humans, and on the other hand, to investigate the effects of Ind as a candidate antibacterial peptide for further development of novel medications (Smirnova et al., 2004). Indolicidin is the best-studied example of genetically encoded antibacterial peptides present in a variety of organisms (Hancock, 1997); they were shown to be active against microorganisms of different taxa: bacteria, fungi, yeasts, and protists. Their ubiquitous presence in the species ranging from bacteria to humans and their antimicrobial activity attract considerable attention of researchers to these promising antimicrobial agents; presumably, microbial resistance to these peptides will develop slowly because of the mechanism of their action (Falla et al., 1996). However, it is currently assumed that survival of bacterial populations treated with antibiotics, carrier state, and recurrent infections depend not as much on genetically determined antibiotic resistance as on constitutive formation of AT persister cells (Lewis, 2007; Gefen and Balaban, 2009; Balaban et al., 2013; Corona and Martinez, 2013). In this work, we have for the first time obtained information on the effects of innate immunity factors (Ind, an antibacterial peptide, and IL-1,

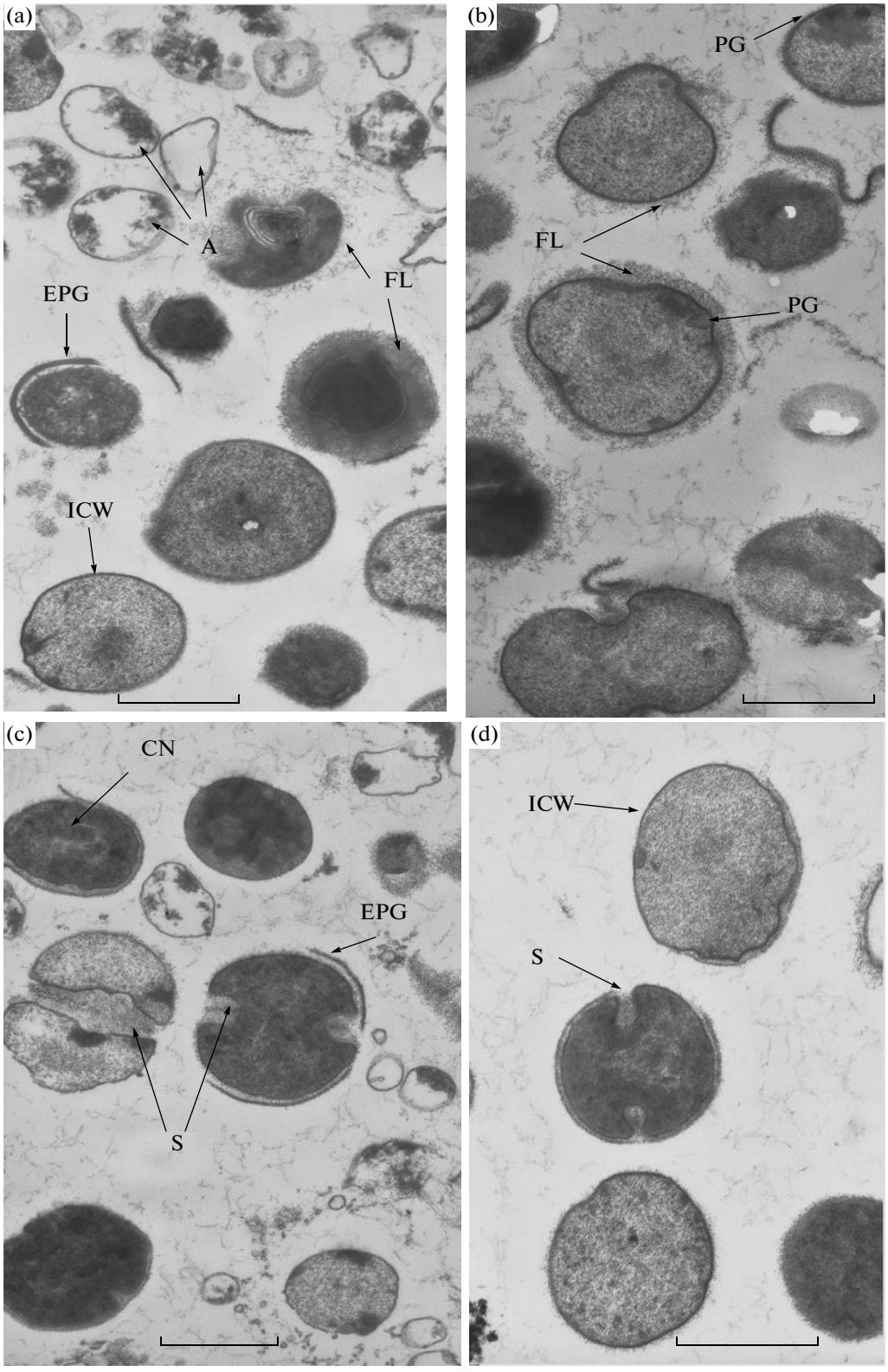

Fig. 7. Electron microscopy of sections obtained from *S. aureus* 209P cells 12 days after Cpf (100 μg/mL) treatment of a 24-h culture (transition between decelerating and stationary growth phases). Notations: A, autolyzing cells; FL, fibrillar layer; CN, compacted nucleoid; EPG, shedding external peptidoglycan layer; DC, dividing cell; PG, peptidoglycan growing within the cell; ICW, internal part of the cell wall; S, septum. Scale bar is 0.5 μm.

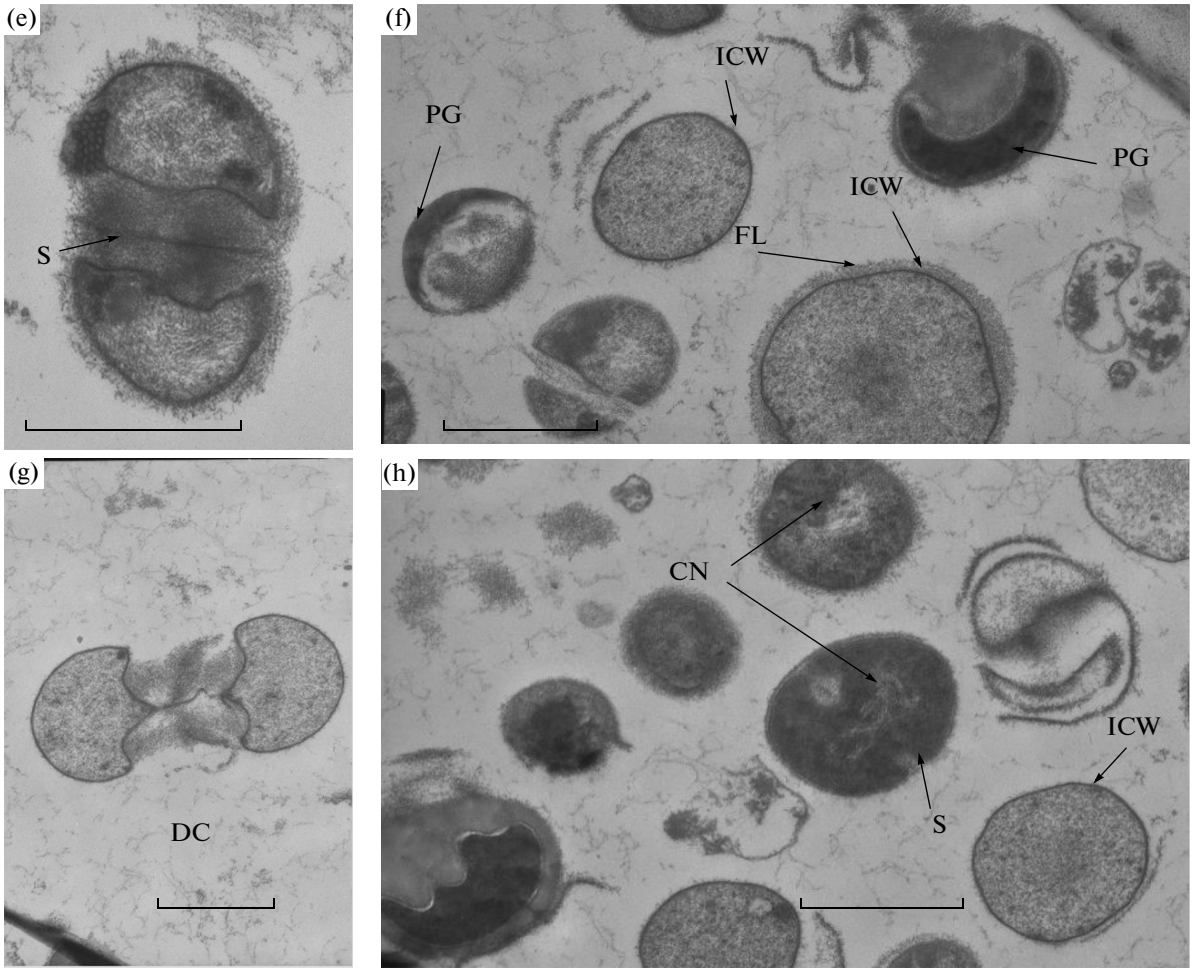

Fig. 7. Contd.

an immunity protein) on the formation of persisters and on survival of a population under antibiotic attack.

Our experiments were designed based on the notion that concentrations of the studied innate immunity factors in infection foci may increase both in the beginning of infection and in the course of bacterial population growth; accordingly, these factors were added to growing tester cultures either in the lag phase (at inoculation) or in the exponential phase. Physiological concentrations of Ind (0.3 or 3.0 μg/mL) added in the lag phase did not affect the growth of test cultures nor the formation of persister cells (Figs. 2a, 3a, 3b), whereas Ind addition to exponentially growing cultures significantly increased the number of type II persisters (Figs. 2b, 3c, 3d) but did not affect the growth of test cultures. The stimulating effect on persister formation caused by Ind addition to exponentially growing cultures was more pronounced in *E. coli* (by two orders of magnitude) and was observed only if the subsequent selection was performed with ampicillin but not with ciprofloxacin. Both in the experimental and control variants with Cpf treatment, the numbers of surviving persister cells were significantly higher. The latter observation agrees with previous reports suggesting that quinolones, including Cpf (Dörr et al., 2009), stimulate persister formation by interfering with DNA synthesis, thus inducing SOS response and a chain of subsequent events (expression of certain toxin–antitoxin modules) that trigger the phenotypic switch of dividing cells into persisters (Lewis, 2007; Gefen and Balaban, 2009; Balaban et al., 2013; Wood et al., 2013).

Our data suggest that exposure of exponentially growing cultures of gram-positive and gram-negative bacteria to physiological Ind concentrations does not affect the division rate and cell counts, but increases the number of persister cells 10- to 100-fold, in agreement with previous works showing that subinhibitory concentrations of antibiotics stimulated the formation of persisters and biofilms, another AT phenotype (Kwan et al., 2013; Mart'yanov et al., 2015).

In the next series of experiments, *S. aureus* cultures were exposed to bactericidal Ind concentrations (9 μg/mL) added at inoculation or during exponential

growth, and the resulting population death curves exhibited characteristic behavior of tolerant subpopulations (Gefen and Balaban, 2009). Although specific Ind dose calculated per cell was much higher when it was added to lag-phase cultures, in contrast to exponentially growing cultures, the share (%) of surviving viable cells (CFU/mL) was tenfold larger in the first variant (Figs. 4a–4c). Therefore, bactericidal efficacy of antibacterial agents depends not only on their specific dose (per cell), but also on the developmental phase of the test culture as a complex multicellular system (Shapiro, 1998; El'-Registan et al., 2006). Importantly, the lag phase should be considered as a stage of outpacing development, determining many aspects of the future behavior of a population (Bukharin et al., 2005). Taking this into account, it should be noted that in both variants of Ind addition the growth of test cultures was similarly restored by 24 and 96 h; but this only occurred when the peptide was added to exponentially growing (4-h and 7-h) cultures and the reversal to growth (persisters → dividing cells) was accompanied by switching of the dominant phenotype into the white variant; this was not observed when Ind was added in the lag phase. This association is extremely important for the understanding of the population heterogeneity (Zgur-Bertok, 2007) and needs to be explained and studied in more detail. A study by Betts et al. (2002) showed that the number of arising persisters was significantly affected by the nitrogen limitation in the medium, which also demonstrated the importance of conditions existing in the lag phase for the subsequent development of bacterial populations. It should be noted that our experiments analyzed only variations in colony morphology, but not other possible phenotypic transitions (e.g., virulence–avirulence), which require special investigations.

Thus, the survival of a bacterial population exposed to an antibiotic attack is determined not only by the number of cells that have retained viability but also by the phenotypic heterogeneity of the surviving fraction (subpopulation).

The most interesting results were obtained in bacterial cultures subjected to a double antibiotic attack: in 3 or 20 h after treatment with Ind (9 μg/mL), they were exposed to Cpf (100 μg/mL). In all variants, the number of persisters in 96-h cultures of *S. aureus* (10^3–10^4 cells/mL) was significantly lower than in cultures treated with Cpf alone (Figs. 5a–5c). However, these similar figures reflected different processes of *S. aureus* population death. In the first variant (Fig. 5a), Cpf was added 3 h after Ind addition to the population that was already being killed by the latter; i.e., the bactericidal effects of the two antibiotics were mutually potentiated. In this case, the number of persisters present in 96-h test cultures reflected their number in the original 4-h culture at the moment of Ind addition, while in control cultures it reflected the number of persisters that had existed in original 7-h cultures ($2–3 \times 10^5$ cells/mL). In the remaining cases (Figs. 5b, 5c), Cpf was added 20 and 24 h after Ind treatment, and it acted, similar to Ind, on an exponential-phase culture, which was, however, composed not of the original but also of the substituting variant regrown after an Ind attack. Accordingly, the number of surviving cells in 96-h cultures reflected the number of persisters (10^3 cells/mL) that had appeared in 15-h cultures of the substituting variant.

This phenomenon observed after a double antibiotic treatment can be considered as an outcome of a tyndallization-like procedure: viable persister cells are allowed to grow into a young vegetative culture sensitive to antibiotics, which is then subjected to a second antibiotic attack. The mutual potentiation of bactericidal effects caused by Ind and Cpf (or other antibiotics) in a lag-phase or growing culture seems to provide a promising approach that can be applied to increase the efficacy of antibiotic therapy and decrease the number of surviving persisters.

Another conclusion of practical importance is that the efficacy of bacterial killing can be increased by using relatively low doses of two different antibiotics (and probably of even a single one) according to a well-calculated algorithm.

The general conclusion from this part of our work is as follows: both physiological and bactericidal concentrations of Ind affect the frequency of persister formation, i.e., it acts as a signaling agent to induce cell differentiation underlying the phenotype switch from normal dividing cells into AT persisters. Moreover, Ind induced phase variant transition of colonial morphology from the dominant R-type into the white type, which occurred as a result of the persister reversal into normal dividing cells; thus we have for the first time demonstrated an association between these phenotype transitions.

A rather unexpected finding was that IL-1 caused an increase in the frequency of the phenotype transition from normal cells to persisters, which was more pronounced in exponentially growing cultures of *S. aureus* (Fig. 6b) and *P. aeruginosa* (Fig. 6d), as well as in lag-phase cultures of *E. coli* (Fig. 6c).

To sum up, it should be noted that the development of antibiotic tolerance and persistence in bacteria is affected by a number of endogenous and environmental factors, as discussed in many reviews and original articles. This study provided further evidence that the frequency of type II persister formation, as well as the size and heterogeneity of subpopulations surviving antibiotic attack, depends on the concentration of human innate immunity factors. Visualization of the structural organization of surviving cells confirmed their morphological heterogeneity and identified significant changes in the external layer of the cell wall, which were apparently associated with the changes of their antigenic properties and affect the recognition of persisters by immune system cells. These data suggest that more comprehensive investigation of the phe-

nomenon is required. A conclusion of practical importance is that it is feasible to use antimicrobial medications according to an optimized algorithm for fractional killing of the populations of opportunistic pathogenic bacteria.

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