The FASEB Journal Express article fj.06-6802com. Published online January 10, 2007.

Impairment of innate immune killing mechanisms by bacteriostatic antibiotics

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ABSTRACT **Antibiotics are designed to support host defense in controlling infection. Here we describe a paradoxical inhibitory effect of bacteriostatic antibiotics on key mediators of mammalian innate immunity. When growth of species including *Escherichia coli* and *Staphylococcus aureus* is suppressed by chloramphenicol or erythromycin, the susceptibility of the bacteria to cathelicidin antimicrobial peptides or serum complement was markedly diminished. Survival of the bacteria in human whole blood, human wound fluid, or a mouse wound infection model was in turn increased after antibiotic-induced bacteriostasis. These findings provide a further rationale against the indiscriminate use of antibiotics.—Kristian, S. A., Timmer, A. M., Liu, G. Y., Lauth, X., Sal-Man, N., Rosenfeld, Y., Shai, Y., Gallo, R. L., Nizet, V. Impairment of innate immune killing mechanisms by bacteriostatic antibiotics. *FASEB J.* 21, 000–000 (2007)**

Key Words: antimicrobial peptides · cathelicidin · complement

The era of antibiotic therapy, now in its seventh decade, has dramatically reduced morbidity and mortality from infectious diseases. Through direct killing (bactericidal) or growth-limiting (bacteriostatic) activities, antibiotics are designed to cure conditions wherein bacteria have bypassed host immunity to establish clinically significant infection. As soluble mediators of innate defense, cationic antimicrobial peptides (AMPs) are "natural antibiotics" produced by epithelial surfaces and leukocytes that play a crucial role in restricting microbial proliferation to skin and mucosal surfaces (1). The cathelicidin family of AMPs is represented by a single member in the mouse (mCRAMP) and humans (LL-37). Mice lacking mCRAMP are more susceptible to invasive bacterial infection of the skin or gastrointestinal tract (2, 3), and differential levels of LL-37 production in skin lesions of atopic dermatitis (low) *vs.* psoriasis (high) correlate inversely with the susceptibility of human patients to bacterial superinfection (4).

We sought to examine how pharmaceutical antibiotics might cooperate with AMPs in bacterial killing. Unexpected results were observed when bacteria were simultaneously exposed to antibiotics with bacteriostatic properties and the innate defense peptides. In essence, certain drugs may be functioning at odds with our immune system rather than in synergy to achieve bacterial killing. Our findings reveal new avenues for investigation of the mechanism of action of key effectors of innate immunity and reinforce caution against the indiscriminate use of certain classes of antibiotics.

MATERIALS AND METHODS

Antibiotics, AMPs, serum, and wound fluid sources

Cell culture tested chloramphenicol (Cm) and erythromycin (Em) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trimethoprim/sulfamethoxazole (TMP/SFX) was obtained from Elkins-Sinn Inc. (Cherry Hill, NJ, USA). LL-37 and mCRAMP were synthesized and purified (>99%) by the Louisiana State University Protein Facility. Piscidin/moronecidin was isolated from hybrid striped bass as described previously (5). Normal human sera were collected from healthy donors. Human C9-deficient sera and C9 were obtained from Quidel (San Diego, CA, USA); 60 μg/ml C9 were used for supplementation. Human surgical wound fluid was obtained from the University of California, San Diego (UCSD) Medical Center. Batches of mCRAMP and human serum with varying antimicrobial activity were used; thus, baseline concentrations used varied slightly in different experimental series.

Bacterial strains and growth conditions

E. coli American Type Culture Collection (ATCC, Rockville, MD, USA) 37535, enterohemorrhagic *E. coli* (EHEC) ATCC 35150, *K. pneumoniae* ATCC 33495 (urinary tract infection isolate), *S. aureus* Newman [wild-type (WT)], its isogenic *dltA* mutant (6), and its random *Tn*917 insertion mutant express-

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doi: 10.1096/fj.06-6802com

ing Em resistance were propagated in Todd-Hewitt broth (THB) media. Broad host range expression plasmid pDCerm (7) conferring Em resistance was introduced into *S. aureus* by electroporation. Cm-resistant *E. coli* ATCC 37535 was generated by passaging on THB agar with stepwise increasing concentrations of Cm (2 to >40 mg/ml). Unless otherwise noted, bacteria were grown overnight in THB without shaking and pelleted and washed twice for 3 min at 3200 *g* in the stated buffers and adjusted to the desired concentration by spectrophotometry confirmed by enumeration of colony-forming units (CFU).

AMP and complement-mediated killing assays

The bacterial WT strains and the Em-resistant *S. aureus Tn*917 mutant were grown overnight in THB; *S. aureus* + pDC*erm* and Cm-resistant *E. coli* were grown in THB + Em 50 μg/ml or Cm 25 μg/ml, respectively. For AMP killing assays, bacteria were washed twice in PBS or 20 mM phosphate buffer + 1 mM $MgCl_2$ (PB) + 1% THB for *K. pneumoniae* and *E. coli* or 10% THB for *S. aureus*. Subsequently, bacteria were incubated at 1×10^7 CFU/ml in PB + the above concentration of THB ± the stated concentration of AMP ± the stated concentration of Cm, Em, or TMP/SFX. For complement killing assays, overnight cultures of *E. coli* and *K. pneumonia* were washed twice in pyrogen-free RPMI 1640, 1% THB (RPMI-THB) ± 5 μg/ml Cm for *E. coli* or ± 7 μg/ml Cm for *K. pneumoniae* and adjusted to 1×10^6 CFU/ml in RPMI-THB ± the stated concentration of serum ± the stated concentration of Cm or Em. For both AMP and complement killing assays, the bacteria were incubated in a total volume of 50–200 μl in individual wells of cell culture tested 96-well microtiterplates shaken on a rotator at 37°C and 200 rpm. Samples were drawn at various time points. Serial dilutions were plated to enumerate CFU.

Measurement and manipulation of *S. aureus* membrane potential

S. aureus was grown overnight in THB, washed twice with PBS, and incubated at 1×10^7 CFU/ml in 200 μl PB, 10% THB ± 25 μg/ml Cm in 96-well microtiter plates shaken at 37°C. For some experiments, the potassium ionophor valinomycin (100 μM in 1% DMSO) was added at a final concentration of 5 μM. Since 0.05% DMSO was present in the final valinomycin-treated samples, control wells with 0.05% DMSO without valinomycin were included. After 30 min, samples were assayed using the BacLight bacterial membrane potential kit (Molecular Probes, Eugene, OR, USA) that uses 30 μM carbocyanine dye 3,3′-diethyloxacarbocyanine iodide ($DiOC_3$) per manufacturer's instructions. $DiOC_3$ exhibits green fluorescence in all bacterial cells, shifting toward red emission when larger membrane potentials cause the dye to self-associate. After being stained for 15 min, bacteria were analyzed by flow cytometry. Forward and side scatters were adjusted with logarithmic (log) signal amplification using a gate set on unstained bacteria. Subsequently, the log green and red fluorescence intensities of 15,000 stained bacterial particles per sample were measured after excitation with a 488 nm laser, and the ratio of red to green mean fluorescence intensity (MFI) was calculated as a size-independent indicator of membrane potential. For each sample, control bacteria were treated with propidium iodide (PI) at 50 μg/ml to determine if Cm affected bacterial viability. PI was excited at 488 nm, and its fluorescence was measured above 600 nm; MFI was determined for 15,000 particles, and the ratio of red fluorescence to particle size (FSC-H) was calculated.

Confocal imaging of peptide binding to *E. coli* membranes

Bacteria in logarithmic phase (*E. coli*, OD_{590}=0.25) were grown ± 3 μg/ml Cm for 30 min and then incubated for 20 min at room temperature with rhodamine-labeled LL-37 peptide (25 μM). Bacteria were then washed twice with PBS. Heat-killed bacteria (45 min; 55°C) served as a control to exclude peptide/antibiotic interaction. Binding of labeled peptide to *E. coli* membrane was determined by Olympus IX70 FV500 confocal laser-scanning microscopy at 12-bit resolution. Rhodamine excitation was set at 543 nm, and fluorescence was recorded from 505–525 nm.

Complement consumption assays

Antibody (Ab)-sensitized sheep erythrocytes (Sigma-Aldrich) were washed three times in gelatin veronal buffer (GVB^{2+}; Sigma-Aldrich) and placed on ice until use. *E. coli* was heat inactivated at 56°C for 60 min, washed twice in RPMI-THB, and adjusted to the desired concentration using a Neubauer-improved counting chamber for bacteria; 500 μl RPMI-THB, 5% normal human serum (NHS) ± 5 μg/ml Cm were incubated with 0 to 2.5×10^8 heat-inactivated *E. coli* cells/ml in sterile microreaction tubes using an overhead rotator. After 30 min of complement activation at 37°C, the bacteria were removed by centrifugation and the supernatants were placed on ice. The supernatants were diluted 1.6-fold in RPMI-THB and subsequently 1:5 into GVB^{2+} containing 5×10^7 Ab sensitized sheep erythrocytes/ml. Samples of 100 μl were incubated in round bottom 96-well microtiter plates at 37°C with gentle shaking on a rotator. After 30 min, the plates were centrifuged at 1000 *g* for 5 min and the supernatants were diluted 1:5 into double-distilled H_20. Subsequently, the optical density (OD) was measured at 405 nm, the absorption maximum of hemoglobin (Hb). The data are shown in percent lysis; 100% lysis was achieved by diluting sheep erythrocytes 1:5 into double-distilled H_20.

Whole blood killing assays

Blood was drawn from healthy volunteers and heparinized. *K. pneumoniae* and *E. coli* were grown overnight as described above; the AMP-susceptible *S. aureus dltA* mutant was grown to the exponential growth phase in THB. Subsequently, bacteria were centrifuged and washed twice in PBS ± 5 μg/ml Cm for *E. coli* and *K. pneumoniae* and in PBS ± 25 μg/ml Cm for *S. aureus*; 800 μl blood and 100 μl bacteria in sterile microreaction tubes were mixed with 100 μl PBS or 100 μl PBS + Cm (70 μg/ml for *K. pneumoniae*, 50 μg/ml for *E. coli*, 250 μg/ml for *S. aureus*) and incubated at 37°C using an overhead rotator. Samples were drawn after 30, 60, 90, and 180 min, and surviving CFU were quantified by plating serial dilutions.

Wound fluid mediated killing

E. coli was grown overnight in THB and washed two times in PBS. Bacteria were incubated at 1×10^6 CFU/ml in RPMI-THB + wound fluid + 5 μg/ml Cm in 200 μl in 96-well plates. CFU were determined as described above.

Murine wound infection model

Both flanks of 11–12 wk old female BALB/C mice (Jackson Laboratories, West Grove, PA, USA) were shaved and treated with Nair. Three days later mice were anesthetized with 75 mg/kg ketamine, 8 mg/kg xylazine, 1.5 mg/kg acepromazine intraperitoneally, and a 0.5-cm incision was made on each

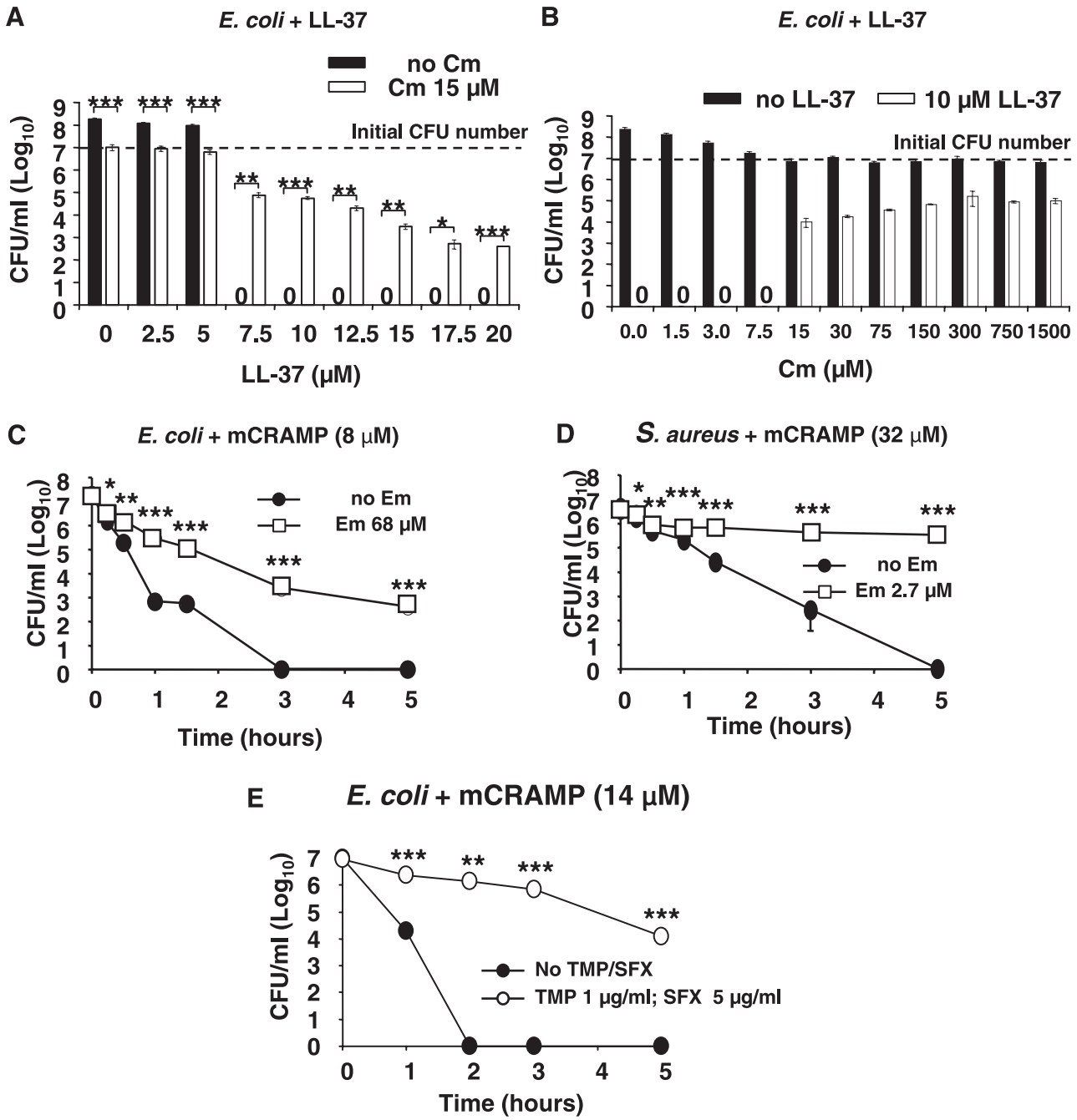

Figure 1. Bacteriostatic antibiotics impair cathelicidin antimicrobial peptide action. *A)* Killing of *E. coli* by different concentrations of human cathelicidin LL-37 after 3 h incubation in the presence or absence of bacteriostatic concentrations of Cm. *B*) Killing of *E. coli* by 10 μM LL-37 after 4 h incubation in the presence of varying concentrations of Cm. Killing kinetics of the murine cathelicidin mCRAMP against *E. coli* (*C*) or *S. aureus* (*D*) in the presence or absence of bacteriostatic concentrations of Em. *E)* Effect of trimethoprim/sulfamethoxazole on mCRAMP-mediated killing of *E. coli.* Data points are mean bacterial CFU ± SD of triplicate measures; experiments representative of 3 performed with similar results. $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$, 2-tailed *t* test.

flank by scalpel. Twenty hours after being wounded, mice were reanesthetized, flanks were disinfected with 70% alcohol, and wounds were reopened by scalpel. One wound was infected with 5 μl of RPMI 1640 + 10^7 CFU of *E. coli* and the other wound with 5 μl RPMI 1640 + 10^7 CFU of *E. coli* + 10 μg/ml Cm. One hour after infection wounds and surrounding tissue were excised, placed in 500 μl ice-cold PBS, and weighed, and 500 μl PBS containing 1 mg/ml cytodex glass beads (Sigma-Aldrich) were added to the samples. The samples were vigorously vortexed for 5 min, and CFU was enumerated as described above.

Statistics

Experimental differences in the *in vitro* assays were evaluated by unpaired Student's *t* test; results of the mouse challenge studies were evaluated by paired Student's *t* test.

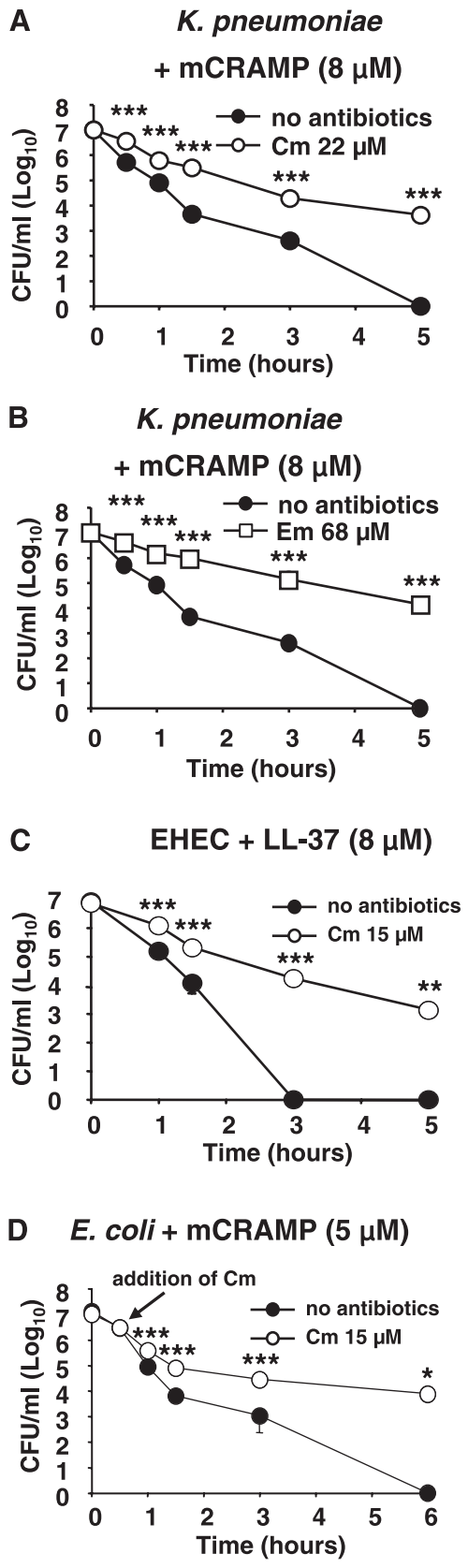

Figure 2. Additional studies of bacteriostatic antibiotic interference with cathelicidin killing. *A, B)* Bacteriostatics antibiotics impair mCRAMP mediated killing of *K. pneumoniae*. *K. pneumoniae* was incubated with 8 μM mCRAMP in the presence or absence of 22 μM Cm or 68 μM Em. *C)* Killing of EHEC isolate by 8 μM human cathelicidin LL-37 ± 15 μM Cm. *D)* Addition of Cm protects *E. coli* from mCRAMP-mediated killing. Bacteria were incubated with 5 μM mCRAMP. After 30 min, Cm at 15 μM (open circles) or buffer (closed circles) was added. Data points are mean bacterial CFU + SD of triplicate measures; experiment representative of 3 performed with similar results. $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$, 2-tailed *t* test.

Assurances

All animal experiments were approved by the UCSD Committee on the Use and Care of Animals and performed using accepted veterinary standards. Experimentation using human blood was approved by the UCSD Human Research Protection Program.

RESULTS

Bacteriostatic antibiotics can inhibit AMP killing

The Gram-negative bacterium *Escherichia coli* was exposed to varying doses of synthetic human LL-37 in the presence or absence of antibiotics. An unexpected result was observed in studies with the broad-spectrum, primarily bacteriostatic antibiotic Cm. As LL-37 concentration was increased into the bactericidal range against *E. coli*, significantly less bacterial killing was observed in the presence of 15 μM Cm than with no antibiotic at all (**Fig. 1*A***). With the use of a fixed bactericidal concentration of 10 μM LL-37, an antibiotic dose-response curve pinpointed the abrupt onset of antagonism to the concentration where Cm began to exert bacteriostatic activity (≥15 μM; Fig. 1*B*). To expand on these observations, kinetic killing curves for synthetic murine mCRAMP against *E. coli* or the Gram-positive bacterium *S. aureus* were performed in the presence or absence of bacteriostatic concentrations of either Cm or the commonly prescribed macrolide antibiotic Em. Em-induced bacteriostasis significantly impaired murine cathelicidin (mCRAMP) killing of *E. coli* and *S. aureus* (Fig. 1*C, D*); similar effects on mCRAMP killing of *E. coli* and *S. aureus* were observed with Cm-induced bacteriostasis (not shown). AMP antagonism was not limited to the ribosomal protein synthesis inhibitors Em and Cm, since mCRAMP killing of *E. coli* was blocked after bacteriostasis induced by TMP/SFX, a commonly prescribed broad-spectrum antibiotic that targets the folate biosynthetic pathway (Fig. 1*E*).

Killing kinetics of mCRAMP against a urinary tract isolate of *Klebsiella pneumoniae* were inhibited by bacteriostatic concentrations of Cm or Em (**Fig. 2*A*, *B***), and human LL-37 killing of an EHEC disease isolate was delayed in the presence of Cm (Fig. 2*C*). In the *E. coli* studies, even if addition of bacteriostatic Cm was delayed for 30 min after initiation of mCRAMP killing, a significant rescue of bacterial survival was seen (Fig. 2*D*). The inhibitory effect of bacteriostatic antibiotics on AMP killing was not restricted to cathelicidins, as addition of Cm or Em impaired the killing of *E. coli*, *K.*

pneumoniae, and *S. aureus* by piscidin (aka moronecidin), a cationic AMP of nonmammalian origin (5, 8; **Fig. 3**).

Antibiotic antagonism of AMP action requires effective bacteriostasis

To further examine the association of antibiotic-induced bacteriostasis with AMP antagonism, the *E. coli* test isolate was passed several times in gradually increasing concentrations of Cm to induce stepwise resistance to the antibiotic. We found that the Cm-resistant isolate of *E. coli* became fully susceptible to mCRAMP in the presence of Cm, *i.e.,* the inhibitory effect of Cm was dependent on effective bacteriostasis (**Fig. 4*A***). Similar findings were observed with an Em-resistant *S. aureus* transposon mutant, as addition of Em did not affect mCRAMP activity; however, AMP killing of the same bacterial strain was strongly impeded by bacteriostatic Cm (Fig. 4*B*). Likewise, when *S. aureus* was transformed with a plasmid containing an Em-resistance gene, susceptibility to mCRAMP in the presence of Em was restored (Fig. 4*C*). Taken together, these experiments indicate that the antagonistic effects of Cm and Em do not involve direct interaction with the cathelicidin peptide; rather, the two bacteriostatic pharmaceutical antibiotics act to decrease bacterial susceptibility to AMP killing.

Cm-induced transmembrane potential shifts in *S. aureus*

A study by Koo *et al.* (9) demonstrated that the bactericidal activity of platelet microbicidal proteins is in part dependent on the transmembrane potential of *S. aureus.* Induced bacteriostasis of *S. aureus* using 77 μM Cm led to a measurable depolarization within 30 min when compared with control cells (**Fig. 5*A***). This treatment did not alter PI uptake, indicating that Cm-induced bacteriostasis did not alter *S. aureus* membrane permeability or viability. The potassium ionophor valinomycin produced hyperpolarization of both Cm-treated and untreated cells. Protection of *S. aureus* from mCRAMP killing by Cm-induced bacteriostasis was observed even after hyperpolarization of the target cell membrane (Fig. 5*B*).

Human AMP LL-37 binds preferentially to dividing bacteria

Cathelicidins kill bacteria by assembly in the cell membrane of the target microorganism to form pores or otherwise damage its functional integrity (10, 11, 12). Confocal microscopy was used to examine the interaction of rhodamine-labeled human LL-37 with *E. coli* in the presence or absence of bacteriostatic concentrations of Cm (fluorescently labeled LL-37 retains anti-

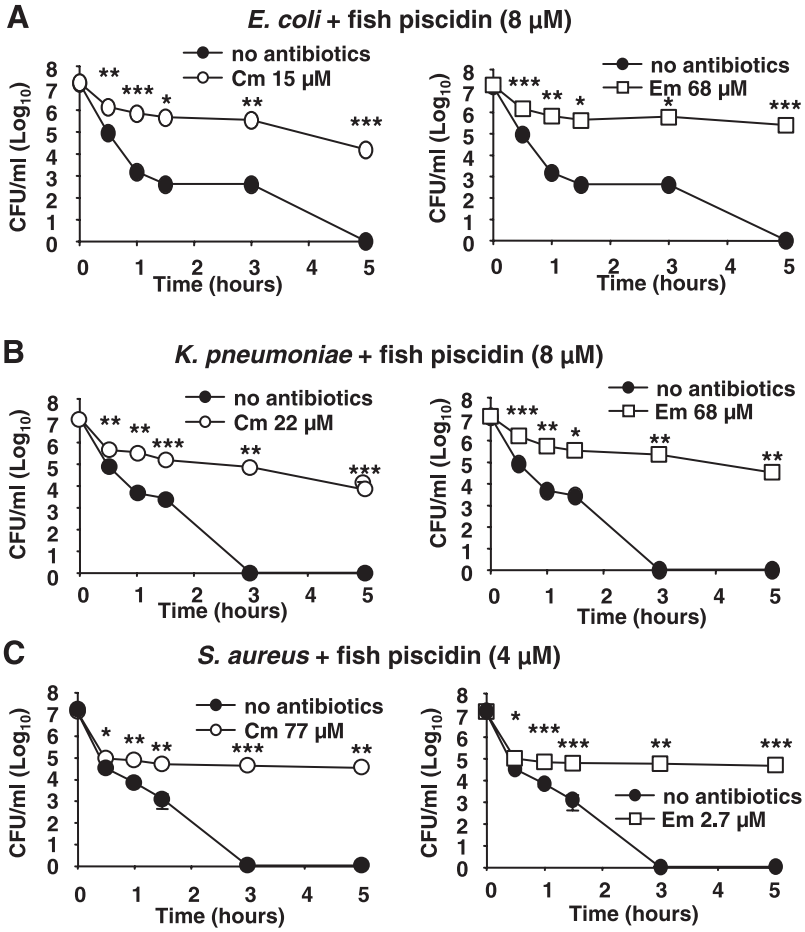

Figure 3. Bacteriostatics impair piscidin antibacterial activity. Killing kinetics of the fish antimicrobial peptide piscidin (aka moronecidin) against *E. coli* (*A*), *K. pneumoniae* (*B*), and *S. aureus* (*C*) in the presence or absence of bacteriostatic concentrations of Cm or Em. Data points are mean CFU ± SD of triplicate measures; experiment representative of 3 performed with similar results. $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$, 2-tailed *t* test.

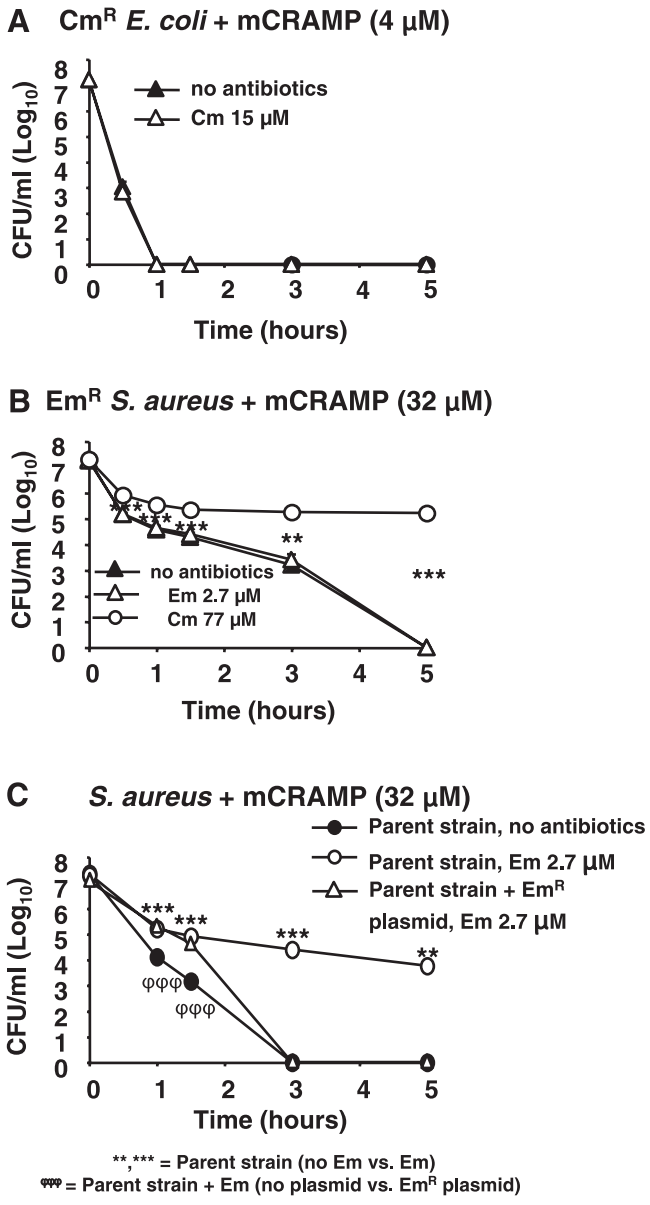

Figure 4. Antibiotic antagonism of cathelicidin action requires effective bacteriostasis. *A)* Selection for Cm resistance in *E. coli* restores full susceptibility of the bacterium to mCRAMP killing in the presence of Cm. *B)* Killing kinetics of LL-37 against an Em-resistant *S. aureus* transposon mutant in the presence or absence of Em and Cm. *C)* Killing of Em-resistant *S. aureus* by mCRAMP. *S. aureus* Newman WT was incubated with 32 μM mCRAMP in the presence (open circles) or absence of erythromycin (closed circles). *S. aureus* bearing pDC*erm* conferring Em resistance was incubated in the presence of Em (open triangles). Data points represent mean bacterial CFU ± SD of triplicate measures; experiments representative of 2 (*B*) or 3 (*A, C*) performed with similar results. $^*P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$, $^{\phi\phi\phi}P < 0.0005$, 2-tailed *t* test.

microbial activity; ref 13). Strong binding of LL-37 to the bacterial surface was noted in the absence of Cm (correlating to bacterial killing), while the addition of Cm markedly reduced LL-37 binding to the bacterium (correlating to bacterial survival; **Fig. 6*A*, *B***). Control experiments demonstrated that LL-37 bound to heat-killed *E. coli* equivalently in the presence or absence of Cm (Fig. 6*C, D*), ruling out a direct interference of Cm with the labeled AMP. Examination of LL-37-treated *E. coli* cultures in the absence of antibiotics demonstrated preferential targeting of dividing bacteria over nondividing bacteria, with maximal staining at the plane of cell division (Fig. 6*E, F*). These results suggest that during cell division, the bacterial enters a phase of heightened vulnerability to AMP killing. Consequently, the antagonistic effect of bacteriostatic antibiotics on

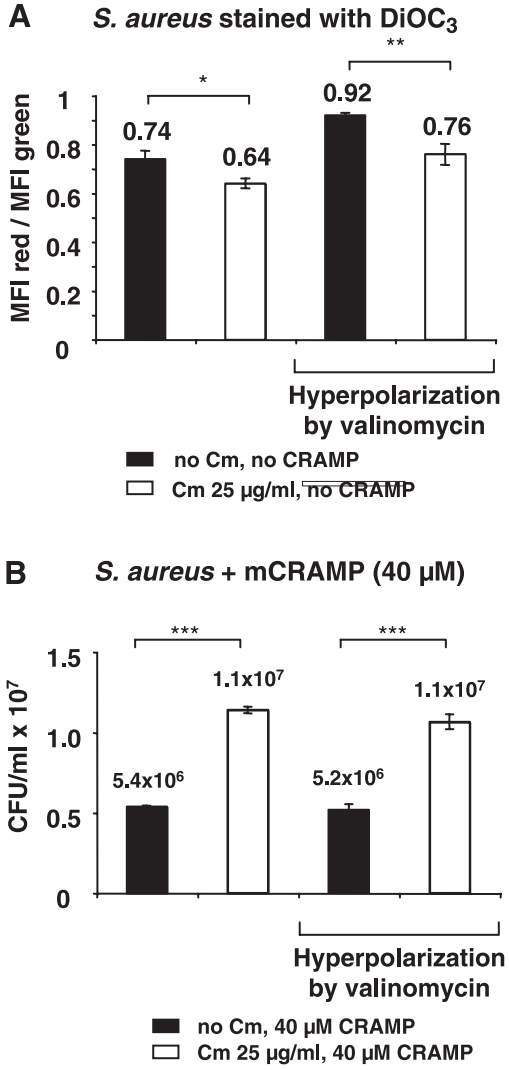

Figure 5. Transmembrane potential shifts in *S. aureus* on treatment with Cm. *A*) Bacteria were incubated for 30 min in the presence or absence of bacteriostatic concentrations of Cm and shifts in transmembrane potential were measured by staining bacteria with the fluorescent dye DiOC$_3$. Red and green MFIs of 15,000 bacterial particles were determined, and ratio of red MFI to green MFI was calculated and plotted. Hyperpolarization of bacterial cells was achieved by addition of the potassium ionophor valinomycin. *B*) In parallel, bacteria were incubated under same conditions with 40 μM mCRAMP for 30 min and number of surviving CFUs was determined. Data points are mean MFI ratios ± SD (*A*) or bacterial CFU ± SD (*B*) of triplicate measures; experiment representative of 3 performed with similar results. $^*P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$, 2-tailed *t* test.

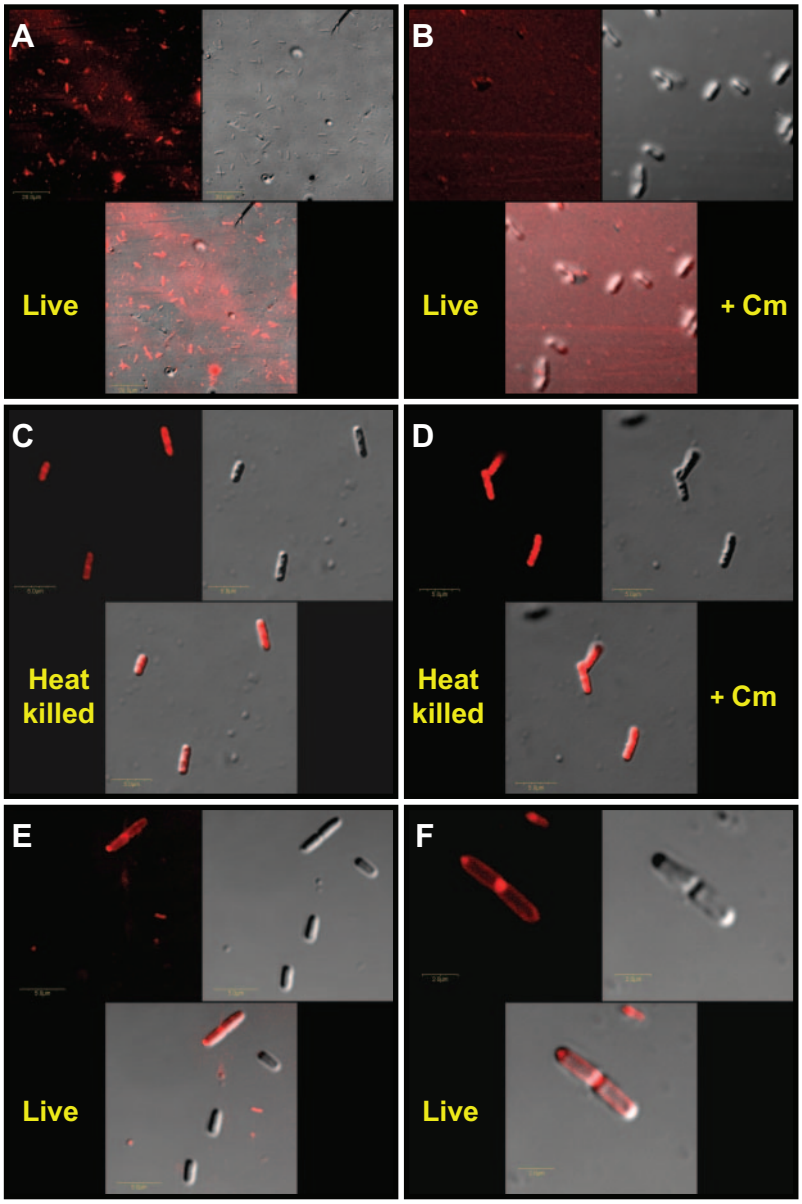

Figure 6. Binding of LL-37 to bacteria is decreased by Cm-induced bacteriostasis. Confocal fluorescence microscopy showing binding of rhodamine-labeled human cathelicidin LL-37 to live *Escherichia coli* in the absence (*A*) or presence (*B*) of bacteriostatic Cm. Binding of LL-37 to heat-killed *E. coli* in the absence (*C*) or presence (*D*) of bacteriostatic Cm. *E, F)* LL-37 preferentially binds dividing *E. coli* and enhanced LL-37 binding is observed at the plane of cell division.

AMP killing may derive from preventing bacteria from entering this condition of heightened susceptibility.

Bacteriostatic antibiotics can impair complement-dependent serum killing of bacteria

Another important component of innate immunity that targets bacterial cell membranes to kill via pore formation is the C5-C9 membrane attack complex of complement generated after activation of the classical, mannose-binding lectin or alternative pathways in mammalian sera. While *E. coli* was rapidly cleared in human sera in a C9-dependent fashion, the addition of bacteriostatic Cm significantly decelerated the rate of bacterial killing (**Fig. 7*A***). Bacteriostatic Cm also exerted an inhibitory effect on serum killing of *K. pneumoniae* (Fig. 7*B*). Similar to our observations in cathelicidin killing, the antagonistic effect of bacterostatic Cm on serum killing was due to an effect on the bacteria rather than an interaction with complement factors. The antagonistic effect of Cm on serum killing was absent when a Cm-resistant *E. coli* strain was tested (Fig. 7*C*), and complement consumption by heat-killed *E. coli* was unaffected by Cm (Fig. 7*D*).

Bacteriostatic antibiotics can impair bacterial killing in whole blood and wound fluid

We next examined whether the phenomenon of bacteriostatic antibiotic interference with innate immune killing mechanisms could be extrapolated to mammalian tissue fluids, which use both AMPs and complement to combat bacterial pathogens. As seen in **Fig. 8*A-C***, *E. coli*, *K. pneumoniae*, and an AMP-susceptible *S. aureus* strain (6) were killed significantly more slowly in freshly isolated human whole blood when bacteriostatic concentrations of Cm were added. A similar antagonistic effect of bacteriostatic Cm on killing of *E. coli* in

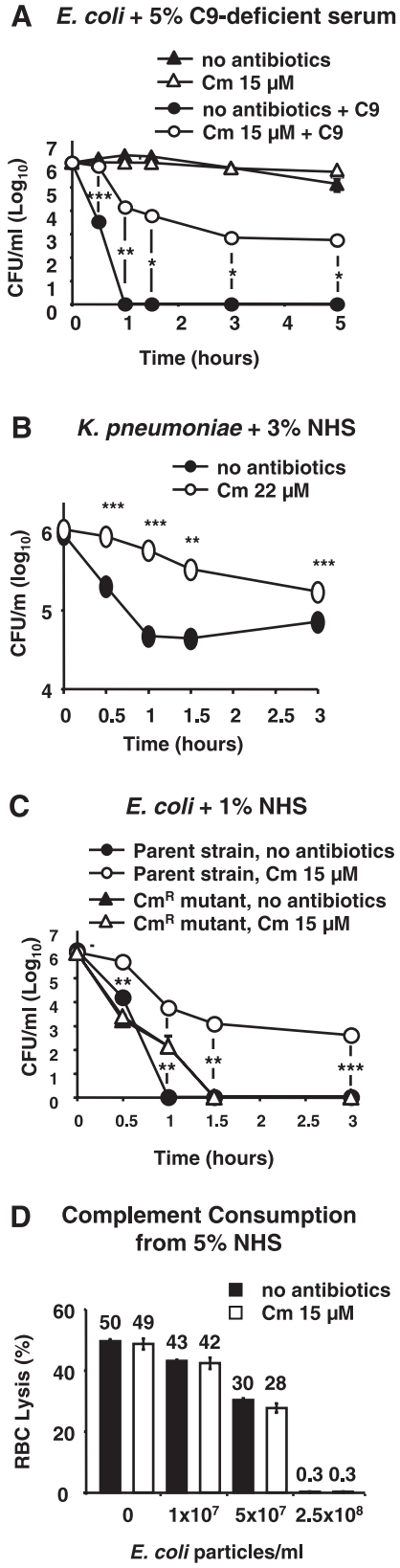

Figure 7. Bacteriostatic antibiotics impair complement-dependent serum killing. Killing kinetics of human serum against *E. coli* in the presence or absence of bacteriostatic concentrations of the antibiotic Cm. *A)* Inhibitory effect of antibiotic bacteriostasis on serum killing of *E. coli* is dependent on the presence of C9 of terminal complement pathway. *B) K. pneumoniae* was incubated with 3% NHS with (open circles) or without (closed circles) 22 μM Cm. *C)* Selection for Cm resistance in *E. coli* restores full susceptibility of bacterium to human serum killing in the presence of Cm. Data points are mean bacterial CFU ± SD of triplicate measures; experiment representative of 3 performed with similar results. $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$, 2-tailed *t* test. *D)* Cm does not affect *E. coli* complement consumption. Heat-killed *E. coli* cells were incubated in 5% NHS in the absence or presence of 15 μM Cm, and ability of collected supernatants to lyse Ab-sensitized sheep erythrocytes was determined. Samples were run in triplicate, and mean percentages of lysis of 1 representative experiment (of 3) are shown.

human wound fluid was observed (Fig. 8*D*). Finally, we challenged mice with equivalent quantities of untreated and Cm-inhibited *E. coli* in a model of acute wound infection. Paradoxically, 1 h after challenge, antibiotic-treated bacteria had enhanced survival in the mouse wound environment when compared to the untreated bacteria (Fig. 8*E*).

DISCUSSION

Our studies demonstrate that bacteria, upon growth suppression by bacteriostatic antibiotics, can become more resistant to mammalian AMPs and complement *in vitro* and to innate immune clearance in specific host biological fluid and tissue environments *ex vivo* and *in vivo*. The effects of antibiotic suppression on bacterial interaction with membrane-active host defense molecules also provide new perspective and context to observations from earlier literature, including the findings that maximal susceptibility of *S. aureus* to thrombin-induced microbicidal protein (tPMP) or *Bordetella pertussis* to complement occurred during exponential growth phase (11, 14) or the observation that neutrophil defensin activity against *S. aureus* was enhanced by cell-wall active antibiotics, while potentially impeded by ribosomally active agents (15).

Antibiotics or combinations of antibiotics with bactericidal activity are often touted as superior to bacteriostatic regimens in complicated infections of bones, heart valves, or the central nervous system (16, 17). Further, because a killed microorganism cannot mutate, the choice of bactericidal over bacteriostatic therapies is proposed to curtail development of antibiotic resistance (18). Our discovery that bacteriostatic antibiotics can impair AMP or complement function suggests that certain pharmaceutical therapies could allow bacteria to persist longer in host tissue environments where innate immune responses are normally summoned. The resultant opportunity for adaptation to such environments could in theory enhance bacterial virulence.

Our observation that cathelicidin peptide preferentially targets replicating bacteria at the plane of cell division likely provides a mechanistic clue to the observed phenomenon of bacteriostatic antibiotic antag-

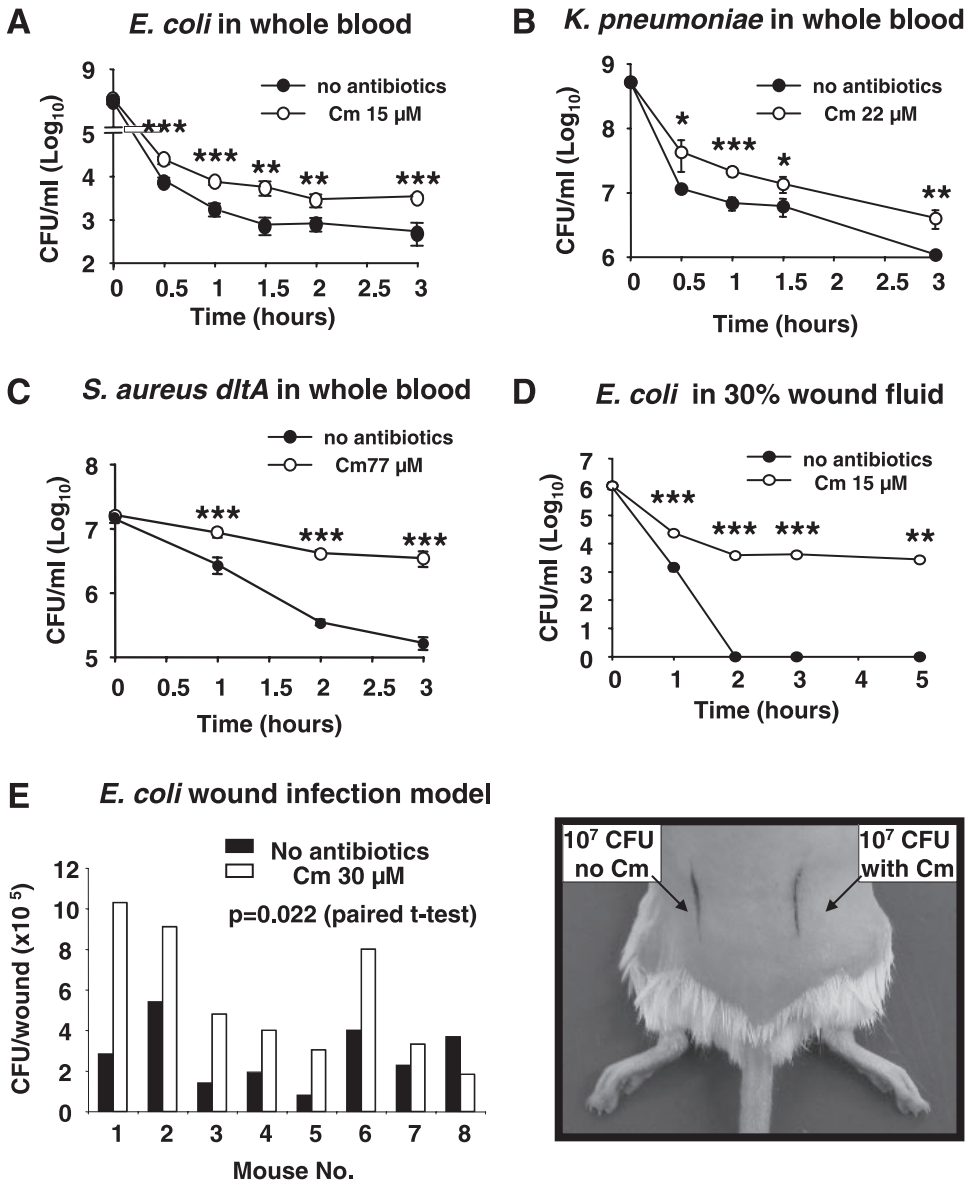

Figure 8. Bacteriostatic antibiotics impair bacterial killing in whole blood and wound fluid. Killing kinetics of *E. coli* (*A*), *K. pneumoniae* (*B*), or *S. aureus* (*C*) 113Δ*dlt*A (AMP-susceptible) in freshly isolated human blood in the presence or absence of bacteriostatic concentrations of Cm. *D*) Killing kinetics of *E. coli* in 30% human wound fluid with or without bacteriostatic concentration of Cm. Data points are mean bacterial CFU ± SD of triplicate measures; experiment representative of 3 performed with similar results. $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$, 2-tailed *t* test. *E*) Survival of untreated and Cm-treated *E. coli* 1 h after inoculation into wounds in a murine infection model.

onism of AMP action. The observation that bacteriostasis can be achieved not only by two different protein synthesis inhibiting antibiotics (Cm and Em) but also the folate biosynthesis inhibitor TMP/SX suggests that the bacteriostasis *per se* is contributing to AMP resistance rather than the decreased synthesis of a target protein molecule. Since the microbicidal actions of cationic molecules such as nisin or insect defensins have been shown to require a threshold electrical potential across the target membrane (19, 20), it is possible that bacteriostatic Cm-induced protection of *S. aureus* from mCRAMP-mediated killing might in part be related to the detectable membrane depolarization. However, when we hyperpolarized the *S. aureus* membane with valinomycin such that the electrical potential after Cm-treatment was similar to untreated cells, significant protection against AMP-mediated killing was still observed.

Small colony variants (SCV) of *S. aureus* area naturally occurring subpopulation with slow growth and other abnormal phenotypic characteristics that can emerge in chronic and persistent infection and on exposure to specific antibiotics (21, 22). We did not observe any such variants during the course of our experiments, but it is interesting to note that parallels may be drawn in the larger scheme, since cell wall-active pharmaceutical antibiotics are less effective against SCV and this diminished activity could theoretically in be related to decreased growth and fewer period of vulnerability during cell division.

Pharmaceutical antibiotics are often tested in combination against pathogenic microbes to probe for synergistic or antagonistic interactions. For example, Cm may be synergistic with the cell-wall active antibiotic ampicillin against bacteria for which Cm is bactericidal but antagonistic to ampicillin against bacteria for which Cm is bacteriostatic (23). Our current findings suggest that similar attention should be paid to the interaction of bacteriostatic antibiotics with key effector molecules of our innate immunity. FJ

We thank V. Kiss for technical assistance with confocal microscopy. This work was supported by National Institutes of Health Grants AI-48176 (R. L. Gallo) and AI-048694 (V. Nizet) and a Veterans Affairs Merit Award (R. L. Gallo).

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Received for publication July 7, 2006.
Accepted for publication October 31, 2006.