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Clindamycin Affects Group A *Streptococcus* Virulence Factors and Improves Clinical Outcome

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Group A *Streptococcus* (GAS) has acquired an arsenal of virulence factors, promoting life-threatening invasive infections such as necrotizing fasciitis. Current therapeutic regimens for necrotizing fasciitis include surgical debridement and treatment with cell wall–active antibiotics. Addition of clindamycin (CLI) is recommended, although clinical evidence is lacking. Reflecting the current clinical dilemma, an observational study showed that only 63% of the patients with severe invasive GAS infection received CLI. This work thus aimed to address whether CLI improves necrotizing fasciitis outcome by modulating virulence factors of CLI-susceptible and CLI-resistant GAS in vitro and in vivo. Treatment with CLI reduced extracellular DNase Sda1 and streptolysin O (SLO) activity in vivo, whereas subinhibitory CLI concentrations induced expression and activity of SLO, DNase, and *Streptococcus pyogenes* cell envelope protease in vitro. Our in vivo results suggest that CLI should be administered as soon as possible to patients with necrotizing fasciitis, while our in vitro studies emphasize that a high dosage of CLI is essential.

Keywords. group A *Streptococcus*; protein synthesis inhibitors; virulence factors; necrotizing fasciitis; clindamycin.

The Gram-positive, β-hemolytic bacterium group A *Streptococcus* (GAS; *Streptococcus pyogenes*) is a leading human pathogen that causes life-threatening invasive infections, such as necrotizing fasciitis and streptococcal toxic shock syndrome (STSS). Standard therapy consists of high doses of penicillin, to which GAS remains exquisitely susceptible, plus, for necrotizing fasciitis, surgical debridement of necrotic tissues. The use of the protein synthesis inhibitor clindamycin (CLI) is strongly recommended by guidelines of the Infectious Diseases Society of America (IDSA) [1]. However, supporting clinical randomized prospective trials are lacking. In contrast to penicillin, CLI acts on stationary growth–phase bacteria [2] in in vitro and murine in vivo models and, in addition, inhibits production of bacterial proteins such as GAS superantigens [3,4]. A recent observational study indicates that complementary treatment with CLI improves the survival of patients with STSS [5], underlining the importance of superantigen inhibition by CLI. However, another observational study showed that only 63% of the patients with severe invasive GAS infection received CLI [6], reflecting the current clinical dilemma. Despite improved medical care, the lethality of necrotizing fasciitis remains high, at 15%–36%, and is 30%–50% when associated with STSS [7,8]. Additional research is necessary to find better quality of evidence for the optimal therapy of severe GAS infections. However, due to the limited number of necrotizing fasciitis patients, prospective studies have not been possible.

Since a broad array of GAS virulence factors has been associated with the pathogenicity of GAS, particularly for necrotizing fasciitis, reducing the virulence factors activity would seem a logical strategy to improve therapeutic approaches for necrotizing fasciitis. So far, benefits of CLI therapy for non-STSS invasive GAS infections remain elusive. In addition, in vitro studies showed that subinhibitory CLI concentration increased the expression of the virulence factors streptolysin O (SLO) and M protein [9–11] and suppressed the expression of streptococcal pyrogenic exotoxin B (SpeB) [12]. This further adds concerns about whether patients with non-STSS invasive GAS infections benefit from CLI therapy. Emergence of CLI-resistant GAS strains worldwide [13–15] might further jeopardize benefits of empirical CLI treatment.

To elucidate potential benefits of CLI in necrotizing fasciitis management, we assessed the effect of CLI against GAS invasive infections with both CLI-susceptible and CLI-resistant GAS M1 clinical isolates in vitro and in vivo. Hyaluronic acid capsule and SpeB were assessed since these GAS virulence factors are crucial during the first stage of infection because they promote adherence to and invasion of host cells [16–18]. We further assessed the activity of SLO, extracellular DNase Sda1 (DNase), and

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S. pyogenes cell envelope protease (SpyCEP) [19–21], all of which are important upon invasion since they interfere with the host's phagocyte function.

MATERIAL AND METHODS

Bacterial Strains

S. pyogenes M1T1 5448 strain (GAS WT) [22] and its isogenic mutants GAS Δ*speB* [23] and animal-passaged (GAS AP) [24] were used. GAS M1 clinical isolates from necrotizing fasciitis (GAS CI417, isolated from the necrotic tissue), pneumopathy (GAS CI416, isolated from blood), and bacteremia originating from recurrent erysipelas (GAS CI529, isolated from blood) were used. They either exhibit constitutive (GAS CI529) or inducible (GAS CI416 and GAS CI417) CLI resistance. GAS CI417 contained a point mutation in the *covS* gene, resulting in a similar phenotype as GAS AP. M-type of the GAS CI777 strain (M75) isolated from the patient was determined following the Center for Disease Control and Prevention guidelines (available at: http://www.cdc.gov/streplab/protocol-emm-type.html, access 14 June 2016). All strains were grown in Todd Hewitt broth (BD) supplemented with 2% yeast extract (THY) at 37° C in a static incubator.

Preparation of Culture Supernatant

Overnight cultures were diluted to OD_{600} 0.1 in THY, grown to OD_{600} 0.4, diluted to a final inoculum of 5×10^5 colony-forming units (CFU)/mL and grown to mid-logarithmic (OD_{600} 0.4–0.5) or late stationary phase (17.5 hours, O/N) in the presence or absence of subinhibitory antibiotic concentrations. Subinhibitory antimicrobial concentrations were defined as a concentration lower than the minimal inhibitory concentration (MIC) required to inhibit bacterial growth. The MIC of antibiotics was determined by the microdilution method, using THY as the growth medium [25]. If not stated otherwise, the antibiotics were used at a concentration corresponding to one fourth of the MIC (Supplementary Tables 1 and 2).

Hemolytic Activity of SLO

Hemolysis assays were performed as described elsewhere [26].

Activity of Extracellular DNase Sda1

Gel electrophoresis to visualize DNase activity [27] and quantitative DNA cleavage assessment [28] were performed as previously described. Recombinant DNase I (Roche) and THY served as positive and negative controls, respectively.

SpyCEP-Mediated Interleukin 8 (IL-8) Cleavage

SpyCEP-mediated IL-8 cleavage was measured by enzyme-linked immunosorbent assay (R&D Systems) as previously described [19]. THY served as a negative control.

SpeB Activity

SpeB activity of bacterial supernatants was assessed by cleavage of the chromogenic substance Bz-Pro-Phe-Arg-Nan (Sigma) as described before [16, 29].

Quantification of GAS Hyaluronic Acid Content

Hyaluronic acid was extracted from bacterial pellets in mid-logarithmic growth phase as described elsewhere [30] and was quantified using a commercial kit (Corgenix; kindly performed by Wolfgang Thormann [Clinical Pharmacology Laboratory, Institute for Infectious Diseases, Bern, Switzerland]).

Murine Infection Model

Either the GAS M1 clinical isolates CI416 (CLI susceptible) or CI529 (CLI resistant) were injected subcutaneously ($3*10^7$ CFU) into the flanks of 10–12-week-old female C57Bl/6 wild-type mice (Janvier, France), followed by treatment with low-dose CLI (75 ng/mouse twice daily intraperitoneally), high-dose CLI (100 μg/mouse twice daily intraperitoneally), or phosphate-buffered saline (PBS). Two independent experiments with 5 mice/group were carried out for both strains, and a third repeat with 2 mice/group was carried out for strain CI416. Skin lesion sizes were monitored daily and measured by multiplying the maximum and minimum diameter of the lesion. Mice were euthanized on day 3 after infection, and bacteria present in the skin lesions were enumerated [19]. In short, skin and adjacent tissue were removed, 2 mL of PBS was added per 1 g of tissue, and the tissues were then homogenized using a tissue lyser (Qiagen). After centrifugation the supernatants were used for DNase and SLO activity assessment. The protocol ZH251/14 was approved by the Institutional Animal Care and Use Committee of the University of Zurich.

CLI Concentrations

CLI concentration in tissue from a patient with necrotizing fasciitis was assessed by liquid chromatography–high-resolution mass spectrometry, using a deuterated internal standard in the clinical chemical laboratory of University Hospital Zürich, Switzerland.

Patient Material

The patient initially presented to an outpatient clinic with a swollen finger and received the β-lactam antibiotic ceftriaxone (2 g intravenously once). After 24 hours, the infection had extended to the entire arm, necessitating immediate hospital admission. Immediate surgery with debridement of the entire arm was required, and ceftriaxone (2 g intravenously) in combination with CLI (4×900 mg intravenously) was given. Further surgical debridement followed on days 2 and 4. Informed consent was obtained in accordance with the ethical committee requirements. Biopsy material was collected during first and second surgical debridements. The tissue was prepared as specified for the mouse tissue described above and in addition was fixed with 10% buffered formalin. The 2-μm–thick slides were stained with standard hematoxylin and eosin and Brown Brenn staining. Whole-slide scanning and photomicrography were performed using the NanoZoomer 2.0-HT Digital Slide Scanner (Hamamatsu) and Cell^P imaging software (Olympus, Life Sciences), respectively.

Statistical Analysis

If not stated otherwise, statistical analyses were performed using the Student *t* test with GraphPad Prism software for Windows (version 5.04). *P* values of < .05 were considered statistically significant.

RESULTS

CLI-Mediated Attenuation of Clinical Severity and Inhibition of Virulence Factors Activity in a Murine Necrotizing Fasciitis Model

To tackle the challenging task of assessing the influence of the protein synthesis inhibitor CLI on GAS virulence factors activity in vivo, we used a murine necrotizing fasciitis model. To gauge the broadness of CLI efficacy in necrotizing fasciitis treatment, we infected mice with a CLI-susceptible (GAS CI416) or a CLI-resistant (GAS CI529) GAS M1 clinical isolate. When infected with GAS CI416, the bacterial burden in the mouse skin was comparable between control and CLI-treated groups (Supplementary Figure 1*A*); however, skin lesion sizes were significantly reduced in the CLI-treated group as compared to that in untreated animals (Figure 1*A*). Treatment with CLI also significantly inhibited DNase and SLO activity (Figure 1*B* and 1*C*). Similarly, infection with the CLI-resistant GAS CI529 produced significantly smaller skin lesions in the CLI-treated group as compared to findings for untreated animals (Figure 1*A*), while bacterial counts were slightly reduced (Supplementary Figure 1*B*). DNase activity was again clearly inhibited by CLI, while SLO activity was barely detectable in both groups (Figure 1*B* and 1*C*). When mice were injected with a CLI dose lower than the MIC of the infecting strain (CI416), the severity of the clinical manifestation was comparable or only slightly reduced as compared to that for the control group (Supplementary Figure 2*A*–2*D*). Overall, empirical treatment of necrotizing fasciitis with a high dose of CLI in a murine infection model proved to be beneficial, even when the infecting strain was resistant to CLI.

Elevated Virulence Factors Activity in GAS M1 Clinical Isolates in the Presence of Subinhibitory Concentrations of Clindamycin

Since virulence factors activity is important for necrotizing fasciitis progression, we assessed the effect of subinhibitory CLI concentrations on virulence factors activity in vitro in the CLI-susceptible GAS CI416 and CLI-resistant GAS CI529 strains. Subinhibitory CLI concentrations permitted assessment of GAS virulence factors activity without affecting bacterial growth. For both strains, a dose-dependent increase of virulence factors activity was observed (Figure 2*A*–2*C* and Supplementary Figure 3*A*–3*C*), together with a sharp decrease in SpeB activity (Figure 2*D*). About 40% of GAS isolated from STSS carry a mutation in the CovR/S regulatory system conferring the AP phenotype, characterized by upregulation of virulence factors and increased virulence [24, 31], which conveys a survival benefit for the invasive bacterium [25]. We therefore tested the effect of subinhibitory CLI concentrations on GAS CI417 displaying such a phenotype. Only a slight increase of SpyCEP and DNase activity and hyaluronic acid content were observed, while SpeB activity was largely reduced (Supplementary Figure 3*D* and 4*A*–4*D*). Taken together, these results show in vitro upregulation of GAS virulence factors activity in the presence of subinhibitory CLI concentrations in CLI-susceptible and CLI-resistant GAS clinical isolates.

Elevated Activity and Expression Levels of GAS M1T1 5448 Virulence Factors in the Presence of Subinhibitory Clindamycin Concentrations

To assess in more detail the effect of CLI on virulence factors activity, we grew the CLI-susceptible and well-characterized GAS M1T1 5448 (GAS WT) in the presence of subinhibitory CLI concentrations. As observed for the clinical isolates GAS CI416 and GAS CI529, subinhibitory CLI concentrations induced the activity of the virulence factors SLO, DNase, and SpyCEP (Figure 3*A*–3*C* and Supplementary Figure 5*A*) and elevated the level of hyaluronic acid content (Supplementary Figure 5*B*), while SpeB activity was suppressed (Figure 3*D*). Consistent with altered virulence factors activity, subinhibitory CLI concentrations increased SLO, DNase, SpyCEP, and hyaluronic acid at the transcript level and SLO at the protein level, while the SpeB protein level decreased (Supplementary Figure 5*C* and 5*D* and Supplementary Table 3). Subinhibitory CLI concentrations thus induced GAS WT to mirror the more virulent AP phenotype. Probably because of already elevated virulence factors activity, subinhibitory CLI concentrations did not affect GAS AP virulence factors expression and activity, as seen for GAS CI417, while GAS Δ*speB* showed the same pattern as GAS WT. Considering the emergence of CLI-resistant GAS, we tested the sub-MIC of various antibiotics (Supplementary Table 2) targeting protein synthesis, such as linezolid, tetracycline, tigecycline, chloramphenicol, and gentamicin. Linezolid, tetracycline, and chloramphenicol led to upregulated activity of SLO and SpyCEP in GAS WT, similarly to CLI. In contrast, gentamicin and tigecycline did not have any effect on the activity of these virulence factors (Supplementary Figure 6*A* and 6*C*). DNase activity was only consistently boosted by tetracycline (Supplementary Figure 6*B*).

Attenuation of Bacterial Load and DNase Activity by Antibiotic Treatment in a Patient With Necrotizing Fasciitis

As a proof of concept, we assessed whether CLI affects virulence factors activity and bacterial count in a patient with necrotizing fasciitis. In the debrided tissue, the number of viable bacteria and CLI concentrations were assessed in both necrotic tissue specimens and apparently healthy tissue specimens obtained adjacent to the necrotic areas. We found CLI concentrations in the necrotic tissue to be about 10 times higher than the MIC of the strain responsible for the infection (GAS CI777; see Supplementary Table 1 for the MIC) already at the first surgery (day 0), immediately after receiving the first dose of CLI, but also after 3 days of antibiotic treatment (day 2).

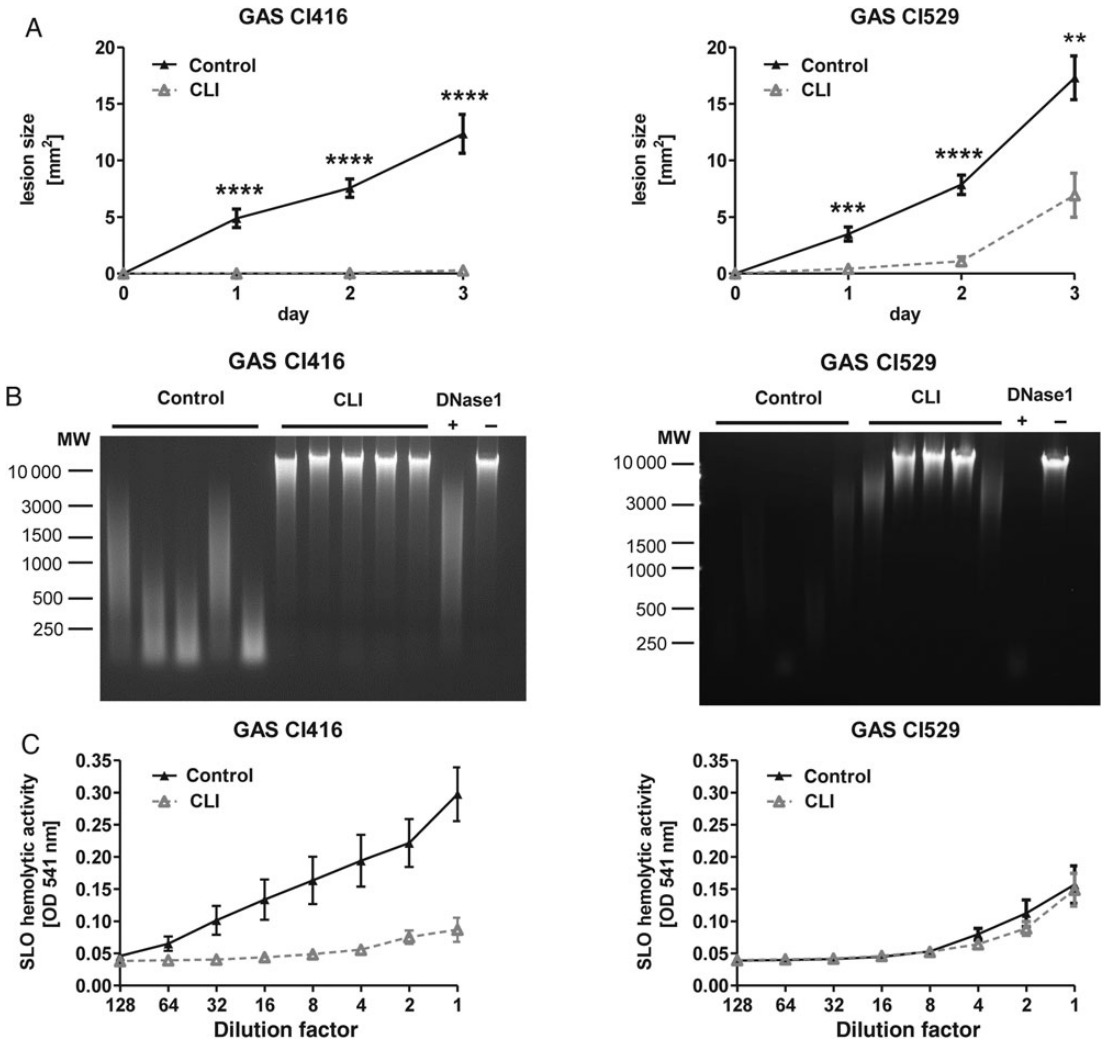

Figure 1. Clindamycin (CLI)–mediated attenuation of clinical severity and inhibition of virulence factors activity in a murine necrotizing fasciitis model. Mice were infected with either the CLI-susceptible (CI416) or CLI-resistant (CI529) group A *Streptococcus* M1 clinical isolate and treated or not with high doses of CLI (100 μg/mouse twice daily intraperitoneally). Skin lesion sizes were monitored over 3 days. Supernatants derived from the infected skin lesions were assessed for extracellular DNase Sda1 and streptolysin O (SLO) activity. *A*, Skin lesion sizes over 3 days after infection. Three independent experiments were performed for the infection with CI416, once using 2 mice/group and twice using 5 mice/group. Two independent experiments (each with 5 mice/group) were performed for the infection with CI529.*B*, DNase activity was visualized by gel electrophoresis; 1 side per mouse is shown. One representative experiment is shown. *C*, SLO activity was assessed by measuring hemolysis of human erythrocytes. One representative experiment is shown. Data show pooled results from 2 (CI529) or 3 (CI416) independent experiments and are presented as mean ± standard error of the mean. $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$, by the unpaired *t* test. Abbreviations: MW, molecular weight; OD, optical density.

The bacterial load in the necrotic tissue was around 10^6 viable CFU/g tissue at the initial surgery despite intravenous ceftriaxone and CLI (Figure 4*A*). Even in the apparently healthy tissue, $>10^3$ viable CFU/g tissue were detected. DNase activity was more pronounced in the tissue with higher bacterial counts. Despite continuous isolation of viable bacteria, on day 2 DNase activity was completely abolished (Figure 4*B*). Histologic analysis of tissue specimens collected on day 0 showed enhanced infiltration of neutrophils and macrophages (Figure 4*C*), and Brown Brenn staining revealed gram-positive cocci intracellularly (Figure 4*D*). After 9 days the wound was closed, and the patient was discharged after 18 days. Together, these data show how difficult it is to reduce the bacterial burden per se despite extensive surgery and the use of bactericidal antibiotics. We can speculate that the activity of the bacterial virulence factors was attenuated by the addition of the protein synthesis inhibitor CLI, which would reflect our observations in the murine necrotizing fasciitis model.

DISCUSSION

In tissue samples from a murine necrotizing fasciitis model, we found a reduction in GAS virulence factor DNase activity and smaller lesion sizes after treatment with CLI despite similar bacterial numbers, reflecting the importance of virulence factors

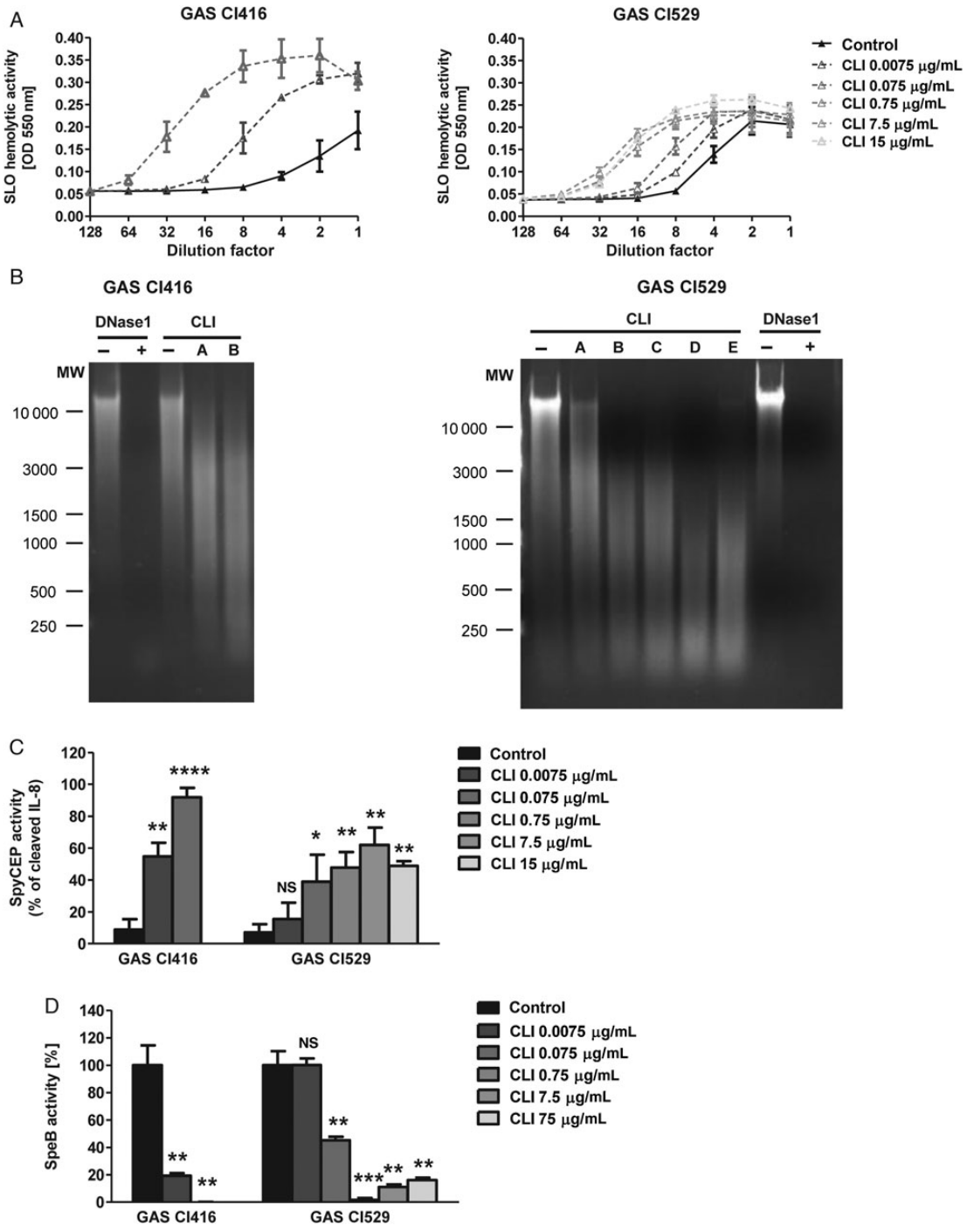

Figure 2. Elevated levels of virulence factors activity in vitro in group A *Streptococcus* (GAS) M1 clinical isolates in the presence of subinhibitory concentrations of clindamycin (CLI). Mid-logarithmic growth-phase supernatants of GAS CI416 and CI529 grown in the presence or absence of subinhibitory CLI concentrations (0.0075 [*A*], 0.075 [*B*], 0.075 [*C*], 0.75 [*D*], and 15 μg/mL [*E*]) were assessed for streptolysin O (SLO), extracellular DNase Sda1, and *Streptococcus pyogenes* cell envelope protease (SpyCEP) activity. Stationary growth–phase supernatants were used for quantification of streptococcal pyrogenic exotoxin B (SpeB) activity. *A*, SLO activity was determined by measuring hemolysis of human erythrocytes. *B*, DNase activity was visualized by gel electrophoresis. One representative gel of 3 independent experiments is shown. *C*, Interleukin 8 (IL-8) cleavage by SpyCEP was assessed by enzyme-linked immunosorbent assay, and the percentage of cleaved IL-8 was calculated relative to the control (IL-8 in THY). *D*, SpeB activity was measured, and the control was set to 100%. *A, C,* and *D*, Data show pooled results from 3 independent experiments and are presented as mean ± standard error of the mean. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001, by the unpaired *t* test. Abbreviations: MW, molecular weight; NS, not significant; OD, optical density.

activity in disease severity. This was true for infections caused by both CLI-susceptible and CLI-resistant GAS M1 clinical isolates. These results were mirrored in a patient with necrotizing fasciitis and treated with a combination of ceftriaxone and CLI. These in vivo observations, showing blockage of GAS virulence factors activity and improved outcome of necrotizing fasciitis in

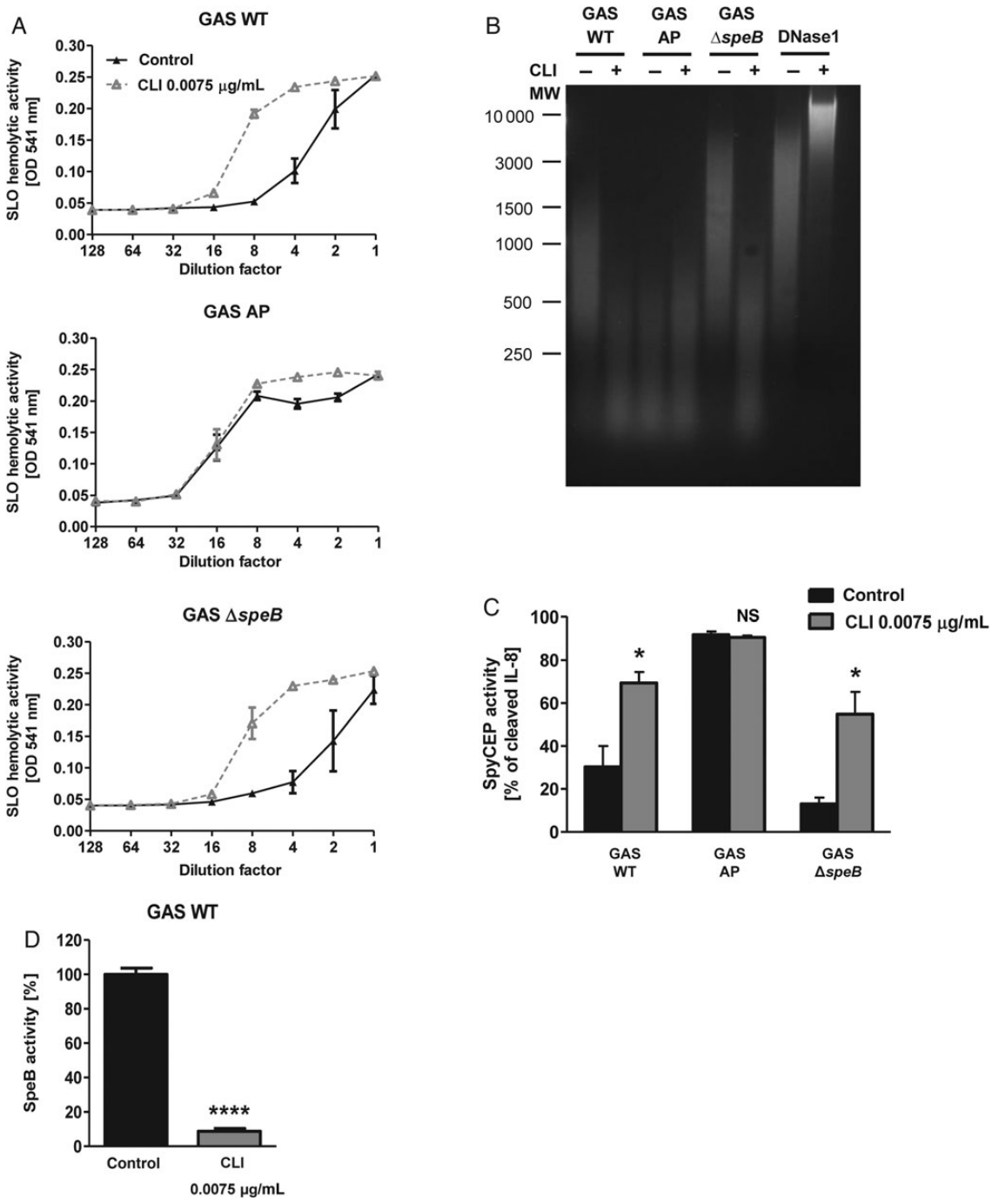

Figure 3. Elevated activity and expression levels of group A *Streptococcus* (GAS) M1T1 5448 strain (WT) virulence factors in the presence of subinhibitory clindamycin (CLI) concentrations. Mid-logarithmic growth-phase supernatants of GAS WT, animal-passaged (AP) GAS, and GAS Δ*speB* grown in the presence (CLI) or absence (control) of subinhibitory CLI concentrations (0.0075 µg/mL) were tested for streptolysin O (SLO), extracellular DNase Sda1, and *Streptococcus pyogenes* cell envelope protease (SpyCEP) activity. Stationary growth–phase supernatants were used for Streptococcal pyrogenic exotoxin B (SpeB) activity quantification. *A*, SLO activity. *B*, DNase activity. One representative gel of 3 independent experiments is shown. *C*, SpyCEP activity. *D*, SpeB activity. *A, C,* and *D*, Data show pooled results from 3 independent experiments and are presented as mean ± standard error of the mean. **P* < .05, ****P* < .001, and *****P* < .0001, by the unpaired *t* test. Abbreviations: MW, molecular weight; NS, not significant; OD, optical density.

mice and a patient, broaden the spectrum of CLI, so far known to act on stationary growth-phase bacteria and to reduce superantigenic activity [2–4]. Our observation that CLI reduced GAS virulence factors activity in vivo, resulting in reduced skin lesion size, shows for the first time the benefits of adding CLI to the treatment regimens of patients with necrotizing fasciitis. This further strengthens the IDSA recommendation that patients with necrotizing fasciitis should be treated with CLI in addition

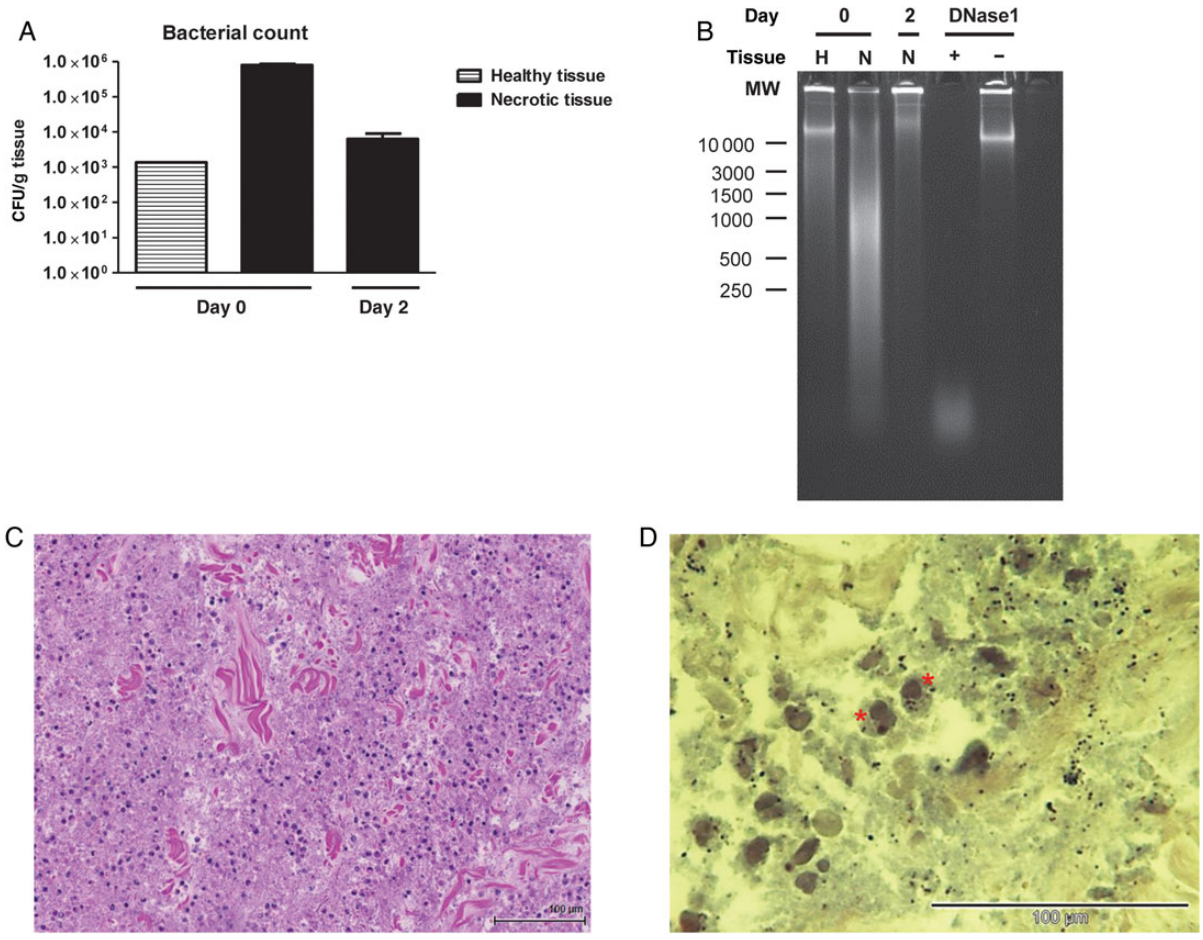

Figure 4. Attenuation of bacterial load and extracellular DNase Sda1 activity by high concentrations of clindamycin (CLI) in a patient with necrotizing fasciitis. Biopsy samples from the patient were lysed in phosphate-buffered saline, using a tissue lyser, and the supernatants were analyzed for bacterial count and extracellular DNase (Sda1) activity. Healthy tissue specimens were collected from regions adjacent to the necrotic tissue. The first surgery was performed on day 0, 32 hours after the start of ceftriaxone treatment and 2 hours after the start of CLI treatment. The second surgery was performed on day 2. *A*, Bacterial counts (in colony-forming units [CFU]) per gram of necrotic or healthy tissue specimens on day 0 and day 2 (data are presented as mean ± standard error of the mean). *B*, DNase activity in tissue supernatant. *C*, Histologic analysis of necrotic tissue on day 0, showing necrosis and diffuse inflammatory infiltrate with residual fascia by hematoxylin-eosin staining. *D*, Histologic analysis of necrotic tissue on day 0, stained with Brown Brenn stain, with intracellular gram-positive cocci in macrophages marked by asterisks. Abbreviations: H, healthy tissue specimens; MW, molecular weight; N, necrotic tissue specimens.

to a β-lactam antibiotic and surgical debridement [1]. We also show that empirical CLI treatment independent of the GAS CLI susceptibility is beneficial, despite the fact that CLI might not be able to completely eradicate the bacteria in the case of inducible and constitutively CLI-resistant strains [32]. Taken together with the fact that low-dose CLI treatment in mice slightly reduced disease severity, resulting in an equivalent or slightly more favorable outcome as compared to that for the control group, CLI should be administered as soon as and at the highest dose possible for correct management of necrotizing fasciitis. Concentrations above the MIC in the affected tissue should be reached, with the aim of blocking virulence factors activity.

The observed inhibition of GAS virulence factors responsible for necrotizing fasciitis disease severity goes in line with a previous in vivo study showing that the use of high doses of protein synthesis inhibitors attenuates *Staphylococcus aureus* virulence factors such as the α-toxin and Panton-Valentine leukocidin [33]. In addition, several studies show reduction of the synthesis of various exoproteins and superantigens in GAS [3, 12, 34, 35] and *S. aureus* at subinhibitory CLI concentrations [28, 36]. In vitro, however, we found that subinhibitory CLI concentrations increased the activity and protein expression of the GAS virulence factors SLO, DNase, SpyCEP, and hyaluronic acid in all GAS strains tested. Thus, subinhibitory CLI concentrations, as may be found after insufficient dosing, did not reduce bacterial growth but increased virulence factors activity and expression in GAS WT, resulting in the more virulent GAS AP–like phenotype. This may explain in part disease progression despite antibiotic administration, stressing again the importance of prompt treatment and a high dose of antibiotics.

We observed persistence of viable GAS in the necrotic tissue of the patient despite high doses of cell wall–active antibiotics ceftriaxone and CLI. Intracellularly located GAS were previously found in patient's tissues despite treatment with a β-lactam antibiotic coupled with CLI [37]. Also, in our murine infection model, high numbers of viable bacteria were isolated after treatment with high doses of CLI. These findings illustrate how difficult eradication of GAS is and make it obvious that further research is required. We are aware of the fact that only 1 patient was examined in this study and that the patient received CLI in combination with a β-lactam antibiotic. Despite this, and taking into account the effect that CLI had in the mouse model, we speculate that CLI treatment is beneficial for necrotizing fasciitis management in patients.

About 40% of GAS isolated from STSS show a distinct AP-like phenotype in which the broad-spectrum protease SpeB is downregulated and several virulence factors are upregulated [31]. Responsible for this phenotypic switch is a point mutation in the sensor kinase CovS gene, involved in the transcription-regulation of virulence factors [20, 31]. The GAS AP M1T1 laboratory strain displays such a phenotype, which conveys a survival benefit to the invasive bacterium [31]. We thus investigated whether subinhibitory CLI concentrations affect the virulence factors activity of GAS AP–like strains and included the isogenic knockout strain GAS Δ*speB* as a control to check for the SpeB dependence of the phenotype [23, 38]. Whereas virulence factors were upregulated in GAS Δ*speB* similarly to GAS WT, GAS AP virulence factors remained unaffected by addition of subinhibitory CLI concentrations. We conclude that the enhancing effect of CLI on virulence factors activity was not detectable, owing to the already elevated virulence factors level. The downregulation of SpeB induced by subinhibitory CLI concentrations in GAS WT might lead to reduced SpeB-mediated cleavage of other virulence factors, thus enhancing their activity. However, since subinhibitory CLI concentrations enhanced messenger RNA transcription of all tested virulence factors, the downregulation of SpeB activity alone cannot explain the effect of subinhibitory CLI concentrations on virulence factors activity observed in GAS WT.

Considering the emergence of CLI-resistant GAS strains [13–15], we investigated additional treatment options for invasive GAS infections and assessed other protein synthesis inhibitors routinely used in clinics. Sub-MICs (ie, concentrations one fourth of the MIC) of linezolid, tetracycline, and chloramphenicol increased virulence factors activity in vitro in a similar fashion to CLI. Our results go along with the findings by Tanaka et al [9]. In contrast, a reduction of virulence factors activity with sub-MICs of linezolid was described in GAS serotype M3 grown to stationary growth phase [39], as was the case for tigecycline and gentamicin. Gentamicin is already routinely used to treat streptococcal endocarditis when the penicillin MIC is >0.1 mg/L [40, 41]. Thus, it may be considered an alternative option for treating severe invasive GAS infections, although nephrotoxicity and ototoxicity are feared complications.

We are the first to show in a comprehensive in vivo analysis that the addition of high doses of CLI, even in the presence of CLI-resistant GAS strains, resulted in improved clinical outcome. These clinical benefits were directly linked to reduced virulence factors activity and therefore illustrate the important implications for the clinical use of CLI. In conclusion, CLI treatment proved to be beneficial for the reduction of disease severity and virulence factors activity in vivo. On the other hand, exposure to subinhibitory CLI concentrations in vitro increased virulence factors activity and abolished SpeB activity in CLI-susceptible and CLI-resistant GAS strains, possibly leading to increased virulence and a substantial survival advantage in the host [20]. This increase in virulence factors activity under subinhibitory CLI concentrations illustrates the importance of using CLI doses that are high enough. It may give an explanation for disease progression despite antibiotic therapy and underlines the importance of aggressive surgical debridement for halting the disease, as recommended in the guidelines [1].

To summarize, the combination of β-lactams and protein synthesis inhibitors seems logical, considering the rapid bactericidal effect of β-lactams on multiplying bacteria, as well as the dual benefit of protein synthesis inhibitors acting both on stationary growth–phase bacteria and inhibiting virulence factors activity. Our data indicate that, in addition to surgical debridement, CLI should be administered as soon as and at the highest dose possible to reach CLI levels above the MIC in affected tissues.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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