MAJOR ARTICLE

Reduced Ability of Penicillin to Eradicate Ingested Group A Streptococci from Epithelial Cells: Clinical and Pathogenetic Implications

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Background. Group A streptococci (*Streptococcus pyogenes;* GAS) invades human epithelial cell lines. Failure of penicillin to eradicate GAS from the throats of patients, especially those who are GAS "carriers," has been increasingly reported. However, there has been no comprehensive evaluation of how effectively antibiotics that are used to treat GAS enter upper respiratory tract epithelial cells and kill internalized GAS. We examined the viability of ingested, intracellular GAS after epithelial cell exposure to antibiotics commonly recommended for therapy of GAS infections.

Methods. A human laryngeal epithelial cell line (HEp-2) was used. Three techniques were used to study antibiotic (penicillin V, erythromycin, azithromycin, cephalothin, and clindamycin) killing of ingested GAS: examination by electron microscopy of ultrathin sections of ingested GAS, qualitative determination of intra–epithelial cell antibiotic, and special stain evaluation of intracellular GAS viability after epithelial cell exposure to antibiotics.

Results. GAS survived intracellularly despite exposure of the GAS-containing epithelial cells to penicillin. In contrast, there was killing of ingested GAS after exposure of epithelial cells to either erythromycin or azithromycin. Electron microscopy confirmed a lack of intracellular GAS fragmentation (cell death) after exposure of epithelial cells to penicillin in contrast to obvious GAS fragmentation after epithelial cell exposure to erythromycin or azithromycin. Cephalothin, a cephalosporin, and clindamycin were more effective in killing ingested GAS than was penicillin, but they were less effective than erythromycin or azithromycin.

Conclusions. These observations strongly suggest that if the GAS upper respiratory tract carrier state results from intra–epithelial cell GAS survival, the failure of penicillin to kill ingested GAS may be related to a lack of effective penicillin entry into epithelial cells. These unique observations may have clinical implications for understanding GAS respiratory tract carriers and managing GAS infections.

During the past decade, studies have demonstrated that group A streptococci (*Streptococcus pyogenes;* GAS) invade human epithelial cell lines in vitro [1–4]. During roughly the same period, there have been clinical reports of the reduced efficacy of penicillin in eradicating GAS from the throats of patients [5, 6]. However, there have been no direct studies of how effectively those antibiotics that are most often recommended to treat GAS infections of the upper respiratory tract enter respiratory epithelial cells and kill ingested GAS. Such information is potentially important for clinical management, especially if intracellular persistence of GAS has a pathogenetic role in the GAS upper respiratory tract carrier state. Difficulty in eradicating GAS from the upper respiratory tract of carriers using penicillin therapy has been documented, but never fully explained [7]. Despite this fact, the almost universal recommendation of penicillin therapy for the treatment of GAS pharyngitis has essentially remained unchanged [8–11].

Eradication of GAS from the throats of carriers has previously not been considered necessary. However, re-

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cently published data indicate that ingested GAS are not only involved in recurrent infections, but have the potential for subsequent dissemination throughout the host [12, 13]. The data from these studies raise the idea that perhaps the GAS upper respiratory tract carrier state should not be clinically or epidemiologically ignored.

We have studied intracellular survival of ingested GAS organisms after antibiotic exposure of the epithelial cells. Our findings, using 3 different in vitro techniques, demonstrate that penicillin does not effectively kill intracellular GAS. In contrast, several antimicrobial agents that readily enter the respiratory epithelial cell (e.g., macrolide/azalide classes) readily kill susceptible strains of GAS.

MATERIALS AND METHODS

Sixteen strains belonging to M-1, M-3, M-6, M-12, M-18, M-49, and M-75 GAS types were used in preliminary studies. On the basis of these studies, the following strains were selected for further examination: isolate A553 (University of Minnesota no. AB1234, from a patient with uncomplicated pharyngitis), an opacity factor (OF)–negative, M-type 1 GAS; isolate A556 (University of Minnesota no. AB1737, from a patient with uncomplicated pharyngitis), an OF-negative, M-type 3 GAS; and isolate A568 (University of Minnesota no. AB1741, isolated from a patient with uncomplicated pharyngitis), an OF-positive, M-type 75 GAS.

Antibiotic susceptibility studies (E-test; AB Bio-disk) revealed strain A553 MICs for penicillin G, erythromycin, cephalothin, and azithromycin to be 0.008 μg/mL, 0.064 μg/mL, 0.125 μg/mL, and 0.38 μg/mL, respectively. For strain A556, the corresponding MIC values were 0.012 μg/mL, 0.094 μg/mL, 0.094 μg/mL, and 0.38 μg/mL, respectively. For strain A568, the corresponding values were 0.016 μg/mL, 24.0 μg/mL, 0.125 μg/mL, and 32.0 μg/mL, respectively.

Antibiotics (penicillin, erythromycin, and cephalothin) were obtained from a commercial source (Sigma-Aldrich). Azithromycin was obtained through the courtesy of Pfizer. In some experiments, clindamycin (Sigma-Aldrich) was also tested.

Cell lines. Human laryngeal epithelial cell line HEp-2 (ATCC CCL23) and a human lung carcinoma cell line (A549; ATCC CCL185) were used. Cells were grown as described previously, with the modification that antibiotic-free Dulbecco's modified Eagle medium (DMEM) was used [14].

Antibiotic penetration into HEp-2 cells. For each antibiotic, 1×10^7 epithelial cells were seeded onto 6-cm Petri dishes and cultivated in DMEM. Cells were grown for 6 h in the presence of 300 μg/mL, 30 μg/mL, and 3 μg/mL of each tested antibiotic. Cells were trypsinized and centrifuged at 4000 U/min for 10 min, resuspended in phosphate-buffered saline (PBS), and centrifuged. To lyse the HEp-2 cells, the pellet was resuspended in 250 μL of ice-cold 0.1% Triton X-100 and incubated for 10 min at −20°C. After centrifugation, 10 μL of the resulting supernatants were dropped onto blood agar plates (Columbia agar with 5% sheep's blood) on which a lawn of either strain A556 or A568 GAS was grown. The plates were incubated in 5% CO_2 for 18 h at 37°C.

Infection assay and live/dead staining. A total of 3×10^5 cells/mL were seeded onto 12-mm glass cover slips in 24-well plates and cultivated in DMEM. The culture medium was replaced with 0.5 mL Hepes-buffered DMEM medium containing 10% fetal calf serum. Overnight cultures of GAS isolates A556 or A568 were grown in tryptic soy broth containing 1% fetal calf serum in 50 mL Falcon tubes at 37°C. Bacterial pellets were resuspended in 0.5 mL PBS and adjusted with PBS to a transmission of 10 at 600 nm. Twenty μL of the suspension was added to each cover slip. After 1 h, cover slips were washed with Hepes-DMEM to remove nonadherent bacteria. Then, each tested antibiotic was added in a concentration of 3 μg/mL. After another 2 h, the washing step and the addition of antibiotic were repeated. After 6 h of infection, the cover slips were washed twice in PBS and stained with the "live/dead" staining kit (LIVE/DEAD *Bac*Light Bacterial Viability Kits; Molecular Probes Europe BV) for 15 min in the dark at room temperature, then washed with PBS and fixed with 1% formaldehyde in PBS for 5 min at room temperature before mounting. Samples were examined with a Bio-Rad MRC1024UV confocal microscope using the 488 nm and 564 nm lines of a krypton/argon laser (Ion Laser Technology) and Lasersharp 2000 software, version 5.2 (Biorad).

Transmission electron microscopy. For morphological analysis of GAS-infected monolayers, 1×10^7 epithelial cells were seeded onto 6-cm Petri dishes and cultivated as earlier described. The infection assay was performed by adding 200 μL of the adjusted bacterial suspension to 5 mL of Hepes-DMEM medium. After 6 h of infection, samples were fixed with a solution containing 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer (0.1 mol/L cacodylate; 0.01 mol/L $CaCl_2$; 0.01 mol/L $MgCl_2$; 0.09 mol/L sucrose; pH, 6.9) for 1 h on ice and washed with cacodylate buffer; the samples were then embedded [15]. Ultrathin sections were cut with a diamond knife, picked up with copper grids (300 mesh), and counter-stained with lead-citrate for 2 minutes before examination with a Zeiss EM 910 transmission electron microscope at an acceleration voltage of 80 kV.

RESULTS

The 16 strains included in this study invaded HEp-2 cells with differing efficiency. Some strains remained within the cells for >24 h without destroying them, whereas others disrupted the cells after a few hours. For more detailed evaluation, we selected the strains that invaded the HEp-2 with high efficiency and led to the death of cells in a time frame that allowed us to perform

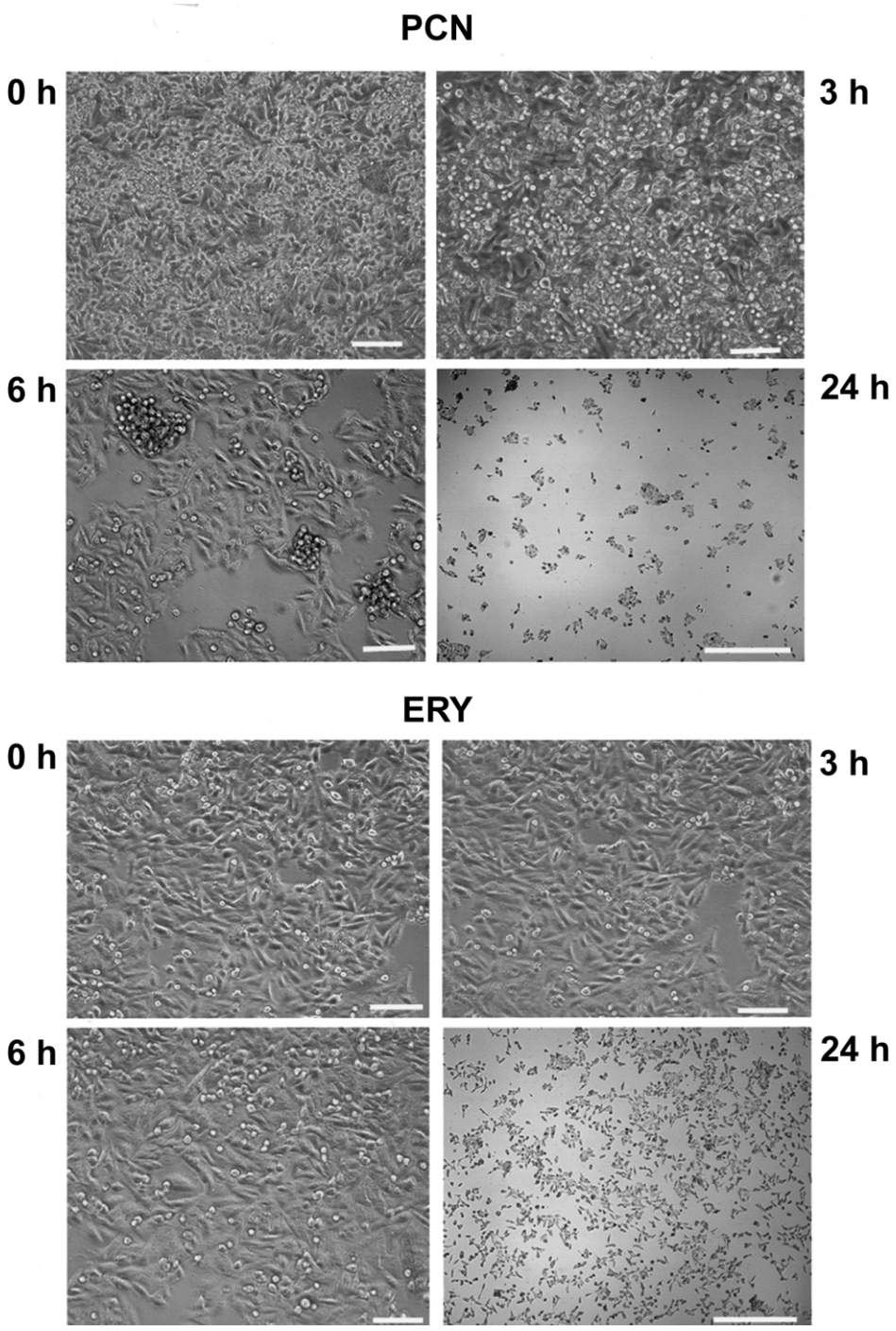

Figure 1. The effects of penicillin (PCN) and erythromycin (ERY) on group A streptococci internalized within confluent HEp-2 cell layers in tissue culture.

the in vitro experiments. In the initial experiments, HEp-2 cells were infected with GAS strain A556 and then exposed to 3 μg/mL penicillin V or to 3 μg/mL erythromycin for various time intervals (figure 1). The confluent cell layer was destroyed after 6–24 h of penicillin exposure, whereas erythromycin exposure of the cell monolayer resulted in an unaltered monolayer after 6 h and a much less disrupted monolayer after 24 h. Because penicillin did not kill the ingested GAS, the still-viable intracellular GAS lead to the death of the HEp-2 cells. Intracellular penetration of erythromycin resulted in death of the ingested GAS, thereby allowing more HEp-2 cell survival with time.

We also studied the morphology of the ingested GAS strains

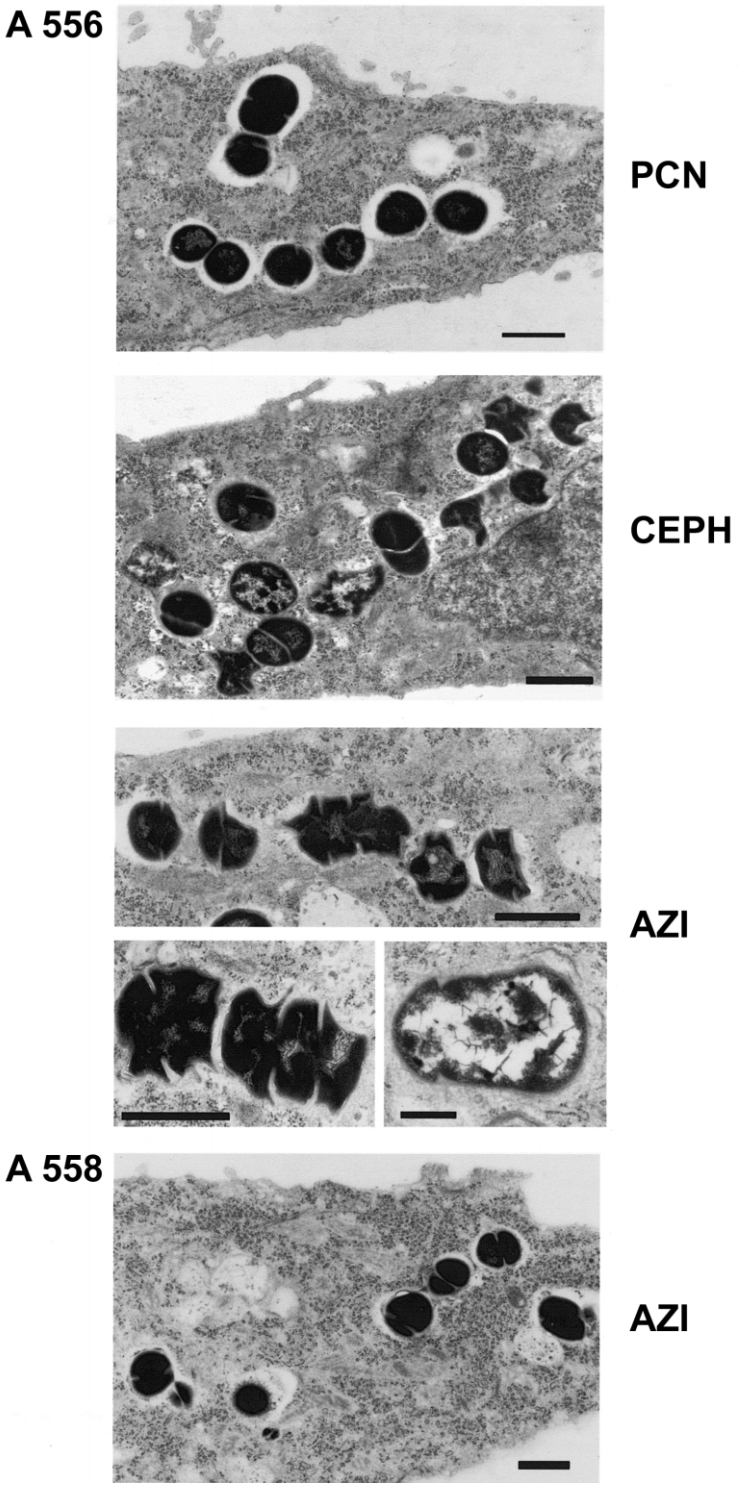

Figure 2. The effects of penicillin (PCN), cephalothin (CEPH), and azithromycin (AZI) that have been added to cell culture media on the morphology of group A streptococci ingested by HEp-2 cells. *Bars,* 1 μm.

after antibiotic exposure within the epithelial cell (figure 2). GAS were allowed to invade HEp-2 cells, and the HEp-2 cells were then exposed to antibiotic. Six h after HEp-2 cell infection with GAS and then following HEp-2 cell exposure to penicillin V, the intracellular streptococci exhibited normal morphological appearance compatible with GAS viability. However, with azithromycin- or erythromycin-exposed, GAS (determined to be susceptible to these antibiotics)–infected HEp-2 cells, there

was marked degradation of intracellular streptococci (i.e., irregularly shaped bacteria and cell envelopes devoid of cytoplasm; data not shown). This observation is consistent with the loss of viability of ingested GAS. Of interest, cephalothin-exposed intracellular GAS revealed only limited intracellular killing when compared with the percentage of ingested GAS killed after erythromycin exposure. An erythromycin-resistant GAS strain (A568) used as a control was not killed by erythromycin; morphologically, the organisms were unaltered and resembled intracellular GAS exposed to penicillin.

Additional experiments were performed to confirm the intracellular presence of antibiotic (figure 3). Uninfected HEp-2 cells were exposed to several concentrations of antibiotics. Then, after HEp-2 cell lysis with Triton X-100, drops of separated intracellular soluble fractions were dropped onto a previously seeded lawn of strain A556 (erythromycin-susceptible) GAS and also on other agar plates with a seeded lawn of strain A568 (erythromycin-resistant) GAS.

Following predisruption exposure of these HEp-2 cells to either erythromycin or azithromycin, the intracellular content of these cells resulted in an hemolysis-free (i.e., absent of GAS growth) zone on the streptococcal lawn; this is because the HEp-2 intracellular content contained macrolide or azalide. In contrast, when penicillin was similarly tested, the intracellular content did not prohibit GAS growth on the lawn, a finding consistent with there being little or no intracellular penicillin in the epithelial cells. After HEp-2 cell exposure to cephalothin, the intracellular content resulted in reduced (but not totally absent) GAS growth on the lawn. This is compatible with limited lethal effect by intracellular cephalothin. Control experiments with the erythromycin-resistant GAS strain (A568) resulted in no effect on lawn growth by the macrolide-containing intracellular content.

To eliminate laboratory-processing artifact as a cause for GAS lawn growth inhibition, additional control HEp-2 cells not previously exposed to antibiotic were lysed and the antistreptococcal effect of the intracellular content (without antibiotic) was studied on a lawn in each experiment. There was no inhibition of GAS growth on any of these control lawns, indicating that the observed zones of inhibited growth during the previously described experiments were caused by the test antibiotics and did not represent artifact.

In a third set of experiments designed to confirm the viability of ingested GAS after GAS-infected epithelial cells were exposed to penicillin (for 6 h), essentially all of the intracellular GAS remained alive (indicated by a green color after staining) (figure 4). In contrast, exposure of GAS-infected HEp-2 cells to either erythromycin or azithromycin resulted in almost total killing of the intracellular erythromycin-susceptible GAS strain (A556); only red-stained (dead) cocci are observed. When a macrolide/azalide-resistant strain (A568) was similarly studied in a control experiment, essentially no lethal effect—either by erythromycin or by azithromycin—was evident; live bacteria (stained green) were seen. In other experiments after similar HEp-2 exposure to cephalothin, both live and dead intracellular GAS are visible in approximately a 50:50 ratio. Similar results to exposure to cephalothin were obtained with clindamycin.

This third group of experiments confirmed the 2 previous sets of experiments; the result was also consistent with the lack of penicillin penetration into HEp-2 cells, the penetration of erythromycin and azithromycin into HEp-2 cells, and a limited intracellular effect of cephalothin.

DISCUSSION

The intracellular effect of the tested antibiotics was confirmed by 3 separate techniques. Phase microscopy was used to observe the disruption of epithelial cells by ingested GAS. A second technique was used to determine the intracellular presence of antibiotics and their effect on a GAS-seeded agar lawn. A third laboratory technique based on direct staining of the ingested GAS by a special live/dead dye was used to examine intracellular GAS viability. These 3 techniques confirmed that erythromycin or azithromycin effectively killed the ingested, susceptible GAS, whereas penicillin had no lethal effect. Cephalothin and clindamycin also killed ingested GAS but were less effective than erythromycin or azithromycin.

Of possible clinical relevance was the "intermediate" result that was obtained when cephalothin (a first-generation cephalosporin) was studied. It has been suggested that cephalosporins may be more effective in eradicating GAS from the upper respiratory tract than penicillin [16, 17]. Our in vitro qualitative findings comparing the effects of 2 β-lactam antibiotics (penicillin vs. cephalothin) are consistent with that possibility. However, we believe that additional and more-quantitative studies are required for confirmation.

We also studied another epithelial cell line (A459); the results were similar to those achieved with the human laryngeal epithelial cell line HEp-2 (data not shown). Studies were also performed with several other antibiotics using $\geqslant$1 of these 3 techniques. Studies with a semisynthetic penicillin had results similar to those achieved in studies performed with penicillin. Clindamycin results were compatible with penetration into the epithelial cells (data not shown).

The fact that viable GAS killed the host epithelial cell would appear to contradict the hypothesis that intracellular streptococci are responsible for the carrier state. However, the preliminary study results obtained with many strains and the published data showed that the ability to destroy the host cell is strain dependent. It has been shown that strains do exist that are capable of surviving in epithelial cells for a long time without destroying them [14].

The data from these studies have potential clinical and path-

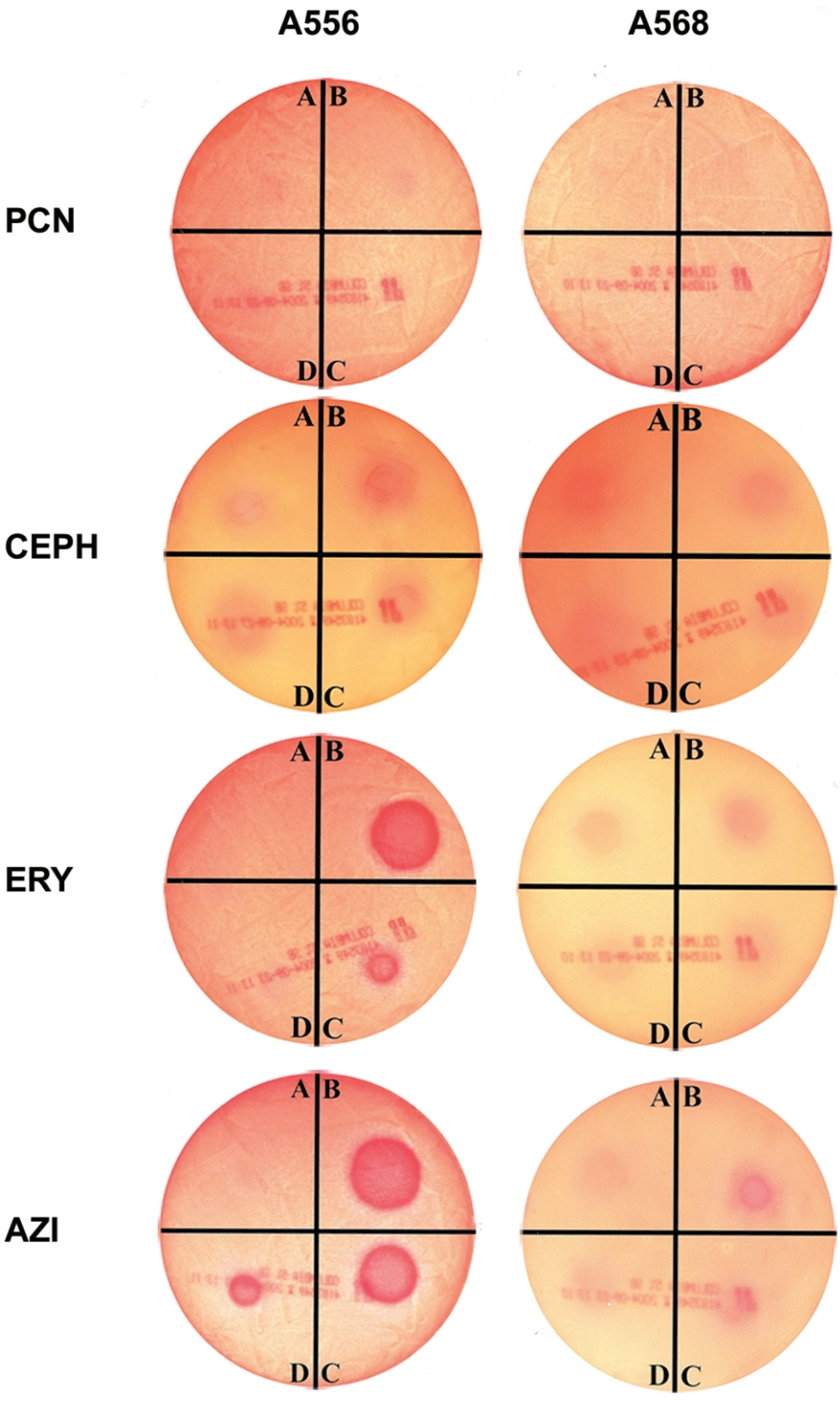

Figure 3. The effects of HEp-2 cell content (soluble fractions) on group A streptococci indicator strain growth on lawns following prior exposure of the HEp-2 cells to antibiotics in different concentrations. *A,* HEp-2 cells not exposed to antibiotics; *B,* HEp-2 cells exposed to 300 μg/mL of the antibiotic; *C,* HEp-2 cells exposed to 30 μg/mL of the antibiotic; and *D,* HEp-2 cells exposed to 3 μg/mL of the antibiotic.

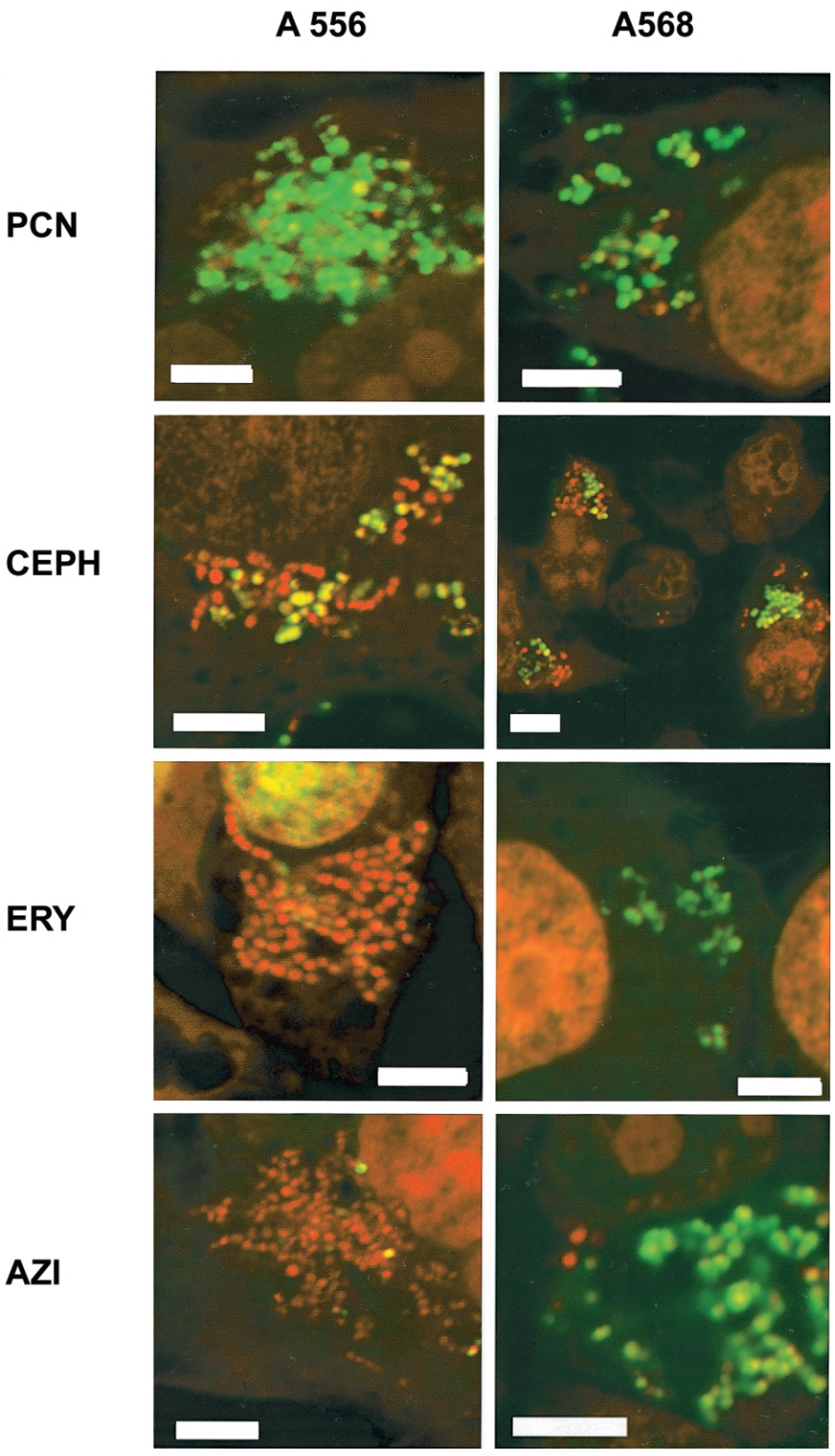

Figure 4. Live/dead staining of previously ingested intracellular group A streptococci following previous antibiotic exposure of the HEp-2 cells. Green cocci are alive; red cocci are not viable.

ogenetic importance. That epithelial cell lines have the ability to ingest GAS has been reported from several laboratories. Our previous studies [2, 4], along with those by LaPenta et al. [1] and Sela et al. [3, 18] have described this. Marouni et al. [3] have also presented limited data that intracellular GAS are not eradicated by prolonged penicillin treatment alone.

The potential clinical importance of our observations relates to the likelihood that in vitro ingestion of GAS by epithelial cell lines is similar to that which occurs in the GAS upper respiratory tract "carrier state" in the human. Our observations of GAS internalization by epithelial cells and inadequate killing effect by penicillin are compatible with published observations of reduced penicillin eradication of GAS from the upper respiratory tracts of GAS upper respiratory tract carriers [7]. Thus, these in vitro observations may suggest a more effective approach for clinical antibiotic management of GAS carriers.

Our data may also have additional clinical implications. Published data with a mouse GAS infection model have demonstrated that ingested GAS may be transported by polymorphonuclear leukocytes to distant parts of the body [12]. The frequent clinical observation that many patients who develop GAS necrotizing fasciitis and/or GAS toxic shock syndrome have no entry wound on or adjacent to the underlying deep tissue site of GAS infection is compatible with the hypothesis that GAS are transported to the site of infection via polymorphonuclear leukocytes. Therefore, these mouse studies imply a need to completely eradicate all (both intracellular and extracellular) GAS from patients through the use of antibiotics that have the capacity to kill intracellular streptococci. More information is needed, and additional studies are in progress to confirm this.

Our data also suggest that the first-generation cephalosporin, cephalothin, might be—at least qualitatively—somewhat more effective than penicillin in killing intracellular GAS in vitro. Whether this is a partial explanation for the observation that some have reported somewhat higher GAS eradication rates from the upper respiratory tracts of patients following cephalosporin therapy than following penicillin therapy [16, 17] cannot be determined solely from our in vitro observations. This important but still unresolved clinical issue requires additional study [19, 20].

Our data, however, should not be interpreted as indicating that macrolides or azalides are clinically more effective in eradicating GAS from the upper respiratory tract. Clinicians must keep in mind that local rates of macrolide resistance remain quite significant in many communities, particularly outside of North America [21].

In summary, these in vitro studies provide data that indicate that GAS ingested by epithelial cells of human origin are not effectively killed when the GAS-containing epithelial cells are exposed to even high concentrations of penicillin in the surrounding media. This finding is in marked contrast to results obtained following exposure of GAS-containing epithelial cells to the macrolide erythromycin or to the azalide azithromycin. The implications might be relevant both for patient care and for public health guidelines for the management of the GAS upper respiratory tract carrier state.

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