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Synergistic Lethal Effect of a Combination of Phage Lytic Enzymes with Different Activities on Penicillin-Sensitive and -Resistant *Streptococcus pneumoniae* Strains

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Pal and Cpl-1, two purified bacteriophage lytic enzymes, were tested for their in vitro activity, alone and in combination, against several serotypes of *Streptococcus pneumoniae*, including penicillin-resistant strains. The enzymes demonstrated synergism in their ability to cleave the bacterial peptidoglycan and thus may be more efficient for the prevention and elimination of pneumococcal colonization.

Streptococcus pneumoniae colonizes the nasopharynx in many adults and in even more children (50% or more), where it is believed to have its reservoir (7). Elimination or reduction of those colonizing organisms could have a major impact on the occurrence of local and systemic pneumococcal disease. New conjugate vaccines for children are being tested extensively and reveal a number of unsolved problems, such as the choice of included serotypes and serotype replacement in colonized children (6). The use of a substance that can specifically eliminate all serotypes of *S. pneumoniae* from the nasopharynx is likely to reduce the bacterial load in the community and enhance herd immunity in combination with a vaccine (1).

We recently proposed a first substance that is able to accomplish this task: Pal, an enzyme from a pneumococcal bacteriophage that can specifically digest any pneumococcal cell wall within seconds, resulting in rapid death of the organism (4). Several such enzymes are known for pneumococcal bacteriophage and are classified into two groups, namely, amidases, which cleave the peptidoglycan between *N*-acetylmuramic acid and L-alanine, and lysozymes, which cleave the glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine (3). Cleavage with either of these enzymes results in a weakening in the cell wall, which leads to the externalization of the cytoplasmic membrane and ultimate lysis. We describe here the killing efficacy of a combination of Pal, an amidase with a molecular mass of 34 kDa, and Cpl-1, a phage lysozyme with a molecular mass of 39 kDa, in vitro. Both enzymes have very different N-terminal catalytic sites and share a similar C-terminal cell wall attachment site, which binds to choline in both cases. We set out to test whether the simultaneous use of both enzymes is competitive and therefore antagonistic or simply additive, or whether it may actually enhance the destruction of the cell wall and hence show synergistic lytic action.

Escherichia coli DH5α(pMSP11) expressing Pal and double-stranded DNA from the pneumococcal phage Cp-1 were obtained from R. Lopez (9). Cpl-1-expressing *E. coli* DH5α(pJML6) was constructed as follows. The Cpl-1 gene was amplified from Cp-1 total phage DNA with a primer pair designed according to the published GenBank sequence number Z47794, flanked by *Xba*I and *Hin*dIII restriction sites, transcriptional start and stop codons, and a ribosomal binding site. To use the same powerful expression system as that used for Pal, which is based on the construct pIN-IIIA and contains a double promoter, we digested the plasmid pMSP11 with *Xba*I and *Hin*dIII, which removes the entire inserted Pal gene (5, 9). The PCR product was subcloned to pMSP11 by using the *Xba*I and *Hin*dIII recognition sites to produce the pJML6 construct. *E. coli* DH5α(pMSP11) and DH5α(pJML6) were grown in Luria-Bertani broth and induced with lactose overnight. Harvest and purification of the enzymes are described elsewhere (4). Both enzymes were stored and assayed in enzyme buffer (50 mM phosphate buffer [pH 7.0] containing 1 mM dithiothreitol and 1 mM EDTA). Activity was measured by exposing *S. pneumoniae* strain DCC 1490 (serogroup 14), grown to log phase and resuspended in sterile saline, to equal volumes of serial twofold dilutions of the enzymes for 15 min in a microtiter plate. The reciprocal of the highest dilution that decreased the optical density by half (from a starting point of 0.5) was defined as the activity in units per milliliter. The specific activity of a freshly produced and purified batch for both enzymes is approximately 1 U/μg.

First we performed time-kill experiments with very short exposures, since the killing of both enzymes can be observed within seconds and a typical application in the nasopharynx would be unique and short. All pneumococcal strains were obtained from A. Tomasz (8). Mid-log-phase cultures of *S. pneumoniae* strains DCC 1355, DCC 1490, DCC 1494, DCC 1335, and DCC 1420 (serotypes 19, 14, 14, 9V, and 23F, respectively, of which the latter three are highly penicillin resistant) were pelleted and resuspended to an absorbance at 600 nm of 1.0 (approximately 10^9 CFU/ml). One hundred fifty microliters of Pal or Cpl-1 at a final concentration of 1 U/ml, or a mixture of both at a concentration of 0.5 U/ml (each), was added to 150 μl of the bacterial solution. Colony counts were performed after 30 s and 10 min and compared to a control exposed to enzyme buffer only, by serially diluting a 10-μl aliquot in saline and plating it on 5% Columbia blood agar, with a detection limit of 10^4 CFU/ml.

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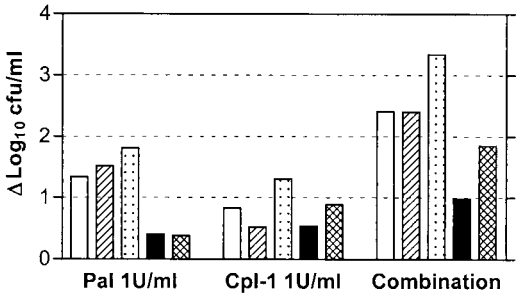

FIG. 1. Killing of five strains of *S. pneumoniae* with 1-U/ml concentrations of Pal, Cpl-1, or a combination of both in 30 s. The combination of the two enzymes shows more than additive killing on a logarithmic scale. Open bar, DCC 1335; hatched bar, DCC 1355; stippled bar, DCC 1420; filled bar, DCC 1494; and cross-hatched bar, DCC 1490.

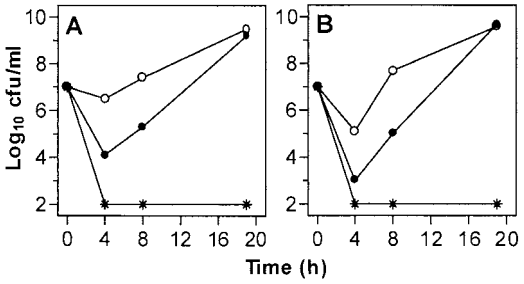

FIG. 2. Time-kill curves for *S. pneumoniae* strains DCC 1355 (A) and DCC 1494 (B) with Pal (filled circles), Cpl-1 (open circles), and a combination of both (asterisks) at 0.25 times the MIC of each (12.5-U/ml concentrations of Pal and Cpl-1 for DCC 1355 and 12.5 U of Pal/ml and 6.25 U of Cpl-1/ml for DCC 1494).

In 30 s, 1 U of Pal per ml reduced the bacterial titer of the four strains by a median of 1.34 $\log_{10}$ CFU/ml (range, 0.38 to 1.81 $\log_{10}$ CFU/ml), while Cpl-1 at 1 U/ml reduced the titers by 0.83 $\log_{10}$ CFU/ml (range, 0.52 to 1.31 $\log_{10}$ CFU/ml). The combination of both enzymes reduced the titers by 2.40 $\log_{10}$ CFU/ml (range, 0.98 to 3.34 $\log_{10}$ CFU/ml) (Fig. 1). After 10 min, the medians and ranges of reduction in $\log_{10}$ CFU/ml were 1.99 (0.73 to 2.54), 1.44 (1.32 to 2.65), and 3.15 (2.50 to 5.28) for Pal, Cpl-1, and the combination, respectively. In other words, mixing 0.5 U of each enzyme/ml increased killing efficacy by a median $\log_{10}$ CFU/ml of 1.07 (at 30 s) to 1.31 (at 10 min) relative to Pal alone and by $\log_{10}$ 1.58 to 1.72 (30 s and 10 min) relative to Cpl-1 alone. By one-way analysis of variance and the Bonferroni posttest for the comparison of the three treatment groups, the killing efficacy of the combination of enzymes was always significantly higher than that of both single enzymes ($P < 0.05$) at both time points, except for that of strain DCC 1494 at 30 s.

Next we performed time-kill studies with longer exposure (up to 19 h), using only strains DCC 1355 and DCC 1494. Bacteria were grown to mid-log phase in cation-adjusted Mueller-Hinton broth with 2.5% lysed horse blood and resuspended in fresh medium at a titer of 1×10^7 CFU/ml. Pal, Cpl-1, or a combination of both were added at 0.25 times the MIC (Pal and Cpl-1 at 12.5 U/ml for strain DCC 1355 and 12.5 U of Pal/ml and 6.25 U of Cpl-1/ml for strain DCC 1494). Samples were taken at 0, 4, 8, and 19 h, serially diluted, and plated on Columbia blood agar for titer determination, with a detection limit of 100 CFU/ml. The usual definition was used for the determination of synergy, namely, a titer reduction by the combination of ≥ 2 $\log_{10}$ CFU/ml more than that by the single most active agent. Figure 2 illustrates the results, which show synergy for both strains.

We then applied the well-described checkerboard broth microdilution method, which allows for the concurrent determination of the MIC of each agent tested (2). Testing was performed repeatedly with all five strains described above in cation-adjusted Mueller-Hinton broth supplemented with 5% lysed horse blood, with a final inoculum of 5×10^4 CFU per well. Both enzymes were assayed at concentrations between 200 and 3.125 U/ml. Plates were incubated at 37°C for 18 h in ambient air and examined visually for growth by using a reflective viewer. The fractional inhibitory concentration index (ΣFIC) was calculated. A ΣFIC of ≤ 0.5 was interpreted as synergy. The MICs were 50 to 200 U/ml for Pal and 25 to 50 U/ml for Cpl-1 in all tested strains. Transcription of the enzyme concentrations along the inhibitory line on the microtiter plate into an isobologram revealed curves with a shape characteristic of synergy. The results for strains DCC 1490 and DCC 1355 are shown in Fig. 3. The ΣFIC for all strains was ≤ 0.5.

The combination of the two lytic bacteriophage enzymes Pal and Cpl-1 appears to have synergistic activity on several *S. pneumoniae* strains, including those that are penicillin resistant, in vitro. This positive interaction could be due to either the increased access of these enzymes to the respective cleavage sites or the enhanced destructive effect of a two-dimensional digestion in the three-dimensional peptidoglycan. Although they are effective in mice, the in vivo efficacy and

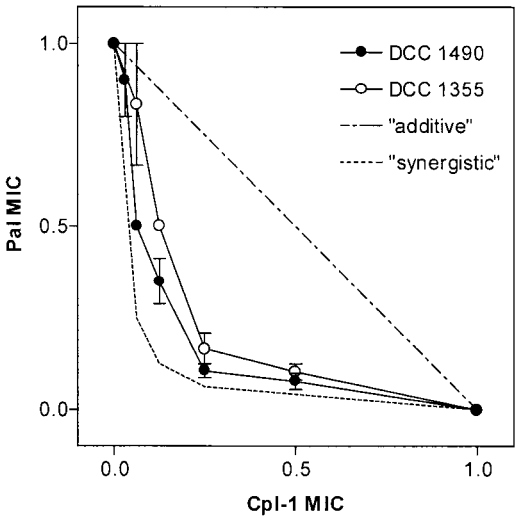

FIG. 3. Isobologram of the checkerboard synergy testing method, showing results for *S. pneumoniae* strains DCC 1490 and DCC 1355. For each well along the inhibitory line, enzyme concentrations (fractions of the enzymes' MICs) were entered in an *x/y* plot. Error bars show standard errors of means. The two dashed lines illustrate theoretical curves.

absence of toxicity of this novel class of antimicrobials remains to be shown in mice.

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REFERENCES

1. **Barbour, M. L., R. T. Mayon-White, C. Coles, D. W. Crook, and E. R. Moxon.** 1995. The impact of conjugate vaccine on carriage of Haemophilus influenzae type b. J. Infect. Dis. **171:**93–98.
2. **Eliopoulos, G., and R. Moellering.** 1991. Antimicrobial combinations. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. Williams & Wilkins, Baltimore, Md.
3. **Garcia, P., A. C. Martin, and R. Lopez.** 1997. Bacteriophages of Streptococcus pneumoniae: a molecular approach. Microb. Drug Resist. **3:**165–176.
4. **Loeffler, J. M., D. Nelson, and V. A. Fischetti.** 2001. Rapid killing of Streptococcus pneumoniae with a bacteriophage cell wall hydrolase. Science **294:** 2170–2172.
5. **Masui, Y., T. Mizuno, and I. Masayori.** 1984. Novel high-level expression cloning vehicles: 10^4-fold amplification of Escherichia coli minor protein. Bio/Technology **2:**81–85.
6. **Pelton, S. I.** 2000. Acute otitis media in the era of effective pneumococcal conjugate vaccine: will new pathogens emerge? Vaccine **19**(Suppl.)**:**S96–S99.
7. **Robinson, K. A., W. Baughman, G. Rothrock, N. L. Barrett, M. Pass, C. Lexau, B. Damaske, K. Stefonek, B. Barnes, J. Patterson, E. R. Zell, A. Schuchat, and C. G. Whitney.** 2001. Epidemiology of invasive Streptococcus pneumoniae infections in the United States, 1995–1998: opportunities for prevention in the conjugate vaccine era. JAMA. **285:**1729–1735.
8. **Sa-Leao, R., A. Tomasz, I. S. Sanches, A. Brito-Avo, S. E. Vilhelmsson, K. G. Kristinsson, and H. de Lencastre.** 2000. Carriage of internationally spread clones of Streptococcus pneumoniae with unusual drug resistance patterns in children attending day care centers in Lisbon, Portugal. J. Infect. Dis. **182:** 1153–1160.
9. **Sheehan, M. M., J. L. Garcia, R. Lopez, and P. Garcia.** 1997. The lytic enzyme of the pneumococcal phage Dp-1: a chimeric lysin of intergeneric origin. Mol. Microbiol. **25:**717–725.