Therapy of Experimental *Pseudomonas* Infections with a Nonreplicating Genetically Modified Phage

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Bacteriophage therapy of bacterial infections has received renewed attention owing to the increasing prevalence of antibiotic-resistant pathogens. A side effect of many antibiotics as well as of phage therapy with lytic phage is the release of cell wall components, e.g., endotoxins of gram-negative bacteria, which mediate the general pathological aspects of septicemia. Here we explored an alternative strategy by using genetically engineered nonreplicating, nonlytic phage to combat an experimental *Pseudomonas aeruginosa* infection. An export protein gene of the *P. aeruginosa* filamentous phage Pf3 was replaced with a restriction endonuclease gene. This rendered the Pf3 variant (Pf3R) nonreplicative and concomitantly prevented the release of the therapeutic agent from the target cell. The Pf3R phage efficiently killed a wild-type host in vitro, while endotoxin release was kept to a minimum. Treatment of *P. aeruginosa* infections of mice with Pf3R or with a replicating lytic phage resulted in comparable survival rates upon challenge with a minimal lethal dose of 3. However, the survival rate after phage therapy with Pf3R was significantly higher than that with the lytic phage upon challenge with a minimal lethal dose of 5. This higher survival rate correlated with a reduced inflammatory response elicited by Pf3R treatment relative to that with the lytic phage. Therefore, this study suggests that the increased survival rate of Pf3R-treated mice could result from reduced endotoxin release. Thus, the use of a nonreplicating modified phage for the delivery of genes encoding proteins toxic to bacterial pathogens may open up a new avenue in antimicrobial therapy.

With the advent of antibiotics, research into phage therapy for bacterial infections was largely abandoned in the West, whereas in the former Soviet Union and in Poland, phage therapy has been used up to the present (33). Animal experiments conducted with scientific rigor showed that phage therapy is effective in treating various bacterial infections (4, 5, 20, 31), and one study (30) indicated that it might even be superior to antibiotics. Several cases in which temperate phage led to increased virulence of the respective pathogens have been reported (23, 34). However, this potential safety risk can be minimized through the use of strictly lytic therapeutic phage and by their detailed molecular characterization. Recent review articles (3, 21) highlight the inherent advantages of phage therapy over antibiotics, such as specific targeting of the pathogen. Nevertheless, phage infections in general culminate in lysis of bacteria and consequently in the release of cell wall components, e.g., endotoxin-release from gram-negative bacteria, which can lead to undesired side effects of phage therapy even when the phage are administered orally (29). Similarly, many β-lactam antibiotics, when used for treatment of systemic gram-negative infections, lead to markedly increased endotoxin levels, which can elicit circulatory shock in patients (15). Hence, there seems to be a need for novel specific and effective bactericidal agents that do not cause this side effect. Toward this goal, it is feasible either to genetically modify lytic phage or to engineer nonlytic phage in such a way that they kill their bacterial target efficiently while leaving the pathogen structurally intact.

In general, large phage possess a dual lysis system consisting of at least a holin and an endolysin (38). At the end of the lytic cycle, the holin compromises the membrane, which allows release of the endolysin to the peptidoglycan. Degradation of this rigid layer is then followed by disintegration of the entire cell envelope, leading to the release of cell wall components. Endotoxin release from gram-negative bacteria can be minimized or avoided through the use or the construction of phage variants with defective endolysin genes. Since the holin function results in dissipation of the membrane potential at the end of the lytic cycle, the bacteria are killed even by endolysin-deficient phage, while the cells retain their structural integrity (25).

Another strategy for avoiding cellular disintegration is the use of nonlytic filamentous phage as a delivery vehicle for genes encoding proteins that are toxic upon their synthesis in the cytoplasm of the host (10, 36). It has recently been demonstrated in a pilot study that in vitro endotoxin release by an M13 variant harboring an endonuclease gene is kept to a minimum relative to that caused by a lytic phage (10). In a conceptually related approach, Westwater et al. (36) have shown that the *Escherichia coli* load in the blood of infected mice can be significantly reduced by using an M13 variant encoding addiction module toxins. In contrast to antibiotics, the concentration of which decreases after administration, the exponential enrichment of phage after lysis or killing of a given
pathogen is generally considered to augment the therapeutic effect (20). However, the use of replicating genetically modified phage can pose a safety risk. The progeny of these phage could be cleared in large quantities from patients to the environment, where they could potentially introduce the toxin genes to otherwise benign bacteria. With this reasoning, in this study we compared the therapeutic efficacy of a nonlytic, nonreplicating phage bearing a toxin gene with that of a lytic phage in treating an experimental Pseudomonas aeruginosa infection of mice.

MATERIALS AND METHODS

Bacterial strains and phage. P. aeruginosa harboring the Rp1 plasmid (designated PAO1 here) and phage Pf3 were kindly provided by A. Kuhn, University of Hohenheim, Stuttgart, Germany. E. coli strain DM1 (18) was used for all cloning procedures. For use as a lysis-inducing phage control, the PAO1 phage Pt1 was isolated from river water by standard procedures. Phage Pt1 formed clear, large plaques on a lawn of PAO1. Only P. aeruginosa strains harboring the IncP1 plasmid RP1 were susceptible, indicating that Pt1 attaches to the same pili and 3/H11032 ORF430 was generated by PCR. A SacI site and a KpnI site were introduced 5/H11032 TTTTTTTGGTACCCTAAAAAAGATCACCAAGG-3/H11032 of the ribosome binding site, and W14 (5/H11032 -TTTTTTTTTTGAG-3/H11032) introduced a PstI site 3/H11032 TTTTTTTTTGCATGCGTCTAATAAAATTTAATATGTCACG-3/H11032 of the stop codon. Next, most of ORF430 was excised from phage Pt1 as described by introducing an NdeI site, allowing in-frame insertion of the BglII restriction endonuclease gene (BglIIIM) while leaving the TGA stop codon of the preceding Pt1 ORF93 intact. This was done by PCR-mediated site-directed mutagenesis using oligonucleotides A20 (5'/GGCCTTTGTGGGAGTCCTCATA-3') and A21 (5'/GCAATAATGAGATGACGTCG-3'). The resulting plasmid was designated pK194ORF430. Plasmid pMBR1 (2) containing BglIIIM was used as a template in a PCR together with oligonucleotides A22 and A23. Oligonucleotide A22 (5'/TTTTTTTTTTCCATATGAAGATTGATATAACGGACT-3') introduced a SacI site 5/H11032 CAGGC-GTAAGAG-3/H11032 of the stop codon of the BglIIIM gene. The KpnI-PstI fragment was inserted into plasmid pUM430, resulting in plasmid pUM430 (Fig. 1).

Construction of Pf3R. First, a 2.593-bp fragment containing ORF430 and a 3,240-bp fragment were obtained by digestion of Pf3 RI DNA with AgeI and BclI. The fragment, containing ORF430, was first ligated into plasmid pSL1180 (8) and then reisolated as a BamHI and HindIII fragment and inserted into the corresponding sites of plasmid pK194 (12). The ATG start codon of ORF430 was modified by introducing an Ndel site, allowing in-frame insertion of the BglII restriction endonuclease gene (BglIIIM) while leaving the TGA stop codon of the preceding Pt1 ORF93 intact. This was done by PCR-mediated site-directed mutagenesis using oligonucleotides A20 (5'/GGCCTTTGTGGGAGTCCTCATA-3') and A21 (5'/GCAATAATGAGATGACGTCG-3'). The resulting plasmid was designated pK194ORF430. Plasmid pMBR1 (2) containing BglIIIM was used as a template in a PCR together with oligonucleotides A22 and A23. Oligonucleotide A22 (5'/TTTTTTTTTTCCATATGAAGATTGATATAACGGACT-3') introduced a SacI site 5/H11032 CAGGC-GTAAGAG-3/H11032 of the stop codon of the BglIIIM gene. The KpnI-PstI fragment was inserted into plasmid pUM430, resulting in plasmid pUM430 (Fig. 1).

Endotoxin assay. Samples (1 ml) of uninfected bacteria and of cultures infected with Pf3R and Pt1 were taken at an optical density at 600 nm (OD600) of 0.2 prior to infection and 90, 240, and 420 min after infection. A multiplicity of infection (MOI) of 50 was used for both phage in this experiment. Supernatant endotoxin levels were determined in triplicate with the QCL-1000 test kit (BioWhittaker, Inc., Walkersville, Md.) according to the manufacturer’s instructions.

Mouse cytokine response. Concentrations of the cytokines tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) were measured in triplicate by using the DuoSet kit (R&D Systems, Inc., Minneapolis, Minn.) as specified by the manufacturer.
Inoculation of mice and serum sample collection. PAO1 cells were diluted in phosphate-buffered saline (PBS) plus 5% mucin, a procedure that reportedly leads to more-reproducible results (37), to yield either the minimal lethal dose (MLD) or three or five times the MLD per 100 μl. Four- to 5-week-old BALB/c mice were inoculated intraperitoneally (i.p.) with 100 μl of bacterial suspension. Phage dilutions in PBS (100 μl) were injected i.p. 45 min, 1 h, or 6 h after infection. Serum samples for measurement of TNF-α and IL-6 levels were obtained by bleeding from the tail vein before infection, shortly before treatment with either Pf3R or Pt1 at 1 h after infection, and 1, 3, and 5 h after treatment. Animals were purchased from Harlan-Winkelmann (Borchen, Germany) and held at InterCell’s animal facilities in accordance with Austrian laws. The mice were allowed to eat and drink throughout the 7-day observation period. Statistical analysis of results was performed by using Fisher’s exact test and forming two-by-two contingency tables, with results for mice receiving no treatment compared to those for the different treatments (26).

RESULTS

Rationale for construction of the Pf3R phage. Pf3 is a filamentous single-stranded DNA phage of PAO1 that does not lyse its host during progeny release (32). In this study, phage Pf3 was equipped by means of recombinant DNA technology with the BglII R gene, whose product does not cleave the replicative form of Pf3 DNA (17). The reasoning was that (i) Pf3R-mediated delivery of the BglII R gene would result in nonrepairable breaks in double-stranded chromosomal DNA and thus in killing of PAO1 and that (ii) the recombinant phage can be stably propagated on a host strain expressing the BglII methylase gene. In addition, the coding region of an export protein gene (ORF430) was replaced with the BglII R gene, whose expression in Pf3R is directed by an upstream phage promoter and by the translational initiation signals of ORF430. This rendered the recombinant Pf3R phage nonreplicative in wild-type PAO1. The Pf3R phage remained genetically stable, and its efficiency at killing PAO1 remained unaltered after 10 passages on strain PAO1(pUM430) (data not shown). High titers of Pf3R were obtained in the production strain harboring a plasmid-borne copy of the phage export protein gene and the BglII methylase gene (Fig. 1).

Pf3R-mediated killing of PAO1. To test whether Pf3R efficiently killed PAO1, and whether the structural integrity of the cells was retained upon killing, we first determined the survival rate and the OD₆₀₀ of PAO1 cultures upon infection with Pf3, Pf3R, or the lytic RNA phage Pt1. Cultures at an OD₆₀₀ of 0.2 were infected with a MOI of 50. The growth of the strain infected with wild-type Pf3 was only slightly retarded relative to that of uninfected bacteria (Fig. 2A). After 90 min, PAO1 cultures infected with either Pf3R or Pt1 showed a >99% decline in CFU (Fig. 2A). As expected, the OD₆₀₀ of the culture infected with the lytic Pt1 phage decreased to almost zero within 90 min. In contrast, the OD₆₀₀ of the culture infected with Pf3R remained constant over 7 h, indicating that the nonviable cells remained structurally intact, whereas Pt1-infected bacteria were almost completely lysed (Fig. 2A).

Endotoxin release upon infection of PAO1 with Pf3R or the lytic phage Pt1. As expected, lysis by Pt1 was accompanied by a rapid increase in endotoxin levels in the supernatant. Compared to the amount present at the time of infection, endotoxin levels were 32- and 60-fold increased at 90 and 240 min, respectively, after infection with Pt1 (Fig. 2B). In contrast, when PAO1 was infected with Pf3R (Fig. 2B), endotoxin levels in the supernatant increased no more than five- and sevenfold at the respective times.

Pf3R and Pt1 therapy of experimental PAO1 infections of BALB/c mice. To establish the MLD in an animal model of infection, BALB/c mice were injected i.p. with varying CFU of PAO1 suspended in PBS supplemented with 5% mucin. The MLD for the PAO1 strain used was found to correspond to 10⁸ CFU. I.p. administration of the MLD of PAO1 resulted in symptoms of ruffled fur, lethargy, and a hunchback posture after 45 min and in severe illness, defined by those symptoms plus exudative accumulation around partially closed eyes, at 6 h postinfection. By 12 h the mice were moribund, and they died within 24 h.

In the initial experiments, the mice were challenged with the MLD following treatment with 10⁶ (MOI, 10), 10⁷ (MOI, 100), 10⁸ (MOI, 1,000), or 10⁹ (MOI, 10,000) PFU of either Pf3R or Pt1 at 45 min postinfection. These studies revealed that an MOI of at least 1,000 of either phage was required to cure the
mice of the lethal infection (data not shown). To obtain a more quantitative picture of the efficacy of phage treatment after infection, in the next experiments, 3 × 10^6 PFU of either Pf3R, Pf3R, or Pt1 phage was administered at 45 min following infection with three times the MLD of PAO1 (MOI, 1,000). Under these conditions, both the lytic RNA phage Pt1 and the Pf3R phage (P < 0.00001), rescued ≥75% of the mice, whereas all untreated and Pt3R-treated mice died within 24 and 48 h, respectively (Fig. 3A). In addition, the mice were challenged with three times the MLD and were then treated at 6 h postinfection with 3 × 10^6 PFU (MOI, 10,000) of either Pf3R or Pt1. Both treatments resulted in rescue of all infected mice; survival was statistically significant, with a P value of 0.0003 (Fig. 3A).

The deleterious effects of endotoxin are expected to increase with higher CFUs of PAO1. Therefore, the mice were next challenged with five times the MLD (5 × 10^7 CFU) of PAO1, and survival was monitored without phage treatment or after treatment with Pf3R or Pt1 (both at 5 × 10^6 PFU) at 60 min postinfection (Fig. 3B). All mice in the negative-control group (n = 15) died within 12 h. At 24 h, 95% of Pt3R-treated mice and 60% of Pt1-treated mice survived (n = 15 for each group). After 48 h, 73 and 20% of the Pt3R- and Pt1-treated mice, respectively, remained viable. No further changes were observed over the following 5 days of observation (Fig. 3B).

FIG. 3. Phage rescue of mice after infection with PAO1. (A) Phage rescue after a challenge with three times the MLD. Mice were monitored for 7 days after i.p. injection with three times the MLD of PAO1 suspended in 100 μl of PBS–5% mucin. Group A (n = 10) did not receive treatment. Groups B, C, and D received phage treatment 45 min after bacterial challenge. Group B (n = 5) received an i.p. injection of 3 × 10^6 PFU of Pf3 suspended in 100 μl of PBS. Group C (n = 15) and group D (n = 15) were treated with 3 × 10^6 PFU of Pf3R or Pt1, respectively. Groups E (n = 5) and F (n = 5) received 3 × 10^6 PFU of phage Pf3R or Pt1, respectively, at 6 h postinfection. (B) Phage rescue after challenge with five times the MLD. Group A (n = 10) was left untreated. One hour after bacterial challenge, groups B and C (n = 15) received i.p. injections of 5 × 10^6 PFU of phage Pt1 or Pf3R, respectively. Results in each panel are representative of two experiments.
experiment (Fig. 2) and the in vivo phage therapy studies of experimental PAO1 infections of mice (Fig. 3) clearly demonstrated the efficacy of Pf3R as an antibacterial agent. A statistically significant difference in survival after phage therapy with Pf3R versus Pt1 was observed upon administration of five times the MLD (Fig. 3B). More than 70% of the mice treated with Pf3R survived the 7-day observation period, whereas only 20% of the mice treated with Pt1 lived past the second day. The Pt1 phage was more effective than Pf3R at killing PAO1 in vitro (Fig. 2) and is able to replicate in PAO1, which is supposed to augment the therapeutic effect. In addition, the Pt1 phage was likewise effective in combating the P. aeruginosa infection when three times the MLD was administered (Fig. 3A). Therefore, we consider it rather unlikely that Pt1 is less efficient than Pf3R at reducing the load of PAO1 in vivo. The low rate of endotoxin after Pf3R-induced killing of PAO1 in vitro (Fig. 2B) was mirrored by the reduced inflammatory response after Pf3R treatment relative to that after Pt1 treatment (Fig. 4).

The mouse inflammatory response to lethal doses of lipopolysaccharide (LPS) has been determined by several groups. Depending on the mouse strain, the source of endotoxin, and the cytokine assays used, considerable variations in the cytokine response were reported (1, 14, 22, 24). TNF-α levels peaked 1 h after LPS injection and ranged from 100 pg/ml (22) to 600 pg/ml (1) to 15,000 pg/ml (14). Reported serum IL-6 levels peaked between 2 and 4 h after LPS injection and ranged from 900 pg/ml (1) to 5,000 pg/ml (14) to 40,000 pg/ml (24). In a murine model of sepsis caused by P. aeruginosa pneumonia, serum IL-6 levels above 3,600 pg/ml were associated with 100% mortality, whereas levels below 1,200 pg/ml were associated with 100% survival (9). Certainly, based on these data, more studies are necessary to assess whether the TNF-α and IL-6 levels upon phage Pt1 treatment determined in this study (Fig. 4) are indicative of endotoxemia. Nevertheless, this study suggests that the higher survival rate of Pf3R-treated mice (see Fig. 3B) could result from reduced endotoxin release. Based on a statistical analysis, it has been suggested that delivery of bactericidal agents by nonreplicating phage may represent a viable antimicrobial strategy (13). In fact, lethal agents delivered by a filamentous M13 phage variant have been shown to reduce the level of E. coli cells in the blood of mice (36). The present study indeed demonstrates that the use of nonreplicative phage in phage therapy of an experimental bacterial infection can be as effective as the use of replicating phage. It remains to be shown whether the same approach can be applied to phage of different bacterial pathogens or whether nonlytic phage are effective against infections where the causative agent is not easily accessible, e.g., in deep tissue infections. However, it seems feasible to use nonreplicative, nonlytic phage for topical treatment or for infections of the gut. Moreover, the use of nonreplicative phage, whether they are genetically modified or not, would allow application of a defined dose of the therapeutic phage rather than a dose that increases exponentially, as in the case of replicating phage. This, in turn, may facilitate the endeavor to obtain certification for phage as pharmaceuticals. A potential problem posed by using a single phage to treat bacterial pathogens is the emergence of phage-resistant bacterial mutants. There appear to be two ways to cope with phage resistance. Either different phage are used to treat a particular pathogen or, alternatively, phage that attach to bacterial surface structures, representing virulence factors, are used (16). For instance, the filamentous P. aeruginosa phage Pf1 adsorbs to type 4 pilii (7), which are required for adhesion to epithelial cells. It is conceivable that resistance to phage may be accompanied by attenuated virulence of the phage-resistant mutants. Furthermore, additional genetic modifications of filamentous phage to broaden their host range are conceivable. It has been shown that filamentous phage containing chimeric Ike and Ff receptor binding domains attach to E. coli possessing either N or F pilii (19). Broadening the host range in this way could be useful for therapeutic purposes. Endotoxin release from gram-negative bacteria is not the only side effect that can be addressed by avoidance of bacterial lysis. Release of superantigens as well as a variety of exotoxins from gram-positive bacteria is likely to increase patient morbidity and prolong recovery. The use of genetically modified lethal but nonlytic phage in therapy could help to avoid these adverse side effects during treatment of infections with gram-positive pathogens.

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