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Long-circulating bacteriophage as antibacterial agents

(phage/bacteria/reticuloendothelial system/toxins/antibiotic resistance)

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ABSTRACT **The increased prevalence of multidrug-resistant bacterial pathogens motivated us to attempt to enhance the therapeutic efficacy of bacteriophages. The therapeutic application of phages as antibacterial agents was impeded by several factors: (*i*) the failure to recognize the relatively narrow host range of phages; (*ii*) the presence of toxins in crude phage lysates; and (*iii*) a lack of appreciation for the capacity of mammalian host defense systems, particularly the organs of the reticuloendothelial system, to remove phage particles from the circulatory system. In our studies involving bacteremic mice, the problem of the narrow host range of phage was dealt with by using selected bacterial strains and virulent phage specific for them. Toxin levels were diminished by purifying phage preparations. To reduce phage elimination by the host defense system, we developed a serial-passage technique in mice to select for phage mutants able to remain in the circulatory system for longer periods of time. By this approach we isolated long-circulating mutants of *Escherichia coli* phage λ and of *Salmonella typhimurium* phage P22. We demonstrated that the long-circulating λ mutants also have greater capability as antibacterial agents than the corresponding parental strain in animals infected with lethal doses of bacteria. Comparison of the parental and mutant λ capsid proteins revealed that the relevant mutation altered the major phage head protein E. The use of toxin-free, bacteria-specific phage strains, combined with the serial-passage technique, may provide insights for developing phage into therapeutically effective antibacterial agents.**

The discovery of viruses that can infect and destroy bacteria was greeted with considerable optimism earlier in this century. d'Hérelle, one of the discoverers of these viruses (bacteriophages), promoted their use as therapeutic agents for the treatment of infectious diseases (1). Despite the efforts of d'Hérelle and other investigators, the use of bacteriophage as an antibacterial therapy was generally abandoned soon after the introduction of antibiotics in the 1940s. To explain the relative failure of bacteriophages as therapeutic agents in human infectious diseases, Stent offered the following suggestions: ". . .the immediate antibody response of the patient against the phage protein upon hypodermic injection, the sensitivity of the phage to inactivation by gastric juices upon oral administration, and the facility with which bacteria acquire immunity or sport resistance against phages" (2). While these factors may have been important, there were also fundamental misconceptions that may have impeded the use of bacteriophage therapy. Perhaps the most serious was d'Hérelle's original belief that there was "but one bacteriophage" (1). We now know that there are many types of bacteriophage, each of which is specific for a specific host range of bacteria. The earlier misconception resulted in applications of phage capable of growing on one bacterial host but with little, if any, ability to influence clinical infections caused by other bacterial strains. Therapeutic failures may also have resulted from the contamination of bacteriophage stocks with debris from bacterial lysis, which typically contain toxins. Such contamination is known to cause symptoms ranging from mild fever to death. In addition, studies concerning the fate of bacteriophage in nonimmune germ-free mice suggested that even in the absence of an antibody response, bacteriophage tend to be rapidly eliminated from the circulation by the reticuloendothelium system (RES) (3), thereby reducing the number of phage available to invade bacteria infecting the patient.

Given the increasing incidence of antibiotic-resistant bacteria, we undertook the current study to determine whether it is possible to increase the efficacy of bacteriophage therapy. This study addressed the concerns noted above by: (*i*) using bacteriophages that are specific for the bacterial strains used to infect the experimental animals, (*ii*) adapting purification methods to reduce toxin concentrations to levels causing minimal side effects, and (*iii*) developing methods to isolate phage mutants that have a capacity to avoid entrapment by the RES. The effects of these methods of improving the *in vivo* antibacterial efficacy of bacteriophages were tested in mice infected with lethal doses of bacteria. In this study, we have used two bacteriophage strains, λ and P22, and their corresponding hosts *Escherichia coli* and *Salmonella typhimurium.* The life cycles of these two phages and their hosts have been characterized both genetically and biochemically in great detail (4–6).

MATERIALS AND METHODS

Bacteria and Bacteriophage Strains. The strains of *E. coli* and *S. typhimurium* and the bacteriophage strains used in this study are described in Table 1.

Preparation of Bacteria and Bacteriophage Stocks and Detection of Toxin Levels in Phage Lysates. For infection of animals, *E. coli* CRM1 or *Sa. typhimurium* CRM3 were grown from a single colony, in 150 ml of LB medium in a shaking incubator at 37°C until the OD_{600} of the culture reached 1.0 [equivalent to 10^9 colony-forming units (cfu)]. After growth, 100 ml of the bacterial culture was harvested by centrifugation at 16,000 × *g* for 10 min at 4°C. The supernatant was discarded, and the bacterial pellet was resuspended in 1 ml of LB at 4°C. From this preparation the bacteria were serially diluted by a factor of 10 in phosphate-buffered saline, from 10^{10} to 10^2. W60, Argo1, and Argo2 phage were grown in CRM1 host and R34, Argo3 and Argo4 phage in CRM3 host, to make high-titer stocks using standard procedures (7). Large-scale preparations and purifications of bacteriophage by cesium chloride density centrifugation were done according to Sambrook *et al.* (8). Toxin levels in phage preparations were measured quantita-

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Abbreviations: RES, reticuloendothelial system; pfu, plaque-forming units; cfu, colony-forming units.

Table 1. Strains of *E. coli* and *Sa. typhimurium* and bacteriophage strains used

Strain number	Relevant genotype	Source
Bacteria		
CRM1	*E. coli* K-12 btuB::Tn10	NIH stock collection
CRM2	*E. coli* K-12 mut D5	NIH stock collection
CRM3	*Sa. typhimurium* LT2	NIH stock collection
Phage		
W60	λcI60cY17	NIH stock collection
Argo1	λcI60cY17*arg1*	This study
Argo2	λcI60cY17*arg2*	This study
R34	P22*vir*3	NIH stock collection
Argo3	P22*vir arg*3	This study
Argo4	P22*vir arg*4	This study

NIH, National Institutes of Health.

tively by the limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA).

Animal Model of Bacterial Infection. For each infection experiment, 1-week-old BALB/c female mice in groups of three were used. To determine the lethal dose of bacteria, serial dilutions (10^2–10^{11} cfu) were injected i.p. into mice, and the animals were observed for 100 hr. In addition, to determine whether bacteria or phage injected into the peritoneal cavity can effectively enter the circulatory system, experiments were done in which either bacteria or phage were injected i.p. followed by the collection of blood samples by puncturing the orbital plexus with a heparinized capillary tube. Approximately 1 ml of blood was collected into a 1.5-ml Eppendorf tube containing 10 μl of heparin (5000 U.S. Pharmacopeia units/ml). The blood samples were centrifuged at $10{,}000 \times g$ for 5 min, and the plasma was collected with sterile pipettes. Plasma from these samples was titered for bacterial cfu and bacteriophage plaque-forming units (pfu). Blood samples were also collected from these mice before injection of phage or bacteria to assure that animals used in these experiments were free of contaminating bacteria and phage. Mice infected with bacteria were scored for levels of illness by monitoring for the following (indicating progressive disease states): decreased physical activity and ruffled fur; general lethargy and hunchback posture; exudative accumulation around partially closed eyes; moribund; and, finally, death.

Experiments to Study the Efficacy of Bacteriophage as Antibacterial Agents. Four groups of mice were infected with 2×10^8 cfu of *E. coli* CRM1 by i.p. injection. We previously found that this dose of CRM1 strain is lethal for BALB/c mice (results not shown). After infection, three of the groups were injected i.p. with 2×10^{10} pfu of bacteriophage λ strain W60. The fourth group was monitored as an untreated control. The mice in this experiment were scored for degree of illness as described above.

Comparison of Protein Profiles of Wild-Type λ and λ Mutants. Preparations of purified bacteriophage were examined by high-resolution two-dimensional protein electrophoresis (9–11) to search for virion protein differences between wild-type and mutant bacteriophage strains. Proteins found to vary by charge or molecular weight were partially sequenced by N-terminal sequencing (12, 13). The protein sequence data were used to search the European Molecular Biology Laboratory data bank to identify phage genes that code for mutant proteins. Genes coding for proteins of interest were cloned and sequenced (8, 14). For PCR and cloning, the two primers selected were 5′-CCA GCG ACG AGA CGA AAA AAC G-3′ and 5′-TTC AGT TGT TCA CCC AGC GAG C-3′, which yield a 1545-bp product from 114 bp upstream to 84 bp downstream of the λ *E* gene.

RESULTS

Experimental Bacteremia in Mice. We followed the presence of *E. coli* strain CRM1 in the mouse circulatory system after i.p. injection of 10^8 and 10^9 bacteria by sampling blood from the orbital plexus. Injection of 10^8 bacteria i.p. resulted in the accumulation of 6.5×10^6 bacterial cfu in the blood by 30 min. The cfu increased to 4.2×10^7 by 3 hr and then decreased to 2.5×10^4 cfu by 24 hr. Similarly, 30 min after i.p. injection of 10^9 bacteria, blood levels of 1.7×10^8 bacterial cfu were observed. These levels rose to 7×10^8 by 3 hr and then decreased to 2.5×10^8 by 7 hr. All of the animals in this latter group died sometime during the next 17 hr.

Use of Virulent Mutants of λ and P22. As models of bacteriophage therapy, we chose to infect mice with a laboratory strain of *E. coli* CRM1 or *Sa. typhimurium* CRM3. The corresponding phage strains specific for these two bacterial species were virulent strains containing specific mutations to assure virulence: λ*vir*(W60) and P22*vir*(R34). The use of virulent bacteriophage strains prevented survival of phage-infected bacteria as lysogens.

Effects of Bacterial Debris and Toxins in Phage Preparations. Injection of 10^9 pfu of filter-sterilized Argo1 phage lysates made in LB broth produced only a mild clinical reaction (ruffled fur) in mice, despite an endotoxin level of 5×10^4 endotoxin units (EU)/ml in the phage preparation as determined by limulus amebocyte lysate assay. In contrast, Argo3 phage lysates prepared in a similar manner on *Sa. typhimurium* had endotoxin levels of 5×10^5 EU/ml, and all the animals died within 12 hr after i.p. injection. However, cesium chloride density-gradient centrifugation-purified stocks of Argo1 and Argo3, with endotoxin levels reduced to 0.3×10^1 EU/ml and 1×10^3 EU/ml, respectively, had no adverse effects at all on mice when injected i.p.

Development and Partial Characterization of Long-Circulating Bacteriophage. Bacteriophage with an enhanced capacity to avoid entrapment by the RES were developed by selecting phage strains that could remain in the circulatory system of mice for progressively longer periods. This selection was started by injecting i.p. into each mouse 10^{11} pfu of W60 grown on either wild-type CRM1 or mutator strain CRM2, followed by collection of blood samples from the mice at 7 hr. The use of the mutator *E. coli* CRM2 bacteria in one set of serial-passage experiments was intended to increase the incidence of mutation in phage λ, so as to enhance the probability that one or more of the phage offspring would have properties that permit evasion of the RES. The phage titers at 7 hr were 10^9 and 10^8 from the mutagenized and nonmutagenized phage, respectively. The phage titers in the circulatory system decreased to 10^2 after 48 hr and to undetectable levels after 120 hr. The residual phage present at 7 hr were grown to high titers in bacteria. These high-titer phage were purified by passing through a 0.22-μm membrane (Millipore, Bedford, MA) filter. This serial cycling of phage, by injection into animals, isolation, and regrowth in bacteria was repeated nine more times. Phage, at titers of 10^{10} pfu, were injected i.p. in the second through the tenth cycles. Of these, only 10^6 pfu remained in the circulatory system at 18 hr in the second cycle, but in subsequent cycles the titers gradually rose, so that 10^9 phage particles remained in the circulatory system at 18 hr in the fourth cycle.

The last six selection cycles provided no significant increase in the number of phage remaining in the circulatory system at 18 hr. After the 10th cycle of the selection process, a single plaque from each of the two experiments was isolated, purified, and grown to high titers on the CRM1 host and designated as Argo1 (cycled on strain CRM1) and Argo2 (cycled on strain CRM2). Both Argo1 and Argo2 displayed similar enhanced capacity to avoid RES entrapment. For example, the 18-hr survival after i.p. injection of Argo1 was 16,000-fold higher and that of Argo2 was 13,000-fold higher than that of the parental λ strain (Fig. 1).

A similar selection process was used to isolate long-circulating variants of the *Salmonella* phage R34. After eight selection cycles, long-circulating single-phage plaques were

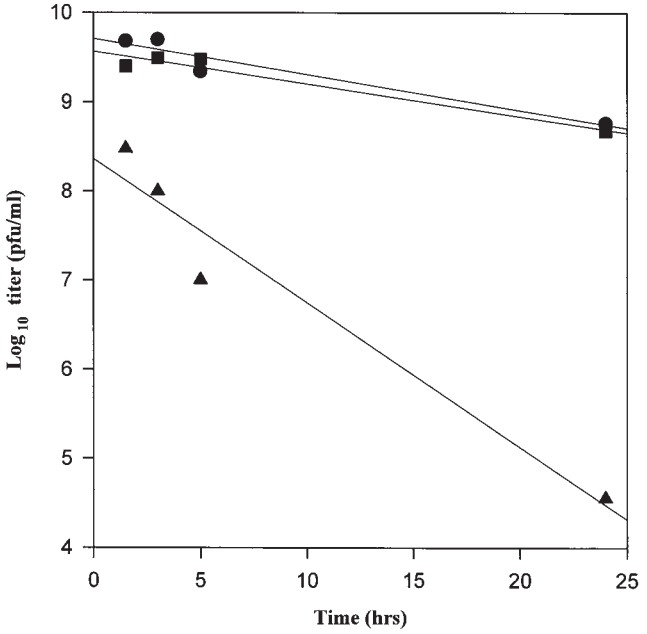

FIG. 1. Capacities of the selected long-circulating phage Argo1 (●) and Argo2 (■) phage to remain in the mouse circulatory system after i.p. injection of 10^{10} pfu into BALB/c female mice were compared to that of the W60 phage (▲). Data are plotted in a semilogarithmic graph in which the logarithm of the phage titer is plotted against the time blood was sampled from the mouse circulatory system in hours. The selected long-circulating mutant Argo1 and Argo2 phage display almost identical enhanced capacities to avoid RES entrapment, in comparison to their parental W60 phage. Regression analysis indicates that the capacity of Argo1 and Argo2 phage to remain in the circulatory system is significantly better ($P = 0.003$) than that of W60 phage.

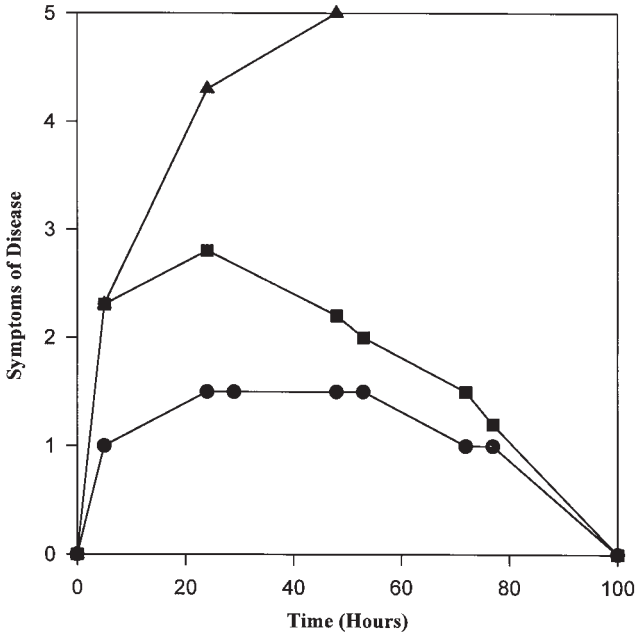

FIG. 2. Comparison of the efficiency of wild-type W60 to the selected long-circulating strains of Argo1 and Argo2 as therapeutic agents for the treatment of bacteremia caused by the i.p. injection of *E. coli* CRM1 into BALB/c female mice. All of the mice were injected i.p. with a lethal dose of *E. coli* CRM1 (2×10^8 cfu). Thirty minutes later the mice in group 2 (■) received an i.p. injection of W60 and the mice in group 3 (●) received an i.p. injection of Argo1. Mice in group 3 (▲) did not receive any phage. A fourth group treated with an Argo2 produced results indistinguishable from those observed with Argo1. The mice were observed and rated according to their condition for a period of 100 hr: 0, normal mouse; 1, decreased activity and ruffled fur; 2, lethargy, ruffled fur, and hunchback posture; 3, lethargy, ruffled fur, hunchback posture, and partially closed eyes with exudate around the eyes; 4, moribund; 5, death. As the observations are categorical condition-stage observations, the actual distances between the states is unknown. For this reason the level of illness indicated is provided simply to record the progression of the disease state. In addition, there was no significant variation of symptoms within any of the experimental groups.

similarly isolated and purified. Two such isolates, designated Argo3 and Argo4, were compared with the parental phage for their rates of clearance from the mouse circulatory system. After an i.p. injection of 5×10^7 pfu, there were no detectable R34 at 24 hr. In contrast, 3×10^2 pfu and 2×10^3 pfu of Argo3 and Argo4, respectively, were detected under the same conditions.

Comparison of Long-Circulating Argo Phage Versus Wild-Type Phage as Antibacterial Agents in an Animal Model. Four groups of mice were injected i.p. with 2×10^8 cfu of *E. coli* CRM1. The mice were scored for degree of illness as described. The first group was a control, with no phage treatment. Within 5 hr these mice exhibited ruffled fur, lethargy, and hunchback posture. By 24 hr they were moribund, and they died within 48 hr. There were three groups treated with 10^{10} pfu of phage. All of the mice treated with phage survived. However, those treated with W60 (group 2) had severe illness before finally recovering, whereas those treated with Argo1 (group 3) and Argo2 (group 4) exhibited only minor signs of illness before complete recovery (Fig. 2).

The following results show that the ability of phage to influence bacterial infections is dose-dependent. Mice were injected i.p. with strain CRM1 (2×10^8 cfu) suspended in phosphate-buffered saline and stored overnight. In contrast to the experiment in Fig. 2, the control group that did not receive any phage in this experiment did not die by 48 hr, an effect that was likely due to reduced virulence of the washed and refrigerated bacteria used in this experiment. However, they did develop moderately severe signs of illness. In the phage treatment groups, 30 min after the bacterial injection the animals received Argo1 phage in doses from 10^2 to 10^{10} pfu. As shown in Fig. 3, at the minimum dose of phage (10^2 pfu) at 30 hr after infection, the animals showed symptoms of disease that were only slightly reduced from that seen in the controls. With increased doses of phage, the animals fared progressively better. At the maximum dose of phage used (10^{10} pfu), the animals showed only minimal illness (decreased physical activity and ruffled fur), and these animals were nearly fully recovered at 48 hr.

Characterization of Argo1 and Argo2 Phage. The capsid proteins of W60 and its two Argo derivatives were analyzed by high-resolution two-dimensional electrophoresis as described. The results revealed an alkaline shift in the 38-kDa major viral protein in Argo1 compared to W60 (Fig. 4). The same electrophoretic protein shift was observed in Argo2. N-Terminal amino acid sequencing of the 38-kDa protein by Edman degradation showed the following sequence for the first 15-amino acid residues for all three phage: S M Y T T A Q L L A A N E Q K. This sequence matched completely with corresponding region of the major λ capsid head protein E (15). Dideoxynucleotide sequencing of the PCR-amplified genes for the λ capsid E protein in Argo1 and Argo2 revealed a G → A transition mutation at nt 6606 in the λ capsid *E* gene in both Argo1 and Argo2. This transition mutation resulted in the substitution of the basic amino acid lysine for the acidic amino acid glutamic acid at position 158 of the λ capsid E protein in both Argo strains. Argo2 protein profiles displayed the presence of an additional altered protein, which also had an alkaline shift. This second protein has a molecular mass of 11.6 kDa. N-Terminal amino acid sequence analysis of the 11.6-kDa protein in W60 and Argo2 phage showed the se-

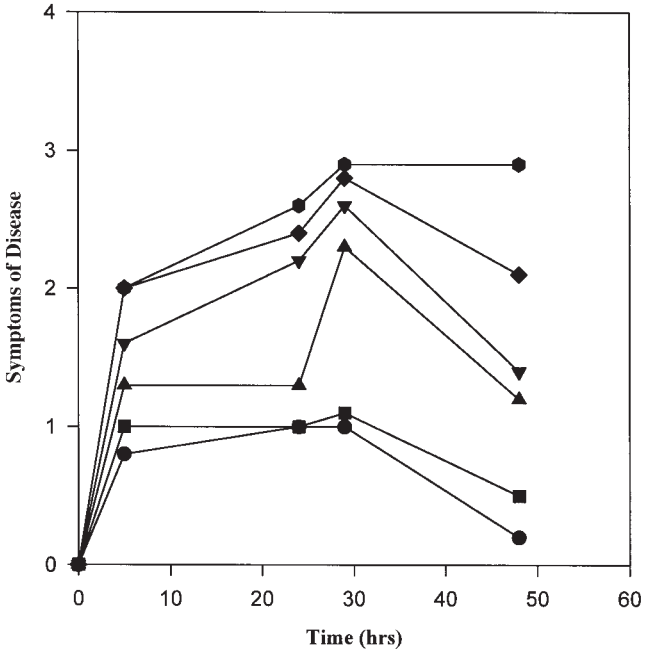

FIG. 3. Dose–response to Argo1 phage in mice infected with *E. coli* CRM1. The experimental design is the same as that used in Fig. 2. In the phage treatment groups, 30 min after bacterial injection the animals received Argo1 phage in the following doses: 10^2 (◆), 10^4 (▼), 10^6 (▲), 10^8 (■), and 10^{10} (●). ●, No phage treatment. Each experimental point represents the results with five animals and had an SEM of 0.2 or less.

quence for the first 16 residues to be M T S K E T F T H Y Q P Q G N S. This sequence is identical with that of the second major capsid head protein D of λ (15). The DNA sequence alteration in the *D* gene of Argo2 was not determined.

DISCUSSION

Despite the enthusiasm with which d'Hérelle and others promoted the use of phage for treatment of infectious disease, such applications are now rare and limited in scope. One of the few remaining, but rarely used, applications of phage in treating infectious disease is based on the use of *Staphylococcus aureus* phage lysates (16–18). Bacterial antigens liberated by lysis in such phage preparations have been reported to have the ability to stimulate cell-mediated immunity and/or delayed hypersensitivity against staphylococcal infections in humans. These lysates are believed to function by enhancing production of antibody in individuals with *St. aureus* infections, rather than by the direct bactericidal effect of the phage.

Although phage therapy by direct bactericidal action was hampered by the misconception that a single strain of phage could be effectively used against many different bacteria (1), some success had been reported when phage strains were used after prescreening for ability to grow on the infectious bacteria (19). In our studies, we chose phage that were specific for the host bacterial strains and that were virulent as well, in order to avoid lysogeny, for the bacteria used in our models of infection. The presence of bacterial toxins in phage preparations may also have limited the effectiveness of phage therapy. Both endotoxins and exotoxins are often released in the lysate during the lytic growth of the phage. Because of this problem, some investigators have administered phage orally to minimize the adverse effects of toxins (20). However, in our studies, no phage particles were detected in the mouse circulatory system after the oral administration of 2×10^9 pfu of W60 (results not shown). We have shown that the toxin content of phage preparations can be diminished >100-fold by purifying phage

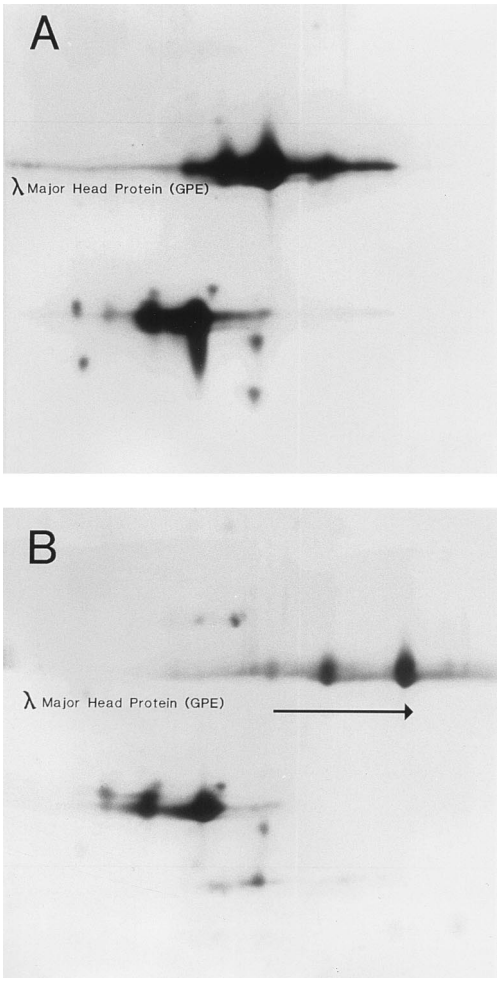

FIG. 4. High-resolution two-dimensional protein electrophoretograms that demonstrate an alkaline shift in charge of the 38-kDa λ capsid E protein found in Argo1 versus the parental W60 phage. The electrophoretic pattern of the region of the gel containing W60 capsid E protein is illustrated in *A*. (*B*) Same region of the electrophoretogram containing the Argo1 capsid E protein, demonstrating the alkaline shift in this protein.

by cesium chloride density centrifugation. While the toxin content of our λ phage preparations grown on *E. coli* CRM1 do not create severe problems and thus do not require extensive purification, in contrast, phage P22 lysates grown on *Sa. typhimurium* CRM3 are lethal in mice and thus require extensive purification before parenteral administration.

The most serious difficulty that may have limited the efficacy of phage therapy is the rapid elimination of >90% of the administered phage from the circulatory system, thereby decreasing the effective dose available for infecting bacteria *in vivo.* Although it was postulated that antibodies may have served as a major factor in the elimination of phage (2), experiments with germ-free animals lacking antibodies against phage have demonstrated that the RES is sufficient for, and highly effective in, the rapid removal of administered phage from the circulatory system (3). Assuming that the removal of phage by the RES depends primarily on the nature of the phage surface proteins, we developed a protocol to select for phage variants that can evade capture by the RES. This protocol involves repeated serial passage of phage through the mouse circulatory system. The procedure selects for phage that can remain in circulation.

In our initial experiments we have isolated by this protocol, variants of *E. coli* phage λ and *Sa. tyhpimurium* phage P22, phage whose wild types have been well characterized physio-

logically and whose genomes have been completely sequenced (ref. 15, and A. R. Poteete, personal communication). In two independent experiments, by passing λ through 10 selection cycles we have isolated two λ variants, designated Argo1 and Argo2. Argo1 and Argo2 displayed, respectively, 16,000-fold and 13,000-fold greater capacity to evade RES entrapment 24 hr after i.p. injection, compared to the parental λ. While Argo1 was the result of a spontaneous mutation, and Argo2 was isolated after mutagenesis, both contained an identical mutation in the λ capsid E protein, which shows the change of glutamic acid to lysine, presumably at the solvent-exposed surface of the phage virion. While the long-circulating Argo2 phage had an additional mutation in the capsid D protein, the presence of a common alteration in the E protein in both of the independently isolated long-circulating phage strains strongly suggests that the change in the capsid E protein is the major factor in diminishing phage entrapment by the RES.

The significance of a double-charge change, from acidic to basic, in avoiding entrapment remains to be determined. Interestingly, both Argo1 and Argo2 displayed an almost identical ability to rescue mice infected with a lethal dose of *E. coli* CRM1, presumably because of their long-circulating nature. The correlation between the ability of the mutant phage to remain in the circulatory system and their ability to rescue bacteremic animals suggests that preventing the capture of phage by the RES may improve the capacity of phage to interact with infecting bacteria.

Because the technique also succeeded in isolating long-circulating mutants of *Sa. typhimuruium* phage P22, the results suggests that our protocol can be used as a general method for obtaining phage capable of reduced capture by the RES. Our results also suggest that such long-circulating phage are useful as antibacterial agents. Although we have addressed a number of problems that may have limited previous applications of phage therapy, phage candidates for therapeutic applications must also be screened against the presence of undesirable phage genes—e.g., the β toxin gene of *Corynebacterium diphtheria* (21), genes encoding antibiotic resistance, and genes that induce lysogeny.

In summary, we have shown that phage can have enhanced therapeutic efficacy when they are (*i*) virulent for the corresponding bacterial host, (*ii*) essentially free of contaminating bacterial toxin, and (*iii*) capable of evading the RES. The development of such phage may provide important tools for the treatment of bacterial diseases.

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1. d'Hérelle, F. (1922) in *The Bacteriophage: Its Role in Immunity*, trans. Smith, G. H. (Williams & Wilkins, Baltimore).
2. Stent, G. (1963) in *Molecular Biology of Bacterial Viruses* (Freeman, San Francisco), p. 8.
3. Geier, M. R., Trigg, M. E. & Merril, C. R. (1973) *Nature (London)* **246,** 221–222.
4. Hendrix, R. W., Roberts, J. W., Stahl, F. W. & Weisberg, R. A. (1993) *Lambda II* (Cold Spring Harbor Lab. Press, Plainview, NY).
5. Poteete, A. R. (1988) Bacteriophage P22, Chapter 11 in *The Viruses, Vol. 2: The Bacteriophages*, ed. Calendar, R. (Plenum Press, NY), pp. 683–724.
6. Neidhardt, F. C., Ingraham, J. L., Low, K. L., Magasanik, B., Schaechter, M. & Umbarger, E. H. (1987) *Escherichia coli and Salmonella typhimurium, Cellular And Molecular Biology* (Am. Soc. Microbiol., Washington, DC).
7. Silhavy, T. J., Berman, M. L. & Enquist, M. L. (1984) *Experiments with Gene Fusion* (Cold Spring Harbor Lab. Press, Plainview, NY).
8. Sambrook, J., Fritch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY) pp. 2.73–2.81.
9. Bjellqvist, B., Pasquali, C., Ravier, F., Sanchez, J.-C. & Hochstrasser, D. F. (1993) *Electrophoresis* **14,** 1357–1365.
10. Hughes, G. J., Frutiger, S., Paquet, N., Ravier, F., Pasquali, C., Sanchez, J.-C., James, R., Tissot, J. D., Bjellqvist, B. & Hochstrasser, D. F. (1992) *Electrophoresis* **13,** 707–714.
11. Merril, C. R., Switzer, R. C. & Van Keuren, M. L. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4335–4339.
12. Hewick, R. M., Hunkapiller, M. W., Hood, L. E. & Dreyer, W. J. (1981) *J. Biol. Chem.* **256,** 7990–7997.
13. Matsudaira, P. (1987) *J. Biol. Chem.* **262,** 10035–10038.
14. Scharf, S. (1990) *PCR Protocols, A Guide to Methods and Applications*, eds. Innis, M., Gelfand, D., Sninsky, J. & White, T. (Academic Press, NY), pp. 84–91.
15. Daniels, D. L., Schroeder, J. L., Szybalski, W., Sanger, F., Coulson, A. R., Hong, G. F., Hill, D. F., Petersen, G. V. & Blattner, F. R. (1983) in *Lambda II*, eds. Hendrix, R. W., Roberts, J. W., Stahl, F. W. & Weisberg, R. A. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 519–676.
16. Salmon, G. & Symonds, M. (1963) *J. Med. Soc. NJ* **60,** 188–193.
17. Mudd, S. & Shayegani, M. (1974) *Ann. NY Acad. Sci.* **236,** 244–251.
18. Dean, J., Silva, J., McCoy, S., Chan, P., Baker, J., Leonard, C. & Herberman, R. (1975) *J. Immunol.* **115,** 1060–1064.
19. d'Hérelle, F. (1930) *The Bacteriophage and Its Clinical Applications* (Thomas, Baltimore), p. 167.
20. Slopek, S., Weber-Dacbrowska, B., Dacbrowski, M. & Kucharewicz-Krukowska, A. (1987) *Archiv. Immunol. Ther. Exp.* **35,** 569–583.
21. Bishai, W. & Murphy, J. (1988) in *The Viruses: The Bacteriophages*, ed. Calendar, R. (Plenum, New York), Vol. 2, pp. 683–724.