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Transduction of *Staphylococcus aureus* to Tetracycline Resistance In Vivo

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ABSTRACT

JAROLMEN, HOWARD (Hahnemann Medical College, Philadelphia, Pa.), AMEDEO BONDI, AND RICHARD L. CROWELL. Transduction of *Staphylococcus aureus* to tetracycline resistance in vivo. J. Bacteriol. 89:1286–1290. 1965.—Staphylophage 80, propagated on a hospital strain of *Staphylococcus aureus* 80/81, has been shown to transduce antibiotic resistance markers to a variety of staphylococcal recipient strains in vitro. In an attempt to demonstrate transduction of penicillin and tetracycline resistance in mice, experiments were performed in which mice were injected intravenously with a pathogenic recipient strain, *S. aureus* N135, and subsequently with transducing phage by the same route. Periodic assays of organs from infected mice revealed that maximal bacterial concentrations were attained in kidneys 6 days after infection, at which time the transducing lysate, containing approximtaely 5×10^{10} plaque-forming particles, was introduced. Isolation of tetracycline-resistant transductants from the kidneys of infected animals was facilitated by the therapeutic administration of tetracycline. In contrast, penicillin-resistant transductants, which produced penicillinase, were not found even when penicillin therapy was administered. Results showed that tetracycline-resistant transductants were recovered from as many as 40% of test animals in repeated experiments. Furthermore, in some of these mice the entire staphylococcal population of the kidneys was found to be tetracycline-resistant. Control infected animals which did not receive phage were uniformly negative for tetracycline-resistant staphylococci. The finding that phage levels were low or undetectable at a time when tetracycline-resistant organisms were recovered from test animals provided evidence that transduction had occurred in vivo.

Ritz and Baldwin (1958) were the first workers to demonstrate transduction of antibiotic resistance in *Staphylococcus aureus*. Their observation opened up new horizons for investigation of the perplexing problem of the frequent development of staphylococcal resistance to the commonly used antibiotics. Pattee and Baldwin (1961, 1962), employing various phages of the International Typing Series, which were propagated on suitable donors, successfully demonstrated transduction of resistance to chlortetracycline, novobiocin, penicillin, and the macrolide antibiotics.

The first demonstration of in vivo transduction of bacteria was that described by Velaudapillai (1960), who showed that transduction of factors controlling synthesis of flagellar antigens could occur between strains of *Salmonella* species in live chick embryos and in mice. There have been no comparable reports of staphylococcal transduction to antibiotic resistance in animals. The objectives of this paper are: (i) to demonstrate that, under appropriate experimental conditions, transduction of staphylococci to tetracycline resistance can be achieved in mice; (ii) to study factors influencing this experimental phenomenon in mice; and (iii) to evaluate the possibility of whether transduction could account for the increase in antibiotic-resistant staphylococci seen in hospitalized patients.

MATERIALS AND METHODS

Strains of S. aureus. S. aureus strain 2800 (phage type 80/81) was employed as the donor of the genetic determinants for both penicillinase production and resistance to tetracycline. This strain was isolated from a clinical infection at the Hahnemann Medical College and Hospital. *S. aureus* strain N135, obtained from the laboratories of Ritz and Baldwin, Ohio State University, Columbus, Ohio, was routinely employed as the recipient of the donor genetic information; it was highly susceptible to both penicillin and tetracycline. Stock cultures were maintained on Trypticase Soy Agar (TSA) slants (BBL), transferred weekly, and stored in the refrigerator. Trypticase soy broth (TSB) and agar were used routinely in all experiments.

Phage. When staphylococcal phage 80, likewise obtained from Ritz and Baldwin, was propagated

on donor strain 2800, the resultant lysate, designated 80/2800, possessed the ability to transduce antibiotic-resistant traits inherent to the donor bacterium. The agar-layer technique of Swanstrom and Adams (1951) was used routinely for preparation of phage stocks. By this procedure, phage titers of 5×10^9 plaque-forming particles (PFP) per milliliter were obtained routinely. For in vivo transduction experiments which necessitated the use of phage suspensions with increased titers, phage stocks were concentrated by centrifugation. Phage, in volumes of 35 ml, was sedimented in the Spinco model L ultracentrifuge at $73,500 \times g$ for 1.5 hr; the pellets were resuspended in 2-ml volumes of TSB, and the filtrate obtained, after passage through Millipore filters mounted in a Swinny hypodermic adapter, provided stock containing titers greater than 5×10^9 PFP per milliliter.

In vitro transduction. The procedure for demonstrating in vitro transduction was essentially that described by Ritz and Baldwin (1961). Transducing phage and *S. aureus* N135 were mixed in reaction tubes, incubated at 37 C for 1 hr, and assayed for transductants on selective TSA plates containing penicillin, 0.12 units per milliliter, or tetracycline, 5 μg/ml.

In vivo transduction. Mice were injected intravenously with 0.2 ml of an overnight broth culture of strain N135. Six days after infection the transducing lysate 80/2800 containing 0.5 to 6×10^{10} PFP per milliliter was introduced intravenously into the surviving animals. (Approximately 30% of the mice had died with staphylococcal infection by this time.) In some experiments, antibiotics were administered subcutaneously to aid in the selection of transductants. Mice were killed at intervals over a course of 6 weeks and their kidneys were assayed for total bacterial and phage content and for number of transductants. Cells of strain N135 which retained their initial phage-susceptibility pattern and acquired the selected antibiotic-resistance trait were considered to be transductants. Groups of infected animals which did not receive transducing phage served as controls to establish that antibiotic-resistant staphylococci did not emerge in the absence of phage.

RESULTS

Standardization of the transducing system in vitro. For the purpose of estimating the frequency of transduction of strain N135 by phage 80/2800 to penicillin and tetracycline resistance, determinations were performed on separate occasions. For these determinations the phage-bacterium ratios were one or less; cells were employed at an approximate concentration of 10^{10} per milliliter. The results of the nine experiments showed that 4.7×10^6 and 1.4×10^6 PFP were required for the production of each penicillin-resistant (pen^r) transductant and each tetracycline-resistant (tetra^r) transductant, respectively. It should be noted that tetra^r transductants were produced at a frequency approximately three- to fourfold greater than that of pen^r transductants.

To determine the effect of phage concentration on production of transductants, increasing concentrations of phage 80/2800 were mixed with a constant number of cells of recipient strain N135 (1.7×10^{10} per milliliter) in a series of reaction tubes and incubated at 37 C for 1 hr. The number of both types of transductants produced was found to be directly proportional to the concentration of phage (Fig. 1). The transduction frequency for both traits derived from these data was comparable to that obtained in previous experiments.

Studies were performed to determine the influence of cell concentration on the frequency of transduction. Increasing cell concentrations of recipient strain N135 were distributed to a number of reaction tubes. Transducing phage 80/2800 in a concentration of 1.3×10^9 PFP per milliliter was introduced into all tubes. The number of transductants in each tube was determined 1 hr later. As the concentration of cells was decreased,

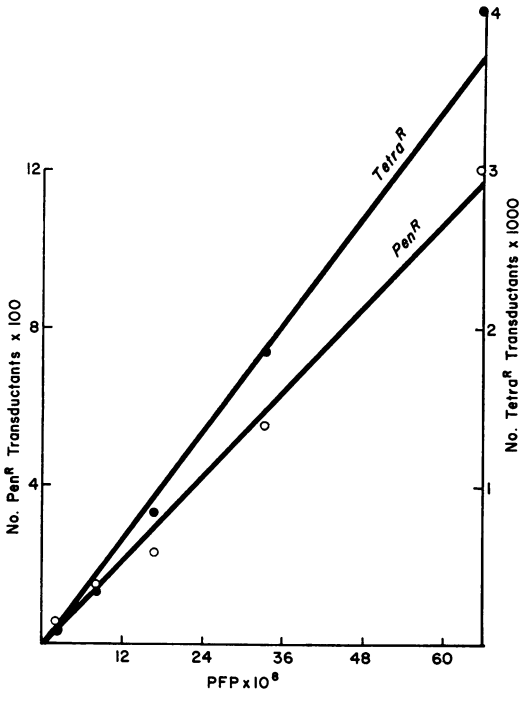

FIG. 1. *Effect of phage concentration on production of transductants. Recipient strain Staphylococcus aureus N135 was employed at a concentration of 1.7×10^{10} cells per milliliter in all reaction mixtures.*

there was a sharp reduction in the incidence of pen^r and tetra^r transductants (Table 1). At a bacterial concentration of less than 3×10^7 cells per milliliter, transduction failed to occur. Absence of transduction at low concentrations of cells was found to reflect inadequate phage attachment during the allotted time period.

Fate of strain N135 in spleen, liver, lungs, and kidneys. Various parameters of the transduction system in vitro having been established, attempts were made to produce staphylococcal transductants in mice. It was concluded on the basis of the in vitro data that a sufficiently high concentration of cells must be achieved in a given organ to allow transduction to occur in mice. The spleen, liver, lungs, and kidneys of mice were cultured for enumeration of viable staphylococcal N135 cells at intervals following intravenous injection. The results (Fig. 2) provided evidence that bacterial multiplication occurred within the kidneys; cell counts increased to a maximum of 10^9 per pair of kidneys 6 days after infection, whereas in the other organs staphylococcal multiplication was not apparent. Consequently, the kidney was selected as the most appropriate organ to study transduction in vivo, and 4 to 6 days after infection was chosen as the optimal time for introduction of transducing phage lysate.

Preliminary attempts at in vivo transduction. The kidneys of numerous staphylococcal-infected mice were studied for phage survival subsequent to injection of phage 80/2800. Results of these studies showed that a concentration of phage considered to be adequate to produce transduction could be achieved for a period of 24 hr after injection. In attempts to demonstrate transductions in vivo, a transducing lysate containing 5×10^{10} PFP was injected intravenously 6 days after infection with strain N135. At intervals, the mice were killed, and the kidneys were assayed for phage and bacterial content as well as for transductants. Many mice yielded transductants at a time when virus concentration was known to be above the level of 10^4 PFP per pair of kidneys (Table 2). Since this amount of phage was shown previously to be adequate for production of transductants in vitro, it was considered that most of the transductants isolated during this period were produced in vitro during the plating procedure. Support for this consideration was

TABLE 1. *Effect of concentration of Staphylococcus aureus N135 on production of transductants**

S. aureus	No. of transductants	
	Pen	Tetra
cells/ml		
3.0×10^{10}	2,050	28,680
3.0×10^9	2,360	33,460
3.0×10^8	145	3,030
3.0×10^7	0	35
3.0×10^6	0	0

* The concentration of phage in all tubes was 1.3×10^9 plaque-forming particles per milliliter.

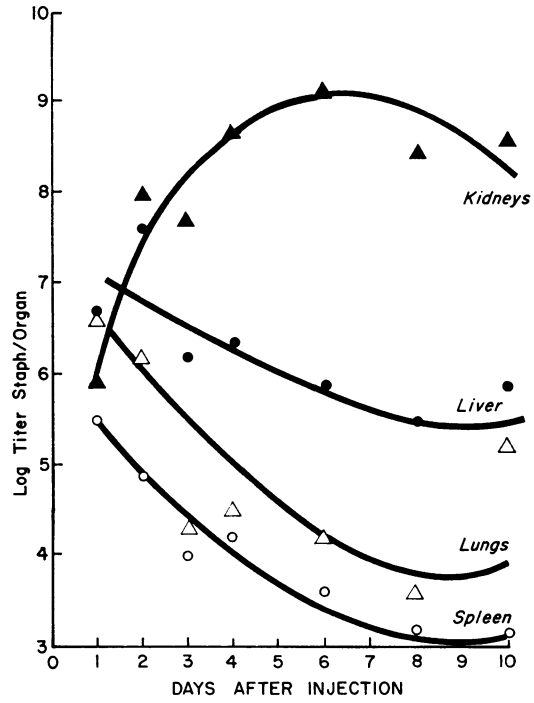

FIG. 2. *Course of Staphylococcus aureus N135 infection in spleen, liver, lungs, and kidneys as revealed by recovery of organisms at intervals after infection. Each count represents an average from four mice.*

TABLE 2. *Staphylococcal transduction of antibiotic resistance in mice*

Time after phage injection*	Phage concn (PFP/kidneys)	Mice with transductants†	
		Pen	Tetra
0–6 hr	10^8–10^6	10/17	16/19
7–30 hr	10^6–10^4	4/15	8/15
2–4 days	10^4–10^2	0/24	6/33
5–14 days	10^2–10^1	0/10	3/20
15–44 days	ca. 10^1	0/18	0/13

* Concentration of phage injected was 5×10^{10} PFP.

† Number of mice positive per number studied.

derived from experiments in which kidneys emulsified in specific antiphage serum showed a greatly reduced incidence of transductants. The transductants, which were isolated from mice at a time when the titer of phage was less than 10^4 PFP per pair of kidneys, however, were considered to have been produced in vivo, since the phage concentration was below that required for in vitro pen^r or tetra^r transduction. Consequently, the tetra^r transductants isolated from the kidneys of 6 of 33 mice 2 to 4 days after phage administration and from 3 out of 20 mice after 5 to 14 days were considered to have been produced in vivo. None of the pen^r transductants isolated was considered to have arisen in vivo.

In vivo selection for resistant transductants. In experiments similar in design to those described previously, the therapeutic administration of tetracycline to test animals facilitated the selection of staphylococci which had been transduced to tetracycline resistance (Table 3). Results showed that transductants were recovered from as many as 40% of the treated animals in repeated experiments. In the untreated group of animals, tetra^r transductants were isolated from only 1 of 20 mice compared to 10 of 26 and 11 of 26 in the 0.2- and 2.0-mg tetracycline-treated groups, respectively. Furthermore, comparatively larger numbers of transductants could be isolated from animals receiving therapy than could be recovered from untreated animals. As many as 10^4 to 10^7 tetra^r transductants per milliliter of kidney suspension were often recovered from treated animals; whereas the number recoverable from untreated animals was rarely greater than 5 to 10/ml of kidney suspension. These results undoubtedly reflect the production by antibiotic therapy of a selective environment which favored the multiplication of drug-resistant transductants over that of drug-sensitive cells. In some of the antibiotic-treated mice the entire staphylococcal population was found to be tetracycline-resistant. Such absolute selection occurred in 2 out of the 10 positive mice which had been treated with 0.2 mg of tetracycline and in 7 of 11 of the 2.0-mg group. Absolute selection was first detected 20 days subsequent to the initiation of therapy. After this period, practically all of the positive mice in the 2.0-mg group exhibited absolute selection. Groups of mice which received staphylococci without phage served as controls. No tetracycline-resistant staphylococci were recovered from the kidneys of any of these animals, regardless of whether or not they received tetracycline treatment. The finding that phage levels were low or undetectable at a time when tetra^r cells were recovered from test animals provided strong evidence that transduction had occurred in vivo.

TABLE 3. *Staphylococcal transduction of tetracycline resistance in mice treated with tetracycline*

Phage injected	Tetracycline*	Mice with transductants†	Mice showing absolute selection†
	mg		
+	—	1/20	0/1
+	0.2	10/26	2/10
+	2.0	11/26	7/11
—	—	0/19	
—	2.0	0/25	

* Tetracycline therapy was initiated 24 hr after phage was injected into the staphylococcal-infected mice; seven injections of tetracycline in concentrations of 0.2 or 2.0 mg were administered subcutaneously at 3- or 4-day intervals.

† Number of mice positive per number studied.

A similar attempt was undertaken to determine whether penicillin therapy would likewise aid in the detection of in vivo pen^r transductants. The results, however, were negative in that no pen^r transductants were recovered from the kidneys of 47 test mice receiving from 82 to 825 units of penicillin, subcutaneously, at 3- to 5-day intervals for nine total injections.

DISCUSSION

The problem of determining whether transductants isolated from kidneys had been produced in vivo or in vitro was resolved when the rate of survival of phage 80/2800 in the kidneys of mice was established. At a time when the concentration of phage in the kidneys was negligible, tetra^r transductants could still be isolated, indicating that they were transduced in vivo. Tetracycline therapy greatly aided the detection of transductants at the expense of susceptible parental cells. In some situations, transduction was obviously masked without the aid of this selective force. A minimum of 20 days subsequent to the initiation of antibiotic therapy was found necessary for demonstration of absolute selection. If a higher dosage of tetracycline had been employed, this period possibly may have been shortened. Resistant isolates resembling transductants were never recovered from treated or untreated control-infected animals which had not received phage.

In vivo pen^r transductants capable of producing penicillinase were not isolated in limited attempts. Since the frequency for production of pen^r transductants in vitro is lower than that for tetra^r transductants, inability to isolate the former type of cells may be due to the use of an

inadequate concentration of phage. It should be recalled that in the case of in vivo transduction of tetracycline resistance, transductants could not be recovered unless the phage concentration administered was high. Failure to recover pen^r transductants may be explained also on the basis that pen^r transductants, individually, may be quite sensitive to the antibiotic and consequently be eliminated. No definitive explanation relative to our inability to isolate pen^r transductants can be made at this time.

Our original goal was to reproduce in mice a situation which would mimic conditions which might be related to those involved in the origin and establishment of strains of resistant staphylococci in a hospital environment. The results of experiments presented in this paper do not favor the role of transduction occurring in vivo in the establishment of resistant strains in humans. To accept the possibility that phage-mediated transduction occurs in vivo naturally, one must account for the presence of an unusually high phage population in the tissues. It is unlikely that concentrations of transducing phage such as those used in this study would be present in the human nares or elsewhere. This conclusion does not exclude, however, the possibility that populations of phage of this magnitude may be released in vivo by induction of lysogenic donor cells in a system such as that reported by Morse (1959).

ACKNOWLEDGMENTS

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