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Phage-Host Interactions and the Production of Type A Streptococcal Exotoxin in Group A Streptococci

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The infection of *Streptococcus pyogenes* nontoxigenic strain $T25_3$ with bacteriophage T12 to form lysogen $T25_3$ (T12) resulted in the production of type A streptococcal exotoxin (erythrogenic toxin or streptococcal pyrogenic exotoxin). Two lines of evidence indicated that lysogeny per se was not sufficient to promote toxigenic conversion of strain $T25_3$. First, a virulent mutant of phage T12, unable to form stable lysogens, was able to affect type A exotoxin production by strain $T25_3$. An unrelated virulent phage A25 did not affect type A exotoxin production after infection of strain $T25_3$. Second, the temperate phage H4489A, which established stable lysogens with strain $T25_3$, did not promote type A exotoxin production. These results suggest that there is a strain specificity to the phage-host interaction which affects type A exotoxin synthesis. Additional evidence is presented which indicates that type A streptococcal exotoxin was not a structural component of phage T12.

Type A streptococcal exotoxin (11), also known as erythrogenic toxin, Dick toxin, and streptococcal pyrogenic exotoxin, is elaborated by certain strains of *Streptococcus pyogenes* and is responsible for the rash that accompanies scarlet fever. In 1964, Zabriskie (21) demonstrated that bacteriophages had a role in the production of type A streptococcal exotoxin; i.e., the infection of a nonlysogenic strain $T25_3$ with a temperate bacteriophage, T12, obtained from a strain of streptococci known to be associated with scarlet fever, resulted in the formation of a lysogen that produced type A streptococcal exotoxin. This observation has been confirmed by Nida et al. (17) and Johnson et al. (9). Preliminary evidence has also been presented by Nida et al. (17) that toxigenic conversion is not limited to the T12 phage-$T25_3$ host system but can be accomplished with a number of other phage-host strains.

In recent years, type A streptococcal exotoxin has been purified and characterized (3, 7, 8, 10, 15, 16); however, little work has been reported concerning the mechanism by which bacteriophages affect the production of type A exotoxin by lysogenic streptococci. In this report, we present evidence that lysogeny itself was not sufficient to promote toxigenic conversion, and furthermore, we suggest that there is a specificity to the phage-host interaction which affects type A exotoxin synthesis. Data are also presented which indicate that type A streptococcal exotoxin was not a structural component of bacteriophage T12.

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MATERIALS AND METHODS

Bacteria and bacteriophages. The primary strains of *S. pyogenes* known to produce type A streptococcal exotoxin which were used in this study were NY5 type 10 (6) and $T25_3$(T12) (21). Two strains known to produce no extracellular type A exotoxin were $T25_3$ and K56. Bacteriophage strains included the temperate phage T12, isolated from its original toxigenic donor (21), and phage H4489A, isolated from a nephritogenic strain of *S. pyogenes*. Virulent phages included strain A25 (14) and T12cp1, a spontaneous mutant of phage T12 which formed clear plaques on a bacterial lawn of strain $T25_3$. Bacterial strain $T25_3$ was relysogenized in this laboratory by phage T12 to construct strain $T25_3$(T12)B and by phage H4489A to produce strain $T25_3$ (H4489A).

Media. Supplemented proteose peptone broth (19) was the standard liquid medium used for bacterial growth and phage propagation. The medium employed for growing toxigenic strains when detection of type A exotoxin was desired was a Todd-Hewitt dialysate medium prepared by dialyzing 100 ml of 10-fold concentrated Todd-Hewitt broth (30 g/100 ml of distilled water) in 500 ml of distilled water for 16 h at 4.0°C. The dialysate was then autoclaved and was completed with the addition of sterile glucose (0.05%). Bacteriophage assays were performed by using the method of Wannamaker et al. (20) on serum Todd-Hewitt agar plates. Serum Todd-Hewitt plates consisted of Todd-Hewitt broth (Difco Laboratories) adjusted to pH 6.9 before addition of 1% bacteriological

agar and 0.03% Na_2HPO_4 and subsequent autoclaving. The sterile mixture was supplemented with 5% normal horse serum and 0.02% $CaCl_2$.

Bacteriophage propagation and phage lysate preparation. Phage propagation was accomplished by simultaneous inoculation of 1 drop of an overnight culture of the sensitive bacterial strain and 1 drop of a phage preparation containing 10^8 plaque-forming units per ml to 5 ml of 6% proteose peptone broth #3 (Difco). To the sterile proteose peptone broth were added (in final concentrations) 0.038% Na_2HPO_4, 0.02% $CaCl_2$, 0.05% glucose, 5% horse serum, and 0.1 mg of hyaluronidase per ml. Incubation was carried out at 37°C for 4 h for most phages and for 6 to 10 h for phage T12. Virulent phages, such as A25 and the mutant T12cp1, resulted in predominantly clear lysates, whereas temperate phages, such as H4489A and T12, resulted in predominantly turbid culture lysates. After the incubation period, the culture was centrifuged at 10,000 × *g* for 15 min, and the phage lysate was filtered through a Millipore membrane (0.45 μm). Phage titers of approximately 10^9 plaque-forming units per ml were obtained for most phages. Concentrated phage preparations were obtained by centrifugation at 101,000 × *g* for 90 min. Disruption of phage T12 was accomplished by resuspension of the phage pellet in distilled water.

Broken-cell extracts. Bacterial cells (50 ml) were grown to the stationary phase, washed in phosphate-buffered saline, and suspended in 10 ml of phosphate-buffered saline. The cells were broken in a Braun cell homogenizer (3 min), and the particulate matter was removed from the extract by centrifugation for 30 min at 6,450 × *g*. Protein concentrations of the broken-cell extract were determined; they ranged between 1 to 2 mg/ml, depending on the extent of cell breakage.

Extracellular culture filtrates. All strains were individually incubated at 37°C in 500 ml of Todd-Hewitt dialysate for 16 h, and growth kinetics were followed turbidimetrically in a Klett colorimeter (660 filter) to insure that all cultures were in the stationary phase of growth. The cells were removed by centrifugation, and the supernatant fluids were filtered through Millipore membranes (0.45 μm). The culture filtrates contained approximately 2.5 mg of protein per ml, about 0.3% of which was type A exotoxin (8). Concentrated samples of culture filtrate were obtained by polyethylene glycol (20,000 M_w) absorption of water from dialysis membranes. Protein concentrations, as determined by the method of Lowry et al. (12), of the concentrated culture filtrates were in the range of 250 mg/ml.

Type A streptococcal exotoxin. The purification of type A streptococcal exotoxin was accomplished by the procedure described by Cunningham et al. (3) with the modification described by us in a previous paper (8).

Preparation of antisera. Hyperimmune rabbit antisera were prepared with culture filtrates of the following streptococcal strains: $T25_3$, $T25_3$ (T12), and NY5 type 10. Immunization of rabbits with culture filtrates was accomplished by biweekly intramuscular and intraperitoneal injection of 1.5 ml of filtrate emulsified in an equal volume of Freund incomplete adjuvant (Difco) for the first 3 weeks. The same procedure was employed for the next 21 days of immunization except that the samples were first suspended in 7.0% polyacrylamide and then polymerized with riboflavin (0.02%) for 30 min in the presence of direct visible light. The gel suspensions were then emulsified with equal volumes (3.0 ml) of Freund incomplete adjuvant. The animals were bled by arterial puncture every 10 days, and precipitating antibody assays were performed by double immunodiffusion (Ouchterlony method). Serum titers were subsequently determined by the tube dilution of antigen in constant serum concentrations.

Polyacrylamide gel electrophoresis. Analytical disk gel electrophoresis was performed on purified type A streptococcal exotoxin and phage T12. The phage preparation was previously sedimented by ultracentrifugation (100,000 × *g*, 90 min) and disrupted by resuspension in distilled water. Electrophoresis of samples in 7.5% polyacrylamide was performed by the method of Ornstein (18) and Davis (4). Protein was detected in the gels by using the Coomassie brilliant blue staining method of Diezel et al. (5).

Immunodiffusion. Ouchterlony double diffusion was performed in gels of 0.8% agarose and 0.3% Noble agar in glycine-saline buffer (0.1 M glycine, 0.15 M NaCl), pH 8.2.

Kinetics of type A exotoxin production. Strain $T25_3$ was grown in Todd-Hewitt dialysate, and phage T12 or phage T12cp1 was added at the early log phase of growth. A sample was removed at each 2-h interval, the cells were removed by centrifugation, and the supernatant fluid was passed through a 0.45-μm Millipore filter. A portion of each sample (0.1 ml) was used to determine the phage titer and to assay type A exotoxin by the erythematous skin test. The backs of New Zealand white rabbits were carefully shaven before injection, and 0.1 ml of the Todd-Hewitt dialysate filtrate was injected intradermally. A positive skin reaction was demonstrated by an erythematous response, which formed the basis of the test. The rabbit skins were observed at 24 to 48 h after injection. The same rabbits were used repeatedly for skin testing.

RESULTS

Phage conversion and production of type A exotoxin. The Ouchterlony immunodiffusion procedure was used to assay for the presence of type A streptococcal exotoxin. An antiserum sample to a culture filtrate of strain NY5 extracellular products was reacted with a sample of the corresponding filtrate and a sample of purified type A exotoxin (Fig. 1). A line of identity between purified type A exotoxin and NY5 extracellular products confirmed the presence of antibody specific to type A exotoxin. Type A exotoxin was also detected in the extracellular filtrate of the lysogenic strain $T25_3$(T12) but not in the nonlysogenic strain $T25_3$ (Fig. 2). These results are consistent with the observations of Zabriskie (21), who first reported lysogenic conversion in these strains of group A streptococci.

To determine whether type A exotoxin was

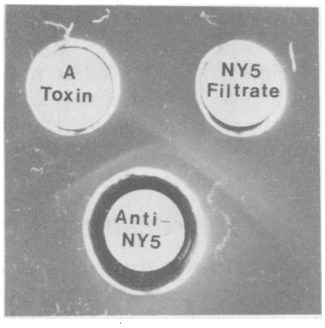

FIG. 1. *Immunodiffusion of purified type A exotoxin and extracellular culture filtrate with antibody to the extracellular culture filtrate of strain NY5 (anti-NY5).*

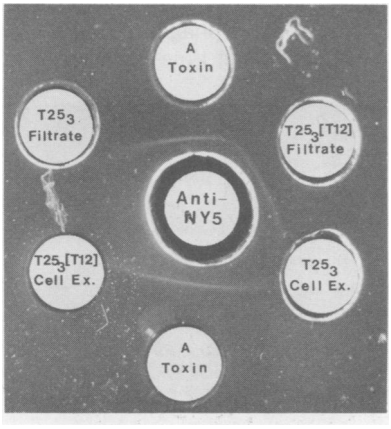

FIG. 2. *Immunodiffusion of antibody to the extracellular culture filtrate of strain NY5 (anti-NY5) with (top well, clockwise direction) purified type A exotoxin, culture filtrate of strain T25$_3$(T12), broken-cell extract of strain T25$_3$, purified type A exotoxin, broken-cell extract of strain T25$_3$(T12), and culture filtrate of strain T25$_3$.*

present intracellularly, we washed bacterial cells, brokethem in a Braun cell homogenizer, and assayed the broken-cell extracts for the presence of type A exotoxin. The broken-cell extracts of neithxr the T25$_3$ (T12) lysogen nor the T25$_3$ nonlysogen contained a level of type A exotoxin that could be detected, whereas toxin was easily detected in the extracellular filtrate of strain T25$_3$(T12) (Fig. 2).

Role of bacteriophage T12 in type A exotoxin production. The mechanism by which phage affects production of type A exotoxin by streptococci is unknown; however, the results obtained in polyacrylamide gel electrophoresis experiments with phage T12 components (Fig. 3) indicate that phage T12 did not have a structural component capable of migrating to the same position as that to which purified type A exotoxin migrated. The lack of immunological reactivity of the phage components to anti-NY5 serum indicated further that type A exotoxin was probably unrelated to any structural component of bacteriophage T12.

The isolation of a clear plaque mutant of phage T12, designated T12cp1, provided the necessary strain for determining whether lysogeny of strain T25$_3$ by phage T12 was essential for type A exotoxin production. After infection of strain T25$_3$ by phage T12cp1 or by the virulent phage A25, the lysates were obtained and were used in the Ouchterlony immunodiffusion assay. Neither the lysate obtained after infection by phage A25 nor the filtrate of the T25$_3$ nonlysogen contained detectable type A exotoxin (Fig. 4). However, the T25$_3$ lysate obtained after infection by the virulent mutant T12cp1 was converted to the toxigenic state, as evidenced by a positive reaction in the Ouchterlony immunodiffusion assay. The possibility that a small percentage of the cells were lysogenized by T12cp1 and were responsible for the detectable type A exotoxin seems remote, because a study of the kinetics of type A exotoxin synthesis revealed that maximum type A exotoxin levels obtained after infection of T25$_3$ by T12cp1 corresponded to the major lytic event, whereas a 6-h lag occurred before maximum extracellular toxin

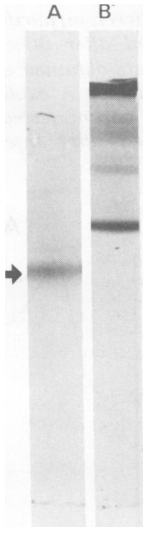

FIG. 3. *Polyacrylamide gel electrophoresis (micrograms of protein) of (A) purified type A exotoxin (30 μg; arrow) and of (B) disrupted phage T12 preparation (200 μg). Gels were aligned by determining R_f values of tracking dye and bands with a Gilford spectrophotometric gel scanning attachment.*

levels were obtained with wild-type T12 (Fig. 5). Lysogeny per se, therefore, was not essential for toxin production. Additionally, phage-mediated lysis alone was not sufficient to account for the appearance of type A exotoxin.

To determine whether a temperate phage unrelated to phage T12 could affect the conversion of strain $T25_3$ to the toxigenic state, we constructed a $T25_3$ lysogen containing temperate phage H4489A as well as a relysogenized strain of $T25_3$ containing phage T12, which was designated $T25_3$ (T12)B. After growth of these strains, the culture filtrates were obtained and were utilized in the Ouchterlony immunodiffusion assay. Whereas the relysogenized strain produced exotoxin identical to purified type A exotoxin, the other lysogen, $T25_3$ (H4489A), was incapable of producing type A exotoxin (Fig. 6). Apparently, there is a specificity to the interaction between phage and bacterial cell to affect a conversion to the toxigenic state.

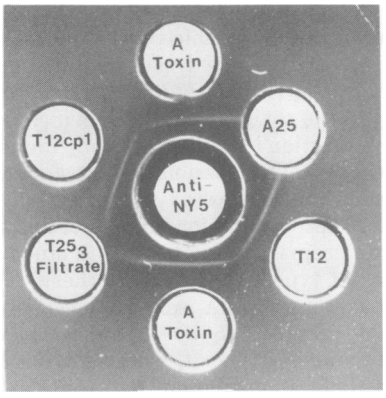

FIG. 4. *Immunodiffusion of antibody to the extracellular culture filtrate of strain NY5 (anti-NY5) with (top well clockwise direction) purified type A exotoxin, $T25_3$ lysate obtained after infection with virulent phage A25, T25 lysate obtained after infection with phage T12, purified type A exotoxin, extracellular culture filtrate from an uninfected $T25_3$ culture, and $T25_3$ lysate obtained after infection with phage T12cp1.*

DISCUSSION

The results of this study provide several lines of new information concerning the role of phage T12 in the conversion of group A strain $T25_3$ to production of type A streptococcal exotoxin. Of special interest is the observation that stable lysogeny and presumptive integration of the phage genome into the host chromosome are not essential for expression of the toxigenic genes. This conclusion was reached when it was observed that virulent phage mutant T12cp1 was capable of producing type A exotoxin after in-

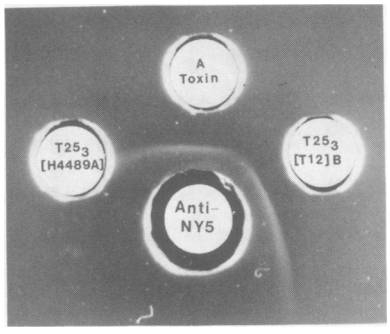

FIG. 6. *Immunodiffusion of antibody to the extracellular culture filtrate of strain NY5 (anti-NY5) with culture filtrates of the lysogen $T25_3$ (H4489A), purified type A exotoxin, and the reconstructed lysogen $T25_3$ (T12)B.*

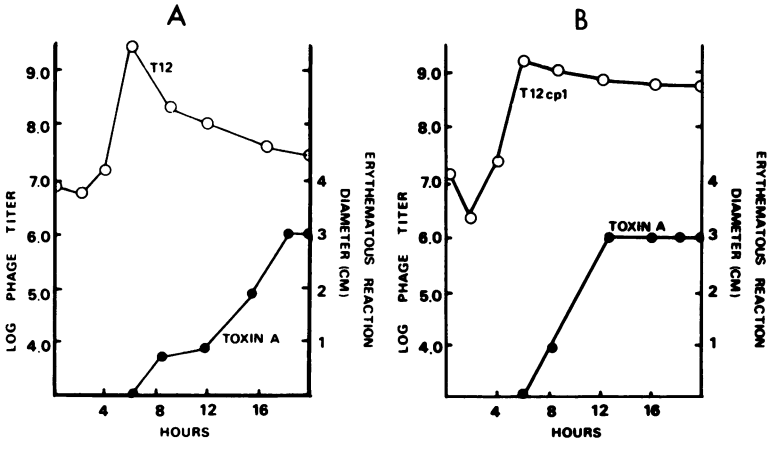

FIG. 5. *Kinetics of type A exotoxin production during infection by phages T12 (A) and T12cp1 (B). Type A exotoxin was assayed by the erythematous skin reaction.*

fection into host bacterial strain $T25_3$. The possibility that a structural component of phage T12 was related to type A streptococcal exotoxin was considered; however, this possibility was reduced by the observation of the absence of protein bands similar to those of type A exotoxin in polyacrylamide gel electrophoresis experiments and the lack of immunological reaction between phage components and antibody specific for type A exotoxin.

One of the most pertinent questions concerning toxigenic conversion in group A streptococci is whether the host bacterial strain or the infecting phage possesses the specificity for type A exotoxin production. Our evidence indicates that phage T12 has a definite role in affecting toxigenic conversion, since two unrelated phages, virulent phage A25 and temperate phage H4489A, were unable to influence strain $T25_3$ to produce type A exotoxin. In another communication, we will present evidence that a number of phages isolated from clinical strains of streptococci known to cause scarlet fever not only determine the type of toxin the host synthesizes but also affect toxigenic conversion in other host bacterial strains.

Whether the gene(s) specifying toxin synthesis is located in the phage genome or in the bacterial chromosome is still unclear. We hypothesize that bacterial strain $T25_3$ possesses the ability to produce at least low levels of intracellular type A toxin and that once introduced into the host strain, phage T12 affects the host to excrete or possibly modify (or both) the intracellular toxin to an extracellular form. Further genetic analysis will be necessary to determine the location of the gene(s) specifying type A exotoxin production and to elucidate the role of phage T12 in toxin production. The development of streptococcal cloning vehicles (1) and recombinant deoxyribonucleic acid techniques in streptococci (2, 13) should facilitate such an analysis.

ACKNOWLEDGMENT

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