Transfer of Group A Streptococcal Pyrogenic Exotoxin Production to Nontoxigenic Strains by Lysogenic Conversion

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Production of group A streptococcal pyrogenic exotoxins (SPE) types A and C was transferred from toxigenic streptococcal strains to nontoxigenic strains by lysogeny. Lysogens were tested for SPE with Ouchterlony immunodiffusion on Todd-Hewitt agar plates; toxin diffusing from isolated colonies reacted with specific hyperimmune antisera to SPE. Phage prepared from strains T253 (T12gl) and 3GL16, both yielding SPE type A, formed plaques on T253 (nonlysogenic) lawns. Over 90% of the colonies picked from the plaque centers yielded A toxin, suggesting SPE type A was transferred by lysogenic conversion. SPE type C formation was transferred to nontoxigenic strains T253 and K56 with supernatant fluids from mitomycin C-induced cultures of CS112, producing SPE types B and C. All lysogens tested were positive for SPE type C, indicating that C toxin induction also was transferred by lysogenic conversion. SPE type B formation was not transferable by lysogeny with the strains tested.

A variety of extracellular toxins and enzymes are produced by group A streptococci, including streptococcal pyrogenic exotoxins (SPE). Three antigenically distinct SPE have been described and purified: A (4, 6, 7, 15), B (1), and C (9, 14). The properties of SPE include pyrogenicity (1, 6, 9, 12, 14), enhancement of susceptibility to lethal endotoxin shock (6, 12), and alteration of blood-brain barrier permeability to other agents, including endotoxin and bacteria, as well as themselves (12). They are potent nonspecific and specific thymus-derived (T)-lymphocyte mitogens (11). Also, SPE are associated with the streptococcal erythrogenic toxins whose activity represents the enhancement of acquired skin reactivity to streptococcal antigens by one or more SPE types (10). Previously it was shown that a filterable agent isolated from scarlatinal strains of hemolytic streptococci induced the formation of erythrogenic toxin by nonscarlatinal strains (5). This was confirmed by Bingel (2) and later by Zabriskie (16) who reported that phage isolated from strain T12gl could induce the formation of A toxin-positive lysogens of T253 (a nontoxigenic group A streptococcus). Recently it was demonstrated that strains T253 (T12gl) and T253 both produce SPE type B (Johnson, Schlievert and Watson, unpublished data). Furthermore, T253 produces very low levels of SPE A (P. M. Schlievert, D. J. Shoettle, and D. W. Watson, J. Infect. Dis., in press). It has been suggested that toxin is transferred by lysogenic conversion rather than transduction (8), but this has not been conclusively established.

Transfer of SPE C production by bacteriophage was recently accomplished (3). However, it was not clear whether the transfer was mediated by transduction or lysogenic conversion. Furthermore, attempts to transfer SPE type B were unsuccessful (3).

This investigation was undertaken to confirm the report of Zabriskie (16) that the production of SPE type A requires the presence of group A streptococcal phage. Also, the role of bacteriophage in production of SPE types B and C was assessed. In addition, studies were undertaken to ascertain whether the transfer of toxin formation was mediated by transduction or lysogenic conversion.

MATERIALS AND METHODS

Bacteria. The following group A streptococcal strains were used for propagation of phage: strain T253 (T12gl), M-type 25, obtained from J. B. Zabriskie at the Rockefeller Institute, producing SPE types A and C; strain 3GL16, M-type 19, obtained from J. B. Zabriskie, yielding SPE type A only; CS112 strain, provisional M-type Truro 12, provided by P. Cleary, Department of Microbiology, University of Minnesota, producing SPE types B and C.

Two group A streptococcal strains were used as indicators: T253, M-type 25, a nonlysogen, producing SPE type B and a small amount of SPE type A not detectable by Ouchterlony immunodiffusion; and K56, M-type 12 not producing SPE.

Stock cultures of all streptococcal strains were maintained lyophilized after growth in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) and suspension in whole defibrinated fresh rabbit blood.

Antiseria. Antisera to SPE were prepared by hyperimmunizing American Dutch rabbits with SPE
embulsified in Freund incomplete adjuvant (9). Toxins used for the immunizations were prepared from culture supernatant fluids of streptococcal strains grown in a beef heart dialyisate medium (14). SPE were purified by differential precipitation with ethanol and resolubilization in acetate-buffered saline (6), followed by preparative thin-layer isoelectric focusing (1, 9). The purified toxins were homogenous when tested by Ouchterlony immunodiffusion against antisera to partially purified SPE (6).

**Bacteriophage assays.** The strains tested for phage were grown overnight at 37°C in a modified number 1 broth (13) in which fetal calf serum was substituted for horse serum. Number 1 broths were then reinoculated (10%, vol/vol) with the overnight cultures. After growth for 1.5 h at 37°C, mitomycin C (0.1 µg/ml) was added. The cultures containing mitomycin C were incubated for 3 h, and the cells were removed by centrifugation (750 x g, 10 min) and filtration (0.22-µm pore size filters). The cell-free supernatant fluids were then tested for phage by spotting 10-µl amounts onto bacterial lawns of the recipient strains prepared on 273 agar. For preparation of 273 agar, 40 g of proteose peptone no. 3, 2 g of yeast extract (Difco), 11 ml of 0.03 M tris(hydroxymethyl)- aminomethane buffer (pH 7.6), 7.2 g of glycerophosphate, and 10 g of agar were added to 1 liter of water. After heat sterilization, 2 g of glucose, 0.3 g of CaCl2, (S. Skjold, personal communication), and 68 mg of hyaluronidase (Sigma Chemical Co., St. Louis, Mo.) sterilized by filtration were added.

After overnight incubation, large plaques appeared on the bacterial lawns. Lysogens were obtained by repeatedly stabbing plaque centers and streaking for isolation on Todd-Hewitt medium with 1% agar.

**Assay for toxigenicity.** Lysogens obtained from plaque centers were incubated overnight at 37°C in 5% CO2 before testing for SPE. Isolated colonies were tested directly for production of SPE types A and B by Ouchterlony immunodiffusion. Each plate contained 10 ml of Todd-Hewitt agar; wells made in the agar, adjacent to individual colonies, were filled with 10 to 20 µl of antisera. For detecting SPE type C, isolated colonies were restreaked heavily on Todd-Hewitt agar plates, incubated overnight at 37°C in 5% CO2, and then tested for toxin by Ouchterlony immunodiffusion. The restreaking procedure for SPE type C facilitated the formation of visible immunoprecipitates.

A representative example of the assay system used is presented in Fig. 1 where toxin diffused from colonies reacted with specific hyperimmune antisera to SPE type A. As shown, the Ouchterlony immunodiffusion assay provided an effective method for screening large numbers of colonies for toxigenicity and utilized small quantities of specific antisera to toxin.

**Assay for transformation.** Supernatant fluids from mitomycin C-induced cultures were treated with 500 µg of deoxyribonuclease I per ml (Sigma Chemical Co., St. Louis, Mo.) for 2 h at 37°C in 0.01 M MgCl2 before spotting on bacterial lawns. Colonies obtained after picking organisms from within plaques were tested for toxin production. The results were compared with controls where supernatant fluids were not treated with the deoxyribonuclease.

**RESULTS**

**Transfer of SPE A.** Strains T253 (T12gl) and 3GL16 produce SPE type A. Supernatant fluids from mitomycin C-induced cultures of these organisms produced plaques on T253 lawns. A high percentage of colonies picked from isolated plaques produced SPE type A when either donor strain was used as the source of bacteriophage (Table 1). All toxin-positive colonies were lysogenic, and no lysogens were obtained that were negative for A toxin. All lysogens tested were found to be immune to infection by phage liberated upon induction. Treatment of supernatant fluids with deoxyribonuclease had no effect on the transfer of toxin production. The Ouchterlony immunodiffusion plate shown in Fig. 1 was a test for toxigenicity of lysogens of T253 when infected by phage in supernatant fluids from T253 (T12gl). The data suggest that SPE

<p>| Table 1. Transfer of SPE A production to a nontoxigenic strain by a temperate bacteriophage |</p>
<table>
<thead>
<tr>
<th>Phage source</th>
<th>Phage strain</th>
<th>Lysogen</th>
<th>No. of colonies tested (%)</th>
<th>A toxin-positive (%)</th>
<th>Phage-positive (%)</th>
<th>Correlation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>T253 (T12gl)</td>
<td>T253 (T12gl)</td>
<td>48</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>3GL16</td>
<td>T253</td>
<td>53</td>
<td>89</td>
<td>89</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

*Percent A-toxin-positive phage positive.

**Fig. 1. Ouchterlony immunodiffusion on Todd-Hewitt agar plate. Wells contain antisera to SPE A and formed an immunoprecipitate with toxin diffusing from isolated colonies of strain T253, lysogenized by phage from strain T253 (T12gl).**
type A was transferred by lysogenic conversion rather than by transduction because of the high frequency of transfer.

**Transfer of SPE C.** Strain CS112 produces SPE types B and C. Supernatant fluids from mitomycin C-induced cultures of CS112 formed large clear plaques on T25, and K56 lawns. All colonies obtained after picking organisms from within plaques of T25, lawns were lysogens, T25(φCS112), and were positive for C toxin. A large proportion (46%) of the K56 colonies, obtained after picking organisms from plaque centers, were also lysogens, K56(φCS112), and all lysogens were positive for C toxin (Table 2). Lysogens were resistant to the phage they produce. Addition of deoxyribonuclease to the supernatant fluids did not affect toxin transfer. The high percentage of C-toxin-positive colonies suggests that C-toxin production was transferred by lysogenic conversion comparable to SPE type A.

SPE type B synthesis in T25, was not altered when the strain was lysogenized by phage from CS112, and no B toxin was detected from any of the K56 lysogens tested.

To demonstrate further that C-toxin transfer was mediated by bacteriophage, supernatant fluids from induced cultures of the lysogenic strain T25,(φCS112) were spotted on K56 lawns. Colonies were picked from plaque centers, and all lysogens tested produced SPE type C.

Similarly, phage prepared from K56(φCS112) formed plaques on T25, lawns, and lysogens from within these plaques were positive for C toxin.

This indicates that phage from strain CS112 can be passed through a number of strains and still retain its ability to transfer C-toxin production.

**DISCUSSION**

The present study was undertaken to determine the role of bacteriophage in the production of SPE types A, B, and C, and to extend the observations of Zabriskie (16). The results confirmed the previous report (16) that ability to produce A toxin can be transferred to nontoxicogenic strains by bacteriophage. The phage were not specific for one M type since phage prepared from a type 19 strain, 3G116, transferred toxin production to strain T25, (M type 25). Whereas Zabriskie used skin tests to evaluate A-toxin production (16), the assay employed in this study was Ouchterlony immunodiffusion which is highly specific for SPE type A. The high percentage of toxin-positive lysogens obtained in this study suggests that A-toxin production is transferred to recipient strains by lysogenic conversion rather than transduction. Treatment of culture supernatant fluids with deoxyribonuclease demonstrated that toxin transfer was not mediated by transformation. If genes responsible for A-toxin production were carried by a phage, all lysogens formed should carry these genes. As indicated in this study, all colonies of T25, producing A toxin were also lysogenic, and no lysogens were observed that did not produce A toxin. Preliminary experiments indicate that A-toxin genes are carried by T12gp phage since cross-reactive mutants have been isolated which give rise to lysogens with altered toxin proteins (Johnson, Schlievert, and Watson, unpublished data). Studies with cross-reactive mutants are under way to investigate further the role of phage and host in expression of SPE.

Recently, it was shown that SPE C induction was controlled by temperate phage, but the investigators were unable to transfer B toxin (3). Comparable results were obtained in this study in which C-toxin production was transferred by bacteriophage from strain CS112 to strains K56 and T25. Furthermore, attempts to transfer B-toxin production by phage from strains CS112 and T25 to the K56 strain were unsuccessful. This study extends the work related to phage regulation of SPE C by showing that the transfer mechanism is by lysogenic conversion rather than transduction.

Although A and C toxin production was regulated by bacteriophage, the nonlysogen strain T25, produces low levels of SPE A. It is possible that these toxin types may be expressed by

**Table 2. Transfer of SPE C production to nontoxicogenic strains by temperate bacteriophage**

<table>
<thead>
<tr>
<th>Phage source</th>
<th>Recipient strain</th>
<th>Lysogen</th>
<th>No. of colonies tested</th>
<th>C toxin positive (%)</th>
<th>Phage positive (%)</th>
<th>Correlation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS112</td>
<td>T25,</td>
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<td>46</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>CS112</td>
<td>K56</td>
<td>K56 (φCS112)</td>
<td>54</td>
<td>46</td>
<td>46</td>
<td>100</td>
</tr>
<tr>
<td>T25, (φCS112)</td>
<td>K56</td>
<td>K56 (φCS112)</td>
<td>47</td>
<td>74</td>
<td>74</td>
<td>100</td>
</tr>
<tr>
<td>K56 (φCS112)</td>
<td>T25,</td>
<td>T25, (φCS112)</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*a* Percent C-toxin-positive phage positive.

*b* Strain T25, lysogenized with phage from strain CS112.

*c* Strain K56 lysogenized with phage from strain CS112.
chromosomal genes, or there is a defective phage operating to give poor phage yields and slight toxin production. Also, attempts to transfer C-toxin production from the highly mucoid T18P strain (9) to a variety of recipient strains (unpublished data) were unsuccessful, although this may have resulted from failure to find an appropriate recipient.

ACKNOWLEDGMENTS

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LITERATURE CITED