Production of Toxic-Shock-Associated Protein(s) in *Staphylococcus aureus* Strains Isolated from 1956 Through 1982

PEGGY S. HAYES,1* LEWIS M. GRAVES,1 JAMES C. FEELEY,1 GARY A. HANCOCK,2 MITCHELL L. COHEN,3 ARTHUR L. REINGOLD,4 CLAIRE V. BROOME,4 AND ALLEN W. HIGHTOWER3

Respiratory and Special Pathogens Laboratory1 and Epidemiology4 Branches, Enteric Diseases Branch,3 and Statistical Services Activity,5 Division of Bacterial Diseases; and Nosocomial Infections Laboratory Branch, Hospital Infections Program,2 Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333

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A total of 281 *Staphylococcus aureus* strains selected from those submitted to the Centers for Disease Control for phage typing between 1956 and 1982 were tested for the production of toxic-shock-associated protein (TSAP) by isoelectric focusing (IEF) and solid-phase radioimmunoassay. The results suggest that the observed temporal trends in the incidence of toxic-shock syndrome were not primarily due to changes in the distribution of TSAP-positive strains of *S. aureus*. Overall, 39 (14%) were TSAP positive by both methods. The earliest positive strain was an isolate submitted in 1957. TSAP-positive strains were most prevalent in the group of isolates submitted in 1976 for which 29% reacted, but the percent positive subsequently declined for isolates submitted in later years. TSAP production was more frequent among strains of phage types 29, 29/52, and 52 than among other strains. The use of IEF to identify TSAP detected false-positive proteins. Seven strains were positive by IEF and negative by solid-phase radioimmunoassay, whereas only one was positive by solid-phase radioimmunoassay and negative by IEF.

Toxic-shock syndrome (TSS) was first described by Todd et al. in 1978 (20), at which time the authors postulated that the disease was due to a toxin produced by *Staphylococcus aureus*. Subsequent epidemiological and laboratory investigations in 1980 confirmed a causal role for *S. aureus* in TSS and demonstrated an association with the use of tampons (11, 19). In 1981, two proteins were identified in *S. aureus* strains isolated from patients with TSS; these proteins were found in 95 to 100% of *S. aureus* strains from patients with TSS but in only 4 to 15% of *S. aureus* strains from non-TSS patients. One protein was designated pyrogenic exotoxin C (18), and the other was designated staphylococcal enterotoxin F (5). Since the molecular weights and isoelectric points of these proteins are similar and most strains positive for one are positive for the other, it is now considered likely that they are closely related or identical (6, 10). However, the role of these proteins, sometimes referred to as toxic-shock-associated proteins (TSAP), in the pathogenesis of TSS has not been clearly established.

Changes in the prevalence of TSAP-positive strains of *S. aureus* over time and a possible relationship between such changes and the incidence of TSS have been incompletely studied. Although a number of studies have examined the prevalence of TSAP-positive strains (1, 3, 4, 7, 12, 13, 16), all but two of these studies have included only isolates collected after 1979 or have not specified the time period during which the strains were recovered. In one of the two remaining studies, Altemeier et al. (1) examined *S. aureus* isolates collected between 1960 and 1979 and found that the earliest TSAP-positive strains were isolated in 1971. The *S. aureus* strains in their study were collected primarily from one hospital and were not a random sample from each year. The second study was a preliminary report of our work (7) that showed a rise in TSAP-producing strains between 1956 and 1979. Our present study was undertaken to define more precisely when the increased prevalence of TSAP-positive strains occurred and to compare two techniques for TSAP detection, isoelectric focusing (IEF) and solid-phase radioimmunoassay (SPRIA), in a group of *S. aureus* strains not known to be associated with TSS.

**MATERIALS AND METHODS**

**Bacterial isolates.** The *S. aureus* strains were chosen from the Staphylococcus Reference Laboratory Collection, Centers for Disease Control (CDC), Atlanta, Ga. This collection consists of all isolates submitted to CDC after 1956 for phage typing. *S. aureus* isolates submitted to CDC before 1956 were not stored. With the use of a random numbers table, a sample of 40 isolates a year was selected for the years 1960, 1964, 1968, 1972, 1976, 1979, 1980, and for the first 3 months of 1982 (266 isolates). When some of the isolates selected were nonviable, additional isolates were selected at random to total 40 per year. Isolates submitted to CDC in 1980 and 1982 from patients suspected of having TSS were excluded, as were strains from animal or environmental sources. No more than one strain was selected from a given patient or related cluster of cases. The strains selected had been isolated from a variety of sites (nose, throat, skin, burns, wounds, abscesses, umbilical cord, eye, blood, etc.).

On the basis of results for the isolates from the years 1960 to 1982, we decided to examine three additional samples of isolates. One sample consisted of all the viable isolates of phage type 29, 52, or 29/52 from the years 1956 to 1959 (15 isolates). Since no vaginal isolates were selected by using a random numbers table, the second sample consisted of all the viable vaginal isolates sent to CDC before 1980 (33 isolates). Because many of the 1976 isolates were nonviable, the third sample consisted of 1976 isolates from another stored collection at CDC (Epidemic Investigations Laboratory Branch) in which viability was not a problem (10 isolates).

The isolates were subcultured in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), grown for 48 h at 37°C, and subcultured twice on plates of Trypticase soy agar prepared with 5% sheep blood (BBL Microbiology Systems).
TSAP extraction. A modification of the method of Schlievert et al. (18) was used for TSAP extraction. S. aureus isolates were inoculated into two 10-ml volumes of fresh beef heart medium in tubes (16 by 150 mm) and incubated at 37°C for 13 to 15 h on a roller tube apparatus in 5% CO₂-95% air. Cells were separated by centrifugation at 2,000 rpm (770 x g) at room temperature for 30 min in a Sorvall RC-5B centrifuge and discarded. Each of the supernatant fluids was precipitated by the addition of 2.3 volumes of 100% isopropanol, followed by incubation at room temperature for 30 min. The precipitate formed was separated at 16,300 x g at 5°C for 30 min, air dried, and suspended in 400 μl of pyrogen-free water. Protein was again precipitated with isopropanol, separated as previously described, and resuspended in 50 μl of pyrogen-free water. The duplicate extracts were then combined to total 100 μl, and any remaining insoluble material was removed by centrifugation for 3 min (Eppendorf centrifuge). This method has been shown in past studies (10) to be as sensitive as the method of Schlievert. Positive and negative control strains of S. aureus (387, 033, 189, and D0318) and a control of un inoculated fresh beef heart medium were included in all runs.

IEF. A 50-μl sample of the extract was subjected to electrofocusing in a thin-layer polyacrylamide gel (Ampholine PAG plates, LKB Instruments, Stockholm, Sweden) with a pH gradient of 5.5 to 8.5, using an LKB Multiphor 2117 apparatus as described by the manufacturer (22). The gels were stained with Coomassie brilliant blue R250, and the isoelectric point was determined both by direct measurement and by comparison of the position of the stained protein band with a hemoglobin standard. An isolate was considered positive for pyrogenic exotoxin C if a stained band was present within 0.5 mm of isoelectric point pH 7.2.

SPRIA. The method of Cohen et al. (9, 10) was used for SPRIA. Briefly, 30 μl of extract was applied to nitrocellulose paper that had been moistened with SSC (0.15 M sodium chloride plus 0.015 M sodium citrate). The nitrocellulose paper was dried and then sequentially soaked in (i) 1% bovine serum albumin in TSGAN (50 mM Tris-hydrochloride [pH 7.5], 0.15 M NaCl, 0.25% gelatin, 0.15% sodium azide, 0.1% Nonidet P-40), (ii) monospecific antibody to staphyloccocal enterotoxin F (S), kindly provided by Merlin S. Bergdoll, and (iii) staphyloccocal protein A labeled with 125I. The nitrocellulose paper was then washed, dried, cut, and counted in a gamma counter. An isolate was considered positive if it had a count equal to or greater than that of the positive controls and at least double that of the negative controls.

RESULTS

In all, 281 isolates (excluding the specially selected vaginal isolates and additional 1976 isolates) were available for testing; although all were tested by IEF, only 222 were tested by SPRIA because we had exhausted our supply of antibody to staphylococcal enterotoxin F. Of the strains tested by both methods, 175 of 176 strains negative by IEF were also negative by SPRIA. We therefore considered the 59 strains (all IEF negative) which were not tested by SPRIA to be SPRIA negative. Of the 281 isolates, 46 (16%) were positive by IEF. Of the 46 IEF-positive strains, 39 (85%) also were positive by SPRIA. Since an SPRIA result is based on a reaction with specific antisera, we believe that SPRIA is a highly specific. However, since IEF depends only on a protein or molecule having an isoelectric point of 7.2 ± 0.5 and does not depend on immunogenicity, we considered the IEF-positive strains that were SPRIA negative to be false-positive results and did not include them in the results discussed below.

Of the 266 isolates that were randomly selected from the years 1960 to 1982, 37 (14%) were positive for TSAP by SPRIA (Table 1). The rate of TSAP-positive strains increased between 1960 and 1976 and subsequently declined. There was no significant difference in the proportion of TSAP-positive strains by year of submission of the strain when isolates were grouped by decade or by pre-1979 versus 1979 and later.

Of the 266 isolates, 62 (23%) were phage type 29, 52, or 29/52; 144 (54%) were other phage types; and 60 (23%) were nontypable. TSAP was found in 16 (26%) of the phage type 29, 52, or 29/52 strains; 11 (8%) of the strains of other phage types; and 10 (17%) of the nontypable strains. Of the 37 positive isolates, 16 (43%) were phage type 29, 52, or 29/52; 11 (30%) were other phage types; and 10 (27%) were nontypable. TSAP was found significantly more often among phage type 29, 52, or 29/52 strains than among other typable strains (P = 0.001; Fisher’s exact test, two-tailed). The proportion of all isolates which were phage type 29, 52, or 29/52 did not vary consistently over time and was not responsible for the variation in the rate of TSAP-positive strains.

There was no significant difference in TSAP production according to body site from which the S. aureus strain was isolated. Of the 51 isolates considered invasive (recovered from wounds, pustules, abscesses, “lesions,” blood, and pleural fluid), 5 (10%) were positive for TSAP. Of the 142 considered noninvasive (recovered from nose, throat, sputum, skin, and urine), 24 (17%) were positive for TSAP. Of the 73 isolates that could not be grouped as invasive or noninvasive (recovered from circumcision sites, burns, drain sites, rectum, eye, trachea, and “source unknown”), 8 (11%) were positive for TSAP.

The 266 strains came from 41 states; the 37 TSAP-positive isolates came from 17 states. There was no significant difference in TSAP production by geographical region or state from which the isolate was submitted.

Of the 15 S. aureus isolates of phage type 29, 52, or 29/52 submitted to CDC during the period 1956 to 1959, 3 (20%) were positive for TSAP (Table 2). The earliest TSAP-positive strain was submitted in 1957.

TABLE 1. Toxic-shock-associated protein production in a random sample of S. aureus strains isolated from 1960 through 1982

<table>
<thead>
<tr>
<th>Yr isolated</th>
<th>Phage type (%)</th>
<th>Other*</th>
<th>Nontypable</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1960</td>
<td>0/9 (0)</td>
<td>0/19 (0)</td>
<td>1/8 (13)</td>
<td>1/36 (3)</td>
</tr>
<tr>
<td>1964</td>
<td>1/5 (20)</td>
<td>1/19 (5)</td>
<td>0/6 (0)</td>
<td>2/30 (7)</td>
</tr>
<tr>
<td>1968</td>
<td>6/17 (35)</td>
<td>0/15 (0)</td>
<td>1/3 (33)</td>
<td>7/35 (20)</td>
</tr>
<tr>
<td>1972</td>
<td>1/10 (10)</td>
<td>1/17 (6)</td>
<td>1/7 (14)</td>
<td>3/34 (9)</td>
</tr>
<tr>
<td>1976</td>
<td>2/5 (40)</td>
<td>2/9 (22)</td>
<td>2/9 (22)</td>
<td>6/21 (29)</td>
</tr>
<tr>
<td>1979</td>
<td>4/9 (44)</td>
<td>1/16 (6)</td>
<td>2/7 (29)</td>
<td>7/32 (22)</td>
</tr>
<tr>
<td>1980</td>
<td>2/6 (33)</td>
<td>1/24 (4)</td>
<td>2/8 (25)</td>
<td>5/38 (13)</td>
</tr>
<tr>
<td>1982*</td>
<td>0/3 (0)</td>
<td>5/25 (20)</td>
<td>1/12 (8)</td>
<td>6/40 (15)</td>
</tr>
</tbody>
</table>

Total 1960 to 1982: 16/62 (26) 11/144 (8) 10/60 (17) 37/266 (14)

* Phage types other than 29, 29/52, and 52, including three phage types from group I, 12 from group II, 27 from group III, 2 from group V, 10 from mixed groups, and 2 from the miscellaneous group of S. aureus.

* Number of strains positive by SPRIA/total tested by IEF or SPRIA or both (%).

* Excludes isolates submitted from possible cases of TSS.
TABLE 2. Toxic-shock-associated protein production by S. aureus strains of phage type 29, 52, or 29/52 isolated from 1956 through 1959

<table>
<thead>
<tr>
<th>Yr isolated</th>
<th>No. of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tested</td>
</tr>
<tr>
<td>1956</td>
<td>1</td>
</tr>
<tr>
<td>1957</td>
<td>4</td>
</tr>
<tr>
<td>1958</td>
<td>6</td>
</tr>
<tr>
<td>1959</td>
<td>4</td>
</tr>
<tr>
<td>Total 1956 to 1959</td>
<td>15</td>
</tr>
</tbody>
</table>

Of the 54 vaginal isolates submitted to CDC during the period 1956 to 1979, 33 were viable. Of these, nine (27%) were positive for TSAP. Five of the nine (56%) were phage type 29, 52, or 29/52; two (22%) were other phage types; and two (22%) were nontypable. The two earliest TSAP-positive vaginal isolates were submitted in 1969, followed by one each in 1970, 1971, 1972, and 1973 and three in 1979. Of the 10 isolates selected from the 1976 collection in the Epidemic Investigations Laboratory Branch, 3 (30%) were TSAP positive.

The discrepancy between IEF and SPRIA testing did not influence our results; the distribution of TSAP-positive strains by year of isolation, phage type, geographic region, and body site did not change materially when the seven IEF-positive, SPRIA-negative strains were counted as TSAP positive.

DISCUSSION

Some authors have postulated that the dramatic increase in the number of cases of TSS in 1978 and 1979 was at least in part due to an increase in the prevalence of TSAP-positive strains at that time (7, 14, 15). Our results show that although changes in the rate of TSAP-positive strains have occurred over time, these changes do not correlate directly with the reported incidence of TSS. We found that the first TSAP-positive strain was isolated in 1957 and that the proportion of S. aureus isolates which produced TSAP peaked in 1976, 4 years before the number of reported cases of TSS peaked (8). Altemeier et al. (1) also found that TSAP positivity among all isolates, irrespective of phage type, peaked in 1976. Thus, an increase in the prevalence of TSAP-positive strains of S. aureus alone does not seem to account for the observed temporal trends in TSS.

The fact that TSAP-producing strains were isolated as long ago as 1957 is not surprising. Vergeront et al. (21) reported that 90% of sera drawn in 1960 from 38 men and women (ages 16 to 24) had anti-staphylococcal enterotoxin F titers of greater than 1:100. This also supports the conclusion that TSAP-producing strains of S. aureus have existed for a considerable time.

Our results are also consistent with those of other investigators in other ways. For example, Schlievert et al. found that 5 (16%) of 31 strains of S. aureus recovered in 1980 and 1981 from wounds and the anterior nares of patients without TSS were pyrogenic exotoxin C positive (17), comparable to our finding that 5 (13%) of 38 isolates from 1980 were TSAP positive. Also, in keeping with previous findings by others (1–3, 12, 13), we found a significant association between TSAP-positive strains and phage types 29, 52, and 29/52. This association of TSAP production with phage types 29, 52, and 29/52 strains was similar for the years 1956 to 1959 (20%) and 1960 to 1982 (26%).

We have interpreted our data with caution for several reasons. First, S. aureus strains submitted to CDC may not be representative of all strains in the U.S. population. Several large states rarely, if ever, send S. aureus isolates to CDC for phage typing. However, the three states (Wisconsin, Minnesota, and California) with the highest number of reported cases of TSS do submit S. aureus isolates to CDC. In addition, the way in which individual physicians, hospitals, or referring laboratories select isolates for forwarding to CDC is occasionally unknown. We are unaware, however, of the existence of a comparable bank of S. aureus strains from the same time period which is more representative of the United States.

Second, it is possible that strains of S. aureus isolated from other body sites are not representative of those found in the vagina. Only 54 vaginal isolates were submitted before 1980. Of the 33 vaginal isolates available for testing, 9 (27%) were positive for TSAP. When comparing TSAP production in S. aureus isolates from the vagina and from other body sites (37 of 266 or 14%), TSAP production appears more likely to occur in vaginal isolates (borderline significance, P = 0.069; Fisher’s exact test, two-tailed).

Third, it is possible that TSAP-positive strains may in some way survive storage better than TSAP-negative strains. However, this does not appear to be true. The only year in which viability was a problem was in 1976, and we found that isolates from 1976 in another stored collection at CDC, in which viability was not a problem, had a similar TSAP-positivity rate (3 of 10 or 30%). Further evidence that survivability does not depend on TSAP production is that the survival rate for 29, 52, or 29/52 isolates was not significantly different from that of other isolates for all years. In addition, we detected no change in TSAP production in our positive control S. aureus isolates after storage in sheep blood at −70°C for 2 years.

Our results suggest that SPRIA testing, based on immunological detection of a protein, is more specific than IEF testing, based on separation by isoelectric point (10). Although the two tests have good comparability when strains from patients suspected of having TSS are being tested (10), comparability is not as good when examining strains from other sources. Consequently, the predictive value of a positive IEF test appears to depend on the source of the S. aureus strains being examined. That is, if most of the isolates being tested are from TSS patients and hence are TSAP positive, a smaller proportion of the positive IEF results will be false-positive. In the group of isolates from non-TSS patients that we tested, 7 (15%) of 46 positive IEF results could not be confirmed by SPRIA.

The hypothesis that changes in the prevalence of TSAP-positive strains of S. aureus accounted for the observed temporal trends in the epidemiology of TSS is not supported by our results. Other hypotheses (15) for these trends, such as the introduction of tampons made from newer synthetic materials, remain credible.

LITERATURE CITED


