Invasive Group A Streptococcal Disease in Taiwan Is Not Associated with the Presence of Streptococcal Pyrogenic Exotoxin Genes

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We reviewed the clinical features of 44 patients with invasive group A streptococcal (GAS) disease who were treated at two teaching hospitals in southern Taiwan from 1991 to 1994. Genes encoding streptococcal pyrogenic exotoxin types A (*speA*), B (*speB*), C (*speC*), and F (*speF*) and serotypes of M1, M6, and M12 were determined by polymerase chain reaction to target specific sequences in the 44 isolates recovered from these patients and in 28 isolates recovered from upper respiratory sites in 28 additional patients during the study period. The protease activity of these isolates was tested by using the casein plate method. Of the 44 patients with invasive diseases, 25 (57%) had no obvious underlying diseases, and 14 (32%) had preexisting neoplastic diseases or had previously used steroids. Twenty-five patients (57%) presented with cellulitis or necrotizing fasciitis, 24 (55%) had bacteremia, and eight (18%) had streptococcal toxic shock syndrome (STSS). Eight patients (18%) died of invasive GAS disease; seven had STSS, and seven had underlying diseases. All eight patients died within 48 hours after hospitalization. The presence of *speA, speC,* or *speF* was not implicated in any particular clinical syndrome in patients with invasive GAS disease. High-level protease activity and the M1 serotype of the isolates were significantly associated with the clinical signs of STSS and with mortality. M1 serotype and protease activity, as well as host immune status, might play significant roles in the pathogenesis of invasive GAS disease in Taiwan.

An increasing incidence of serious invasive infection caused by *Streptococcus pyogenes* (group A streptococcus) has been noted worldwide in recent years [1–8]. Clinical features that result in life-threatening sequelae of these infections include streptococcal toxic shock syndrome (STSS), bacteremia, and necrotizing fasciitis [1–10]. Such infections have been associated with death for 30% of patients and have occurred predominantly in otherwise immunocompetent hosts [2–4, 7]. The upsurge in the severity of group A streptococcal (GAS) disease has been attributed to the increasingly aggressive nature of bacteria and the subsequently exaggerated host response due to cytokine production or T cell activation [11–15]. Since the mid 1980s, strains of group A streptococci isolated from patients with invasive disease in North America and Europe have been mainly restricted to a few M serotypes and have been associated with the production of streptococcal pyrogenic exotoxins (SPEs) [2, 3, 5, 6, 8, 16]. However, recent reports have suggested that the serotype distribution of GAS strains and the pathogenic role of the SPEs in causing invasive disease and STSS vary in different geographic areas [3, 4, 6, 8, 16].

To determine whether the spectrum of invasive GAS disease in Taiwan parallels the increasing incidence and severity of GAS disease seen in other countries and to characterize the microbiological nature of the isolates causing this invasive disease, we conducted a 4-year review of all invasive GAS infections that were diagnosed at two teaching hospitals in southern Taiwan. We attempted to correlate the clinical parameters of disease severity with the serotypes and protease activity of the isolates and with the presence of genes encoding for SPEs to compare these factors in isolates causing invasive disease with those in isolates causing noninvasive disease.

Patients and Methods

Case Definition and Review

From January 1991 to December 1994, all culture records in the bacteriology laboratories of National Cheng Kung University Hospital (800 beds) and Taiwan Provincial Tainan Hospital (400 beds) in Tainan (population, approximately 0.6 million), located in southern Taiwan, were reviewed. All cultures positive for group A streptococci were collected, and the medical charts of the patients were reviewed to obtain clinical data. For comparison, isolates from normally sterile sites were considered invasive, whereas isolates from nonsterile sites were classified as noninvasive [7, 17]. Forty-four invasive GAS isolates were available and were recovered from blood cultures (24 isolates), aspirates of cutaneous bullae fluid (11) or deep-

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seated-abscess fluid (two), synovial fluid (five), ascitic fluid (one), or pleural effusion (one). Noninvasive GAS isolates were cultured from upper respiratory sites, including throat swabs (20 isolates) and sputum specimens (eight), for 28 additional patients.

Definition of Diseases

The criteria used to define STSS were in accordance with those described by the Working Group on Severe Streptococcal Infections [17]. Necrotizing fasciitis or soft-tissue necrosis was defined in accordance with the description of Talkington et al. [18]. An infection that occurred within the first 72 hours after admission to the hospital was considered to be community acquired. Previous steroid use was defined as receipt of steroid therapy or use of a Chinese herbal drug for at least 2 weeks before the development of GAS disease. Antimicrobial therapy was considered to be appropriate if the drug had in vitro activity against the isolate, as revealed by routine disk diffusion susceptibility testing, and was administered intravenously according to the manufacturer's recommended dosages. A rapidly fatal outcome was defined as the onset of illness <48 hours before admission, with death occurring within 2 days after hospitalization.

Bacterial Strains

All GAS isolates were identified on the basis of colony morphology, bacitracin susceptibility, and the pyrrolidonyl arylamidase test and were confirmed by commercial latex agglutination (Oxoid; Basingstoke, Hants, England) [19]. *S. pyogenes* NZ131, 86J28a, and 87/433 were kindly provided by D. R. Martin, New Zealand Communicable Disease Center, Porirua, New Zealand. All isolates were stored at −70°C in Todd-Hewitt medium (Difco Laboratories, Detroit) with 15% glycerol until testing.

Determination of *speA, speB, speC, speF,* and M Protein Genes

Genomic DNA preparation. Genomic DNA was prepared by using a modification of the method described previously [18]. Bacteria were grown overnight in 30 mL of Todd-Hewitt broth at 37°C, harvested by centrifugation, and resuspended in TE buffer (pH, 8.0). Mutanolysin and lysozyme (Sigma, St. Louis) were added to final concentrations of 10 ng/mL and 5 mg/mL, respectively. The mixture was incubated at 37°C for 1 hour. Cells were lysed with 5% SDS at 65°C for 30 minutes and then extracted twice with phenol-chloroform, followed by two volumes of ethanol. The DNA pellet was collected by centrifugation, washed twice with 70% ethanol, and dried. The pellet was then dissolved in 500 μL of TE buffer and stored at 4°C. The DNA concentration was measured from OD260 and run in 0.8% agarose gel along with standard markers (Promega, Madison, WI).

PCR. PCR was performed to detect *spe*A, *spe*B, *spe*C, *spe*F and serotypes M1, M6, and M12. The corresponding oligonucleotide primers were based on the previous descriptions [15, 20, 21]. PCR was performed in a total volume of 50 μL of reaction mixture containing 100 ng of template DNA, 50 p*M* of each primer, 0.1 m*M* dNTP, 1.25 m*M* $MgCl_2$, 1 × of *Taq* buffer, and 1 unit of *Taq* polymerase (Amersham; Arlington Heights, IL) in a thermal cycler (Perkin-Elmer Corporation, Norwalk, CT). The PCR conditions were programmed for 30 cycles of 2 minutes at 94°C, 2 minutes at 55°C, and 2 minutes at 72°C for *speA, speB,* M1, M6, and M12; 2 minutes at 94°C, 2 minutes at 45°C, and 1 minute at 72°C for *speC;* and 2 minutes at 94°C, 2 minutes at 50°C, and 2 minutes at 72°C for *speF.* The final amplification products were analyzed by gel electrophoresis in 2% agarose gel and stained with ethidium bromide. *S. pyogenes* NZ131, 86J28a, and 87/433 were used as control strains for determination of *speA, speB,* and *speC* (strain NZ131 was positive only for *speB,* 86J28a for *speA* and *speB,* and 87/433 for *speB* and *speC*).

Plasmid constructs. All plasmids were maintained in *Escherichia coli* Novablue unless otherwise stated. The PCR products of 353 bp (*speA*), 257 bp (*speB*), 130 bp (*speC*), 142 bp (M1), 150 bp (M6), and 142 bp (M12) were cloned into a pT7 blue T-vector (Novagen, Madison, WI), according to the instructions of the manufacturer, to construct plasmids pMW138, pMW139, pMW140, pMW141, pMW154, and pMW166, respectively. All constructions were confirmed by analyzing digestion patterns with appropriate enzymes and DNA sequencing (Sequenase version 2.0, USB; United States Biochemical Corp., Cleveland).

Southern blotting. The PCR products of the isolates were electrophoretically separated on 2% agarose gel. The gel was denatured with use of the method described by Reed and Mann [22]. DNA was completely transferred to nylon membranes (Amersham) with a pressure blotter (Posi-Blot; Stratagene, La Jolla, CA) and fixed to the membrane by ultraviolet cross-linking with a Stratalinker (Stratagene). The blot was incubated with 10 mL of prehybridization solution (5 × Denhardt, 5 × SSPE, 0.1% SDS, and 200 μg of denatured salmon sperm DNA per mL) at 68°C for at least 2 hours. The gene fragments of *speA* (pMW138), *speB* (pMW139), *speC* (pMW140), M1 (pMW141), M6 (pMW154), and M12 (pMW166) were labeled by random oligonucleotide priming (Promega) as a probe and were then hybridized overnight at 68°C. Blots were washed twice with 2 × standard saline citrate (SSC) for 30 minutes and then washed with 0.5 × SSC and 0.1% SDS for 30 minutes at room temperature. Finally, the membrane was washed in 0.1% SDS at 50°C for 30 minutes before autoradiography was performed.

Dot blotting. Dot blotting was performed to detect M protein genes (*emm* 1, *emm* 6, and *emm* 12). The oligonucleotide probes of the *emm* genes adopted in this study were in accordance with those described by Kaufhold et al. [23]. Ten micrograms of DNA were transferred to nylon membrane by dot

Table 1. Type of infection in 44 patients with invasive group A streptococcal disease.

Type of infection	No. (%) of patients
Cellulitis	18 (41)
Bacteremia	10 (23)
No bacteremia	8 (18)
STSS	1 (2)
Necrotizing fasciitis	7 (16)
Bacteremia	5 (11)
No bacteremia	2 (5)
STSS	4 (9)
Primary bacteremia	7 (16)
STSS	2 (5)
Septic arthritis	5 (11)
Pyelonephritis and bacteremia	2 (5)
Pneumonia	1 (2)
Peritonitis and STSS	1 (2)
Retroperitoneal abscess	1 (2)
Tubo-ovarian abscess	1 (2)
Mastoiditis	1 (2)

NOTE. STSS = streptococcal toxic shock syndrome.

blotter (Hybri-Dot Maniflod, GIBCO BRL, Life Technologies, Gaithersburg, MD). The probes were 5′ end labeled with T4 polynucleotide kinase, according to the method described by Sambrook et al. [24]. The hybridization conditions and washed blots were the same as those for Southern blotting, except for the incubation temperature of 50°C for hybridization and room temperature for washed blots.

Determination of Protease Activity

The protease activity of each isolate was determined twice by using the casein plate assay described previously [25, 26]. The protease activity was defined as the arithmetic average of two values, calculated by dividing the square of the diameter of the zone of casein hydrolysis by the square of the colony diameter [26].

T-protein Typing

The T-protein patterns of the isolates were determined by the slide agglutination method, as previously described, and were derived from a previous study [27].

Statistical Analysis

All analyses were performed with use of Scheffe's posteriorial comparison [28].

Results

Patients' Characteristics and Clinical Features

During the 4-year study period, 44 patients with invasive GAS disease were identified (table 1). Eight individuals were identified in 1991, 3 in 1992, 16 in 1993, and 17 in 1994. None of the GAS isolates appeared to reflect any clustering of cases, and we were not aware of any outbreaks of GAS disease at schools or hospitals during this period. The mean age of these patients was 47 years (range, 2–90 years); 20 (45%) of them were older than 60 years, and 25 (57%) were male. The clinical features included skin and soft-tissue infections (25 patients [57%]), bacteremia (24 [55%]), and STSS (8 [18%]). All the patients had community-acquired invasive diseases. Of nineteen patients (43%) with underlying diseases, seven had neoplastic diseases; seven had histories of steroid use (use of steroids for at least 2 weeks before admission); and one each had diabetes mellitus, liver cirrhosis plus diabetes mellitus, nephrotic syndrome, systemic lupus erythematosus, and chronic renal insufficiency. Of the seven patients with primary bacteremia, six had solid tumors, and two had complications of STSS. The deaths of eight patients (18%) were directly attributed to invasive GAS disease; six were older than 60 years, seven had underlying disease, and seven had STSS. All deaths occurred within 48 hours after hospitalization, regardless of whether appropriate antimicrobial therapy had been administered (table 2).

Microbiological Characteristics of the Isolates

None of the colonies of GAS isolates were mucoid on sheep blood agar (BBL Microbiology Systems, Cockeysville, MD). Table 3 shows that all isolates possessed genes for *speB.* The frequencies of *speA, speC,* and *speF* were similar among invasive and noninvasive isolates. In addition, no significant difference in the presence of *speA, speC,* or *speF,* respectively, was evident in association with the clinical signs of soft-tissue necrosis or shock or with mortality. Twenty-six (36.1%) of the 72 isolates belonged to the three M serotypes tested in this study. There was no significant difference in the frequency of serotypes M1, M6, or M12 between invasive and noninvasive GAS isolates. However, six (75%) of the patients with invasive M1 isolates (including three with M1T1 isolates, two with M1T12, and one with M1T23) that caused shock and mortality vs. none of the patients with invasive M6 and M12 isolates causing infections developed any septic complication or died. Statistically significant differences ($P < .05$) in protease activities between invasive and noninvasive isolates were noted (table 4). Significantly higher protease activities ($P < .01$) in the invasive isolates were also observed for patients with clinical signs of soft-tissue necrosis and shock and for those who died. Only one of the eight isolates from patients who died possessed *speA* (table 2).

Discussion

It is evident that there was an increase in the number of cases and in the severity of invasive GAS infections in southern Taiwan from 1991 to 1994. These findings were in accord with

Table 2. Clinical and microbiological features of eight patients with invasive group A streptococcal disease that resulted in rapidly fatal outcomes.

Patient no.	Age (y)/sex	Underlying condition	Type of infection	M/T serotype	Presence of *speA*/*speC*/*speF*/PA	Complication(s)
1	69/M	Liver cirrhosis	Necrotizing fasciitis, STSS	N/27	−/+/−/36.0	S, ARDS, R, H
2	62/F	Cushing's syndrome	Necrotizing fasciitis, STSS	N/5	−/+/+/30.3	S, R, H, C
3	68/F	Steroid use	Necrotizing fasciitis, STSS	1/1	−/+/+/36.0	S
4	7/M	None	Necrotizing fasciitis	1/12	−/+/+/25.0	S
5	70/F	Cervical carcinoma	Bacteremia, STSS	1/23	−/+/+/25.0	S, R, H, C
6	74/F	Diabetes mellitus	Bacteremia, STSS	1/1	+/−/−/36.0	S, ARDS
7	68/M	Steroid use	Bacteremia, STSS	1/1	−/+/+/36.0	S, R, H
8	54/F	Diabetes mellitus	Peritonitis, STSS	1/12	−/+/+/30.3	S

NOTE. ARDS = adult respiratory distress syndrome; C = coagulopathy; H = hepatic dysfunction; N = M serotype other than M1, 6, or 12; PA = protease activity; R = renal impairment; S = shock; STSS = streptococcal toxic shock syndrome; + = positive by PCR reaction; − = negative by PCR reaction.

those reported in the North America and Europe [1–10]. In the present study, more than half of the patients with invasive GAS infections had no underlying diseases, and approximately half of the patients were elderly. The overall mortality among the patients with invasive GAS disease was similar to that reported in other series [1–4, 7]. Contrary to the 30% mortality rate and low incidence of underlying medical conditions among patients with STSS described by other investigators [2, 5, 7, 9], all of our patients with STSS died, and the majority of those patients were elderly individuals with severe underlying diseases. These findings reflect the fact that regardless of the presence or absence of some virulence factors in these invasive GAS isolates, the preexisting immunocompromised statuses of our patients might have played an important role in the poor clinical outcome.

Previous investigations have suggested that the clinical outcome of invasive GAS disease, especially STSS, was due to the interaction between bacterial virulence factors (M protein and SPEs) and the immune status of the hosts [5, 6, 14]. In several reports, it has been documented that the presence of *speA* and its encoding toxin SPEA is strongly correlated with invasive GAS disease, including STSS [1–10, 12, 21, 29–31]. However, it has also been reported that there is no significant association between the presence of *speA* and GAS bacteremia in children, although the number of cases was small, and the attributable mortality was relatively low [8]. The same study also indicated that *speA* was present in 80% of STSS-associated bacteria and 50% of all GAS isolates causing STSS or death [8]. These findings are not consistent with the results of the present study, which showed that *speA* was present in only 39% of invasive GAS isolates and only 13% of STSS- and mortality-associated isolates. The role of *speB, speC,* and *speF* in the pathogenesis of invasive GAS disease is obscure, although each of these toxin genes has been reported to be impli-

Table 3. Associations between microbiological characteristics of group A streptococcal (GAS) isolates and clinical features of GAS disease.

	No. (%) of isolates					
	Invasive isolate					
Characteristic	Soft-tissue necrosis (n = 7)	Shock (n = 14)	Mortality (n = 8)	Total (n = 44)	Noninvasive isolate (n = 28)	Total (n = 72)
Gene						
speA	1 (14)	4 (29)	1 (13)	17 (39)	9 (32)	26 (36)
speB	7 (100)	14 (100)	8 (100)	44 (100)	28 (100)	72 (100)
speC	7 (100)	13 (93)	7 (88)	40 (91)	25 (89)	65 (90)
speF	5 (71)	10 (71)	6 (75)	28 (64)	18 (64)	46 (64)
Serotype						
M1	2 (29)	6 (43)	6 (75)	8 (18)	6 (21)	14 (19)
M1T1	1 (14)	3 (21)	3 (38)	3 (7)	1 (4)	4 (6)
M1T12	1 (14)	2 (14)	2 (25)	4 (9)	5 (18)	9 (13)
M1T23	0	1 (7)	1 (13)	1 (2)	0	1 (1)
M6	0	0	0	4 (9)	0	4 (6)
M12	0	0	0	6 (14)	2 (7)	8 (12)

Table 4. Association between protease activity of group A streptococcal (GAS) isolates and clinical characteristics of GAS disease.

Characteristic	No. of isolates	Mean protease activity ± SEM*	*P* value
Invasive isolates			
Yes	44	17.81 ± 1.53	
No	28	13.32 ± 1.28	<.05
Shock			
Yes	14	24.70 ± 2.16	
No	58	13.98 ± 1.08	<.01
Mortality			
Yes	8	31.83 ± 1.73	
No	64	14.78 ± 1.09	<.01
Soft-tissue necrosis			
Yes	7	28.37 ± 2.67	
No	65	15.38 ± 1.11	<.01

* Protease activity of each isolate was determined by dividing the square of the diameter of the zone of hydrolysis by the square of the colony diameter.

cated in invasive GAS disease in different geographic areas [8, 9, 15, 21, 28, 32, 33]. Similarly, the presence of any of these toxin genes was not significantly associated with the invasive nature of GAS isolates in southern Taiwan.

Previous reports have indicated that the majority of invasive GAS isolates, especially STSS-associated organisms, was limited to several M serotypes (M1, M3, M6, M12, and M18), many of which have a mucoid phenotype [6, 8, 11, 16]. Several studies have also implied that a unique M1T1 clone is strongly associated with the majority of invasive GAS disease [8, 34]. Although M1 and M6 serotypes occurred in a higher proportion of our invasive isolates than noninvasive isolates, we were unable to draw definitive conclusions on the correlation between invasive disease or *speA, speC,* and *speF* and any M serotype isolate because only three M serotypes were determined. However, the fact that six of the eight isolates that caused fatal invasive GAS disease were M1 serotype, and three of them were M1T1, is impressive. These data support previous observations [6, 8, 11, 16, 33]. However, in addition to M1T1, M1 isolates other than T1 might also be considered important pathogens that cause disastrous sequelae of invasive GAS disease in Taiwan.

Our results showed that among the invasive isolates, those associated with death had significantly higher protease activities than those not associated with death. Investigators have previously observed that protease activity was significantly associated with necrotizing fasciitis and M1 serotype GAS isolates [1, 6, 18, 31]. Our findings were consistent with these observations. Significant differences in this enzyme activity were also apparent between the STSS-associated and non-STSS-associated invasive isolates.

Our data support previous observations that the prognosis for patients with GAS disease complicated by septic shock was frequently less favorable [1, 10, 11, 35]. Contrary to reports from other investigators, up to half of our patients with septic shock had STSS. Twenty-nine percent of our bacteremic patients had primary bacteremia, a rate for primary bacteremia that is within the range reported in the literature (0–41%). Three-fourths of our patients with primary bacteremia had malignant diseases, a rate slightly higher than the 50% reported by Moses et al. [36].

In summary, during a 4-year retrospective study of invasive GAS disease in southern Taiwan, we found a trend toward an increase in the number of these infections and an apparent increase in their severity. The lack of association between the presence of *speA, speB, speC,* or *speF* in the isolates implicated in any clinical syndrome in patients with invasive GAS disease does not confirm previous observations. M1 serotype and protease activity of GAS isolates, as well as host immune status, might play significant roles in the pathogenesis of invasive GAS disease in southern Taiwan.

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