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Effect of Group A Streptococcal Cysteine Protease on Invasion of Epithelial Cells

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Cysteine protease of group A streptococci (GAS) is considered an important virulence factor. However, its role in invasiveness of GAS has not been investigated. We demonstrated in this study that two strains of protease-producing GAS had the ability to invade A-549 human respiratory epithelial cells. Isogenic protease mutants were constructed by using integrational plasmids to disrupt the *speB* gene and confirmed by Southern hybridization and Western immunoblot analyses. No extracellular protease activity was produced by the mutants. The mutants had growth rates similar to those of the wild-type strains and produced normal levels of other extracellular proteins. When invading A-549 cells, the mutants had a two- to threefold decrease in activity compared to that of the wild-type strains. The invasion activity increased when the A-549 cells were incubated with purified cysteine protease and the mutant. However, blockage of the cysteine protease with a specific cysteine protease inhibitor, E-64, decreased the invasion activity of GAS. Intracellular growth of GAS was not found in A-549 cells. The presence or absence of protease activity did not affect the adhesive ability of GAS. These results suggested that streptococcal cysteine protease can enhance the invasion ability of GAS in human respiratory epithelial cells.

Group A streptococci (GAS) (*Streptococcus pyogenes*) are commonly associated with pharyngitis, cellulitis, and impetigo, but the recent resurgence in blood infections has generated interest in their potential to invade deeper tissues (35). However, so far, their pathogenic factor is poorly defined. Streptococcal pyrogenic exotoxin B (SPE B) is among several extracellular products produced by GAS that may cause severe infections. The gene encoding SPE B is found in all isolates and is assumed to be chromosomal (43). SPE B is a cysteine protease produced extracellularly as a zymogen of about 40 kDa (13). The 28-kDa mature protease is formed by autoproteolytic truncation of the zymogen (42). In 1945, Elliott (10) first reported that streptococcal cysteine protease (SCP) had fibrinolytic activity, and later it was reported that intravenous injection of SCP into rabbits, guinea pigs, and mice caused myocardial necrosis (21). Patients with severe streptococcal infections had lower titers of antibody to SCP than patients with less severe infections (15, 25). Immunization with SCP protected mice from lethal challenge (3, 18). Inactivation of SCP significantly decreased lethality to mice (23). Moreover, purified SCP not only can cleave inactive human interleukin 1β precursor, monocytic cell urokinase receptor, and fibronectin but also can degrade vitronectin (19, 20, 41). SCP can release biologically active kinins and C5a peptidase as well as activate a 66-kDa human endothelial cell matrix metalloprotease (2, 4, 14). In addition, SCP acts synergistically with other *S. pyogenes* products to increase tissue injury (32). These pieces of evidence suggest that SCP plays multiple roles in GAS infection.

Recently, LaPenta et al. (22) have shown that GAS are efficiently internalized and can persist in cultured human respiratory epithelial cells, suggesting that the organisms actually enter the cell during the course of infection. In addition, Molinari et al. (24) and Cue and Cleary (7) demonstrated that the fibronectin-binding protein of GAS and fibrinogen are involved in the internalization of GAS. In this study, we found that cysteine protease of GAS also enhanced the ability of the microorganism to invade A-549 cells in vitro.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and tissue culture cells. One GAS strain, A-20 (type M1, T1, opacity factor negative), was isolated from a blood culture and collected from National Cheng Kung University Hospital. *S. pyogenes* NZ131 (type M49, T14) was a gift from D. R. Martin, New Zealand Communicable Disease Center, Porirua. All *S. pyogenes* cultures were grown in tryptic soy broth supplemented with 0.5% yeast extract (TSBY). *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was grown in Luria broth (LB). Plasmids pSF151 and pDL286 were kindly provided by L. Tao, University of Missouri, Kansas City (37). All strains were stored at −70°C in LB with 15% glycerol until testing. Human alveolar carcinoma epithelial cell monolayers (A-549; American Type Culture Collection CCL-185) were used for all invasion assays and were grown in Dulbecco's modified Eagle medium (DMEM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (GIBCO Laboratories), penicillin (100 μg/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). The cells were subcultured every second day.

Protease assay. Detection of protease activity was based on the method of Hynes and Tagg (16). GAS isolates were grown on Columbia agar base (Difco Laboratories, Detroit, Mich.) containing 3% skim milk for detection of protease production. After aerobic incubation at 37°C for 24 h, the zone of casein hydrolysis was measured. For the azocasein assay, bacterial supernatant was collected and assayed by a modified method of Ohara-Nemoto et al. (26). The reaction was initiated by addition of 200 μl of 24-h-growth supernatant of GAS to 400 μl of reaction mixture containing 2.7 mg of azocasein (Sigma Chemical Co., St. Louis, Mo.) per ml in 50 mM Tris-HCl (pH 8.0) which had been prewarmed at 37°C. After incubation at 37°C for 20 min, the reaction was stopped by addition of 100 μl of 15% ice-cold trichloroacetic acid. The reaction mixture was held on ice for 15 min and then centrifuged, and an equal volume of 0.5 M NaOH was added to the supernatant. The absorbance at 450 nm of the sample was measured with a kinetic microplate reader, V-max (Molecular Devices Corporation, Menlo Park, Calif.), to determine the amount of azopeptides not precipitated with trichloroacetic acid.

Adhesion assay. Adhesion of ^{3}H-labeled GAS to A-549 cells was measured by the method of Wang and Stinson (40). Bacteria were suspended in DMEM to a density of 4×10^8 CFU/ml. Aliquots of the bacterial suspension (250 μl) were added to prewashed confluent A-549 monolayer cultures containing about 5×10^5 cells per well. Bacteria were centrifuged onto the monolayer for 10 min at

183 × *g* at room temperature to accelerate the contact of bacteria and A-549 cells. After 90 min, nonadherent bacteria were removed by washing the monolayer three times with phosphate-buffered saline (PBS) (15 mM Na_2HPO_4, 145 mM NaCl [pH 7.2]). The monolayer with adherent bacteria was suspended in 200 μl of 1% Triton X-100, and radioactivity was determined by liquid scintillation spectrometry (Beckman Instruments, Somerset, N.J.).

Invasion assay. The assay of streptococcal invasion of A-549 monolayers was based on the method of Isberg and Falkow (17). Briefly, A-549 cells (5×10^5) were seeded into each well of 24-well tissue culture plates (Costar Co., Cambridge, Mass.) containing DMEM with antibiotics. To remove the antibiotics, the cells were washed three times with PBS before inoculation. The bacterial cultures were washed three times with PBS and resuspended in the original volume. Before inoculation onto monolayers, bacterial suspensions were retained at room temperature and then diluted to the appropriate concentration in DMEM. Confluent monolayers were infected with approximately 5×10^7 CFU of streptococci (infection rate, 1:100). After 90 min of incubation at 37°C in 5% CO_2, extracellular bacteria were removed by five washes with PBS. DMEM containing penicillin (0.8 μg/ml) was then added to the infected monolayer at 37°C in 5% CO_2 and the monolayer was incubated for 90 min to eliminate extracellular bacteria. The infected monolayers were washed three times with PBS to remove the antibiotics. The cells were lysed by addition of 200 μl of 1% Triton X-100. The lysate was collected, and the number of intracellular bacteria was determined by plating on TSBY agar. All experiments were done at least three times for each strain. Wells without epithelial cells served as negative controls.

An invasion kinetics assay was done as described above except that the infected monolayers were incubated for 1.5, 2, 3, 4, 5, and 10 h before the addition of penicillin to eliminate extracellular bacteria (33). An intracellular multiplication assay was done as described above except that the infected monolayers were incubated for 1.5, 2, 3, and 4 h after the addition of penicillin to the infected monolayer (34).

Purification of SPE B from strain A-20. The method for purification of SPE B was based on the methods of Kapur et al. (20) and Ohara-Nemoto et al. (26), with modifications. Bacteria were grown overnight at 35°C in TSBY medium. A 10-ml aliquot of the overnight growth was added to 500 ml of TSBY medium, which was then incubated at 35°C for 22 to 24 h. The cells were removed by centrifugation, and the supernatant was filtered through a 0.45-μm-pore-size membrane filter. The filtrate was diluted with 4 volumes of cold distilled water, the pH was adjusted to 8.0, and then a 1/20 volume of DEAE-Sephadex (Pharmacia Biotech, Sweden) equilibrated with 20 mM Tris-HCl (pH 8.0) was added. The suspension was left for 30 min with occasional mixing, and then unbound material was collected by filtration. The filtrate was concentrated to 100 ml by use of a 3-kDa-cutoff ultrafiltration cartridge (Amicon Division, W. R. Grace & Co., Beverly, Mass.). Buffer exchange by ultrafiltration was conducted with 1 liter of 20% ethanol–20 ml Tris-HCl (pH 7.0) (buffer A) at 4°C. The ultrafiltration solution was passed through a matrix gel Red A column (1.5 by 15 cm; Amicon Division) equilibrated with buffer A. The column was washed with buffer A until the absorbance (280 nm) returned to baseline, and the bound protein was eluted with buffer A containing 2 M NaCl. The eluted material was collected as one fraction and concentrated to 4 ml by ultrafiltration, and the buffer was exchanged with PBS (pH 7.2) by ultrafiltration. The proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The desired proteins contained in the gel slices were identified by amino acid sequencing (Applied Biosystems 477A autosequencer) after the proteins had been eluted and renatured.

Preparation and purification of anti-SPE B antibody. Fifty micrograms of the purified SPE B was emulsified with complete Freund's adjuvant and used to immunize rabbits. Four subsequent immunizations with 25 μg of SPE B emulsified with incomplete Freund's adjuvant were given at 2-week intervals. The titers of the antisera were determined by an enzyme-linked immunosorbent assay using microdilution plates coated with SPE B. The immunoglobulin G fractions of the antisera were purified with a protein A column (Zymed Laboratories, Inc., South San Francisco, Calif.). The neutralizing activity of anti-SPE B immunoglobulin G was determined by azocasein assay according to the procedures described above.

Reconstitution and neutralization in the invasion assay. For reconstitution studies, the purified SPE B was diluted to the appropriate concentration with 1× PBS buffer and added together with GAS to A-549 cells. Purified SPE B was also heated in a boiling water bath for 10 min and then subjected to the invasion assay. For neutralization, 8 and 16 μM E-64 (Sigma Chemical Co.) was used with purified SPE B and GAS at room temperature for 10 min. The rest of the invasion assay was carried out as described above. Control wells were preincubated with tissue culture medium without SPE B.

Cloning of *speB*. The *speB* gene was amplified by PCR using *S. pyogenes* A-20 DNA as a template with the first primer (5′ GATCAAAACTTTGCTCGTA ACG 3′) corresponding to bases 81 to 103 of the *speB* gene and the second primer (5′ CTAAGGTTTGATGCCTACAACAG 3′) designed to be complementary to *speB* nucleotides 1175 to 1195 in the published sequence (13). PCR was performed with a total volume of 50 μl containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100, 100 ng of DNA, 50 pM primers, 10 mM deoxynucleoside triphosphates, and 1 U of *Taq* DNA polymerase (New England Biolabs Inc., Beverly, Mass.). Amplification was performed in a DNA thermal cycler (Perkin-Elmer Corporation, Norwalk, Conn.) that had been programmed for 4 cycles of 1 min at 94°C, 1 min at 37°C, and 2 min at 72°C followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. Following amplification, the 1.1-kb PCR product was digested with either *Kpn*I and *Pst*I or *Hin*dIII and *Pst*I to obtain 0.4- and 0.7-kb fragments of *speB* from nucleotides 578 to 1005 and 347 to 1005, respectively (13). The 0.4- and 0.7-kb fragments of *speB* were ligated into plasmids pSF151 and pDL286 to construct plasmids pMW152 and pMW153, respectively. The constructions were checked by analyzing digestion patterns obtained with appropriate endonucleases and DNA sequencing.

DNA preparation and sequencing. Plasmid DNA was prepared from *E. coli* as described previously (29). Chromosomal DNA of GAS was prepared by the method of Cleary et al. (6). DNA sequencing was performed by the dideoxy-chain termination method of Sanger et al. (30). A Sequenase reagent kit (United States Biochemical Corp., Cleveland, Ohio) was used according to the manufacturer's protocol.

Transformation. *E. coli* was transformed by the method of Hanahan (11). *S. pyogenes* was electroporated by the method of Schalén et al. (31); selection for kanamycin and spectinomycin resistance was done with 50 and 100 μg/ml, respectively.

DNA-DNA hybridization. Chromosomal DNA preparations from GAS were digested with restriction endonucleases and electrophoresed through a 0.8% agarose gel. DNA was transferred to nylon membranes (Amersham, Arlington Heights, Ill.) as previously described (29). The purified *speB* DNA fragment to be used as a probe was labeled with [α-^{32}P]dCTP (Amersham) by use of a random-primer DNA labeling kit (Promega Corp., Madison, Wis.) according to the manufacturer's instructions. Hybridizations were performed as follows: prehybridization and hybridization were carried out for 2 and 18 h, respectively, at 65°C in 5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–0.02% SDS–1% (wt/vol) blocking reagent for nucleic acid hybridization (Boehringer, Mannheim, Germany)–0.1% *N*-lauryl-sarcosine, followed by two washings in 2× SSC–0.1% SDS at room temperature for 5 min and two washings in 0.2× SSC–0.1% SDS at 65°C for 15 min.

TEM. Bacteria were prepared for the invasion assay as described above. Samples for electron microscopy were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 4 h, washed in sodium cacodylate buffer with 3 mM $CaCl_2$, and postfixed in 1% osmium tetroxide at 4°C for 1 h. After being rinsed in cacodylate buffer, the cells were dehydrated in an ascending series of alcohol concentrations (50, 70, 85, 95, 100, 100, and 100%; each step for 15 min). After being rinsed in propylene oxide and then subjected to infiltration in propylene oxide-epoxy resin mixtures, the cells were embedded in epoxy resin and polymerized at 60 to 70°C for 48 h. Sections were cut 0.5 μm thick with a Reichert OM UIII ultramicrotome and stained with 1% toluidine blue in 1% borax. Areas suitable for ultrathin sectioning (approximately 90 nm) were selected. Sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and then examined with a Hitachi H-7000 transmission electron microscope (TEM).

Susceptibility test. The MIC of penicillin was determined by the Etest method (AB Biodisk, Piscataway, N.J.) according to the manufacturer's instructions.

Statistical analysis. The difference in invasion ability of the wild type and the isogenic mutant was analyzed by *t* test. A P value of <0.01 was considered statistically significant.

RESULTS

Disruption of *speB*. Integrational plasmids were used to disrupt the *speB* gene. Plasmids pMW152 and pMW153 were constructed by cloning into vectors pSF151 and pDL286 an insert of *S. pyogenes* DNA that contained part of the *speB* coding region. These plasmids replicated autonomously in *E. coli* but not in *S. pyogenes* when transformed into *S. pyogenes* with selection for the vector-determined resistance; transformants arose because of integration of the plasmid into the homologous region of the recipient chromosome by a Campbell-like mechanism. When the region cloned on an integrational plasmid was entirely internal to the *speB* transcriptional unit, it disrupted that transcriptional unit. Plasmid pMW152 integrated into A-20 to obtain an isogenic protease mutant was designated SW507; plasmid pMW153 integrated into NZ131 was designated SW510. Transformant colonies were initially screened on the basis of proteolytic activity on casein plates. Protease activity was present in the extracellular proteins secreted by strains A-20 and NZ131, while strains SW507 and SW510 had no extracellular protease activity (data not shown). As analyzed by Southern hybridization, 5.5- and 1.3-kb hybridization bands were obtained with *Nsi*I and *Hin*dIII digestion, respectively, while a 1.0-kb fragment was seen in an *Nsi*I-

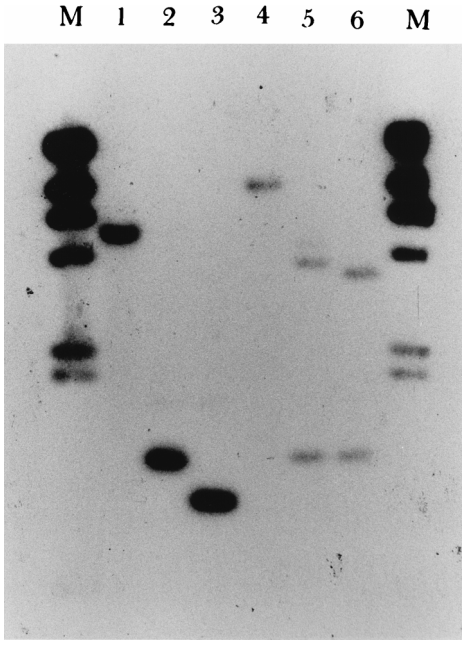

FIG. 1. Southern blot analysis of genomic DNA extracted from GAS A-20 and SW507. The probes used were specific for the *speB* gene and λ DNA. The DNAs were digested with *Nsi*I (lanes 1 and 4), *Hin*dIII (lanes 2 and 5), and *Hin*dIII-*Nsi*I (lanes 3 and 6). Lanes 1 to 3, GAS A-20 genomic DNA; lanes 4 to 6, GAS SW507 genomic DNA; lanes M, λ *Hin*dIII marker used as a molecular size standard.

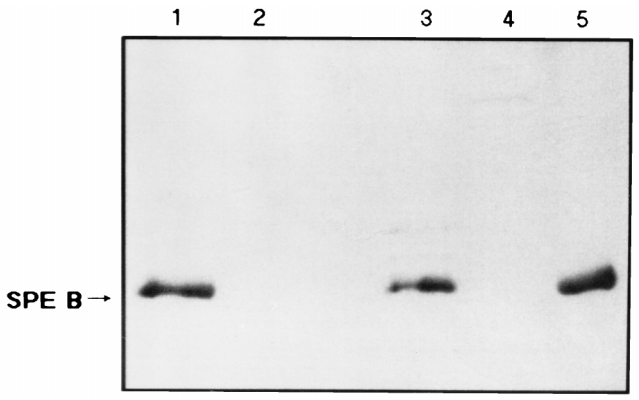

FIG. 2. Western immunoblot analysis of SPE B present in the supernatants of wild-type GAS (A-20 and NZ131) and mutants (SW507 and SW510) after electrophoresis on an 8% polyacrylamide gel. The blot was developed with rabbit anti-SPE B polyclonal antibodies. Lane 1, A-20; lane 2, SW507; lane 3, NZ131; lane 4, SW510; lane 5, purified 28-kDa SPE B from A-20 used as a standard. The mature SPE B protein is indicated (arrow).

*Hin*dIII double digestion of wild-type strain A-20 (Fig. 1, lanes 1 to 3). For mutant SW507, hybridization bands of 1.3 and 3.9 kb were seen in the *Hin*dIII digestion, a 9.4-kb fragment was seen in the *Nsi*I digestion, and both 3.6- and 1.3-kb fragments were seen in the *Nsi*I-*Hin*dIII double digestions, confirming that integration occurred by a Campbell-like mechanism (Fig. 1, lanes 4 to 6). In the same way, integration into strains NZ131 and SW510 was also confirmed by Southern hybridization (data not shown). To demonstrate that strains SW507 and SW510 lost their ability to express SPE B protein, the supernatants from these strains were collected and the absence of SPE B protein was confirmed by Western immunoblot analysis using rabbit anti-SPE B antibody. As shown in Fig. 2, the antiserum recognized the mature form of SPE B (28 kDa) in supernatants of strains A-20 and NZ131, while no SPE B protein bands (either 40 or 28 kDa) were observed in the supernatants of SW507 and SW510. No difference in growth rates was observed between mutants and the wild-type strains in TSBY and DMEM (Fig. 3). No differences in streptokinase expression and hemolysis on blood agar were found (data not shown). In addition, the MIC of penicillin for strains A-20 and SW507 was 0.023 μg/ml, whereas that for strains NZ131 and SW510 was 0.016 μg/ml.

Adhesion assay. The ability of GAS to adhere to monolayers of A-549 cells is shown in Table 1. All wild-type strains and isogenic mutants adhered to the A-549 monolayer, regardless of protease activity.

Penicillin protection assay. A penicillin-protected bacterium is shown in Fig. 4. A-549 monolayers were infected with GAS for 2 h before the addition of penicillin to eliminate extracellular bacteria. TEM demonstrated the intracellular presence of GAS in the vacuoles of A-549 cells.

Effect of cysteine protease on GAS invasion. To examine the effect of cysteine protease on GAS invasion, the protease-positive strains and mutants were tested by an invasion assay based on the method of Isberg and Falkow (17). Strains A-20 and NZ131, both protease positive, had invasive activities two- to threefold greater than those of isogenic mutant strains SW507 and SW510 (Table 2 and Fig. 5). Statistically significant differences ($P < 0.01$) in invasion activities between wild-type

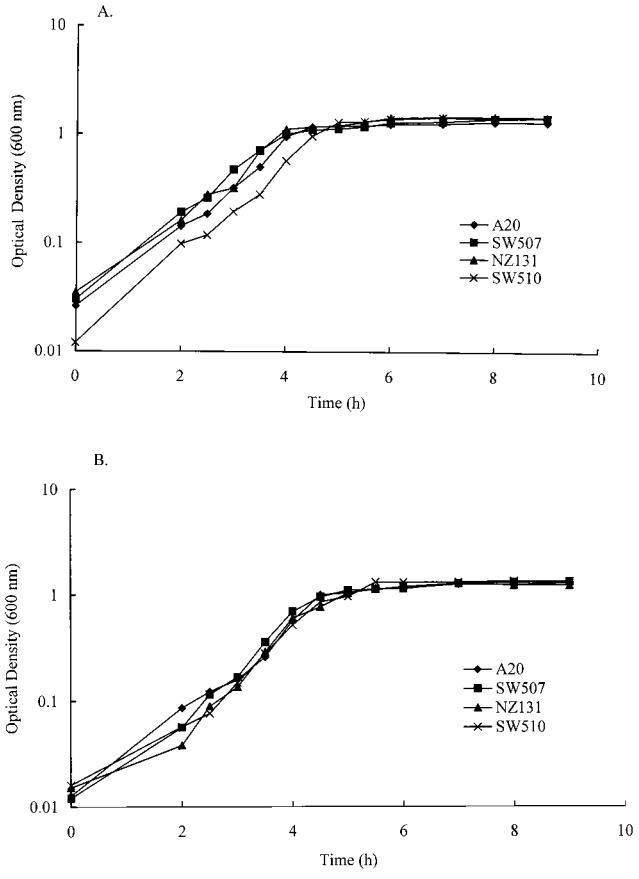

FIG. 3. Growth curves for wild-type strains (A-20 and NZ131) and isogenic mutants (SW507 and SW510) in TSBY (A) and DMEM (B).

TABLE 1. Adhesion abilities of protease-positive and -mutant strains of GAS

Strain	Proteolytic activity[a]	Adhesion[b] (10^8 CFU/ml)
A-20	+	4.88 ± 0.13
SW507	−	6.20 ± 0.21
NZ131	+	3.13 ± 0.34
SW510	−	5.39 ± 0.47

[a] The proteolytic activity was determined by use of a casein plate. +, activity; −, no activity.
[b] Adhesion ability was assayed as described in the text. Results are means ± standard errors of the means of three experiments.

strains and mutants were noted. The invasion kinetics assay showed that when the incubation time was extended from 1.5 to 10 h, the invasion activity for both protease-positive strains and mutants increased significantly (Fig. 5).

To further examine the role of cysteine protease on the invasion of GAS, purified SPE B protein was tested in vitro. Identification of the purified protein as SPE B was confirmed via N-terminal sequence analysis of the protein from the Red A column; the first 10 amino acids (Gln-Pro-Val-Val-Lys-Ser-Leu-Leu-Asp-Ser) were confirmed exactly as predicted from the DNA sequence (13). The purified cysteine protease was mixed with GAS prior to the invasion assay. After a 1.5-h incubation, the invasion activities of the mutants increased to levels similar to those of protease-positive strains ($P < 0.01$) (Table 2). The SPE B-mediated enhancement of invasion activity was significantly inhibited by the addition of a specific cysteine protease inhibitor, E-64, for both SW507 and SW510 mutants ($P < 0.01$).

Intracellular growth of GAS. The intracellular multiplication of GAS was determined by addition of penicillin to the infected monolayers after GAS had invaded the cells for 1.5 h. Infected cells were collected for 4 h after the bacterial invasion, the cells were lysed, and the numbers of intracellular bacteria were determined by plating on TSBY agar. As shown in Fig. 6, there was no significant increase in the number of intracellular bacteria over the 4-h incubation period. Control experiments showed that no viable bacteria were recovered from wells without A-549 cells after incubation in culture medium containing 0.8 μg of penicillin per ml.

DISCUSSION

Recently, LaPenta et al. (22) have demonstrated for the first time that GAS, which represent typically extracellular pathogens of epithelial surfaces, can enter and persist in host cells. Invasion might play a role in the pathogenicity of GAS by facilitating tissue invasion or helping to protect the bacteria from extracellular defense mechanisms. Although evidence

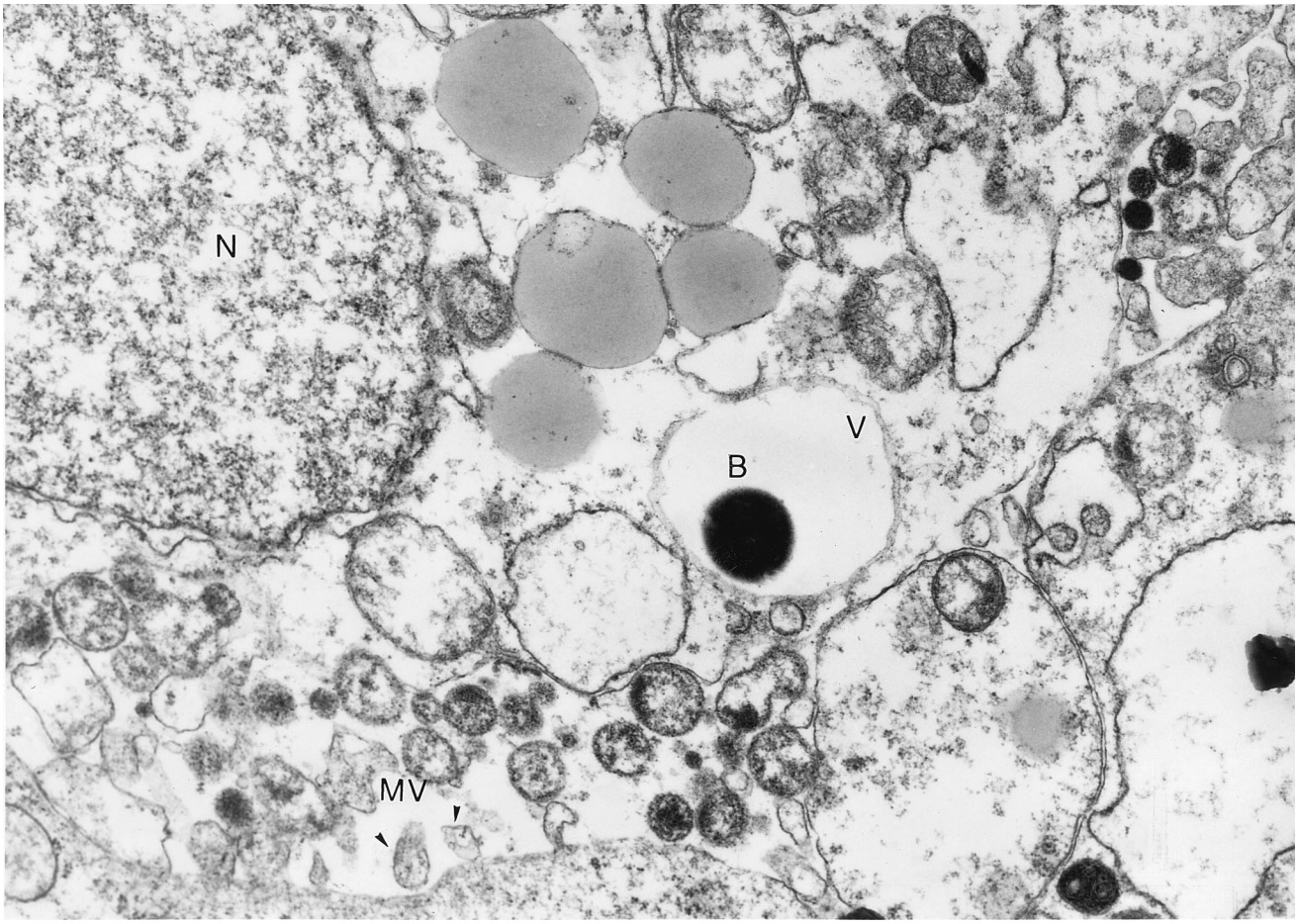

FIG. 4. Electron micrograph of GAS strain A-20 entry into cultured A-549 cells. Monolayers were infected with 5×10^7 CFU for 120 min before they were washed and exposed to penicillin. A bacterium (B) is enclosed in a vacuole (V). The nucleus (N) and microvilli (MV) are indicated. Magnification, ×10,000.

TABLE 2. Invasion abilities of protease-positive and -mutant strains of GAS

Strain	No. of streptococci (10^4 CFU/well) grown within A-549 cells reconstituted with[a]:					
	None	SPE B[b]	Heated SPE B	SPE B + E-64 (8 μM)	SPE B + E-64 (16 μM)	E-64 (16 μM)
A-20	9.68 ± 0.14	12.6 ± 0.11	10.02 ± 0.37	10.2 ± 0.19	9.68 ± 0.30	9.88 ± 0.65
SW507	4.87 ± 0.11	9.58 ± 0.29	5.32 ± 0.58	5.15 ± 0.17	4.38 ± 0.09	4.75 ± 0.39
NZ131	10.79 ± 0.24	11.17 ± 0.39	ND[c]	ND	ND	ND
SW510	4.43 ± 0.08	9.21 ± 0.29	5.40 ± 0.21	6.65 ± 0.81	6.40 ± 0.56	4.43 ± 0.05

[a] Results are demonstrated as the number of intracellular bacteria ± standard error after 1.5 h of incubation.
[b] The invasion assay was done with reconstituted purified SPE B.
[c] ND, not done.

suggests that cysteine protease plays an important role in the pathogenesis of streptococcal infection, it is not known whether it plays a role in the invasion of epithelial cells. In this study, we used gene disruption of *speB*, protease reconstitution, and neutralization experiments to study the role of cysteine protease of GAS in the invasion of respiratory epithelial cells in vitro.

On the basis of the finding that penicillin-protected bacteria were inside the cells, we have provided data which demonstrate the ability of cysteine protease to enhance the entrance of GAS into eukaryotic cells. Strains A-20 and NZ131, both protease-positive GAS, displayed two- to threefold increases in invasive activity over isogenic protease mutants (SW507 and SW510). The effect was also observed when purified cysteine protease and the mutant were incubated together with A-549 cells prior to the invasion assay. Blockage of the cysteine protease with E-64 decreased the invasion activity (Table 2). These data suggest that cysteine protease not only enhanced bacterial uptake by the cells but also had a role in receptor-ligand interaction involved in the entry of GAS into the respiratory epithelial cells. However, we cannot rule out the possibility that a substance(s) other than cysteine protease present in the purified fraction may also have enhanced the invasive activity. No significant changes were observed if GAS were treated with E-64 alone prior to the invasion assay. The results suggest that cysteine protease was not present on the bacterial surfaces. How cysteine protease interacts with the host and the nature of the receptor recognized by cysteine protease require further study.

Factors other than cysteine protease may also have influenced the results of the invasion assay. The MICs of penicillin are the same for both mutants and wild-type strains, suggesting

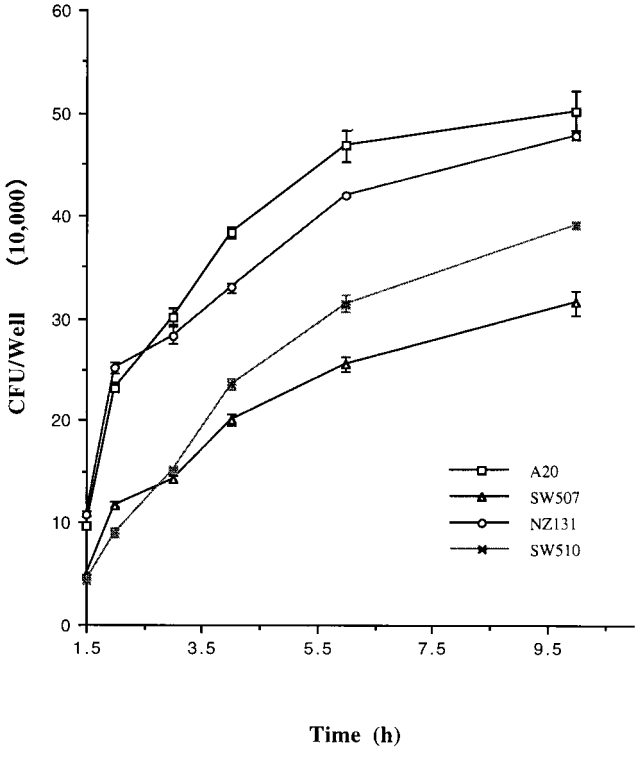

FIG. 5. Invasion kinetics of the wild-type strains (A-20 and NZ131) and isogenic mutants (SW507 and SW510). Confluent cell monolayers were infected with 5×10^7 bacteria in 24-well plates. The cells were incubated for 1.5, 2, 3, 4, 5, and 10 h, washed with PBS, and incubated for another 1.5 h in medium containing 0.8 μg of penicillin per ml. GAS cell numbers were determined by serial dilutions of cell lysates and plating on agar. Results are means ± standard errors of the means (SEM) of the numbers of GAS per well from four separate experiments; at each time point, triplicate samples were analyzed.

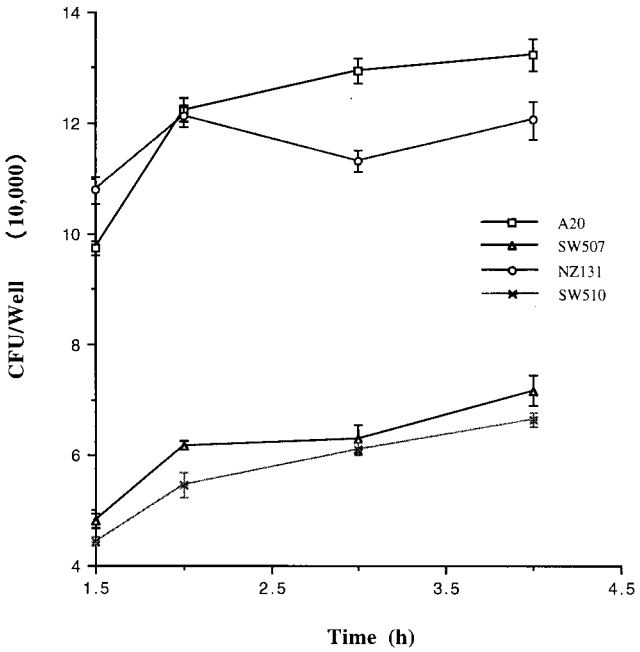

FIG. 6. Intracellular growth of *S. pyogenes* in A-549 cells. Confluent cell monolayers were infected with 5×10^7 bacteria in 24-well plates. The cells were incubated for 90 min, washed, and incubated for another 1.5 to 4 h in medium containing 0.8 μg of penicillin per ml. GAS cell numbers were determined by serial dilution of cell lysates and plating on agar. Results are the means ± SEM of the numbers of GAS per well from four separate experiments; at each time point, triplicate samples were analyzed.

that the mutants were no more susceptible to penicillin than the wild-type strains. In addition, the growth rates of A-20 and NZ131 and their mutants in TSBY and DMEM appeared to be similar (Fig. 3). Therefore, the growth rates of the two isogenic GAS strains did not explain the differences in the invasion of A-549 cells. A previous report (22) showed that as GAS enter the stationary phase, they may invade cells more efficiently. Recently, Chaussee et al. (5) demonstrated that cysteine protease is produced to a greater extent during the stationary phase than during the other growth phases. Taken together, the differences in invasion ability observed reflected the differences in cysteine protease rather than growth characteristics.

As the incubation time increased, a drastic increase in the recovery of intracellular bacteria from cells was observed, suggesting that bacterial entry increased as the infection progressed. The increase in recovery rate could be due to greater production of a bacterial product that stimulated bacterial uptake or that allowed GAS to replicate inside the cell. However, our results have demonstrated that once internalized, wild-type strains did not grow inside A-549 epithelial cells; neither did the mutants (Fig. 6). A similar observation was made for group B streptococci (28). Whether this effect is due to intracellular starvation or is a result of culturing cells in a medium containing antibiotics is not clear.

Since mutants displayed a 0.1% invasive activity, it seems that factors other than the cysteine protease are important for invasion. Recently, Molinari et al. (24) showed that the binding of SfbI protein to eukaryotic cells via fibronectin can trigger bacterial internalization in HEp-2 cells. In addition, Cue and Cleary (7) demonstrated that fibrinogen and peptides containing the sequence Arg-Gly-Asp could promote bacterial invasion.

Studies of other pathogens have shown that adherence and invasion of host cells involve interactions between specific components on the surfaces of bacteria and the eukaryotic cells (1, 12, 17, 36, 39). *S. pyogenes* colonizes the epithelial surface of the nasopharynx and the skin of humans, often resulting in infections. Several investigators have shown that M^+ or protein F-containing strains of *S. pyogenes* adhere more easily to human buccal and pharyngeal epithelial cells or HEp-2 cells than M^- strains (8, 38, 40). Since SCP can enhance the invasion of GAS, it is of interest to determine the role of SCP in adhesion to epithelial cells. Our results indicated no striking differences in adhesion between protease-positive strains and isogenic mutants. Although there was an increase of adhesion in the mutants compared to that of the wild-type strain, it was not investigated further.

Although the protease isogenic mutant showed invasion activity lower than that of the wild-type strain, it was not known whether this was due to the polarity effect which was required for invasion. Based on the published data (13, 27), *speB* is transcribed via a monocistronic message. The transcript size is about 1.8 kb, which corresponds to the length predicted from the *speB* gene. In addition, there is no known gene either at least 600 bp upstream from the *speB* start codon (27) or at least 270 bp downstream from the *speB* termination codon (13). On the basis of this information, we believe that the decreased invasion activity was caused by a lack of *speB* gene product (cysteine protease). A similar protease isogenic mutant was constructed by Lukomski et al. (23).

In conclusion, cysteine protease is not the only factor responsible for invasion of respiratory epithelial cells by GAS; other bacterial factors may also be responsible. However, cysteine protease can enhance the ability of GAS to invade respiratory epithelial cells.

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