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C5a Peptidase Alters Clearance and Trafficking of Group A Streptococci by Infected Mice

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Group A streptococcal C5a peptidase (SCPA) specifically cleaves the human serum chemotaxin C5a at the polymorphonuclear leukocyte (PMNL) binding site. This study tested the proposal that SCPA contributes to virulence by retarding the influx of inflammatory cells and clearance of streptococci during the first few hours after infection. To investigate the specific contribution of SCPA to the virulence of group A streptococci, *scpA* insertion and deletion mutants were created by directed plasmid insertion into *scpA* and gene replacement. The precise locations of insertion and deletion mutations were confirmed by PCR and DNA sequence analysis. The impact of mutation on virulence was investigated with a mouse air sac model of inflammation. Experiments evaluated clearance of streptococci from the air sac within 4 h after infection. SCPA$^-$ streptococci were cleared more efficiently than wild-type bacteria. Localization of streptococci in lymph nodes and spleens of infected mice revealed a significant difference between mutant and wild-type streptococci. PMNLs and other granulocytes that infiltrated the air sac were quantitated by single-color flow cytometry. The total cellular infiltrate was greater and PMNLs dominated the granulocytic infiltrates of air sacs inoculated with SCPA$^-$ mutant bacteria. The data obtained are consistent with the possibility that SCPA$^-$ streptococci are initially cleared from the site of infection primarily by PMNLs. Moreover, mutant and wild-type streptococci followed different paths of dissemination. SCPA$^-$ bacteria were transported to lymph nodes, whereas wild-type streptococci avoided transport to the lymph nodes and rapidly spread to the spleen.

Group A streptococcus (GAS) is one of the most common primary human pathogens. This gram-positive organism is responsible for a variety of diseases, including pharyngitis, impetigo, erysipelas, and systemic infections associated with toxic shock (33). Several bacterial surface factors are associated with virulence. The genes that encode these proteins are clustered on the chromosome, and their expression is positively controlled by the *mga* gene, previously termed *virR* (30). Surface proteins associated with virulence include the C5a peptidase (*scpA*), M protein (*emm*), and immunoglobulin-binding protein (*fcrA*) genes. The M protein is studied most and is known to interfere with phagocytosis of streptococci in nonimmune hosts by blocking the deposition of C3b onto the bacterium's surface (15). Several other M-like proteins have been identified and shown to bind immunoglobulins, but their role in virulence is less clear (16, 17). Since its original discovery by our laboratory, group A streptococcal C5a peptidase (SCPA) was shown to be highly specific for the complement peptide C5a (13, 35). C5a is cleaved between the His-67 and Lys-68 residues of C5a, a region of the chemotaxin which binds to receptors located on the surface of polymorphonuclear leukocytes (PMNLs) (13, 35). A similar peptidase was also shown to be expressed by human isolates of group B (11, 18), C (unpublished data), and G (12) streptococci.

The ability of bacteria to colonize mucosal surfaces with pathological consequences is profoundly influenced by the initial inflammatory response. Activation of the alternative complement pathway produces C5a, which is one of the primary mediators of chemotaxis in human tissue, attracting PMNLs to sites of infection (22, 31). We postulated that SCPA helps streptococci to colonize a host by inhibiting the influx of PMNLs, thereby impeding initial clearance of the streptococci. Preliminary experiments with transposon Tn*916* and chemically induced mutants supported this proposal but were inconclusive (25). Therefore, the impact of SCPA on streptococcal virulence was further investigated with strains with well-defined mutations in the protease structural gene. SCPA mutants were constructed by targeted plasmid insertion and by replacement of the wild-type gene with *scpA* containing a specific internal deletion. A mouse air sac model was successfully used to investigate inflammation, C5a leukocyte chemotaxis (5, 9), and virulence of a mouse-adapted group A streptococcal isolate (26). An advantage of this model is that the air sac remains inflated for several days and free of inflammation, unless an irritant is injected. Thus, injected bacteria and the resulting inflammatory response remain localized over short periods of infection. The air sac model was modified to compare clearance of wild-type and SCPA$^-$ streptococci and to analyze the cellular infiltrate at an early stage of infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Streptococcus pyogenes* CS101 is a serotype M49 and OF$^+$ strain. CS159 is a clinical isolate with a deletion which extends through the M gene cluster and *scpA*. A spontaneous, streptomycin-resistant derivative of strain CS101, named CS101Sm, was selected by plating streptococci from a stationary-phase culture on tryptose blood agar containing streptomycin (200 μg/ml). CS101::pG$^+$host5 is strain CS101 with pG$^+$host5 integrated into the chromosome at an unknown location but outside *scpA* and the *emm* gene cluster. *Escherichia coli* ER1821 (from New England Biolabs, Inc., Beverly, Mass.) was used as the recipient for the suicide vector, plasmid pG$^+$host5. Plasmid pG$^+$host5 (1) was obtained from Appligene, Inc., Pleasanton, Calif. Streptococci were grown in Todd-Hewitt broth supplemented with 2% neopeptone or 1% yeast extract (THY) or on tryptose agar plates with 5% sheep blood. *E. coli* ER1821 containing plasmid pG$^+$host5 was grown in Luria-Bertani broth with erythromycin (ERM; 300 μg/ml). Streptococci with plasmid pG$^+$host5 were cultured in THY containing 1 μg ERM per ml.

Construction of the *scpA* insertional mutant. A *Bgl*II-*Hin*dIII fragment of *scpA* (8) was ligated into the *Bam*HI-*Hin*dIII sites of plasmid pG$^+$host5 to form plasmid pG::scpA1.2 and transformed into *E. coli* ER1821. Recombinant plasmid pG::scpA1.2 was electroporated into CS101 recipient cells as described previously (1). Transformants were selected on THY agar plates containing 1 μg of ERM per ml at 30°C. Chromosomal integrants which resulted from recombination between the plasmid insert and the chromosomal *scpA* gene were

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selected for ERM resistance (ERM^r) at 39°C. Revertants of insertion mutants were obtained by passaged in THY with no antibiotic at 30°C and finally plated at 37°C without ERM selection. Colonies that had lost plasmid pG$^+$host5 were identified by growth on media with and without ERM.

Construction of a defined *scpA* deletion mutant. A defined deletion in the *Bgl*II-*Hin*dIII fragment of *scpA* was produced by inside-out PCR with primers 1 (5′ GGGGGG**GAATTC**GTAGCGGGTATCATGGGAC 3′) and 2 (5′ GGG GGG**GAATTC**GGGTGCTGCAATATCTGGC 3′). Underlined nucleotides correspond to *scpA* sequences with coordinates 2398 and 2322, respectively, and boldface nucleotides correspond to an *Eco*RI recognition site. Plasmid pG::scpA1.2 was used as the template DNA. The primers were selected to produce an in-frame deletion in the *scpA* gene. The amplified product was digested with *Eco*RI and ligated. The resulting plasmid, pG::ΔscpA1.1, contained a 76-bp deletion internal to *scpA* and was transformed into *E. coli* ER1821. Colonies were selected for ERM^r and then screened for the appropriate *scpA* deletion with miniprep plasmid DNA restricted by *Eco*RI. The precise boundaries of the deletion were confirmed by DNA sequencing. Plasmid pG::ΔscpA1.1 was electroporated into strain CS101Sm as described above, and then integrants were selected by growth on ERM at 39°C. Integration of the plasmid resulted in duplication of the *Bgl*II-*Hin*dIII *scpA* fragment; one copy contained the deletion. Excision of the plasmid by homologous recombination within the duplicated DNA can produce wild-type *scpA* or leave behind the deleted form. To isolate the appropriate segregant, insertional mutants were serially transferred six times in THY with no antibiotic at 30°C and then streaked onto THY agar plates. After overnight incubation, colonies were replica plated onto plates with and without ERM. ERM^s colonies were purified and confirmed to contain the deletion by their failure to hybridize to oligonucleotide probe scpFor2374 (5′ GGAACTAGTATGTCTGCGCC 3′), which was labeled by 3′ tailing with digoxigenin-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Streptococcal colonies were printed onto a positively charged nylon membrane (Boehringer GmbH, Mannheim, Germany). The cells were lysed by placing membranes on Whatman paper saturated with 2× lysis buffer (200 mM Tris, 100 mM EDTA, 4% sodium dodecyl sulfate [SDS]) at 65°C for 30 min. Hybridizations were performed at 57°C for 2 h as described in the *Genius System User's Guide* for filter hybridization (Boehringer Mannheim Biochemicals). Colonies that did not hybridize were presumed to contain a deletion in *scpA*.

Analysis of mutants. Chromosomal DNA was isolated by a microwave method (3). An M13 reverse primer (5′ AACAGCTATGACCATG 3′; specific for the plasmid) and an scpRev3941 primer (5′ GAGTGGCCCTCCAATAGC 3′; specific for the chromosome) were used to amplify insertional mutants. *Taq* DNA polymerase was obtained from Promega, Heidelberg, Germany. PCR products were digested with *Bgl*II, *Eco*RV, *Hae*III, *Hin*dIII, *Nco*I, and *Xho*II to confirm the origin of the PCR fragments. Primers scpFor1033 (5′ CCCCCCGGATC CACCAAAACCCCACAAACTC 3′) and scpRev3941 were used to amplify the chromosomal gene containing the pG::ΔscpA1.1 insertion. PCR products were digested with *Eco*RI.

Southern blot analyses were performed by using the entire *scpA* gene as a labeled probe. Negative controls consisted of chromosomal DNAs from strains CS101Sm and CS159. To precisely define the *scpA* internal deletion in chromosomal DNA, the *scpA* gene was amplified by using primer scpForW (5′ CCCCCCGGATCCAAGGACGACACATTGCGTA 3′) and a biotinylated scpRev4358 primer (8). A single-stranded PCR product was isolated by solid-phase separations with Dynabeads M-280 streptavidin and the protocols described by the manufacturer (Dynal Inc., Great Neck, N.Y.). The sequencing primer was scpForp2L1 (5′ TGCCTGCGGCCTTTATCAG 3′).

SDS-polyacrylamide gel electrophoresis (PAGE), Western blot (immunoblot), and colony immunoblot techniques. Streptococci were incubated in 100 ml of THY at 37°C overnight. The culture pellet was washed twice in 5 ml of cold 0.2 M Na acetate (pH 5.2) and then suspended in 1 ml of TE-sucrose buffer (20% sucrose, 10 mM Tris, 1 mM EDTA, pH 7.0) and 40 μl of mutanolysin. The mixture was rotated at 37°C for 2 h and then centrifuged for 5 min at 1,500 × *g*. Supernatants were supplemented with 100 mM phenylmethylsulfonyl fluoride. The electrophoresis and Western blotting methods used were previously described (21). For colony blots, colonies were grown on THY agar plates, printed onto a nitrocellulose membrane (BioBlot-Nc; Costar, Cambridge, Mass.), fixed under an infrared lamp for 10 min, and exposed to antibody as described previously (25). The primary antiserum used to detect SCPA protein on Western and colony blots was prepared by immunization of a rabbit with purified recombinant SCPA protein. Binding was detected by an anti-rabbit antibody-alkaline phosphatase conjugate (25).

PMNL adherence assay. SCPA activity was measured by using a PMNL adherence assay (4). After incubation of recombinant human C5a (rhC5a; C5788; Sigma, St. Louis, Mo.) with streptococcal extracts or purified protease, residual rhC5a can activate PMNLs to become adherent to bovine serum albumin (BSA)-coated wells. First, microtiter wells were coated with 0.5% BSA in phosphate-buffered saline (PBS) and incubated for 1 h at 37°C. Human PMNLs were isolated by centrifugation in Ficoll Hypaque (Sigma). A 40-μl volume of intact streptococci or protein extracts was incubated with 20 μl of 5 μM rhC5a in 340 μl of PBS with 1% glucose and 0.1% $CaCl_2$ at 37°C for 45 min. BSA-coated wells were washed with PBS, and resuspended PMNLs and residual rhC5a were added to the wells. The mixture was incubated for 45 min at 37°C in 7% CO_2. Finally, the wells were washed to remove nonadherent PMNLs. Adherent PMNLs were stained with crystal violet, and the optical density at 570 nm (OD_{570}) was read in an enzyme-linked immunosorbent assay reader. The OD_{570} is proportional to the amount of residual rhC5a or inversely proportional to the amount of SCPA activity.

PMNL chemotaxis. Mouse PMNL chemotaxis to zymosan (Sigma)-activated mouse serum (ZAMS) was determined in 48-well microchemotaxis chambers (Neuroprobe, Cabin John, Md.) with polycarbonate membranes (Poretics Corp., Livermore, Calif.) as previously described (2). Mouse PMNLs were prepared by centrifugation of 5 ml of heparinized whole blood through Ficoll-Hypaque. ZAMS was prepared as previously described (28). ZAMS, diluted to 50% in Hanks balanced salt solution–1% BSA, was incubated with 5 μg of purified SCPA protein (7) per ml–5×10^8 CFU of CS101Sm or MJ3-15 bacteria per ml, respectively, at 37°C for 1 h. After bacteria were removed by centrifugation, the supernatant was added to the lower wells of the chamber in triplicate. A 50-μl volume of mouse PMNLs (2.5×10^6/ml in 1% glucose–PBS with calcium) was added to each upper well. The chamber was incubated for 1 h at 37°C in 5% CO_2, and the membrane was removed and stained with crystal violet. The number of PMNLs that had migrated through the membrane was counted under a microscope at a magnification of ×400.

Mouse air sac model. CD1 male outbred mice (25 g) obtained from Charles River Breeding Laboratory, Wilmington, Mass., were used for all experiments. A connective tissue air sac was generated by injecting 0.9 ml of air and 0.1 ml of GAS diluted in PBS with a 25-gauge needle under the skin on the back of the mouse (5). In some experiments, SCPA$^+$ CS101::pG$^+$host5 was used as a positive control. In other experiments, strain CS101Sm was used as the positive control. Mice were euthanized by cervical dislocation 4 h after infection. As indicated in the Results, all four inguinal lymph nodes, the spleen, and the air sac were removed from each animal and homogenized in PBS. Tissue suspensions were assayed for viable CFU on blood agar plates containing 1 μg of ERM per ml or 200 μg of streptomycin per ml.

Single-color fluorescence-activated cell sorter (FACS) analysis. Dispersed cell populations of air sacs were prepared by grinding the air sacs in PBS and passing them through Nylon monofilament mesh (TETKO Co., New York, N.Y.). The cells were pelleted by centrifugation for 5 min at 300 × *g* and resuspended at 5×10^6/ml in FACS buffer (Hanks balanced salt solution without phenol red, 0.1% NaN_3, 1.0% BSA fraction V). Cells (1.0×10^6) were stained directly with 1 μg of fluorescein isothiocyanate–anti-mouse Mac-1 or indirectly with 1 μg of biotin-conjugated anti-mouse Gr-1, followed by 1 μg of streptavidin-fluorescein isothiocyanate (6, 32). Monoclonal antibodies Mac-1 and Gr-1 were obtained from Pharmingen, Inc. Labeled cells were fixed in 1.0% paraformaldehyde. Fluorescence profiles were generated with a FACScan flow cytometer and Consort 32 software (Becton Dickinson). Mouse PMNLs were purified from whole blood by Ficoll-Hypaque density gradient centrifugation and used as a standard to define PMNLs in mixed populations. For measurement of specifically labeled cells, the mean fluorescence for each antibody marker was determined and gates were set to reflect intensely labeled cells. Controls included unstained cells and cells exposed to only streptavidin-fluorescein isothiocyanate.

RESULTS

Construction of an insertional *scpA* mutant. To further investigate the role of SCPA in the virulence of GAS, we first constructed plasmid insertional *scpA* mutants. The insertion target, an internal 1.2-kb *Bgl*II-*Hin*dIII fragment of *scpA*, was ligated into thermosensitive shuttle vector pG$^+$host5 [strain CS101(pG::scpA1.2)]. This vector contains a pBR322 origin of replication that is active in *E. coli* at 37°C; a temperature-sensitive, gram-positive origin of replication (active at 30°C and inactive at 39°C in streptococci); and an ERM^r-encoding gene for selection (1). High temperature forced the plasmid to integrate into the chromosomal DNA of GAS by homologous recombination at frequencies ranging from 10^{-2} to 10^{-3}. Integrants were selected by ERM^r at 39°C. Strain CS101 (pG$^+$host5), which contained the plasmid without a homologous fragment of streptococcal DNA, produced fewer than 10^{-8} CFU when plated at 39°C. Among the 16 high-temperature survivors isolated, two mutants, M14 and M16, were further analyzed. ERM^s revertants of strains M14 and M16 which had lost the plasmid were subsequently isolated to confirm that the mutant phenotype resulted from insertion of the plasmid into *scpA* rather than from a simultaneous, unrelated mutation.

Introduction of a defined deletion into *scpA*. We constructed a strain with a defined deletion internal to *scpA* to eliminate the possibility that insertions in *scpA* could be polar and reduce

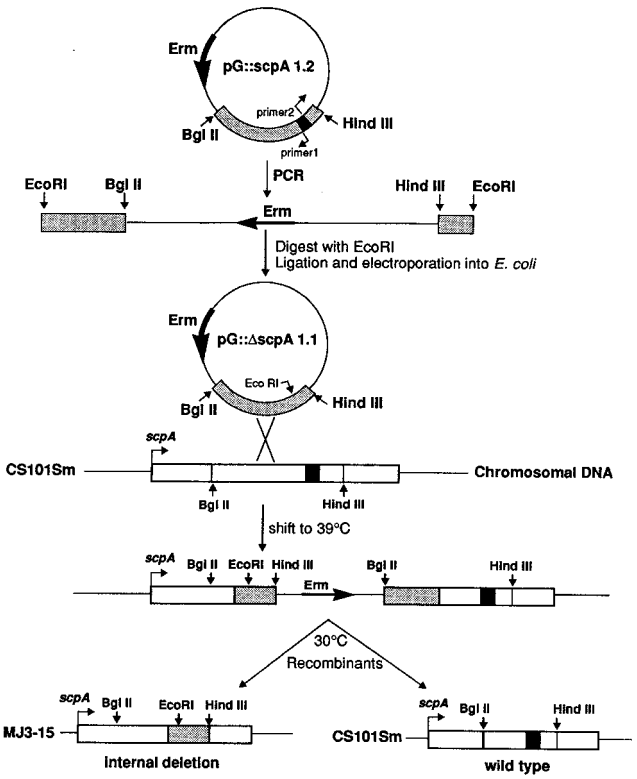

FIG. 1. Construction of *scpA* internal deletion mutants. A portion of the active center of *scpA* was deleted from pG::scpA1.2 by inverse PCR with scpRev2322 (primer 1) and scpFor2398 (primer 2). PCR products were digested with *Eco*RI and ligated to form plasmid pG::ΔscpA1.1. This plasmid DNA was electroporated into CS101Sm. Insertional mutants were obtained by growth at 39°C. Excision of the plasmid was enhanced by growth at 30°C. The plasmid was excised by a crossover event between duplicated sequences. Recombination either restored the wild-type phenotype or left behind the deletion. The black boxes represent the *scpA* fragment to be deleted. The shaded boxes represent the *scpA* fragment which is the target of insertion. The open boxes represent the chromosomal *scpA* gene.

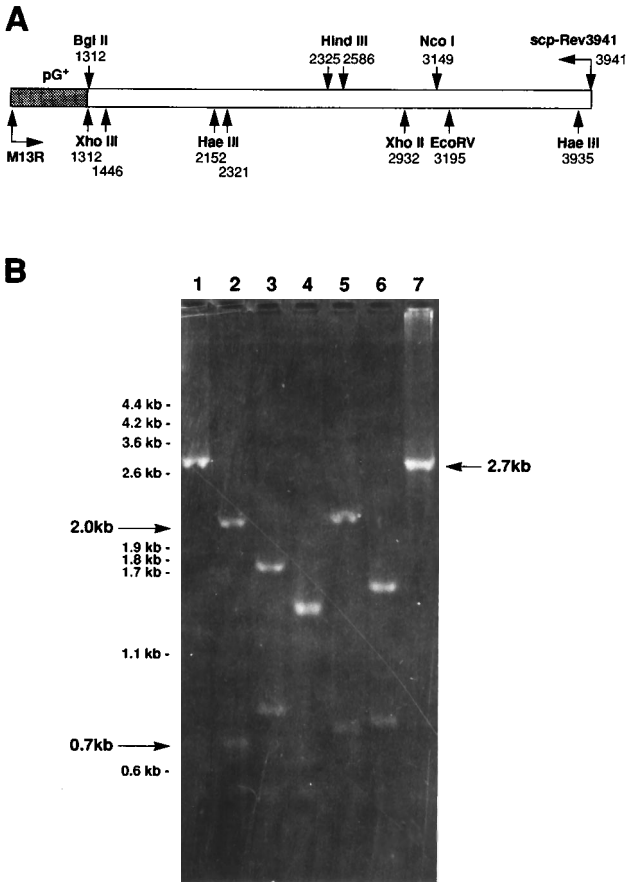

FIG. 2. Verification of plasmid DNA insertion into *scpA* by PCR with primers M13Rev and scpRev3941. Each enzyme recognition site in the *scpA* target fragment is represented in panel A. The shadowed box represents a segment of pG$^+$host5 DNA. The open box represents the insertion target of *scpA*. In panel B, lanes 1 to 6 contained the PCR product digested with *Bgl*II, *Eco*RV, *Hae*III, *Hin*dIII, *Nco*I, and *Xho*II, respectively. Lane 7 shows the PCR product without digestion with a restriction endonuclease. Molecular size markers are given on the left.

expression of genes downstream from *scpA* which could also contribute to the organism's virulence. First, a defined 76-bp deletion internal to the *scpA* sequence was introduced into pG::scpA1.2 by inverse PCR (Fig. 1). Plasmid pG::scpA1.2 was used as the template DNA. Primers scpFor2398 and scpRev2322 were designed to flank the sequence which encodes the amino acid sequence GQDILSSVANNKYAKLSGT SMSAPL. These amino acids contribute to the catalytic center of the protease (8). After amplification and ligation, pG::ΔscpA1.1, containing the deletion, was transformed into *E. coli*. Plasmid DNA from one transformant was sequenced and shown to contain the expected in-frame deletion (data not shown). This plasmid, pG::ΔscpA1.1, was isolated and electroporated into strain CS101Sm as described in Materials and Methods. Insertion of pG::ΔscpA1.1 into the strain CS101Sm chromosome was accomplished by growth at high temperature as also described in Materials and Methods. The insertion location was confirmed by PCR. Passage of CS101 Sm(pG::ΔscpA1.1) at low temperature without ERM selection resulted in high-frequency segregation of ERM^s revertants which had lost the plasmid by random deletion events or by excision due to recombination between the duplicated *scpA* sequences created by the insertion (unpublished observation). ERM^s revertants (208 CFU) were probed by colony hybridization with a labeled oligonucleotide that corresponded to the sequence to be deleted. Among 18 colonies which failed to hybridize to the oligonucleotide, two deletion mutants, MJ2-5 and MJ3-15, were studied further.

Characterization of SCPA$^-$ mutants. PCR analysis demonstrated that plasmid pG::scpA1.2 DNA had been inserted into *scpA* in the chromosomes of mutants M14 and M16. The expected 2.7-kb PCR product was obtained by using primers M13Rev and scpRev3941. The correct origin of this PCR fragment was confirmed by restriction endonuclease analysis of the 2.7-kb PCR fragment (Fig. 2). The *scpA* fragment should have one internal *Eco*RV site at 3,195 bp. Upon digestion with *Eco*RV, two bands of 1,983 and 746 bp were obtained, as expected (8).

The chromosomal deletion left behind by recombination excision of the plasmid insertion was also defined by Southern hybridization and sequencing. Hybridization to only one *Eco*RI fragment of digested chromosomal DNA from the wild-type CS101 strain was expected, because the *scpA* gene does not contain an *Eco*RI site. In contrast, the *scpA* probe bound to two *Eco*RI fragments from strain MJ2-5 and MJ3-15 chromosomal digests (Fig. 3). This was expected, because an *Eco*RI recognition site was created by the PCR primers used to construct the *scpA* deletion. DNA sequencing across the deletion

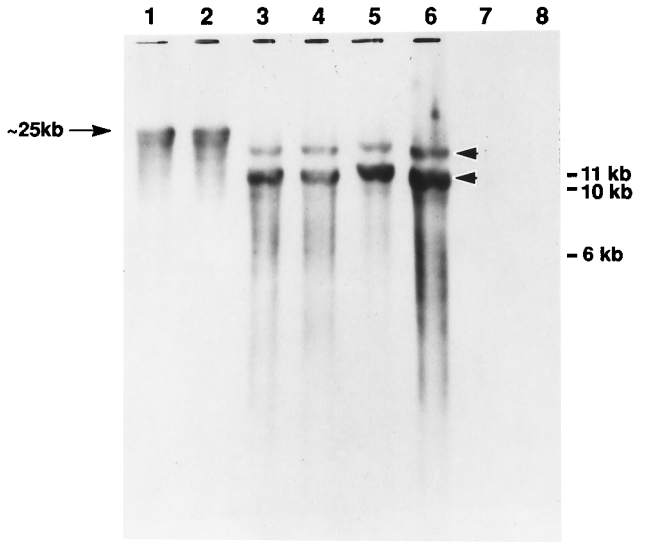

FIG. 3. Southern blots of DNA from strain CS101Sm and an SCPA$^-$ deletion mutant. Chromosomal DNAs from strains CS101Sm (lanes 1 and 2), MJ2-5 (lanes 3 and 4), MJ3-15 (lanes 5 and 6), and CS159 (lanes 7 and 8) were digested with *Eco*RI and probed with the digoxigenin-labeled *scpA* gene. The two arrows adjacent to lane 6 show the two expected *Eco*RI fragments, 14 and 11 kb, produced by cleavage of the PCR-incorporated *Eco*RI site. DNA molecular size markers are given on the right.

confirmed that the reading frame was not altered by the deletion in strain MJ3-15 (data not shown).

The impact of insertion and deletion on expression of SCPA antigen and peptidase activity was assessed by using Western blot and PMNL adherence assays. SDS-PAGE and Western blots confirmed that *scpA* insertion mutants M14 and M16 (Fig. 4, lanes 5 and 6 and lanes 7 and 8, respectively) lack SCPA protein, a band of 130 kDa (Fig. 4, lanes 1 and 2). Because the deletion encompasses the middle third of the *scpA* gene, strain MJ3-15 could still produce a truncated form of the peptidase. In fact, Western blots of whole-cell protein extracted from MJ2-5 and MJ3-15 revealed a small amount of a peptide which reacted with anti-SCPA serum and which was approximately the same size as SCPA. Colony immunoblots of mutants also showed that MJ2-5 and MJ3-15 produced SCPA antigen (data not shown).

Neutrophil adherence assays were performed to confirm

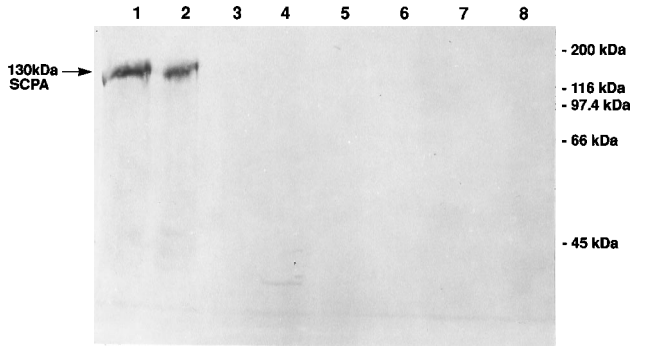

FIG. 4. Western blot analysis of mutanolysin extracts of *scpA*$^+$ and *scpA* mutant streptococci. Protein extracts of the following strains were analyzed: CS101 (lanes 1 and 2), CS159 (lanes 3 and 4), M14 (lanes 5 and 6), and M16 (lanes 7 and 8). The molecular size markers are biotinylated SDS-PAGE standards.

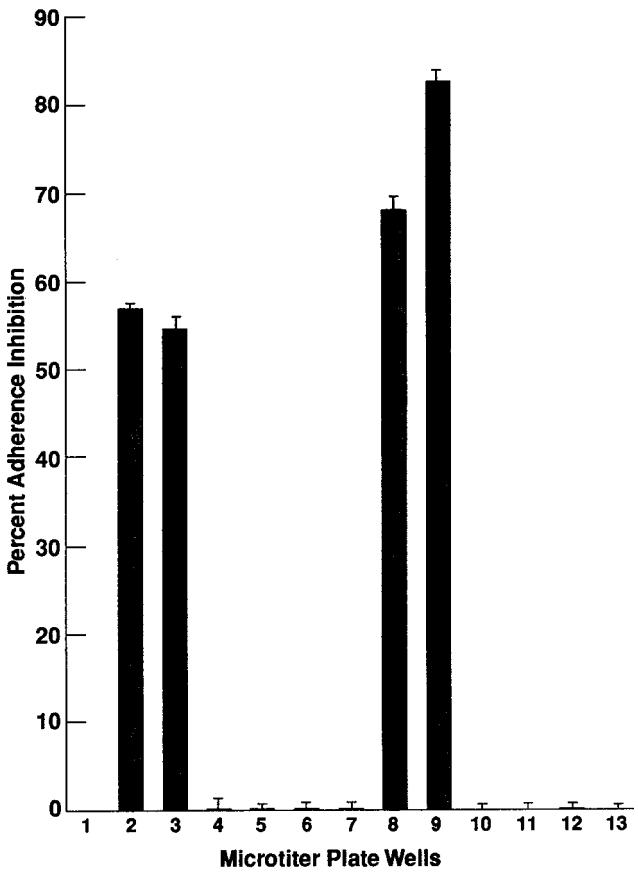

FIG. 5. PMNL adherence assay for functional SCPA activity. Whole, intact bacteria (lanes: 2, CS101; 3, CS101Sm; 4 to 7, mutant strains M14, M16, MJ2-5, and MJ3-15) or mutanolysin protein extracts from strains CS101 (lane 8), CS101Sm (lane 9), M14 (lane 10), M16 (lane 11), MJ2-5 (lane 12), and MJ3-15 (lane 13) were incubated with 20 μl of 5 μM rhC5a for 45 min. Bacteria were removed by centrifugation, and supernatants were incubated with freshly isolated PMNLs in microtiter plate wells coated with BSA. The nonadherent PMNLs were removed, and adherent PMNLs were stained with crystal violet. OD_{570} was read with an enzyme-linked immunosorbent assay reader. Lane 1 contained rhC5a incubated with PMNLs. Percent inhibition was calculated by the following formula: % adherence inhibition = [(OD_{570} of C5a incubated with PMNLs − OD_{570} of residual C5a incubated with PMNLs)/OD_{570} of C5a incubated with PMNLs] × 100%.

that mutant strains M14, M16, MJ2-5, and MJ3-15 lacked SCPA activity (Fig. 5). In this assay, rhC5a was incubated with washed streptococci or mutanolysin extracts of streptococci. Residual rhC5a was quantitated by its potential to activate human PMNLs which become adherent to BSA-coated microtiter plate wells (4). rhC5a alone was taken as 100% adherence or no inhibition. rhC5a exposed to CS101 or CS101Sm whole cells or protein extracts from these bacteria showed 54.5 to 81.5% inhibition of adherence, indicating that much of the rhC5a was destroyed. In contrast, mutant strains M14, M16, MJ2-5, and MJ3-15 lacked functional SCPA activity. Less than 1.5% of the rhC5a was eliminated. Both whole cells and mutanolysin protein extracts from M14, M16, M2-5, and MJ3-15 lacked the ability to destroy rhC5a-activated adherence. The 1.0 to 1.5% inhibition associated with mutant extracts may be due to toxic effects of the extract on the neutrophils.

Because Bohnsack et al. (2) reported that group B streptococcal C5a-ase could not inactivate mouse C5a, it was necessary to determine if mouse C5a could be inactivated by SCPA in vitro. Modified Boyden chambers were used to measure the

TABLE 1. Chemotaxis inhibition of ZAMS by SCPA with mouse PMNLs[a]

Chemoattractant condition	Mean no. of PMNLs attracted ± SEM	% Inhibition
Buffer alone	4 ± 1	
50% ZAMS	72 ± 7	0
50% ZAMS + SCPA (5 μg/ml)	12 ± 2[b]	83
50% ZAMS + CS101Sm	49 ± 1[c]	30
50% ZAMS + MJ3-15	71 ± 6	1

[a] The chemotaxis assay was conducted twice. Representative data were analyzed statistically with an unpaired *t* test.
[b] $P < 0.001$ compared with untreated ZAMS.
[c] $P < 0.05$ compared with untreated ZAMS.

chemotactic response to yeast ZAMS with a PMNL chemotaxis assay. With this assay, we found that 50% ZAMS was optimal for attraction of mouse PMNLs (data not shown). As shown in Table 1, 50% ZAMS increased the number of PMNLs migrating through the membrane by nearly 20-fold. To test for SCPA destruction of C5a, 50% ZAMS was incubated with purified SCPA protein or washed streptococci. When ZAMS was incubated with SCPA protein, PMNL chemotaxis was inhibited by 83% (Table 1). Also, when ZAMS was incubated with $SCPA^+$ wild-type strain CS101Sm, chemotaxis was reduced by 30%. In contrast, when ZAMS was incubated with $SCPA^-$ mutant MJ3-15, chemotaxis was not inhibited. These experiments confirm that SCPA can inactivate mouse C5a.

Virulence of SCPA-deficient streptococci in mice. Boyle et al. used a mouse connective tissue air sac model to study recruitment of neutrophils in response to C5a and to examine the role of M-like immunoglobulin receptors in the virulence of GAS (5). Although overwhelming numbers of streptococci were required to cause lethal infection by this route of inoculation (26), our first experiment compared the capacities of $SCPA^+$ and $SCPA^-$ streptococci to cause lethal infection following inoculation of air sacs (Table 2). The wild-type M^+ $SCPA^+$ culture killed four of five mice within 4 days after inoculation. Four of five mice inoculated with the highest dose of $SCPA^-$ strain M16 also died. Fewer mice died when inoculated with 3.5×10^8 $SCPA^-$ CFU; however, this difference was not statistically significant relative to mice inoculated with approximately the same number of wild-type streptococci. In contrast to the $SCPA^-$ strain, a *mga* insertion mutant which is down regulated for at least three surface proteins, the M49 protein, the FcRA49 immunoglobulin G-binding protein, and the SCPA protein and serum opacity factor (23), was significantly less virulent than wild-type streptococci. Overall, 8 of 10 mice survived infection with this strain, compared with 2 of 10 mice inoculated with wild-type streptococci.

TABLE 2. Survival of mice after air sac infection with wild-type and mutant streptococci

Strain	Infection dose (CFU/mouse)	No. of survivors/total no. of mice			
		Day 1 (21 h)	Day 2 (45 h)	Day 3 (69 h)	Day 4 (93 h)
CS101(pG^+)	3.0×10^9	5/5	4/5	3/5	1/5
	3.0×10^8	5/5	5/5	3/5	1/5
M16 ($SCPA^-$)	3.5×10^9	5/5	5/5	3/5	1/5
	3.5×10^8	5/5	5/5	4/5	2/5
1-Int (*mga*)[a]	4.5×10^9	5/5	5/5	5/5	4/5
	4.5×10^8	5/5	5/5	4/4[b]	4/4

[a] 1-Int was isolated and characterized by McLandsborough and Cleary (23).
[b] One mouse was sacrificed because of an open wound on the back.

TABLE 3. Distribution of $SCPA^+$ and $SCPA^-$ streptococci 4 h after air sac infection

Strain (phenotype)	No. of mice[a]	No. of positive cultures		Mean no. of CFU/ml in homogenized air sac ± SEM[b]
		Spleen[c]	Lymph node	
CS101pG ($SCPA^+$)	8	7	2	$1.3 \times 10^8 \pm 2.2 \times 10^7$
M16 ($SCPA^-$)	8	0	5	$6.0 \times 10^7 \pm 1.3 \times 10^7$
CS101Sm ($SCPA^+$)	8	6	2	$1.6 \times 10^8 \pm 2.6 \times 10^7$
MJ3-15 ($SCPA^-$)	8	1	5	$3.7 \times 10^7 \pm 1.5 \times 10^7$

[a] Each mouse was inoculated with 3×10^8 CFU of stationary-phase streptococci.
[b] Differences between the numbers of CFU isolated from homogenized air sacs were significant for strains CS101pG ($SCPA^+$) and M16 ($SCPA^-$) ($P < 0.05$) and strains CS101Sm ($SCPA^+$) and MJ3-15 ($SCPA^-$) ($P < 0.001$) in each experiment by unpaired *t* test.
[c] The difference in the frequency of isolation of $SCPA^+$ streptococci from spleens relative to $SCPA^-$ streptococci was statistically significant ($P < 0.05$) for each experiment by the Fisher exact test.

The ability to colonize and cause lethal infection is surely dependent on the expression of multiple surface and extracellular products. Therefore, failure to observe a significant difference between the capacities of $SCPA^-$ and $SCPA^+$ streptococci to cause lethal infection was not surprising. Our model predicts that SCPA functions very early after infection, i.e., to retard initial clearance of the organism. To further investigate this possibility, we compared $SCPA^+$ and $SCPA^-$ streptococci for persistence in the air sac just 4 h after inoculation. Moreover, we examined the dissemination of streptococci to lymph nodes and the spleen after this short period of infection (Table 3). In a preliminary experiment, air sacs were fixed on slides, stained with Wright's stain, and examined microscopically. Although counts of granulocytes by this method were unreliable, there appeared to be significantly fewer residual $SCPA^-$ streptococci in fixed tissue than in tissue infected with wild-type streptococci at 4 h postinfection (data not shown). Two additional experiments were performed to measure this difference. The first compared *scpA* insertion mutant strain M16 to its $SCPA^+$ parent culture, strain CS101::pG, and the second compared *scpA* deletion mutant strain MJ3-15 to its parent, strain CS101Sm (Table 3). In both experiments, the homogenized air sacs from mice inoculated with $SCPA^-$ streptococci contained fewer streptococci than did air sacs inoculated with wild-type streptococci. The first experiment showed twofold fewer streptococci, and the second showed a fourfold difference. These differences were statistically significant at $P < 0.05$ and $P < 0.001$, respectively, with an unpaired *t* test.

Other interesting differences were also observed. Wild-type $SCPA^+$ streptococci were found in spleen homogenates from seven of eight mice and six of eight mice, whereas the $SCPA^-$ mutants were rarely found in the spleen by 4 h postinfection. For unexplained reasons, the number of streptococci in spleen homogenates varied greatly from mouse to mouse. The opposite was true for lymph node homogenates, however. Nodes from 10 of 16 mice infected with $SCPA^-$ streptococci harbored viable streptococci, whereas only 4 of 16 nodes from mice infected with wild-type streptococci contained viable bacteria. This difference was determined to be statistically significant at $P < 0.05$ with the Fisher exact test.

Compositions of the cellular infiltrate in air sacs inoculated with $SCPA^+$ and $SCPA^-$ streptococci. Our previous studies

TABLE 4. Analysis of cell population by single-color FACS

Strain (phenotype)	Dose (CFU)	No. of mice	Mean total no. of cells[a]/ air sac (10^4)	% of Gr-1-positive cells[b]	% of Mac-1-positive cells[b]
CS101 ($SCPA^+$)	1.0×10^6	3	4.7 ± 0.9	6.1 ± 2.3	21.0 ± 1.6
	1.0×10^8	3	7.5 ± 2.7	23.4 ± 7.7	26.5 ± 3.4
MJ3-15 ($SCPA^-$)	1.5×10^6	3	8.2 ± 2.8	20.8 ± 8.3	19.0 ± 5.6
	1.5×10^8	3	19.6 ± 3.5	36.7 ± 7.6	34.5 ± 5.7

[a] Values are means from three mice.
[b] Fluorescence data were analyzed by gating on PMNLs. A second gate was set to define high-staining cells in the populations circumscribed by the first gate.

(25) predicted that more rapid clearance of $SCPA^-$ streptococci from air sacs, as observed here, resulted from more intense recruitment of PMNLs. To test this prediction, the total cell population, the number of Mac-1-positive granulocytes, and the number of Gr-1-positive PMNLs in air sacs were compared by single-color FACs analysis (Table 4). Air sacs infected with the $SCPA^-$ deletion mutant contained twice as many inflammatory cells as those inoculated with $SCPA^+$ streptococci. A 100-fold increase in the inoculum size did not alter this difference. As predicted, air sacs infected with 10^6 cells of $SCPA^-$ strain MJ3-15 contained three times more Gr-1-positive cells than did those inoculated with the $SCPA^+$ culture. Within air sacs inoculated with $SCPA^+$ streptococci, approximately 6% of the cells were PMNLs and 15% were other kinds of leukocytes, including monocytes and macrophages. In contrast, air sacs inoculated with $SCPA^-$ streptococci contained predominately PMNLs; i.e., the number of Gr-1-positive cells was equal to or greater than the number of Mac-1-positive cells. With a 100-fold increase in inoculum size, 1.6-fold more Gr-1-positive cells had infiltrated air sacs receiving $SCPA^-$ streptococci than those inoculated with $SCPA^+$ bacteria. However, after inoculation with a larger streptococcal dose, PMNLs dominated infiltrates, no matter whether the air sac was inoculated with $SCPA^+$ or $SCPA^-$ streptococci. Flow cytometer gates were set to measure only high-staining granulocytes. We presume that the remaining 50 to 60% of cells not stained with either antibody were either low-staining granulocytes or lymphocytes. Large numbers of lymphocytes were observed microscopically in Wright-stained air sac preparations.

DISCUSSION

The fact that all serotypes of GAS tested to date (8, 10) and human isolates of group B (18, 11) and G (12) streptococci produce a C5a peptidase or carry the *scpA* gene is reason to believe that specific cleavage of C5a chemotaxin contributes to the virulence of these streptococci. Earlier experiments in our laboratory with Tn*916* and nitrosoguanidine mutagenesis did not conclusively establish that the peptidase is required for full virulence (25). This prompted us to readdress the role of the C5a peptidase in streptococcal virulence by using strains with well-defined mutations in *scpA*. Mutant strains were compared to wild-type streptococci in a mouse model of infection which is known to reflect the elaboration of C5a at the site of inflammation (5, 26).

The thermosensitive shuttle vector pG^+host5, originally developed and described by Biswas et al. (1), proved to be very useful for construction of site-specific knockout mutants of GAS. We also used this vector for gene replacement in which a segment of the wild-type gene was replaced with a fragment containing a defined deletion. Appropriate constructs were made in vitro and then maintained and characterized in *E. coli*. The target sequence carried by the plasmid was 1.2 kb long. Although fragments with 1.0-kb or greater homology increased the frequency of insertion by Campbell-like recombination, we obtained insertions in *scpA* and *emm-12* promoter sequences with only 200 bp of sequence homology on the plasmid. Initial problems with genetic instability of the vector and the insertions were minimized by growing *E. coli*(pG^+host5) at 38°C and streptococcal cultures with chromosomal inserts at 39°C. We presume that the thermosensitive, gram-positive Rep function is somewhat leaky at 37°C and those small amounts of activity are detrimental to maintenance of the plasmid.

Because streptococcal virulence is genetically unstable (29), we were obligated to isolate ERM^s revertants from insertion mutants to prove that the mutant phenotype resulted from plasmid insertion rather than an undefined spontaneous genetic event. Passage of streptococcal cultures with chromosomal inserts at 30°C without ERM selection greatly enriched the culture for ERM^s revertants. Most ERM^s segregants had regained the wild-type $SCPA^+$ phenotype.

A strain was constructed with a defined deletion in *scpA* to eliminate the inherent problem of instability, the potential for polarity on downstream genes, and the need to maintain infected animals on a selective antibiotic. A defined region of *scpA*, known to encode the reactive serine, among other residues presumed to be components of the active site of the enzyme (8), was deleted by inside-out PCR. Primers were chosen such that the correct reading frame was maintained. This was accomplished by manipulating a fragment of *scpA* carried by plasmid pG^+host5. The deleted construct was then integrated by high-temperature selection. Integration resulted in duplication of the *scpA* fragment carried by the plasmid, except that one copy contained the deletion. Excision of the plasmid by recombination between the wild-type copy and the deletion copy with a crossover between the deletion and the plasmid sequence replaced the wild-type sequence with the deleted form. This segregant was ERM^s and lacked SCPA activity.

Streptococcal virulence has been assessed in studies with both mice (14, 34) and rats (19) in which persistence of streptococci for several days after infection or death defined the end point of an experiment. Streptococcal infection, like infection with most pathogens, is a complex dynamic process that is presumed to involve differential expression of many gene products. O'Connor and Cleary showed that passage of streptococci in mice enriches cultures for organisms that produce SCPA (24), and others showed that M protein (19) and immunoglobulin-binding proteins (27) are similarly affected. We proposed that SCPA eliminates the C5a chemotactic gradient where it is formed by activation of complement at the cell surface and that this, in turn, delays the initial influx of granulocytes and subsequent clearing of streptococci.

Interpretation of mouse experiments was confounded by the failure of Bohnsack et al. (2) to demonstrate cleavage of rodent C5a, including mouse C5a, by group B streptococcal C5a peptidase (SCPB). The nucleic acid sequence encoded by the SCPB gene (*scpB*) is highly similar to that encoded by *scpA* (11; unpublished data). However, Bohnsack et al. did not find that $SCPB^+$ cells can inactivate mouse C5a. In contrast, we found that SCPA can inactivate mouse C5a (Table 1). One possible explanation for these different results is that SCPA is more highly expressed than SCPB on streptococci (unpublished data). The other possible explanation is that we prepared PMNLs from mouse blood, whereas, Bohnsack et al. isolated mouse PMNLs from peritoneal exudates (2).

We believe that use of death as an indicator of virulence or

the end point of infection may obscure more subtle roles of virulence factors. Numbers of streptococci sufficient to kill mice could overwhelm local defenses and mask the impact of SCPA on early events. To minimize these potential problems, we adapted a mouse air sac model to study the effect of SCPA on the initial clearance of M^+ streptococci from infected tissue. As with previous experiments (25), when air sacs were inoculated with sufficient numbers to cause death, little difference between $SCPA^+$ and $SCPA^-$ streptococci was observed. In contrast, down regulation of the *vir* regulon, including SCPA, M49 protein, FcRA49 immunoglobulin-binding protein, and serum opacity factor, by plasmid insertion into the positive regulator, *mga* (23), significantly reduced the capacity of streptococci to cause lethal infection. This confirms that maximal virulence requires the coordinated expression of several surface proteins.

Examination of air sacs just 4 h after infection demonstrated interesting differences between those inoculated with wild-type streptococci and those inoculated $SCPA^-$ streptococci. FACS analyses of the cellular infiltrate confirmed that surface C5a peptidase reduces the initial inflammatory response. As predicted, air sacs injected with $SCPA^+$ streptococci contained significantly fewer total cells and fewer PMNLs than did those injected with $SCPA^-$ streptococci. PMNLs were the dominant granulocytes present in air sacs inoculated with mutant bacteria.

Elimination of *scpA* by mutation had a more subtle influence on virulence. By 4 h postinfection, wild-type M^+ $SCPA^+$ streptococci were prevalent in the air sac and had disseminated to the spleen. Colonies of $SCPA^+$ streptococci that emerged from spleen homogenates were highly encapsulated, resembling water drops. This observation again points to the importance of the hyaluronic capsule in mouse virulence (34). In contrast, $SCPA^-$ mutants were partially cleared from the air sac and had not disseminated to the spleen. Viable $SCPA^-$ streptococci were detected in lymph nodes significantly more often than $SCPA^+$ bacteria. The basis for differential trafficking of mutant and wild-type streptococci has not been investigated, but we speculate that the latter directly invade blood and then become sequestered by the spleen. Because of the more vigorous influx of phagocytic cells, $SCPA^-$ bacteria may be more rapidly engulfed by macrophages and/or skin dendritic cells and delivered to lymph nodes by these antigen-processing cells. Reduction of mutant streptococci relative to wild-type streptococci is an unexpected finding, because $SCPA^-$ streptococci are M^+ and resistant to phagocytosis by human neutrophils in vitro. This finding is consistent with the possibility that monocytes and/or macrophages play an important early role in clearing streptococci from a site of infection. Alternatively, the antiphagocytic function of M protein could be diminished by proteases released locally from inflammatory cells.

Expression of a C5a peptidase by human isolates of at least four species of human beta-hemolytic streptococci is clearly not the remnant of an outmoded catabolic pathway. Data presented here show that SCPA not only retards the infiltration of phagocytic cells to the site of infection but directly or indirectly alters dissemination of streptococci within the animal. The pathway to lethal infection is obviously complex, involving several gene products of the organism. Apart from adherence, the bacterium's response to early inflammatory events is critical to the outcome of infection. Several new chemokines have been discovered in the past few years, adding more complexity to our vision of the inflammatory response. Consistent with this idea, Ivey et al. recently observed that C5a initially attracts neutrophils, which subsequently produce interleukin 8 in ischemic myocardia of rabbits (20). Our data suggest that C5a is critical. Perhaps it initially recruits granulocytes to the site of infection. These initial recruits may then amplify inflammation by releasing other chemokines. Accordingly, pathogenic streptococci delay the response by eliminating C5a.

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