Survival of *Streptococcus pyogenes* within Host Phagocytic Cells: A Pathogenic Mechanism for Persistence and Systemic Invasion

Eva Medina, Oliver Goldmann, Antonia W. Toppel, and Gursharan S. Chhatwal

Department of Microbial Pathogenesis and Vaccine Research, German Research Centre for Biotechnology, Braunschweig, Germany

*Streptococcus pyogenes* is generally an extracellular pathogen that can survive and persist within the host by circumventing the host defense mechanisms. To achieve this, *S. pyogenes* has developed a number of strategies to circumvent the host immune system (e.g., virulence factors directed to prevent phagocytosis). By use of a murine model of skin infection, it was shown that survival within host phagocytic cells constitutes an additional strategy used by *S. pyogenes* to evade the host defenses and disseminate. Viable microorganisms were isolated from mouse phagocytic cells after in vitro or during in vivo infection. The capacity of intracellularly located bacteria to establish infection was demonstrated by the efficiency of gentamicin-treated neutrophils isolated from infected mice to transfer infection when injected intravenously into naive mice. The ability of *S. pyogenes* to exploit the inflammatory response of the host by surviving inside phagocytic cells may constitute an additional virulence mechanism of this pathogen.

*Streptococcus pyogenes* is an important human pathogen that causes a variety of diseases, ranging from uncomplicated pharyngitis and pyoderma to severe and life-threatening invasive infections [1]. An increased global incidence of severe invasive diseases due to *S. pyogenes* has been observed over past decades [2–6] and often has been associated with tissue invasion, bacteremia, streptococcal toxic shock syndrome (STSS), and high mortality [7–9]. Although the causes of this change in the virulence of group A streptococcus have not yet been clarified, the emergence of new bacterial serotypes or clones with increased virulence has been suggested [10]. However, despite significant studies, no single bacterial component has been clearly identified to be responsible for the recent increase in streptococcal virulence, and many aspects of the pathophysiological processes underlying host-pathogen interactions during *S. pyogenes* infection remain poorly understood.

Severe invasive streptococcal infections are often associated with systemic dissemination of the infecting microorganism from the initial focus of infection. In fact, *S. pyogenes* can be isolated from the bloodstream in most patients with invasive infection. However, the mechanisms used by invasive strains of *S. pyogenes* to disseminate from the primary site of infection remain unclear. This can be due, at least in part, to the lack of appropriate animal models of infection. Although *S. pyogenes* is not a mouse pathogen and only a very few streptococcal strains can establish infection in mice, the mouse model still has been extensively used for the study of streptococcal pathogenesis [11–14], and various virulence factors, such as C5a peptidase, extracellular cysteine protease, and streptococcal protective antigen, among others, have been identified to play a role in bacterial virulence by using these models of infection [12, 15, 16].

For the present study, we have examined the strategies used by *S. pyogenes* to survive and systemically...
disseminate from a local infection focus by use of a mouse model of streptococcal infection. Our results show that \textit{S. pyogenes} has developed pathogenic mechanisms directed not only at circumventing the host defenses but also at exploiting the host inflammatory response for persistence and systemic dissemination.

**MATERIALS AND METHODS**

**Bacterial strains.** \textit{S. pyogenes} strain A271 (serotype M1T1) was a clinical isolate provided by Dr. Allison McGeer (Mount Sinai Hospital, Toronto), which was originally obtained from a patient with STSS. \textit{S. pyogenes} strain A20 (M type 23) was a human isolate obtained from the German Culture Collection (DSM 2071). Stock cultures were maintained at $-70\,^\circ\text{C}$ and were cultured at $37\,^\circ\text{C}$ in Todd-Hewitt broth (Oxoid) supplemented with 1% yeast extract. Bacteria were collected during the midlog phase, washed twice with sterile PBS, and diluted to the required inoculum, and the number of viable bacteria was determined by counting colony-forming units after diluting and plating in blood agar plates (Gibco) containing 5% horse blood.

**Mice.** C3H/HeN female mice were purchased from Harlan-Winkelmann and were used in experiments when they were between 8 and 10 weeks of age. They were housed in microisolator cages and given food and water ad libitum. All studies were approved by the local Animal Committee Board.

**Infection models.** Only a few strains of \textit{S. pyogenes} can establish infection in mice. In the present study, we used a streptococcal strain already shown to be virulent for mice (strain A20) \cite{17} and a new mouse virulent strain (strain 271). For the skin infection, mice were anesthetized, and the hairs were partially removed from a portion of their backs. Streptococci were harvested during the midlog phase and were adjusted to an inoculum of $10^8$ cfu/100 $\mu$L. The inoculum was injected subcutaneously into the backs of mice. The actual number of colony-forming units injected was verified for each experiment by performing colony counts on blood agar plates. At selected times after infection, mice were killed by CO$_2$ inhalation and subjected to peritoneal lavage by washing the peritoneal cavity with 5 mL of Dulbecco’s modified Eagle medium (DMEM). Peritoneal exudates were washed with sterile PBS, centrifuged, resuspended in DMEM, and incubated in tissue culture plates for 2 h at $37\,^\circ\text{C}$ in a 5% CO$_2$ incubator. Nonadherent cells ($\sim70\%$ PMNs, as determined by FACScan analysis) then were collected and used for in vitro assays. These PMNs expressed levels of RB6 and CD11b molecules, comparable to those isolated from the peritoneal cavity of mice infected with \textit{S. pyogenes} and retained their phagocytic capacity.

**In vitro phagocytic assay.** Murine PMNs were isolated as described above and were adjusted to a final concentration of $10^6$ cells/mL in DMEM supplemented with 1% of normal mouse serum. Streptococci were harvested at the midlog growth phase, washed with PBS, and resuspended in DMEM at a final concentration of $5 \times 10^7$/mL. Equal volumes (250 $\mu$L) of PMNs and bacteria were mixed, yielding a final bacteria:PMN ratio of 5:1, and were incubated for 3 h at $37\,^\circ\text{C}$ in a rotary shaker. To monitor extracellular bacterial growth, samples containing bacteria, but not PMNs, were included in each experiment. PMNs then were lysed by the addition of 1 mL of deionized H$_2$O (dH$_2$O), and viable bacterial counts were determined by plating diluted samples onto blood agar plates.

For the determination of bacterial intracellular survival, PMNs were incubated for 2 h with \textit{S. pyogenes}, washed, resuspended in fresh medium containing 100 $\mu$g/mL of gentamicin (Sigma) to kill extracellular bacteria, and further incubated for 1 h at $37\,^\circ\text{C}$. Gentamicin kills extracellularly located \textit{S. pyogenes} but is limited in its ability to gain access to intracellular organisms \cite{18,19}. Subsequently, the samples were extensively washed with PBS, and cells were lysed by the addition of 1 mL of dH$_2$O, serially diluted in PBS, and plated to determine the number of viable intracellular bacteria. Samples containing only bacteria were used to estimate the efficacy of antibiotic killing.

**Gentamicin protection assay for in vivo detection of viable intracellular \textit{S. pyogenes}**. To determine the viability of \textit{S. pyogenes} microorganisms associated with host cells in infected tissue, a gentamicin protection assay was performed. For this purpose, mice were infected with \textit{S. pyogenes} either intravenously or subcutaneously, and spleens were removed from infected mice at 48 h after inoculation. Single-cell suspensions were performed by placing portions of spleen on a 200-$\mu$m mesh nylon screen and by pressing the pieces against the mesh with the plunger of a syringe until most fibrous tissue remained. Cell-free bacteria were separated from cell-associated bacteria by differential centrifugation at 115 g for 10 min. Both pellet (cell-associated bacteria) and supernatant (cell-free bacteria) were treated with 100 $\mu$g/mL gentamicin for 1 h at $37\,^\circ\text{C}$. After 1 h, cells were washed 3 times with PBS to eliminate traces of gentamicin, and aliquots either were taken to assess intracellular bacterial viability (log$_{10}$ colony-forming units of \textit{S. pyogenes}) or were injected intravenously into naive mice.

598 \ JID 2003:187 (15 February) \ Medina et al.
**Systemic dissemination of S. pyogenes in a murine model of skin infection.** C3H/HeN mice were challenged subcutaneously with $10^7$ cfu of S. pyogenes strains A271 or A20 and monitored for survival over a period of 7 days. The results (see figure 1A) confirmed the capacity of these streptococcal strains to infect and kill 100% of infected animals within an interval of 48–96 h after inoculation. The mortality of mice was associated with the ability of these strains to disseminate from a local skin lesion to the bloodstream and to establish infection in systemic organs (figure 1B).

**Resistance of S. pyogenes to killing mechanisms of PMNs.** Despite the fact that S. pyogenes strains produce a wide range of antiphagocytic virulence factors [20–24], PMNs still represent an important first line of effector cells in the control of this infection. Therefore, it could be anticipated that the capacity of these strains of S. pyogenes to establish infection in mice also might be associated with a better ability to resist the killing mechanisms of inflammatory PMNs. To assess this possibility, the ability of S. pyogenes A20 and A271 to survive in murine granulocytes was assessed in vitro in a gentamicin protection assay. PMNs were isolated from mouse peritoneal exudates and were incubated with S. pyogenes. Determination of bacterial killing by PMNs was performed after 3 h incubation by lysing PMNs with dH$_2$O and by plating diluted samples onto blood agar plates. No significant bacterial killing was observed in samples that contained PMNs, compared with that in control samples that contained medium alone (figure 2, upper panels, black bars).

For the determination of bacterial intracellular survival, PMNs were infected in vitro with S. pyogenes and gentamicin was added after 2 h incubation to kill extracellularly located bacteria. Gentamicin added to the culture supernatant does not generally access the intracellular milieu; therefore, intracellular located bacteria are protected from the antibiotic activity [18, 19]. PMNs then were washed and lysed with dH$_2$O, and viable bacterial counts were determined by plating diluted samples onto blood agar plates. As shown in the upper panels of figure 2 (white bars), a considerable proportion of microorganisms was associated with PMNs and was intracellularly situated, given that they remained viable after gentamicin treatment. Bacteria incubated in the absence of PMNs were almost completely eradicated after antibiotic treatment, corroborating the susceptibility of these microorganisms to gentamicin.

To assess whether viable S. pyogenes could be detected inside PMNs isolated from a site of infection, spleen cells isolated from mice infected with S. pyogenes A20 or A271 were subjected to gentamicin treatment. Spleens were isolated from infected mice at 48 h after infection and turned out into a single-cell suspension. The results (see figure 2, lower panels, black bars) confirmed the capacity of these streptococcal strains to infect and kill 100% of infected animals within an interval of 48–96 h after inoculation with S. pyogenes and were converted into a single-cell suspension. Total spleen cells or purified PMNs were treated with 100 μg/mL gentamicin for 1 h and were injected into naive recipient mice after extensive washing. Mice were either monitored for survival or killed at different times after inoculation, and the bacterial burden was determined in blood and peripheral organs by counting the colony-forming units of S. pyogenes in blood agar plates.

**Figure 1.** A, Survival curves of mice infected with different strains of Streptococcus pyogenes. C3H/HeN mice were challenged subcutaneously with $10^7$ cfu of S. pyogenes strain A271 (squares) or A20 (triangles), and mortality was recorded over time. Each experimental group consisted of 10 mice. B, Bacterial counts in systemic organs of C3H/HeN mice after subcutaneous infection with $10^7$ cfu of S. pyogenes strain A271 (black bars) or A20 (white bars). The results are expressed as log$_{10}$ viable count (mean ± SD) obtained from groups of 5 mice per data point.
they remain viable inside the phagocytic cells (figure 2, bottom panels).

Spleen cells isolated from mice infected with S. pyogenes are sufficient to establish infection in naive mice. To assess whether cells containing live bacteria were capable of transferring infection, we injected naive C3H/HeN mice intravenously with gentamicin-treated spleen cells isolated from S. pyogenes–infected or uninfected mice. The results (see figure 3) show that gentamicin-treated infected spleen cells were able to establish infection in recipient mice to a similar extent as an intravenous infection with a similar number of broth-grown bacteria. Mice injected either with gentamicin-treated infected spleen cells or with broth-grown bacteria died from infection between days 3 and 5 after inoculation. Mice injected with an equivalent number of uninfected spleen cells were used as controls. Extent of bacteremia (figure 3B) and bacterial loads in reticuloendothelial organs (figure 3C) also were comparable between both groups. The results (see figure 3) represent experiments carried out with S. pyogenes A20, and identical results were observed with S. pyogenes A271.

Purified PMNs isolated from mice infected with S. pyogenes can transfer infection to naive mice. To confirm the phenotype of the cell population responsible for the transmission of infection during the adoptive transfer of spleen cells into naive mice, additional experiments were performed with purified PMNs. For this purpose, PMNs were purified from the spleen of mice infected with S. pyogenes and were inoculated into naive mice. The results (see figure 4A) show that all mice inoculated with PMNs purified from spleen of infected mice died between 2 and 3 days after inoculation. Mortality was associated with S. pyogenes infection, as demonstrated by the high levels of bacteremia (figure 4B) and significant bacterial loads in systemic organs at 48 h after inoculation (figure 4C). Mice inoculated with PMNs purified from the spleen of uninfected donors did not develop infection or other side effects and survived the treatment.
Pathogenic Mechanisms of S. pyogenes

DISCUSSION

The success of pathogenic microorganisms depends on their ability to colonize host tissue and persist by ensuring their long-term survival. S. pyogenes is generally an extracellular pathogen that can cause severe invasive infection by gaining access to the systemic compartment of the host, where they propagate and persist. For this purpose, S. pyogenes has developed a broad array of virulence functions directed to circumvent the host immune mechanisms [20–24]. Thus, the surface-associated M protein confers resistance to phagocytosis by inhibiting activation of the alternative complement pathway [20, 21]. The antiphagocytic capacity of the hyaluronic acid capsule also has been demonstrated [22]. The streptococcal C5a peptidase interferes with the recruitment of PMNs to the site of the infection by inactivating the chemotactic component C5a of the complement system [15, 23] and, finally, the recently identified Mac protein that inhibits professional phagocyte function by binding to FcγRIIB on the surface of PMNs [24]. Other studies, however, have shown variations in the relative importance of some of these factors in the resistance of S. pyogenes to phagocytic killing [25, 26]. Nevertheless, PMNs still represent an important first line of effector cells in the control of S. pyogenes infections, and activated PMNs have been shown to be able to phagocytose...
and kill S. pyogenes that were resistant to the Lancefield bactericidal assay [27]. Therefore, the survival and persistence of S. pyogenes within the host may be ensured by a combination of strategies that allows these microorganisms not only to evade but also to resist the killing mechanisms of the nonspecific host immune system. In the present study, we have demonstrated that an additional strategy of S. pyogenes to circumvent the host defences is to avoid the killing mechanisms and to survive intracellularly within phagocytic cells. This was clearly demonstrated by the ability of PMNs isolated from infected mice to transfer infection after being inoculated into naive mice. Furthermore, the resistance of S. pyogenes to intracellular killing by PMNs also might contribute to their systemic dissemination by their being transported across the blood vessel during leukocyte trafficking. Intracellular bacteria then could establish a new site of infection by eventual escape from the short-lived neutrophils. This form of bacterial invasion have been demonstrated for other microorganisms such as Listeria monocytogenes [28], Staphylococcus aureus [18], and Streptococcus suis in swine [29].

The ability of S. pyogenes to exploit the inflammatory response of the host by surviving inside phagocytic cells may constitute, therefore, an additional virulence mechanism of this pathogen. Studies are under way to elucidate the mechanism(s) used by S. pyogenes to avoid the killing activities of PMNs. Nevertheless, it is important to mention that the pathogenesis of S. pyogenes in a murine model of infection may not exactly reflect human infection. However, some aspects of streptococcal infection, such as the rapid accumulation of PMNs at the site of infection, are common features to both systems, which suggests that the identification of bacterial interactions with host PMNs in the murine model might provide valuable information to investigate these particular interactions in the much more complicated human system.

Our results suggest that S. pyogenes has developed pathogenic mechanisms directed not only at circumventing the host immune system but also taking advantage of the leukocyte trafficking to be transported and spread from primary sites of infection. Our data do not dismiss a role for cell-free bacteria to cross the endothelium and to establish an invasive infection. It seems likely that the combined actions of cell-free and -associated bacteria might contribute to the invasive capacity of S. pyogenes.

**Acknowledgment**

We thank Allison McGeer (Mount Sinai Hospital, Toronto) for providing the streptococcal toxic shock syndrome clinical isolate S. pyogenes A271.

**References**

23. Weder DE, Chenoweth DE, Cleary PP. Mechanism of action of the


