Fate of *Streptococcus pyogenes* and epithelial cells following internalization

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The fate of GAS and epithelial cells following internalization was determined in this study. HEP-2 cells harbouring intracellular bacteria were treated with antibiotics to kill extracellular adherent bacteria, washed, and the fate of bacteria and epithelial cells was assessed up to 24 h post-infection. In the absence of antibiotics, massive bacterial growth was apparent in the cell medium, accompanied by extensive cell death, suggesting that intracellular bacteria had multiplied and damaged the monolayer. Addition of the internalization inhibitor, cytochalasin D, either pre- or post-internalization prevented bacterial growth and cell injury; post-internalization treatment with chloramphenicol had the same effect. Analysis of three apoptotic markers in HEP-2 cells – chromatin condensation, DNA laddering and translocation of phosphorylatedserine onto the cell-surface membrane – indicated that HEP-2 cells underwent apoptosis. Taken together, the data presented here support a model in which internalized bacteria can induce their own externalization into the medium by a process that requires both an intact host-cell cytoskeleton and de novo synthesis of bacterial proteins. Concomitantly, intracellular and, apparently, extracellular free bacteria induce apoptosis through their cytotoxic activity, and release essential nutrients required for their growth.

INTRODUCTION

Group A streptococcus (GAS) is a major human pathogen that is responsible for a wide range of diseases, ranging from mild pharyngotonsillitis and pyodermas to life-threatening invasive infections (reviewed by Cunningham, 2000). Although GAS was considered an extracellular pathogen, it has been established that the bacterium can efficiently enter a variety of mammalian cells (LaPenta \(^et\) \(al.,\) 1994; Greco \(et\) \(al.,\) 1995; Jadoun \(et\) \(al.,\) 1995; Österlund \& Engstrand, 1995; Molinari \& Chhatwal, 1999). Internalization of GAS strains by epithelial cells occurs via Fn-dependent or Fn-independent mechanisms that involve different bacterial ligands and cellular membrane receptors (Molinari \(et\) \(al.,\) 2000).

Much of the knowledge regarding GAS internalization and intracellular survival has been gained from studies that employed the conventional antibiotic-protection assay, in which cultured epithelial cells are infected with bacteria for a predetermined time. The infected cells are then washed to remove unattached bacteria and antibiotics that cannot enter the cells are added to the culture medium. In this way, extracellular adherent bacteria are killed, while intracellular protected bacteria remain viable (LaPenta \(et\) \(al.,\) 1994). By means of the conventional antibiotic-protection assay, earlier studies demonstrated that the number of intracellular bacteria gradually decreases with time until no viable bacteria are recovered. Various GAS strains were shown to survive intracellularly from 24 h up to 7 days (LaPenta \(et\) \(al.,\) 1994; Greco \(et\) \(al.,\) 1995; Österlund \& Engstrand, 1995). Furthermore, when an infected monolayer, incubated for 7 days with antibiotic-containing medium, was washed and incubated with fresh antibiotic-free medium, bacteria reappeared in the fluid, which suggested that GAS is capable of cell externalization (Österlund \& Engstrand, 1995). It was hypothesized that an increased internalization rate and prolonged intracellular survival are associated with persistent GAS carriage and recurrent infections (Österlund \& Engstrand, 1995; Österlund \(et\) \(al.,\) 1997; Neeman \(et\) \(al.,\) 1998; Sela \& Barzilai, 1999; Sela \(et\) \(al.,\) 2000). Although the antibiotic-protection assay seems to be a valuable test for measuring internalization, it might suffer from limitations in the study of intracellular survival during long periods following internalization. Since, the majority of survival studies used media that contained antibiotics, it is possible that differences among strains in their survival rates were actually related to differences in their capacity to escape from the cell into the extracellular medium, rather than to bona fide intracellular viability.

To gain better understanding of the interaction between GAS and epithelial cells, the fate of the bacterium and the HEP-2 cell following internalization was examined in an antibiotic-free medium.

Abbreviation: GAS, group A streptococcus.
METHODS

Bacterial strain and tissue culture cells. GAS strain SP3832 is an M12 serotype that was originally isolated from a carrier who continued to carry GAS in his throat following antibiotic therapy (Neeman et al., 1998). GAS was grown in Todd–Hewitt broth supplemented with 0.2 % yeast extract (THY). Human epidermoid carcinoma epithelial cell monolayer (HEp-2) was used for all assays. Cells were grown in RPMI 1640 medium supplemented with 10 % fetal calf serum, penicillin (100 μg ml⁻¹), streptomycin (100 μg ml⁻¹) and amphotericin B (0.25 μg ml⁻¹). The cells were grown at 37 °C in 5 % CO₂ and 95 % humidity, and were subcultured every second day, as follows: the monolayer was washed with PBS and then with 1 ml pre-warmed Versen-Trypsin (1 : 1 : 8 g NaCl, 0.4 g KCl, 1 g glucose, 0.5 g EDTA, 2 g NaHCO₃, 1 ml 1% phenol red solution, 25 g trypsin). Following the removal of trypsin, the cells were incubated at 37 °C until lifted off; and then resuspended in a fresh medium and seeded onto new plates.

Internalization assay. The antibiotic-resistance assay was used to determine entry of GAS into the HEp-2 cells, essentially according to Jadoun et al. (1998). Briefly, HEp-2 cells were seeded in 24-well tissue culture plate, and grown for 24 h in RPMI 1640 supplemented with 10 % of fetal calf serum (FCS) without antibiotics. Each monolayer was washed with PBS and then with 1 ml pre-warmed Versen-Trypsin (1 : 1 : 8 g NaCl, 0.4 g KCl, 1 g glucose, 0.5 g EDTA, 2 g NaHCO₃, 1 ml 1% phenol red solution, 25 g trypsin). Following the removal of trypsin, the cells were incubated at 37 °C until lifted off; and then resuspended in a fresh medium and seeded onto new plates.

Trypan blue exclusion assay. Infected and non-infected (control) HEp-2 cells were washed, trypsinized and mixed with an equal volume of trypan blue (0.5 %, v/v, in PBS). Ten microliters of the mixture were plated on a Neubauer chamber, and the numbers of total and stained cells were determined under a light microscope. The percentage of dead cells was calculated by dividing the mean number of dead (stained) cells by the total number of cells in 50 microscopic fields, and multiplying by 100.

Statistical analysis. All assays were performed in triplicate, and repeated at least three times on different days. Student’s t-test was applied to the results, which were considered significant if P < 0.05.

RESULTS AND DISCUSSION

Fate of GAS following internalization

One of the initial events in GAS pathogenesis is the interaction of the bacterium with epithelial cells of the upper respiratory system or skin. Internalization of the bacterium by eukaryotic cells has been demonstrated both in vitro (LaPenta et al., 1994; Greco et al., 1995; Österlund & Engstrand, 1995) and ex vivo (Österlund & Engstrand, 1997); however, the role of internalization in GAS pathogenesis remains unclear. LaPenta et al. (1994) hypothesized that entry into cells might serve as an initial step toward tissue invasion. In contrast, others have suggested that internalization is an innate immune response mechanism, which results in bacterial killing (Schrager et al., 1996). Yet, another hypothesis is that internalization represents a bacterial mechanism, evolved to subvert the host’s defence mechanism by providing the bacterium with a protective niche against the host’s immune response and against antibiotics that do not penetrate into cells (Österlund & Engstrand, 1995; Österlund et al., 1997; Neeman et al., 1998; Sela & Barzilai, 1999; Sela et al., 2000). The observation that strains recovered from patients with pharyngitis were ingested by epithelial cells more efficiently than strains derived from invasive diseases (Jadoun et al., 1998; Molinari & Chhatwal, 1998, 1999) might support the last two hypotheses. The findings that intracellular bacteria remained viable inside epithelial cells that were covered with antibiotic-containing medium for up to 7 days (Österlund & Engstrand, 1995; Marouni & Sela, 2004), and emerged into the external medium when the antibiotic was removed (Österlund & Engstrand, 1995) suggest that intracellular GAS are able to externalize following internalization. To investigate the de-
tails of this process we initially determined the number of viable intracellular bacteria in infected monolayer in the presence of antibiotics, in a serum-free cell culture medium. In the continuous presence of antibiotics, the number of intracellular viable bacteria declined with time, and after 24 h less than 1% of the internalized bacteria were recovered (Table 1). However, when antibiotics were removed from the medium after internalization had occurred, substantial bacterial growth was observed in the wells, as indicated by colour change (yellow) and turbidity of the medium (not shown). Similar observations were previously reported by Österlund & Engstrand (1995). In order to quantify the bacterial growth, samples were taken from the medium and plated for c.f.u. enumeration. Until 12 h post-infection only a minimal increase in the number of extracellular c.f.u. was observed, but at 24 h the medium contained $4.6 \times 10^6$ c.f.u. per well (Fig. 1). Since the antibiotics used were found to be sufficient to kill $10^8$ bacteria ml$^{-1}$ (data not shown), we hypothesize that the small number of c.f.u. detected at time 0 might represent bacteria that had just been externalized and were immediately plated on THY, thus diluting the antibiotics. Still another explanation might be that the antibiotics failed to kill a small proportion of the extracellular bacteria that were ‘hiding’ in protected microenvironmements on cell surfaces and were later released to the medium. This explanation, however, is less likely, since it was found that pre-internalization treatment of the cells with cytochalasin D (which inhibits GAS internalization, but has no effect on adherence) resulted in a significant reduction (half log, $P < 0.05$) in the number of extracellular GAS at time 0 (Fig. 1).

Since, GAS were found unable to multiply in serum-free RPMI 1640 (unpublished data), several explanations could account for this observation: (i) intracellular bacteria multiplied within the cells and escaped into the medium, where they were killed in the presence of antibiotics, or remain viable in their absence; (ii) intracellular bacteria lysed the cells (rather than multiplying intracellularly) and then, in the absence of antibiotics, grew extracellularly by feeding on nutrients available in the cell lysate; (iii) there was a combination of these two possibilities, i.e. intracellular bacteria first multiplied and then lysed the cells, and the released bacteria continued to multiply extracellularly by feeding on the cell lysate.

Recent studies which demonstrated that some GAS strains are capable of intracellular multiplication (Molnari et al., 2000, 2001) support the first and last hypotheses.

**Epithelial cells are injured following GAS internalization**

It is likely that in order to escape from epithelial cells, intracellular GAS need to lyse the cell membrane. To extend our study of the fate of HEP-2 cells after internalization, cell death was quantified. At various time points HEP-2 cells were tested for viability by determining their capacity to exclude trypan blue dye from their cytoplasm. The advantage of this assay is that it can detect the minimal cell damage that is inflicted in the early stages of attack by pore-forming toxins, such as streptolysin O and streptolysin S. As shown in Fig. 2, cell injury occurred whether or not antibiotics were present in the medium, but it was much more prominent in their absence. It is worth noting that during 12 h post-infection, cell injury increased to 18% (Fig. 2), whereas there was only a small increase in the number of external bacteria (Fig. 1). These findings suggest that cell injury preceded bacterial escape and extracellular multiplication. Furthermore, it is obvious from these results that the sharp decrease in the number of intracellular bacteria (at 12 h) in the standard internalization assay (Table 1) is not associated with bacterial

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**Fig. 1.** Emergence of free-living GAS in infected epithelial cells. HEP-2 cells were infected for 2 h with GAS, followed by antibiotic treatment, as described in Methods. Cells were washed and overlaid with RPMI 1640, with or without antibiotics. The effects of pre-internalization and post-internalization treatment with cytochalasin D (Cyt. D, 1 μg ml$^{-1}$) or chloramphenicol (Cm, 20 μg ml$^{-1}$; post-internalization only) on bacterial release was tested. The number of c.f.u. in the cell culture medium was determined at various time-points. Time 0 indicated the measurement immediately after internalization (4 h post-infection). Values are means of c.f.u. per well in three independent experiments $\pm$ SE. Asterisks indicate significant difference ($P < 0.05$), compared to control, as determined by Student’s t-test.
release from the cells, but rather is related to cell injury and penetration of antibiotics into epithelial cells, as was proposed by Molinari et al. (2001). Cell injury increased rapidly, and at 18 and 24 h post-infection the injury rates reached about 50 and 78%, respectively. In agreement with these findings, light microscopy showed no significant morphological damage in the presence of antibiotics (Fig. 3a), whereas there was extensive cellular destruction in their absence (Fig. 3b).

**GAS internalization and de novo protein synthesis are necessary for cell damage induction**

It could be hypothesized that a small number of extracellular adherent bacteria were not killed by the antibiotics during the internalization assay; they would then have resumed their metabolic activity when the antibiotics were removed from the medium, and induced external cell injury. To test this unlikely possibility, cytochalasin D, an inhibitor of GAS internalization (Greco et al., 1995) was added to epithelial cells prior to the internalization assay. Treated cells were incubated with bacteria in the presence of antibiotic-free medium, and cell cytotoxicity was determined by trypan blue staining. After 24 h only a slight increase (up to 25%) in the number of dead cells was observed among the cytochalasin D-treated cells, compared with about 80% among the untreated cells (Fig. 2). Concomitantly, only a few hundred

![Fig. 2. Effect of cytochalasin D and chloramphenicol on GAS-induced cell injury. HEP-2 cells were infected with GAS for 2 h, followed by antibiotic treatment, as described in Methods. Cells were washed and overlaid with RPMI 1640, with or without antibiotic. The effects of pre-internalization and post-internalization treatment with cytochalasin D (Cyt. D, 1 μg ml⁻¹) or chloramphenicol (Cm, 20 μg ml⁻¹; post-internalization only) on cell injury were tested. The percentage of injured cells was determined at various time-points, with time 0 representing the measurement immediately after internalization (4 h post-infection). Cells were stained with trypan blue and counted under a light microscope. Data are presented as the mean percentage ± SE of injured (stained) cells among the total number of cells in 50 microscopic fields. Asterisks indicate significant difference (P < 0.05), compared to control, as determined by Student’s t-test.]

![Fig. 3. Morphology of HEP-2 cells at 24 h following infection, with or without antibiotics in the medium. HEP-2 cells grown on glass coverslips were infected with GAS for 2 h, followed by additional incubation for 2 h with penicillin and gentamicin. Cells were then incubated for 20 h, with (a) or without (b) antibiotics. Coverslips were stained with Giemsa prior to visualization under light microscopy. In the presence of antibiotics, intact cell morphology was observed (a), whereas in the absence of antibiotics, signs of cell degradation were seen throughout the monolayer (b).]
free-living bacteria were recovered at 24 h post-infection from the cell culture fluid of treated cells, compared with > 10^6 from that of the untreated control cells (Fig. 1). Although we observed that in the presence of antibiotics the number of intracellular bacteria declined with time (Table 1), we found that the level of cell injury increased (Fig. 2). A possible explanation could be that the progressive increase in the cell injury level resulted from continuing activity of preformed enzymes (such as SpeB) which had been secreted by intracellular bacteria before they were killed.

These results support the notion that intracellular, rather than extracellular bacteria were responsible for the observed cell injury and the re-emergence of free-living bacteria into the external medium. To determine whether the host cell cytoskeleton is also involved in GAS externalization, cytochalasin D was added to the culture medium following internalization (4 h post-infection). Interestingly, this treatment also resulted in a small number of extracellular bacteria and inhibition of epithelial cell injury, which suggests that the cytoskeleton was involved in the processes leading to bacterial release and cell death.

In order to determine whether de novo protein synthesis in GAS is required for inducing cytotoxicity and bacterial release, cell injuries and the numbers of c.f.u. in the external medium were examined in the presence of chloramphenicol, a bacteriostatic antibiotic that inhibits prokaryotic protein synthesis. When chloramphenicol was added at 4 h post-infection (after internalization), cell injury was significantly reduced (Fig. 2), as was the number of extracellular free-living bacteria (Fig. 1).

Taken together, these data could indicate that cell injury and bacterial externalization require both GAS internalization and de novo protein synthesis. Our results agree well with recent findings that, following internalization, GAS escape from endocytic vacuoles into the cytosol, where they can multiply (Molinari et al., 2000).

**HEp-2 cell damage is caused by programmed cell death**

Many bacterial pathogens are capable of inducing cell death in the host via apoptosis (reviewed by Zychlinsky & Sansonetti, 1997; Weinrauch & Zychlinsky, 1999; Grassme et al., 2001). Apoptosis – programmed cell death – is a fundamental cellular mechanism which ensures homeostasis of the organism. It is characterized initially by a series of stereotypic morphological changes, such as DNA fragmentation, nuclear condensation and fragmentation, and membrane blebbing. GAS express a variety of cytotoxic enzymes and toxins, which might be responsible for epithelial cell injury, therefore, the cell deaths that are observed following GAS internalization could be a result of bacterial activity alone, and/or induced apoptosis. Recent studies have revealed that GAS are capable of inducing apoptosis in epithelial cells and in monocyte-like cells. Streptococcal pyrogenic exotoxin B (SpeB), SpeA, streptolysin O and streptolysin S have all been implicated in this process (Tsai et al., 1999; Molinari et al., 2001; Kuo et al., 1999; Bricker et al., 2002). To determine whether HEp-2 cells undergo apoptosis after internalization, we looked for several indicators of apoptosis at various time points. Infected HEp-2 cells were washed, fixed and stained with DAPI. Microscopic examination of uninfected (control) cells showed uniform nuclear morphology, whereas infected cells showed a highly condensed nucleus (Fig. 4a), characteristic of apoptotic cells. Another general hallmark of apoptotic cells is internucleosomal DNA fragmentation. DNA isolated from infected and uninfected cells was assessed by looking for presence of a 180-bp laddering pattern on agarose gels. Gel electrophoresis of chromosomal DNA revealed a characteristic fragmentation of DNA derived from infected but not of that derived from control cells at 24 h post-infection, with partial fragmentation appearing as early as 12 h (Fig. 4b).

An earlier indicator of apoptosis is the detection of phosphatidylserine on the outer leaflet of the plasma membrane bi-layer (Raffray & Cohen, 1997). During the early stages of apoptosis, the asymmetry of plasma membrane phospholipids is lost, which leads to the external exposure of phosphatidylserine. To obtain further confirmation of the nature of the cell death, the appearance of phosphatidylserine on the surface of HEp-2 cell membrane was determined at 8

**Fig. 4.** Characteristics of apoptosis in infected HEp-2 cells: (a) HEp-2 cells were incubated with cell culture medium alone (panel 1) or infected with GAS (panel 2). Following GAS internalization, the cells were incubated with antibiotic-free medium for 20 h, fixed with 4 % paraformaldehyde and then stained with DAPI; (b) agarose gel electrophoresis of genomic DNA extracted from non-infected HEp-2 cells (lane 1) or from GAS-infected cells at 12 h (lane 2) and 20 h (lane 3) post-infection. DNA was stained with ethidium bromide.
and 12 h post-infection. Annexin-V-FLOUS, a specific and sensitive phosphatidylserine binding protein, was used, and showed that at 8 h a significantly higher proportion of the infected cells than of the control cells expressed phosphatidylserine on their surface: about 35 and 5%, respectively. At 12 h post-infection, the percentage of infected cells expressing phosphatidylserine on their surface increased to 60%, which was significantly higher than the 7% of the control cells that did so (data not shown). These findings support the notion that cell death following GAS internalization is mainly caused by programmed cell death.

GAS internalization has previously been shown to be necessary and sufficient for inducing apoptosis in mammalian cells (Tsai et al., 1999; Nakagawa et al., 2001). Similarly, internalization has been reported to be a prerequisite for inducing apoptosis in cells infected with *Staphylococcus aureus* (Nuzzo et al., 2000; Kahl et al., 2000; Menzies & Kourteva, 1998; Bayles et al., 1998), suggesting that the induction of programmed cell death by facultative intracellular bacteria is a common occurrence in host cell–bacterium interactions. Tsai et al. (1999) reported that 11–13% of HEP-2 cells showed indications of apoptosis at 20 h following GAS infection, and that about 40% of the cells exhibited membrane injury. The relatively low percentage of injured cells, compared with our present data, might be a reflection of the differing experimental conditions used in the two studies. Tsai et al. (1999) measured cytotoxicity in cells overlaid with antibiotic-supplemented medium, whereas no antibiotics were present in the culture medium in our study. Hence, it is conceivable that apoptosis was enhanced by the presence of extracellular multiplying bacteria. The recent finding that extracellular GAS are also capable of inducing apoptosis by insertion of NAD+-glycohydrolase into the cytoplasm of infected keratinocytes (Bricker et al., 2002), supports such a notion.

It has been suggested that apoptosis of intestinal epithelial cells following pathogen infection may be a means of eliminating infected and damaged epithelial cells, and restoring epithelial cell growth regulation and cell integrity (Kim et al., 1998). Likewise, apoptosis may lead to eradication of engulfed streptococci via desquamation of infected cells and/or ingestion by resident macrophages. Phagocytosis of apoptotic cells does not elicit bacterial activities and therefore provides a protective intracellular niche, shielded against exogenous host immune defences, as well as against extracellular antibiotics. It is noteworthy that electron microscopy has revealed that eight out of 11 tonsils removed from asymptomatic GAS carriers contained intracellular GAS residing in macrophage-like cells (Österlund et al., 1997), an observation that supports the notion that some GAS strains may have developed a unique survival strategy to subvert the host defence mechanism and to persist within the human host. It is yet to be determined whether GAS strains derived from other sources interact in the same way with epithelial cells.

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