BACTERIAL PATHOGENICITY

Relationship between asymptomatic carriage of *Streptococcus pyogenes* and the ability of the strains to adhere to and be internalised by cultured epithelial cells

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This study was undertaken to determine whether the ability of group A streptococci to persist in the throat following antibiotic therapy corresponded with their capacity to adhere to and be internalised by epithelial cells. The study employed a HEP-2 cell model to examine the adherence and internalisation capacities of 42 strains (13 from asymptomatic patients with bacteriological eradication failure and 29 from patients with bacterial eradication). The adherence and internalisation efficiencies of strains from symptomless carriers were significantly higher. The average adherence efficiency of the carriers' strains was 53 (SEM 6)% versus 35 (SEM 5)% in control strains. The average internalisation efficiency of the carriers' strains was 13.4 (SEM 4)% compared with 4.4 (SE 1.6)% in the control group. The results are in agreement with the hypothesis that, in a significant number of cases, streptococcal internalisation might contribute to eradication failure and persistent throat carriage.

Introduction

Group A streptococcus (GAS) is a major human pathogen that commonly causes throat and skin infections [1]. In recent years, an increase in streptococcal invasive diseases, including necrotising fasciitis, myositis and toxic shock-like syndrome has been reported [2, 3]. It has been suggested that pyrogenic toxins may play an important role in these infections; however, no specific component of the bacterium has been implicated in the invasiveness of these infections [3–5]. GAS is generally considered to be an extracellular pathogen that adheres to epithelial cells. Nevertheless, recent studies have established that many GAS strains are capable of epithelial cell internalisation [6–11]. The nature of some of the bacterial components involved in cell internalisation is just beginning to be elucidated. Both M protein (serotypes M1, M6) and the homologous fibronectin-binding proteins F1 and SfbI were shown to be involved in

the entry of certain strains into cultured epithelial cells [8, 10, 12–14].

Failure to eradicate bacteria from the throat following antibiotic therapy occurs in 5–30% of cases with streptococcal pharyngotonsillitis [15, 16]. It has been speculated that a strain that is internalised efficiently is more likely to be carried after penicillin therapy, as intracellular streptococci are protected from the bactericidal effect of β -lactam agents that do not penetrate eukaryotic cell membranes [4]. More recently, Österlund and co-workers have documented that GAS is found, *in vivo*, inside pharyngeal epithelial and macrophage-like cells. Based upon their findings, they have proposed that an intracellular reservoir of GAS might be a possible explanation for recurrent pharyngotonsillitis [17].

A previous study examined the prevalence of *prtF1*, a streptococcal gene involved in GAS internalisation [8, 10], among various GAS isolates from paediatric patients with acute pharyngotonsillitis who were treated with β -lactam antibiotics [18]. A higher prevalence of the *prtF1* gene was found in strains from patients with asymptomatic carriage following therapy than in strains from patients from whom bacteria were eradicated [18].

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The significant correlation between the presence of *prtF1* gene and GAS carriage is consistent with the hypothesis that internalisation might be involved in the events leading to the development of the carrier state.

To further examine this hypothesis, the present study extended the earlier investigation and employed an epithelial cell-culture model to compare adherence and internalisation capacities among streptococcal strains from patients with successful eradication and with eradication failure following β -lactam antibiotic therapy.

Materials and methods

Bacterial isolates

Streptococcal isolates used in this study were described previously [18]. Briefly, strains were isolated from patients with pharyngotonsillitis who participated in a study in which the efficacy of two β -lactam antibiotics was compared. The double-blind study was randomised at a ratio of 1:1. GAS isolates from patients' throats were divided into two groups based upon the bacteriological response of the patients. Both groups of isolates were from patients who were clinically well, i.e., no signs or symptoms of pharyngotonsillitis were recorded. The first group (I) consisted of 29 pre-treatment isolates from patients in whom GAS was successfully eradicated from the throat following therapy, and was not present at 24–27 days after the commencement of treatment. The second group (II) consisted of strains (both pre- and post-treatment isolates) from 13 patients who continued to carry GAS in their throat at 24–27 days after the onset of therapy. The two groups were comparable with regard to age and treatment received [18]. Streptococci were grown in Todd-Hewitt Broth (Difco laboratories, Detroit, MI, USA) supplemented with yeast extract 0.2% w/v (THY).

Internalisation assay

Internalisation assays were performed as described previously [10]. Confluent HEp-2 cells were infected with 5×10^5 cfu of stationary phase streptococci resuspended in tissue-culture medium and incubated for 2 h at 37°C in air with CO₂ 5%. Non-adherent bacteria were removed by washing with 1 ml of warm tissue-culture medium. Extracellular adherent bacteria were killed by adding 0.5 ml of fresh tissue-culture medium supplemented with gentamicin 100 μ g/ml and penicillin 5 μ g/ml and incubating for 2 h. Infected monolayers were washed twice with medium and lysed with ice-cold water (15 min at 4°C). Viable intracellular streptococci were counted by plating serial dilutions (in duplicate) of cell lysates and determining the number of cfu. Results were read as the average percentage (SD) of inoculum that survived antibiotic

treatment in three infected wells. Each experiment was repeated independently at least three times.

Adherence assay

The method described by Okada *et al.* was used to measure bacterial adherence, with minor modifications [19]. Briefly, confluent HEp-2 cells grown on glass coverslips were incubated in a six-well plate with stationary phase bacteria (10^7 cfu/well), for 30 min at 37°C in air with CO₂ 5%. The coverslips were then washed three times with tissue-culture medium, stained with Giemsa and examined by light microscopy. Five-to-eight randomly chosen microscopic fields were examined and the data were presented as the mean percentage of epithelial cells with at least two adhered bacteria or chains in one microscopic field. This method was shown to provide consistently reproducible results [19].

Statistical analysis

Average adherence and internalisation data of the various groups are presented as mean (SEM). Differences between the various groups of strains were compared by Student's *t* test, following square-root transformation, or Fisher's exact test, where appropriate. *p* values were considered significant if *p* < 0.05.

Results

Adherence and internalisation of GAS isolates

Adherence and internalisation data from the test strains varied considerably. Nevertheless, GAS isolates from the group II (eradication failure) consistently adhered to and were internalised by HEp-2 cells at a higher efficiency than group I (eradication) isolates with an average adherence efficiency of 53 (SEM 6)% versus 35 (SEM 5)%, respectively (*p* = 0.005; Student's *t* test, following square-root transformation, equal variances not assumed). The difference in the internalisation efficiencies between the two groups of strains were also significant. The average internalisation efficiency of the carriers' strains was 13.4 (SEM 4)% compared with an average of 4.4 (SEM 1.6)% in the eradication group (*p* = 0.006; Student's *t* test, following square-root transformation, equal variances not assumed). It should be noted that the presence of GAS at 24–27 days after treatment could result from persistence of the original pre-treatment strain, recolonisation with the same strain, or re-infection with another strain, shortly after treatment. A previous study [18] found that in three of 13 carriers described, the post-treatment isolates differed from the pre-treatment isolates [18], which suggested re-infection by a new strain. The other 10 isolates represented true eradication failure, although the data concerning these strains do not preclude a possible eradication of the original strains with subsequent recolonisation by the same strain. The average

adherence and internalisation efficiencies of the 10 strains were 51 (SEM 6)% and 15.4 (SEM 5.2)%, respectively, still significantly higher than those of the eradication group ($p = 0.011$, and $p = 0.015$, respectively; Student's t test, following square-root transformation, equal variances not assumed). Based on a previous study [10], adherence and internalisation efficiencies were subdivided into 'low' and 'high' categories (Fig. 1). Significantly, a higher proportion of group II strains (both $n = 13$ and $n = 10$) were assigned to the 'high' (>30%) adherence category than group I strains (Fig. 1a). Similarly, a higher proportion of group II strains were assigned to the 'high' (>5%) internalisation category (Fig. 1b).

Fig. 1. (a) Adherence of GAS isolates to HEp-2 cells. Isolates were divided into low (0-30%) and high (>30%) percentage of adherence. *Significant difference ($n = 13$: $p = 0.002$; $n = 10$: $p = 0.01$; Fisher's exact test). (b) Internalisation of GAS isolates into HEp-2 cells. Isolates were divided into 'low' (0-5%) and 'high' (>5%) efficiency of internalisation. *Significant difference ($n = 13$: $p = 0.008$; $n = 10$: $p = 0.019$; Fisher's exact test). Group I strains from patients with bacterial eradication (■). Group II ($n = 13$): strains from patients following antibiotic therapy (▨). Group II ($n = 10$) represents strains from patients with apparent 'true' bacterial eradication failure (▩). Numbers above each column represent the number of isolates from each group.

To test whether efficient internalisation is a product of the carrier state, the internalisation of pre- and post-treatment isolates among group II-strains was compared. No significant differences in the efficiency of internalisation were observed (data not shown).

Discussion

The finding that intracellular GAS are resistant to penicillin [6, 7] has led to a novel hypothesis regarding the failure of β -lactam antibiotics to eradicate GAS from the throat of some patients [4, 17, 18]. GAS eradication failure is a well-known phenomenon that is currently explained by a number of mechanisms. Among these are the co-existence of β -lactamase-producing bacteria within the tonsillar crypts that inactivate penicillin [20]; penicillin 'tolerance', i.e., a significantly decreased bactericidal effect of β -lactam antibiotics [21]; bacterial interference, i.e., the eradication of the normal oral flora especially the 'viridans group' that usually compete with GAS and prevent colonisation [22, 23]; and non-compliance of the patient with therapy [24]. This study tested another hypothesis that may explain convalescent streptococcal carriage, at least in a portion of the cases. It was found that GAS strains isolated from asymptomatic carriers adhered to and internalised into cultured epithelial cells significantly better than strains from patients in whom antibiotic treatment successfully eradicated bacteria from the throat. Thus, these findings support the hypothesis that GAS internalisation might be associated with the establishment of persistent colonisation in the carrier, regardless of the time of colonisation. It is important to note that the carrier state is apparently the product of complex multifactorial interactions between the parasite and the host. Thus, it is not surprising that in some cases, other streptococcal or host factors, or both, might be more critical to establish persistent colonisation.

Recent studies by Österlund and co-workers, employing both electron microscopy and immunohistochemistry, have demonstrated the presence of intracellular GAS in epithelial cells from 13 of 14 patients with tonsillitis. The researchers also found intracellular streptococci in macrophage-like cells in 8 of 11 tonsils removed from asymptomatic GAS carriers [17]. Thus, this study suggested that GAS internalisation also occurs *in vivo*. Both studies support a putative role for streptococcal internalisation (at least in a portion of the cases) in the aetiology of streptococcal carriage following therapy.

A previous study examined the entry of a limited number of GAS strains into HEp-2 cells. It was found that strains of M types 1, 3 and 18 that were isolated from patients with severe invasive disease and acute rheumatic fever were internalised with a much lower efficiency than a laboratory strain [10]. More comprehensive studies were recently performed by several

groups of investigators [11, 25]. Both studies reported that skin and throat isolates exhibited the highest internalisation efficiencies, whereas blood isolates exhibited poor internalisation [11, 25]. In contrast, another group of investigators reported that GAS isolates from invasive and non-invasive diseases adhered to and penetrated HEP-2 cells equally well [26]. However, the conclusion was based upon a rather small number of invasive strains tested [26].

The accumulated data support the hypothesis that, in a significant number of cases, streptococcal adherence and internalisation are associated with persistent throat colonisation, and might contribute to bacterial eradication failure and asymptomatic carriage.

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