Update

Group A Streptococcus and its antibiotic resistance

Lo Strepotococco beta-emolitico di gruppo A e la sua resistenza alla terapia antibiotica

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SUMMARY

Acute pharyngo-tonsillitis caused by beta-haemolytic group A Streptococcus is a common disease in childhood. Epithelial cells are the initial sites of the host invasion by group A Streptococcus. Although group A Streptococcus has been considered an extracellular pathogen, recent studies have demonstrated that strains of this bacterium can internalize into epithelial cells both *in vitro* and *in vivo*. As adherence to and internalization into host cells significantly contributes to the pathogenesis of group A Streptococcus infections, internalization of group A Streptococcus by human epithelial cells has been extensively studied during the past decade. Multiple mechanisms are involved in this process. Most strains of *Streptococcus pyogenes* express the fibronectin-binding proteins F1 and F2, which promote bacterial adherence to and entry into human cells. Strains containing the gene for the protein F1 have been proved to be responsible for the failure of antibiotic treatment to eradicate *Streptococcus pyogenes*. Thus, in a significant number of cases, streptococcal internalization might contribute to eradication failure and persistent throat carriage. Since treatment failure, asymptomatic group A Streptococcus carriers and recurrent group A Streptococcus infections represent the main group A Streptococcus reservoir, from which the bacteria are spread in the general population, the choice of antibiotic is crucial. Beta-lactams select a large number of F1-positive organisms: therefore, macrolides, and, possibly, last generation molecules, are the best and first choice for antibiotic treatment against group A Streptococcus.

KEY WORDS: Pharyngo-tonsillitis • Group A Streptococcus internalization • Antibiotic resistance • Beta-lactams resistance • Macrolides

RIASSUNTO

La faringo-tonsillite acuta causata dallo Streptococco beta-emolitico di gruppo A è una patologia comune nell'età infantile. Le cellule epiteliali sono il sito iniziale di invasione da parte dello Streptococco, che viene classicamente considerato un patogeno extracellulare. Recenti studi hanno però dimostrato che alcuni ceppi di questo batterio sono in grado di internalizzarsi all'interno delle cellule epiteliali sia in vitro che in vivo. Dal momento che sia l'aderenza che l'internalizzazione nelle cellule dell'ospite contribuiscono in modo significativo alla patogenesi delle infezioni da Streptococco beta-emolitico di gruppo A, l'internalizzazione di quest'ultimo è stata oggetto di numerosi studi nell'ultima decade. Molteplici meccanismi sono implicati in questo complesso processo. Diversi ceppi di Streptococcus pyogenes esprimono le fibronectin-binding proteins (proteine leganti la fibronectina) F1 e F2, le quali promuovono l'aderenza e l'ingresso dei batteri nelle cellule umane. È stato dimostrato che i ceppi dotati del gene per la proteina F1 sono responsabili del fallimento del trattamento antibiotico. Quindi in un numero significativo di casi l'internalizzazione dello Streptococco potrebbe contribuire al fallimento della eradicazione del germe e allo stato di portatore sano dello stesso in faringe. Dal momento che il fallimento terapeutico, lo stato di portatore asintomatico e le infezioni ricorrenti da Streptococco rappresentano la principale riserva dalla quale i batteri vengono diffusi all'interno della popolazione, la scelta dell'antibiotico è cruciale. Gli antibiotici beta-lattamici selezionano un gran numero di microrganismi F1-positivi, pertanto i macrolidi e possibilmente le molecole di ultima generazione, sono la prima e la migliore scelta per la terapia antibiotica contro lo Streptococco beta-emolitico di gruppo A.

PAROLE CHIAVE: *Faringo-tonsilliti • Streptococco Gruppo A • Internalizzazione batterica • Antibiotico-resistenza • Resistenza ai Beta-lattamici • Macrolidi*

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Introduction

Streptococcus pyogenes (*S. pyogenes*) (group A Streptococcus (GAS)) is exclusively a human pathogen, the T antigens of which form the basis of a major serological typing scheme, an alternative or supplement to M typing. GAS is well known as a highly adhesive extra-cellular organism, the virulence of which is related to the production of exotoxins and the presence of particular surface components. Its extraordinary biological diversity becomes evident in the

wide range of diseases and in the antigenic heterogeneity present on its surface. As a prerequisite for colonization or causing local infections, *S. pyogenes* needs to adhere to eukaryotic cell surfaces. Recently, the capacity of *S. pyogenes* to invade eukaryotic cells has been reported. A better understanding of the surface proteins involved in the interaction between group A Streptococci and epithelial cells will be helpful in the development of new strategies to fight infections. In fact, antibiotic treatment has been demonstrated to be unable to eradicate group-A Streptococci in up to 30% of patients with pharyngotonsillitis [1]. Thus an antibiotic, targeted against the strains of group-A Streptococci that have entered into the respiratory epithelial cells, which is unable to penetrate the cell membrane (such as penicillins), would fail to eradicate the bacteria.

In the present review, an attempt is made to answer the following questions, referring to the clinical concern regarding GAS resistance to antibiotics:

1. how can *S. pyogenes* internalize into human cells?
2. what is the fate of *S. pyogenes* following internalization?
3. why do antibiotics fail in the treatment of group A Streptococcus infections?

How can *Streptococcus pyogenes* internalize into human cells?

Bacterial pathogens have evolved various strategies for their survival. *S. pyogenes* produces several proteins that influence host-pathogen interactions.

In the adherence process, the host extracellular matrix (ECM) proteins act as prime targets. GAS may express several microbial surface components recognizing adhesive matrix molecules, which attach to fibronectin (Fn), a matrix protein, or collagen. Fn was discovered by Harvard's Plasma Protein Program as plasma "cold-insoluble globulin", in the 1940s. It is able to change shape in response to various environmental conditions and interactions with other substances found in the extra-cellular space.

Fn-binding proteins are known as mediators for adherence of Streptococci to eukaryotic cells and play an important role in the adherence of *S. pyogenes* to human epithelial cells. The Fn-binding proteins F1 and F2, which are considered to be major group A streptococcal virulence factors, are functionally related but, structurally dissimilar.

The interaction of *S. pyogenes* Fn-binding protein (SfbI = F1) with Fn on epithelial cells triggers bacterial internalization [2]. Blocking of the SfbI adhesin by either antibodies against the whole protein or antibodies against the Fn-binding domains of SfbI, prevents both *S. pyogenes* attachment and internalization. Inert latex beads, pre-coated with the purified SfbI protein, are ingested by eukaryotic cells, demonstrating that SfbI is sufficient to trigger the internalization process.

Entry of serotype M1 *S. pyogenes* into host cells depends on binding of the host glycoprotein Fn by the bacterial M1 protein. Deletion of both the N-terminal A and B repeats regions of M1 abrogated Fn binding and intracellular invasion. Deletion of either the A domain (M1DeltaA) or B repeats (M1DeltaB) significantly reduced, but did not completely eliminate Fn binding [3].

Both the surface proteins F1and M6 are required for efficient internalization: the protein F1 mediates streptococcal internalization and the M6 protein is required for more efficient entry of the bacterium [4 5]. The functional upstream domain (FUD) of *S. pyogenes* (protein F1) interacts with the amino-terminal type I modules of Fn to unveil the cell-binding region of Fn [6].

Rocha and Fischetti [7] identified a new *S. pyogenes* Fn-binding protein (PFBP). This 127.4 kDa protein shows a structure characteristic of cell wall-associated proteins in gram-positive organisms. Its gene (pfbp) is transcribed during cell growth. The amino acid sequence exhibits a variable N-terminal region (105 amino acid with no homology with previously described Fn-binding molecules) and a conserved C-terminal region.

Brandt et al. [8] carried out the characterization of 40 consecutive *S. pyogenes* isolated from 18 patients with pharyngitis and bacteriological treatment failure. They reserved a special reference to prtF1 (the gene encoding the Fn-binding protein F1) and sic (streptococcal inhibitor of complement)/drs (distantly related sic).

On the basis of their data, the Authors suggest that neither the presence of prtF1 nor the diversification of sic/drs is required for the persistence of *S. pyogenes* in pharyngitis. GAS strains differ in the genetic potential to express Fn binding proteins SfbI, SfbII and PrtF2: strains possessing two or more of the genes for these FBPs bound Fn significantly more than strains possessing none or one of the genes [9]. As no correlation was found between Fn binding ability and the avidity of the strains to adhere to epithelial cells, the Authors suggest that while Fn binding is important for adhesion, for many GAS strains, the extent of Fn binding is not the critical determinant of adherence. Further studies came to other conclusions. The spe B (streptococcal pyogenic exotoxin B) is a secreted cysteine protease erythrogenic toxin. Inactivation of the spe B gene enhanced Fn-dependent uptake of the pathogens compared to that in the isogenic wild-type strain. The effect was attributed to an abrogation of Fn binding to the surface of the bacteria, not involving the streptococcal Fn-binding protein SfbI [10 11]. The spe B protease specifically alters bacterial cell surface proteins and, thereby, influences pathogen uptake. The influence of group A streptococcal acid glycoprotein (SAGP) on the expression of major virulence factors and internalization by epithelial cells had been investigated. SAG was studied using an isogenic mutant containing an in-frame deletion within the sagp gene [12]. The sagp mutant displayed slower growth-rate and 5-fold higher internalization efficiency than the parent strain. A down-regulation of the spe B was the most striking effect of the sagp mutation. When the Authors treated the SAGP mutant cells with the exogenous mspe B (mature protease), their susceptibility to internalization was only partially reduced. Overall, results show that SAGP modulates the expression of spe B, but also of other genes that facilitate *S. pyogenes* internalization. The cysteine proteinase spe B and the protein F1 played a correlate role in streptococcal entry into human cells [13]. In fact, the protein F1 was degraded by spe B also when in complex with Fn. Therefore, the removal of protein F1 from the bacterial surface reduced internalization. In conclusion, spe B modulates Fn dependent internalization of *S. pyogenes* by efficient proteolysis of cell-wall-anchored protein F1. Fn, bound to F1/SfbI, acts as a bridging molecule towards host cell integrins, which, in turn, initiate the uptake process that leads to GAS inter-

nalization. In their intracellular niche, GAS can persist, protected from antibiotics and host defence [14].

Recently, Gorton et al. [15] investigated an association between the presence of prtF1 and prtF2 genes (the genes of proteins F1 and F2, respectively) and internalization efficiency in group A streptococci isolated from patients with invasive disease. The prtF2-positive isolates internalised up to three times more efficiently than isolates that had prtF1 alone ($p < 0.001$), and 1.5-fold better than isolates that had neither gene. No significant association was found between internalization efficiency and presence of the prtF1 gene. According to Kreikemeyer et al. [16] protein F2 expression act as an indispensable virulence factor for both earlier and later pathogenetic stages of GAS superficial infections.

In transgenic mice lacking plasma fibronectin, Nyberg et al. [17] introduced the protein F1 gene in a *S. pyogenes* strain lacking this gene. Protein F1-expressing bacteria were less virulent to normal mice, but when these bacteria were used to infect mice lacking plasma Fn the virulence was partly restored. Plasma Fn bound to the bacterial surface down-regulates *S. pyogenes* virulence by limiting bacterial spread. From an evolutionary point of view, reducing virulence by binding Fn adds selective advantages to the bacterium [17]. According to Wang et al. [18], entry of *S. pyogenes* into host cells is mediated by Fn bound to protein F1. The consequent bridge to alpha5beta1 integrins leads to cytoskeletal rearrangement and uptake of Streptococci. The Authors demonstrated that integrin-linked kinase (ILK) is the universal link between integrins and several bacterial pathogens: in fact, the inhibition of ILK reduces the invasion of epithelial cells.

The main adhesin genes are contained in a genotype-specific pattern within the FCT region of the GAS genome. The chromosomal region, designated as FCT region, is approximately 11 to 16 kb in length and contains genes encoding a collagen-binding protein (Cpa) and the second Fn-binding protein (PrtF2 or PfbpI). The highly recombinatorial FCT region of the *S. pyogenes* genome is under strong selection for change in response to the host environment [19]. Nakata et al. [20] identified MsmR in the FCT region as a positive regulator of the major Fn-binding adhesin protein F2 in a serotype M49 strain. Altered levels of Fn-binding proteins affect eukaryotic cell attachment and internalization into human pharyngeal epithelial cells.

The expression of GAS MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) is under the control of several transcriptional regulators and two-component signal transduction systems. These mechanisms are crucial, since interference with MSCRAMM function alone, or in conjunction with specific manipulations of regulators, is an attractive goal for novel anti-infective strategies [16].

Another molecule involved in adherence and internalization is the M3 protein. Strains of the M3 serotype form the second largest group isolated from patients with severe invasive diseases and fatal infections. Eyal et al. [21] demonstrated that the M3 protein is involved in both adherence to and internalization by epithelial cells (HEp-2 cells), but it is not essential for the maturation of spe B. Inhibition of *S. pyogenes* internalization by spe B is not related to the presence of an intact M3 protein. Thus, other proteins which serve as targets for spe B activity play a major role in the internalization process. Surface-associated M proteins show an anti-phagocytic property. Studying phagocytosis by means of human neutrophils of wild-type *S. pyogenes* and strains deficient in expression of M protein and/or the M-like protein H., Staali et al. [22] demonstrated that all strains of *S. pyogenes* tested were phagocytosed by human neutrophils. However, the wild-type strain could survive inside neutrophils, whereas mutant strains were rapidly killed. The Authors concluded that bacterial evasion of host defences occurs intra-cellularly and that survival inside human neutrophils may contribute to the pathogenesis and recurrence of *S. pyogenes* infections.

Lipoteichoic acid (LTA) was demonstrated to play a role in the interactions between *S. pyogenes* and host cells [23]. LTA inhibited bacterial entry into HEp-2 epithelial cells, but had only a slight inhibitory effect on adherence. On the basis of confocal laser microscopy imaging and analysis, LTA might exert a cytotoxic effect that interferes with bacterial internalization. A possible target for LTA activity might be the actin cytoskeleton, which is known to be essential for bacterial uptake. The LSP, a member of the LraI-lipoprotein family in *S. pyogenes*, was demonstrated to be involved in eukaryotic cell adhesion and internalization [24].

The *S. pyogenes* Fas two-component signal transduction system could be involved in local tissue destruction and bacterial aggressiveness towards host cells [25]. In fact, the activity of the *S. pyogenes* Fas-system promotes high adherence and internalization rates, massive cytokine gene transcription and cytokine release, host cell apoptosis via a caspase-2 activation pathway, and cytotoxicity.

Which will be the fate of *Streptococcus pyogenes* following internalization?

Secretory IgA (sIgA) against group A Streptococci inhibits streptococcal adherence to pharyngeal cells [26]. However, in case of adherence, we might ask what will the fate of GAS be following internalization.

In the attempt to answer this question Marouni and Sela [27] treated epithelial cells (HEp-2) harbouring intracellular bacteria with antibiotics to kill extra-cellular adherent bacteria and washed. In the absence of antibiotics, massive bacterial growth was apparent in the cell medium, accompanied by extensive death of the epithelial cells harbouring intracellular bacteria. This result suggests that intracellular bacteria had multiplied and damaged the monolayer. When they add either cytochalasin D (an internalization inhibitor) or chloramphenicol, bacterial growth and cell injury were both prevented.

Analysis of three apoptotic markers in HEp-2 cells indicated that the latter underwent apoptosis. The Authors explained the data obtained by the following model:

1. 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;
2. intracellular and, apparently, extra-cellular free bacteria induce apoptosis through their cytotoxic activity, and release essential nutrients required for their growth.

The adhesion of *S. pyogenes* stimulates nuclear translocation of the transcription factor NF-kB. However, bacterial internalization is required for a sustained nuclear translocation of this transcriptional factor [28].

Nakagawa et al. [29] demonstrated that human pharyngeal epithelial HEp-2 cells become apoptotic with DNA fragmentation by invasion of GAS. The internalization of GAS can be followed by the induction of apoptosis, which is initiated by mitochondrial dysfunction. The mechanism of GAS-induced apoptosis is different from that induced by other intracellular invasive bacteria, e.g. *Shigella* and *Salmonella* species.

Two distinct pathways for the invasion of *S. pyogenes* in non-phagocytic cells are known [30]. According to the first pathway, in the invasion process Fn acts as a bridging molecule and the alpha5beta1 integrin as cellular receptor. The uptake process is characterized by the generation of large membrane invaginations at the bacteria-cell interface without evidence of actin recruitment or cellular injury. The bacterial cells are located into phagosomes and, 24 h after infection, a consistent part of the bacterial population reaches the cytoplasm. Following an alternative pathway, the uptake requires major rearrangements of cytoskeletal proteins underneath attached bacteria. Bacterial attachment stimulates elongation and massive recruitment of neighbouring microvilli, which surround streptococcal chains. They lead to the generation of large pseudopod-like structures, as a consequence bacteria are rapidly released and replicated in the cytoplasm.

Why do antibiotics fail in the treatment of group A Streptococcus infections?

The failure of penicillin to eradicate Streptococci from the throat occurs in up to 35% of patients with pharyngo-tonsillitis. Several explanations have been advanced, such as coexistence of oropharyngeal beta-lactamase-producing bacteria, interference by aerobic and anaerobic commensals, penicillin tolerance, reinfection. At present, many studies support the hypothesis that the intracellular niche may protect group A Streptococcus from penicillin, which does not reach high intracellular concentration. GAS strains were shown to survive 4-7 days inside cultured epithelial cells and strains isolated from patients with eradication failure contained the internalization-associated gene, prtF1/sfbI, in higher prevalence than the strains recovered from patients with successful eradication [31]. Thus, internalization and intracellular survival represent a novel explanation for penicillin eradication failure. In fact, the ability of group A Streptococci to persist in the throat following antibiotic therapy corresponds with their capacity to adhere to and be internalised by epithelial cells.

Studying 13 strains from asymptomatic patients with bacteriological eradication failure and 29 from patients with bacterial eradication, Sela et al. [32] observed that the adherence and internalization efficiencies of strains from carriers were significantly higher.

In 355 Italian paediatric pharyngitis patients, 127 (35.8%) erythromycin-resistant GAS isolates were detected [33]. The Authors found 126 of the 127 macrolide-resistant isolates serum opacity factor (sof) gene positive. Thus, a strong association between macrolide resistance and the presence of sof among GAS isolates was demonstrated. The Fn-binding protein F1, which is needed to enter the respiratory epithelial cells, is linked to the ability of some *S. pyogenes* strains to persist in the throat in spite of antibiotic therapy. Neeman et al. [1] hypothesised that an intracellular reservoir of group-A Streptococci could account, at least in part, for failure to eradicate throat carriage. They investigated the frequency of prtF1-containing strains in 67 patients with pharyngo-tonsillitis. As they found 12 (92%) of the 13 patients with symptomless carriage had prtF1-containing strains in the throat, compared with 16 (30%) of the 54 patients with successful eradication ($p = 0.0001$), they suggested that protein-F1-mediated entry to cells is involved in the causative process of the carriage state.

According to Musumeci et al. [34]. the rate of *S. pyogenes* strains carrying the pfbpI gene was significantly higher among asymptomatic carriers (80%) than among children with pharyngitis (53%; $p < 0.05$). A significant association between macrolide resistance and protein F/SfbI ($p < 0.001$) was detected in both groups by the same Authors. These results confirm that the presence of the pfbpI gene can be linked to the ability of *S. pyogenes* to persist in the throat of asymptomatic carriers.

The bacterial population isolated from baseline pharyngeal swabs by Cocuzza et al. [35] showed an overall prtF1 rate of 33%. Following antibiotic therapy, the average level of prtF1 increased among erythromycin-resistant strains (45%). The prevalence was higher (84%) in strains belonging to inducible resistance phenotype (iMLS) and in constitutive resistance phenotype (cMLS) (67%) as compared to the efflux pump resistance phenotype (M phenotype) (15%) ($p < 0.001$). The prevalence of the prtF1 gene was significantly lower ($p = 0.04$) in strains belonging to M resistance phenotype as compared to erythromycin-susceptible strains (28%).

The association of prtF1with macrolide resistance and with bacteriological treatment failure was studied in 713 paediatric patients presenting with acute GAS pharyngo-tonsillitis before and after antibiotic treatment [35]. Failed bacterial eradication was demonstrated in 124 patients. The prtF1 rate was unchanged in patients treated with macrolides (9/54); in contrast it was significantly higher ($p = 0.015$) in strains isolated after therapy with beta-lactams (21/70) as compared to baseline isolates (6/70).

The association between resistance to erythromycin and the presence of the Fn binding protein F1 gene (prtF1) was studied in *Streptococcus pyogenes* isolates from 301 German paediatric patients also by Haller et al. [36]. The prtF1 gene was present significantly more often in macrolide-resistant isolates.

Streptococcal pyrogenic exotoxin (spe) genes speA and speC were studied in *Streptococcus pyogenes* isolates, recovered from paediatric patients with pharyngitis, asymptomatic children and GAS-invasive disease [37]. The speA gene was detected in 13.9% of asymptomatic children, 16.8% of pharyngitis isolates and 25% of invasive cases. The speC gene incidence was higher in paediatric populations (55.4% in pharyngitis isolates and 65.8% in asymptomatic children) than in invasive isolates (30%; $p < 0.0001$). Macrolide resistance was detected in 38.0% of asymptomatic children, 37.6% of pharyngitis populations and 26.6% of invasive cases. The Authors suggest that the incidence of exotoxin and antibiotic-resistance genes among populations may have no clinical significance.

These results allow us to conclude that beta-lactams select

more prtF1-positive organisms than macrolides. Macrolides are the best and first choice for the antibiotic treatment against GAS as supported by the result of a high overall eradication rate (88%) of prtF1-positive isolates, belonging to both the erythromycin-susceptible and -resistant phenotypes [35].

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