Evaluation of the ability of erythromycin-resistant and -susceptible pharyngeal group A *Streptococcus* isolates from Spain to enter and persist in human keratinocytes

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The macrolide-resistance rate among group A *Streptococcus* (GAS) isolates has increased in some European countries. To investigate the reasons for this increase, the ability of 60 erythromycin-resistant and 61 erythromycin-susceptible, genetically unrelated, pharyngeal GAS isolates from Spain to enter and persist within human keratinocytes was evaluated. It was observed that erythromycin resistance was associated with the presence of *prtF1*, a gene related to invasiveness, whereas no association was observed with the ability to enter human keratinocytes. However, the ability to enter human keratinocytes was not associated with the presence of *prtF1* or with the *emm* type, suggesting that interaction with keratinocytes depends on several characteristics of the isolate. Almost all strains (95.9 %) were capable of persisting within human keratinocytes. However, most of them (91.7 %) exhibited a decline in viability over time. Interestingly, the ability to persist within keratinocytes protected from the action of the β -lactams was higher among erythromycin-resistant isolates and correlated with their ability to avoid the induction of cellular lysis. These observations suggest that if the carrier state results from intracellular GAS survival, the association between erythromycin resistance and intracellular persistence may represent a serious problem for the eradication of these isolates.

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INTRODUCTION

Streptococcus pyogenes or group A *Streptococcus* (GAS) is the most common cause of bacterial pharyngitis and colonizes up to one-half of all school-age children in non-epidemic periods. Although penicillin remains the drug of choice for the treatment of streptococcal infections, macrolide antibiotics are recommended as suitable alternatives for patients allergic to penicillin.

In the last decade, the incidence of macrolide resistance has increased dramatically in different countries and nowadays represents an important problem, particularly in southern European countries (Bingen *et al.*, 2004; Dicuonzo *et al.*, 2002; Melo-Cristino & Fernandes, 1999), including Spain (Albertí *et al.*, 2003), where in some regions the incidence is very high (62.3 %) (Pérez-Trallero *et al.*, 2005). Resistance to macrolides is based on the presence of an active drug efflux pump encoded by *mefA* (M phenotype) or on target site modification by methylases encoded by

ermB and *ermA* and expressed constitutively (constitutive resistance, cMLS phenotype) or due to the presence of an inducing antibiotic (inducible resistance, iMLS phenotype) (Leclercq, 2002).

It is well established that increased resistance to macrolides may be due to the extensive use of these antimicrobials. Thus the relationship between macrolide consumption and spread of resistance in GAS has been described (García-Rey *et al.*, 2002; Pérez-Trallero, *et al.*, 2005). However, the increased incidence of macrolide-resistant GAS isolates may also be due to the presence of certain virulence factors in these isolates that would confer advantages for colonizing the pharynx.

Attachment to and entry of GAS into epithelial cells may be considered the initial steps in pharynx infection. Several virulence factors are involved in these steps, including the M protein (Courtney *et al.*, 2002; Schrager *et al.*, 1998) and the fibronectin-binding protein F1 (Molinari *et al.*, 1997; Ozeri *et al.*, 1998). The M protein, encoded by the *emm* gene, promotes the entry of the micro-organism through

Abbreviation: GAS, group A *Streptococcus*.

the recognition of different receptors on the surface of the eukaryotic cells depending on the M serotype (Courtney *et al.*, 2002). The fibronectin-binding protein F1, encoded by gene *prtF1*, mediates GAS adherence to cells and the extracellular matrix, and efficient cell internalization via integrins (Ozeri *et al.*, 1998).

Facinelli *et al.* (2001) reported the association between erythromycin resistance in GAS and the ability to enter human respiratory cells, as demonstrated by the presence of the fibronectin-binding protein F1. This study raised a serious concern since strains combining erythromycin resistance and ability to enter human respiratory-tract cells may be able to escape treatment with both β -lactams, by virtue of intracellular location, and macrolides, by virtue of resistance. More recently, Haller *et al.* (2005) confirmed the association between resistance to erythromycin and the presence of the fibronectin-binding protein F1 in GAS isolates from German paediatric patients.

In this report, we characterized the ability of a large number of erythromycin-susceptible and erythromycin-resistant GAS strains isolated in Spain to invade and persist in human keratinocytes to better understand the reasons for the increased frequency of erythromycin-resistant GAS in Spain.

METHODS

Bacterial strains and their characterization. Sixty erythromycin-resistant ($\text{MIC} \geq 1 \mu\text{g ml}^{-1}$) and 61 erythromycin-susceptible ($\text{MIC} < 1 \mu\text{g ml}^{-1}$) GAS strains were included in this study. They were isolated from patients with community-acquired acute pharyngitis and belonged to a large collection of isolates obtained between 1996 and 1999 at eight different hospitals from different geographical locations in Spain (Albertí *et al.*, 2003). The genetic relatedness of the isolates was verified by *emm* typing and PFGE following protocols previously described (Albertí *et al.*, 2003). In the case of identity, only one isolate was used in this study. Isolates were maintained frozen and cultured on 5% sheep blood agar plates before testing.

The MIC for erythromycin was determined according to the guidelines of the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) (NCCLS, 2000). Erythromycin-resistant strains were differentiated into resistance phenotypes by the double-disc test (Seppala *et al.*, 1993) and screened for the presence of *ermB*, *ermTR*, *mefA* and *prtF1* genes as previously described (Neeman *et al.*, 1998; Seppala *et al.*, 1998; Sutcliffe *et al.*, 1996).

Cell culture assays. For the invasiveness studies, we followed the protocol described by Schrager *et al.* (1996). Monolayers of human dysplastic oral keratinocytes [European Collection of Cell Cultures (ECACC), reference 94122104] were grown to confluence in DMEM (Sigma) supplemented with 2 mM glutamine, 5 μg hydrocortisone ml^{-1} and 10% fetal bovine serum plus penicillin and streptomycin in 24-well tissue culture plates ($\sim 5 \times 10^5$ cells per well). Monolayers were washed three times to remove unattached cells, then inoculated with approximately 10^5 c.f.u. of GAS per well. After incubation for 2 h at 37 °C in 5% CO_2 , the monolayers were washed with PBS and then incubated for 1 h with fresh medium containing gentamicin (100 $\mu\text{g ml}^{-1}$) to kill extracellular bacteria. A 50 μl aliquot of supernatant fluid was removed for quantitative culture to confirm killing of

extracellular bacteria. To recover intracellular bacteria, the gentamicin-containing medium was removed, the keratinocytes were washed again, then lysed, and the contents of each well were vortexed for 15 s to evenly disperse the bacteria. Serial dilutions in water were done and an aliquot was spread on 5% sheep blood agar plates for quantitative culture. Cell-invasion efficiency was established as previously described (Facinelli *et al.*, 2001), on the basis of the percentage of the intracellular bacteria (with reference to the initial inoculum) recovered after the incubation of infected cells with gentamicin, which is unable to enter into the cells. Depending on cell-invasion efficiency (>10%, 1–10% or <1%), strains were defined as highly invasive, weakly invasive or non-invasive, respectively.

For the persistence assays, we used ampicillin to kill extracellular bacteria and the incubation step was prolonged up to 18 h. After that, the antibiotic-containing medium was washed away, the keratinocytes were lysed, and intracellular bacteria were recovered as described above. Persistence was calculated as the percentage of intracellular bacteria recovered after 18 h of incubation with respect to the intracellular bacteria recovered 2 h after inoculation. Depending on persistence (>50%, 1–50% or <1%), strains were defined as highly persistent, weakly persistent or non-persistent.

Cytotoxicity was estimated by the method described by Tsai *et al.* (1999). In brief, keratinocytes were infected as described above and after 3 and 18 h of incubation with ampicillin-containing medium this was replaced with fresh culture medium containing the dye WST-1 (Boehringer Mannheim) and incubated at 37 °C for 30 min. The plates were read on a microplate reader and the per cent cytotoxicity was calculated with respect to uninfected monolayers (100% viability). Depending on the cytotoxicity ($\geq 90\%$ or <90%), strains were defined as non-toxic or toxic, respectively.

Each streptococcal strain was tested in duplicate in three independent experiments done on different days. Results were analysed using Fisher's test and found significant when $P < 0.05$.

RESULTS AND DISCUSSION

The detection of *prtF1*, cell-invasion efficiency and, for erythromycin-resistant strains, the resistance phenotype and genotype are shown in Table 1.

Overall, the proportion of *prtF1*-positive strains was higher among erythromycin-resistant strains than among erythromycin-susceptible strains [55/60 (91.7%) vs 46/61 (75.4%); $P < 0.01$]. These data confirm the results obtained by Facinelli *et al.* (2001) in Italy and Haller *et al.* (2005) in Germany. However, the rate of *prtF1*-positive isolates, among both erythromycin-resistant and erythromycin-susceptible isolates, was markedly higher in our study than in these studies. Since most of the resistant strains included in our study exhibited the M phenotype (88.3%), we could not study whether the presence of *prtF1* was associated with a particular resistance phenotype (M or MLS phenotype) as described by Cocuzza *et al.* (2004).

The efficiency of bacterial entry into keratinocytes varied widely among isolates, although the behaviour of each individual isolate was quite reproducible. Interestingly, the proportion of highly invasive isolates was higher among those that were erythromycin-resistant than among erythromycin-susceptible isolates [32/60 (53.3%) vs 27/61 (44.3%); $P = 0.2$] but the difference was not significant as

Table 1. Characteristics of erythromycin-resistant and erythromycin-susceptible GAS strains included in this study

Resistance phenotype	n	Resistance genotype			prtF1 gene		Cell-invasion efficiency*		
		ermB+ mefA	ermB	mefA	+	−	High	Weak	Non-invasive
Erythromycin-resistant									
All resistant	60	9 (15 %)	4 (6.7 %)	42 (70 %)	55 (91.7 %)		32 (53.3 %)	19 (31.7 %)	4 (6.7 %)
		1 (1.6 %)	2 (3.3 %)	2 (3.3 %)		5 (8.3 %)	3 (5 %)	2 (3.4 %)	0
M	53	8	0	42	50		29	17	4
		1	0	2		3	3	0	0
cMLS	3	0	3	0	3		1	2	0
		0	0	0		0	0	0	0
iMLS	4	1	1	0	2		2	0	0
		0	2	0		2	0	2	0
Erythromycin-susceptible									
	61				46 (75.4 %)		27 (44.3 %)	14 (22.9 %)	5 (8.2 %)
						15 (24.6 %)	8 (13.1 %)	6 (9.8 %)	1 (1.6 %)

*Cell-invasion efficiency was established on the basis of the percentage of intracellular bacteria (with reference to the initial inoculum). Depending on cell-invasion efficiency (>10 %, 1–10 % or <1 %), strains were defined as highly invasive, weakly invasive or non-invasive.

described by Facinelli *et al.* (2001). This discrepancy may have different explanations. The clonal independence of the macrolide-resistant isolates was not assessed in the original study. In fact, in a later study, Spinaci *et al.* (2004) reported that three major clones accounted for 47 % of the erythromycin-resistant isolates included in the study of Facinelli *et al.* (2001). In contrast, in our study, we used genetically unrelated strains as assessed by *emm*-typing and PFGE. Also, in our invasion assays, we used monolayers of human keratinocytes, the major cell type of pharyngeal epithelium, which are more representative of the pharynx infection caused by GAS than the tumoral cell line A549 of type II pneumocytes used by Facinelli *et al.* (2001). Finally, in our study, most of the erythromycin-resistant isolates exhibited the M phenotype of resistance (88.3 %), while in the study of Facinelli *et al.* (2001), only 40.5 % of the isolates exhibited this phenotype. It is likely that this difference may contribute to explaining our results, although, to date, it is unclear whether the resistance phenotype of the isolates is associated with their cell-invasiveness ability.

We did not find a direct correlation between the presence of *prtF1* and the ability to invade keratinocytes, suggesting that other bacterial components are involved in the entry of GAS into these human cells. M protein, encoded by *emm*, is critical in causing pharyngitis (Ashbaugh *et al.*, 2000), and may recognize different receptors on the surface of the eukaryotic cells depending on the M type (Courtney *et al.*, 2002). We investigated whether the *emm* type was associated with the invasion efficiency. As shown in Table 2, we did not find a clear association between the *emm* type of the isolates and their ability to enter into keratinocytes. Moreover, isolates with the same *emm* type showed differences in their ability to enter into keratinocytes. Thus isolates expressing the prevalent *emm* types among the erythromycin-resistant strains from Spain (*emm* types

4, 12, 75 and st1815; Albertí *et al.*, 2003) presented a wide heterogeneity in their ability to invade cells. These results are consistent with those reported by Molinari & Chhatwal (1998), who demonstrated that the ability of GAS to adhere to and invade epithelial cells seemed to depend on the isolate, not on the M type. Probably other factor(s) besides the M protein are involved in the bacteria–cell interaction.

Table 2. Cell-invasion efficiency among GAS with different *emm* types

<i>emm</i> type	n	Cell-invasion efficiency*		
		High	Weak	Non-invasive
1	6	1	4	1
2	4	2	1	1
3	8	4	4	0
4	22	17	5	0
6	6	6	0	0
9	6	2	3	1
11	2	1	1	0
12	19	8	7	4
13	3	2	0	1
22	11	9	2	0
28	6	4	2	0
41	1	1	0	0
44	3	0	3	0
50	1	1	0	0
75	8	2	6	0
st1815	16	10	5	1

*Cell-invasion efficiency was established on the basis of the percentage of intracellular bacteria (with reference to the initial inoculum). Depending on cell-invasion efficiency (>10 %, 1–10 % or <1 %), strains were defined as highly invasive, weakly invasive or non-invasive.

For instance, the hyaluronic acid capsule may modulate the ability of GAS to attach to and invade human keratinocytes (Schrager *et al.*, 1998).

The biological significance of GAS entry into host cells is not well defined. Entry into epithelial cells generally leads to their lysis. However, it is not clear whether this mechanism represents a strategy of the pathogen to spread and invade deeper tissues or a host mechanism to initiate inflammation and control the infection (Cywes & Wessels, 2001; Tsai *et al.*, 1999). Also, entry into epithelial cells may represent a reservoir for persistent throat carriage in a site where the bacteria are protected from the actions of the immune system and antibiotics, particularly penicillin, the drug of choice in the treatment of streptococcal pharyngitis (Facinelli *et al.*, 2001; Kaplan *et al.*, 2006). Recently, Spinaci *et al.* (2006) investigated the intracellular persistence of 31 erythromycin-resistant isolates. They demonstrated that the resistant isolates were capable of persisting in A549 cells. In this study, we compared the intracellular persistence of erythromycin-resistant and erythromycin-susceptible isolates. For this purpose, we studied recovery of the intracellular bacteria from infected human keratinocytes after 18 h of incubation in cell monolayers in the presence of a β -lactam (ampicillin) that fails to kill intracellular GAS. Overall, the proportion of highly persistent strains was higher among erythromycin-resistant strains than among erythromycin-susceptible strains [16/60 (26.7 %) vs 7/61 (11.4 %); $P=0.019$] (Table 3). However, we recovered viable intracellular bacteria after 18 h of exposure to ampicillin in 111 (91.7 %) of the isolates, either resistant or susceptible to erythromycin. We found that in most of the cases (116; 95.9 %), intracellular bacteria were not able to multiply within keratinocytes and showed a decline in their viability over time. Interestingly, five erythromycin-resistant isolates exhibited the highest persistent rates. These data are consistent with those reported recently by Spinaci *et al.* (2006).

Although ampicillin cannot enter into eukaryotic cells, clearance of bacteria may be due to exposure of the micro-organism to the ampicillin present in the medium since

GAS may induce apoptosis and lysis of epithelial cells (Tsai *et al.*, 1999). To investigate this hypothesis, we determined the viability of keratinocytes after 18 h of exposure to intracellular bacteria. All isolates were able to reduce the viability of the human keratinocytes, suggesting that the decline of bacterial viability was due to the exposure of bacteria to extracellular ampicillin after lysis of the keratinocytes by GAS. However, we found a large heterogeneity in the ability of the isolates to induce the lysis of the cells (Table 3). Interestingly, we found an inverse correlation between the ability of the isolates to persist intracellularly and their ability to lyse the cells. Thus the isolates with a higher ability to persist within the cells were less cytotoxic than those with a lower ability [21/23 (91.3 %) vs 7/23 (30.4 %), $P=0.03$], suggesting that persistence may be associated with the ability of the isolate to remain intracellularly without the induction of cell lysis. These results are in agreement with those reported by Molinari *et al.* (2001), who demonstrated that intracellular persistence depends on the efficient expression of the Nra regulator, which decreases the expression of genes involved in host cell damage, such as those for streptolysin S and SpeB (Beckert *et al.*, 2001).

In summary, our study shows that in GAS isolates from Spain, resistance to erythromycin is associated with the presence of the *prtF1* gene but not with the ability to enter human keratinocytes, which probably depends on characteristics of the isolate other than the *emm* type or the presence of *prtF1*. Interestingly, the ability to persist within keratinocytes, and therefore be protected from the action of the β -lactams, is higher among erythromycin-resistant isolates and it seems to be related to their ability to avoid the induction of cellular lysis. These observations suggest that if the state of the upper respiratory tract in GAS carriers results in intracellular GAS survival, the association between erythromycin resistance and intracellular persistence may represent a serious problem for the eradication of these isolates because they will be able to escape both β -lactams, by virtue of intracellular location, and macrolides, by virtue of resistance.

Table 3. Ability of erythromycin-resistant or erythromycin-susceptible GAS to persist in and induce keratinocyte lysis

Erythromycin phenotype	Persistence*			Cytotoxicity†	
	High	Weak	Non-persistent	Toxic	Non-toxic
Erythromycin-resistant	2 (3.3 %)	6 (10 %)	7 (11.7 %)	15 (25 %)	0
	14 (23.3 %)	28 (46.7 %)	3 (5 %)	0	45 (75 %)
Erythromycin-susceptible	0	16 (26.2 %)	7 (11.5 %)	23 (37.7 %)	0
	7 (11.5 %)	27 (44.3 %)	4 (6.6 %)	0	38 (62.3 %)

*Persistence was calculated as the percentage of intracellular bacteria recovered after 18 h of incubation with respect to the intracellular bacteria recovered 2 h after inoculation. Depending on persistence (>50 %, $1-50$ % or <1 %), strains were defined as highly persistent, weakly persistent or non-persistent.

†Cytotoxicity was calculated with respect to uninfected monolayers (100 % viability). Depending on the cytotoxicity (≥ 90 % or <90 %), strains were defined as toxic or non-toxic, respectively.

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REFERENCES

- Alberti, S., García-Rey, C., Domínguez, M. A., Aguilar, L., Cercenado, E., Gobernado, M., García-Perea, A. & Spanish Surveillance Group for Respiratory Pathogens (2003). Survey of *emm* gene sequences from pharyngeal *Streptococcus pyogenes* isolates collected in Spain and their relationship with erythromycin susceptibility. *J Clin Microbiol* **41**, 2385–2390.
- Ashbaugh, C. D., Moser, T. J., Shearer, M. H., White, G. L., Kennedy, R. C. & Wessels, M. R. (2000). Bacterial determinants of persistent throat colonization and the associated immune response in a primate model of human group A streptococcal pharyngeal infection. *Cell Microbiol* **2**, 283–292.
- Beckert, S., Kreikemeyers, B. & Podbielski, A. (2001). The group A streptococcal *rofA* gene is involved in the control of several virulence genes and eukaryotic cell attachment and internalization. *Infect Immun* **69**, 534–537.
- Bingen, E., Bidet, P., Mihaila-Amrouche, L., Doit, C., Forcet, S., Brahimi, N., Bouvet, A. & Cohen, R. (2004). Emergence of macrolide-resistant *Streptococcus pyogenes* strains in French children. *Antimicrob Agents Chemother* **48**, 3559–3562.
- Cocuzza, C. E., Lanzafame, A., Sisto, F., Broccolo, F. & Mattina, R. (2004). Prevalence of the internalization-associated gene *prtF1* in a bacterial population of *Streptococcus pyogenes* isolated from children with acute pharyngotonsillitis before and after antibiotic therapy. *Microb Drug Resist* **10**, 264–268.
- Courtney, H. S., Hasty, D. L. & Dale, J. B. (2002). Molecular mechanisms of adhesion, colonization and invasion of group A streptococci. *Ann Med* **34**, 77–87.
- Cywes, C. & Wessels, M. R. (2001). Group A streptococcus tissue invasion by CD44-mediated cell signalling. *Nature* **414**, 648–652.
- Dicuonzo, G., Fiscarelli, E., Gherardi, G., Lorino, G., Battistoni, F., Landi, S., De Cesaris, M., Petitti, T. & Beall, B. (2002). Erythromycin-resistant pharyngeal isolates of *Streptococcus pyogenes* recovered in Italy. *Antimicrob Agents Chemother* **46**, 3987–3990.
- Facinelli, B., Spinaci, C., Magi, G., Giovanetti, E. & Varaldo, P. E. (2001). Association between erythromycin resistance and ability to enter human respiratory cells in group A streptococci. *Lancet* **358**, 30–33.
- García-Rey, C., Aguilar, L., Baquero, F., Casal, J. & Martín, J. E. (2002). Pharmacoepidemiological analysis of provincial differences between consumption of macrolides and rates of erythromycin resistance among *Streptococcus pyogenes* isolates in Spain. *J Clin Microbiol* **40**, 2959–2963.
- Haller, M., Fluegge, K., Arri, A. J., Adams, B. & Berner, R. (2005). Association between resistance to erythromycin and the presence of the fibronectin binding protein F1 gene, *prtF1*, in *Streptococcus pyogenes* isolates from German pediatric patients. *Antimicrob Agents Chemother* **49**, 2990–2993.
- Kaplan, E. L., Chhatwal, G. S. & Rohde, M. (2006). Reduced ability of penicillin to eradicate ingested group A streptococci from epithelial cells: clinical and pathogenetic implications. *Clin Infect Dis* **43**, 1398–1406.
- Leclercq, R. (2002). Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. *Clin Infect Dis* **34**, 482–492.
- Melo-Cristino, J. & Fernandes, M. L. (1999). *Streptococcus pyogenes* isolated in Portugal: macrolide resistance phenotypes and correlation with T types. *Microb Drug Resist* **5**, 219–225.
- Molinari, G. & Chhatwal, G. S. (1998). Invasion and survival of *Streptococcus pyogenes* in eukaryotic cells correlates with the source of the clinical isolates. *J Infect Dis* **177**, 1600–1607.
- Molinari, G., Talay, S. R., Valentin-Weigand, P., Rohde, M. & Chhatwal, G. S. (1997). The fibronectin-binding protein of *Streptococcus pyogenes*, SfbI, is involved in the internalization of group A streptococci by epithelial cells. *Infect Immun* **65**, 1357–1363.
- Molinari, G., Rohde, M., Talay, S. R., Chhatwal, G. S., Beckert, S. & Podbielski, A. (2001). The role played by the group A streptococcal negative regulator Nra on bacterial interactions with epithelial cells. *Mol Microbiol* **40**, 99–114.
- NCCLS (2000). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, 5th edn. Approved Standard. NCCLS document M7-45. Wayne, PA: National Committee for Clinical Laboratory Standards.
- Neeman, R., Keler, N., Barzilai, A., Korenman, Z. & Sela, S. (1998). Prevalence of internalisation-gene, *prtF1*, among group-A streptococcus strains isolated from asymptomatic carriers. *Lancet* **352**, 1974–1977.
- Ozeri, V., Rosenshine, I., Mosher, D. F., Fassler, R. & Hanski, E. (1998). Roles of integrins and fibronectin in the entry of *Streptococcus pyogenes* into cells via protein F1. *Mol Microbiol* **30**, 625–637.
- Pérez-Trallero, E., García-de-la-Fuente, C., García-Rey, C., Baquero, F., Aguilar, L., Dal-Ré, R., García-de-Lomas, J. & Spanish Surveillance Group for Respiratory Pathogens (2005). Geographical and ecological analysis of resistance, coresistance, and coupled resistance to antimicrobials in respiratory pathogenic bacteria in Spain. *Antimicrob Agents Chemother* **49**, 1965–1972.
- Schrager, H. M., Rheinwald, J. G. & Wessels, M. R. (1996). Hyaluronic acid capsule and the role of streptococcal entry into keratinocytes in invasive skin infection. *J Clin Invest* **98**, 1954–1958.
- Schrager, H. M., Alberti, S., Cywes, C., Dougherty, G. J. & Wessels, M. R. (1998). Hyaluronic acid capsule modulates M-protein mediated adherence and acts as a ligand for attachment of group A *Streptococcus* to CD44 on human keratinocytes. *J Clin Invest* **101**, 1708–1716.
- Seppala, H., Nissinen, A., Yu, Q. & Huovinen, P. (1993). Three different phenotypes of erythromycin-resistant *Streptococcus pyogenes* in Finland. *J Antimicrob Chemother* **32**, 885–891.
- Seppala, H., Skurnik, M., Soini, H., Roberts, M. C. & Huovinen, P. (1998). A novel erythromycin resistance methylase gene (*ermTR*) in *Streptococcus pyogenes*. *Antimicrob Agents Chemother* **42**, 257–262.
- Spinaci, C., Magi, G., Zampaloni, C., Vitali, L. A., Paoletti, C., Catania, M. R., Prenna, M., Ferrante, L., Ripa, S. & other authors (2004). Genetic diversity of cell-invasive erythromycin-resistant and -susceptible group A streptococci determined by analysis of the RD2 region of the *prtF1* gene. *J Clin Microbiol* **42**, 639–644.
- Spinaci, C., Magi, G. E. V. P. & Facinelli, B. (2006). Persistence of erythromycin-resistant group A streptococci in cultured respiratory cells. *Pediatr Infect Dis J* **25**, 880–883.
- Sutcliffe, J., Grebe, T., Tait-Kamradt, A. & Wondrack, L. (1996). Detection of erythromycin-resistant determinants by PCR. *Antimicrob Agents Chemother* **40**, 2562–2566.
- Tsai, P.-J., Lin, Y.-S., Kuo, C.-F., Lei, H.-Y. & Wu, J.-J. (1999). Group A streptococcus induces apoptosis in human epithelial cells. *Infect Immun* **67**, 4334–4339.