Synergistic effect of erythromycin on polymorphonuclear cell antibacterial activity against erythromycin-resistant phenotypes of Streptococcus pyogenes.[*A.M.Cuffini is the corresponding author]

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Synergistic effect of erythromycin on polymorphonuclear cell antibacterial activity against erythromycin-resistant phenotypes of *Streptococcus pyogenes* ☆

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ABSTRACT

To evaluate the synergistic activity of erythromycin and human polymorphonuclear cells (PMNs) on the binomial erythromycin-resistant (ERY^R) *Streptococcus pyogenes*/host, the phagocytic and bactericidal activities of PMNs against ERY^R streptococcal strains ($cMLS_B$, M, and $iMLS_B$ A, B and C phenotypes) were assessed in the presence of the macrolide. The results showed that when erythromycin, PMNs and streptococci [both erythromycin-sensitive (ERY^S) and ERY^R] were simultaneously present in the culture medium, PMN phagocytic activity was similar to that of drug-free controls. In contrast, the results emphasised a significant high increase in intracellular killing by PMNs in the presence of erythromycin not only for ERY^S streptococci but also for ERY^R *S. pyogenes* with high ($cMLS_B$, $iMLS_B$ A and $iMLS_B$ B phenotypes) and moderate (M and $iMLS_B$ C phenotypes) erythromycin resistance compared with controls without drug. From literature data it emerged that, even if intracellularly concentrated, erythromycin is relatively inactive because of its instability. The results indicate that the enhanced intra-PMN streptococcal killing detected is mainly attributable to PMN bactericidal systems that synergise with intracellular erythromycin in eradicating ERY^R *S. pyogenes* strains (both with high and moderate resistance). These data confirm that the antibiotic resistance detected in vitro does not always imply a failure of antimicrobial treatment.

1. Introduction

Streptococcus pyogenes, a common human pathogen that can cause a wide variety of infections, is globally susceptible to β-lactams. Macrolides represent a good alternative in the treatment of *S. pyogenes* infections in patients who are allergic to penicillin or its derivates [1,2]. Unfortunately, macrolide-resistant *S. pyogenes* have been isolated in many countries, including Europe and Asia [3ï 6]; in Italy the rate of erythromycin resistance in group A streptococci (GAS), which notably increased in the 1990s, is now 25% [7,8]. Two principal mechanisms are responsible for acquired resistance to macrolideï lincosamideï streptogramin B (MLS_B) antibiotics for GAS, namely target site modification and active efflux. Target site modification is due to a methylase, which can be constitutive ($cMLS_B$ phenotype) or inducible ($iMLS_B$ phenotype), that prevents the antibiotic binding to its ribosomal target. Active efflux (M phenotype) is related to a membrane protein responsible for efflux-mediated resistance [9,10].

However, this alarming in vitro erythromycin resistance does not always correlate with poor clinical efficacy in vivo as standard susceptibility testing methods do not take into account several host defence mechanisms that play a key role during infection in preventing the triggering and spread of a bacterial infection process [11ï 13]. In fact, the ability of professional phagocytes such as polymorphonuclear cells (PMNs) to ingest and kill microorganisms is central to innate immunity and host defence [14ï 16]. Thus, the current trend in therapy requires the use of antibiotics that combine a high level of antibacterial activity and optimal pharmacodynamic and

pharmacokinetic properties with the capacity to act in concert with the immune system in a way that potentiates the hostôs defence mechanisms. The literature reports much evidence regarding the impact of erythromycin on the primary functions of phagocytes, namely human PMNs and macrophages [17ï 20]. Since antibiotics that can interact positively with host defences might significantly contribute to improving the outcome of bacterial infection, this study focused on the potential synergy between human PMNs and erythromycin for antimicrobial activity, especially against erythromycin-resistant (ERY^R) *S. pyogenes*, in order to underline the differences in bacterial susceptibility to PMNs related to different antibiotic resistance phenotypes, e.g. $cMLS_B$, M, and $iMLS_B$ subtypes A, B and C. The excellent penetration of erythromycin via an active (energy-requiring) process and its highly effective concentration within PMNs displaying a stimulating effect on phagocytic activities is already well known and largely documented in the literature [17ï 22]. However, even if highly concentrated intracellularly, relative inactivity of erythromycin in intracellular killing has been detected compared with that of other macrolides owing to its instability at low intracellular pH values.

2. Materials and methods

2.1. Bacteria

Erythromycin-sensitive (ERY^S) and ERY^R clinical isolates of *S. pyogenes* were cultured on Columbia agar supplemented with 5% sheep blood (Biolife Italiana Srl, Milan, Italy). Young colonies (18ï 24 h) were picked up to ca. 3ï 4 McFarland standard and were inoculated into cryovials containing both cryopreservative fluid

and porous beads to allow bacteria to adhere (Microbank; bioMérieux, Rome, Italy). Following inoculation, cryovials were kept at ï 80 °C for extended storage [23,24].

2.2. Antimicrobial activity of erythromycin against Streptococcus pyogenes

Solutions of erythromycin (Sigma, St Louis, MO) were freshly prepared for each experiment and were shown to be free from endotoxin in a standard *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, MD). Determination of the minimum inhibitory concentration (MIC) of erythromycin was carried by the microdilution broth method according to the latest Clinical and Laboratory Standards Institute (CLSI) guidelines [25]. Interpretation of the results was basically as outlined in the abovementioned CLSI guidelines [25].

2.3. Resistance phenotypes

ERY^R phenotypes were determined by triple-disk diffusion testing [26] on Muellerï Hinton agar supplemented with 5% sheep blood (Oxoid Ltd., Cambridge, UK). Commercial disks (Oxoid Ltd.) of erythromycin (15 μg), clindamycin (2 μg) and josamycin (30 μg) were used. The erythromycin disk was placed at the centre of the plate, with the clindamycin disk on the right and the josamycin disk on the left. A disk of penicillin G (10 units) (Oxoid Ltd.) was added on the bottom of the plate to confirm susceptibility of the GAS isolated strains. The antibiotic disks were placed 15ï 20 mm apart. After 18 h of incubation at 37 °C in a 5% CO_2 atmosphere, absence of a significant zone of inhibition around the three disks was taken to indicate constitutive resistance ($cMLS_B$ phenotype). The presence of a zone of inhibition around the

clindamycin and josamycin disks and growth around the erythromycin disk were taken to indicate the M phenotype. A blunting zone of inhibition of clindamycin and josamycin proximal to the erythromycin disk was taken to indicate inducible resistance ($iMLS_B$ phenotype). The $iMLS_B$ A strain was characterised by absence of any zone of inhibition around both erythromycin and josamycin disks; the $iMLS_B$ B strain was characterised by blunting of the josamycin zone of inhibition proximal to the erythromycin disk and by no zone of inhibition around the erythromycin disk; and the $iMLS_B$ C strain was characterised by blunting of the josamycin zone of inhibition proximal to the erythromycin disk with a restricted zone of inhibition around the erythromycin disk [27].

To induce erythromycin resistance in $iMLS_B$ *S. pyogenes* strains, bacteria were incubated in Toddï Hewitt broth (BD Becton Dickinson Italia S.p.A., Milan, Italy) containing an inducing subinhibitory concentration (0.25× MIC) of drug for 3.5 h at 37 °C in a 5% CO_2 atmosphere [28].

2.4. Polymorphonuclear cells

Peripheral venous blood was pooled from healthy donors negative for the presence of microbial and viral diseases (A.O.U. San Giovanni Battista, Turin, Italy). Blood was collected into sterile evacuated blood-collecting tubes containing lithium heparin (150 USPU/10 mL blood) and was settled at room temperature by gravity for 30 min in 2.5% dextran (500 000 molecular weight; Pharmacia S.p.A., Milan, Italy) in normal saline (1:1 ratio). The leukocyte-rich plasma supernatant was carefully layered on

Ficoll-Paque (Pharmacia S.p.A.) and was centrifuged twice at 1200 rpm for 15 min. To obtain pure PMNs, residual erythrocytes were lysed by hypotonic shock for 30 s in sterile distilled water and then centrifuged further [29-31]. After counting in a Bürker cell counting chamber (Bürker, Marienfield, Germany), the density of PMNs was adjusted to 10^6 cells/mL in phosphate-buffered saline (PBS) supplied with 0.1% glucose and 0.1% human albumin (Sigma). PMNs were placed in sterile plastic-capped tubes treated with RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (Gibco Laboratories) and were incubated at 37 °C in a shaking water-bath before addition of streptococci [10^7 colony-forming units (CFU)/mL]. Viability assayed by trypan blue exclusion before and after each experiment was >95%. The time between collection of blood and the beginning of the experiments did not exceed 3 h. The interval between PMN harvest and the start of experiments was <30 min [29-31].

2.5. Radioactive labelling protocol

A total of 200 μL of the frozen culture was placed in fresh Toddï Hewitt broth containing 150 μCi of ^{3}H-uracil (specific activity 1.27 TBq/mmol) (NEN Products, Milan, Italy) at 37 °C. Radiolabelled streptococci were centrifuged twice at 1200 rpm for 10 min with Toddï Hewitt broth and were re-suspended in fresh medium and adjusted to yield 10^7 CFU/mL as confirmed by colony counts in triplicate [29-31].

2.6. Phagocytosis assay

In all experiments, the bacterium:PMN ratio was 10:1. Aliquots of 1 mL of *S. pyogenes* in RPMI 1640 with 10% FCS were added to PMNs in sterile plastic tubes (10^6 cells) and the tubes were then incubated at 37 °C in a shaking water-bath. After incubation for a period of 30, 60 or 90 min, the tubes were centrifuged at 1200 rpm for 5 min. The pellet suspended in PBS was centrifuged at 1200 rpm for 5 min to remove free bacteria. Cells were then suspended in 1 mL of sterile distilled water for 5 min and 100 μL samples of this suspension were placed in scintillation fluid (Atomlight; NEN Products) and counted by liquid scintillation spectrophotometry. Radioactivity was expressed as the counts/min (cpm) per sample. The percentage of phagocytosis at a given sampling time was calculated as follows: phagocytosis (%) = (cpm in PMN pellet/cpm in total bacterial pellet) × 100 [29-31].

2.7. Measurement of antimicrobial activity of polymorphonuclear cells

In all the experiments, the bacterium:PMN ratio was 10:1. Aliquots of 1 mL of *S. pyogenes* (10^7 CFU) and PMNs in sterile plastic tubes (10^6 cells) were incubated in RPMI 1640 at 37 °C in a shaking water-bath for 30 min to allow phagocytosis to proceed. The PMN/bacterium mixtures were centrifuged at 1200 rpm for 5 min and washed with phosphate saline to remove free extracellular bacteria. An aliquot of PMNs containing bacteria was taken, lysed by adding sterile water and then a viable count of intracellular GAS was performed (time zero). PMNs were then incubated further and at intervals (time *x*) viable counts of the surviving intracellular bacteria were measured in the same way. PMN killing values were expressed as the survival

index (SI), which was calculated by adding the number of surviving microorganisms at time zero to the number of survivors at time *x* and dividing by the number of survivors at time zero [29-31].

2.8. Influence of erythromycin on phagocytosis and intracellular killing

The effects of erythromycin on either the phagocytosis of *S. pyogenes* strains or intracellular bacterial killing by PMNs were investigated by incubating the bacteria and the phagocytes (bacteria:PMN ratio 10:1) at 37°C in a shaking water bath for periods of 30, 60, or 90 min in the presence of the drug (MIC and 0.25× MIC) [29-31].

2.9. Statistical analysis

Each test was performed in triplicate and the results were compared with those obtained with the control systems and expressed as the mean and standard error of the mean for 10 separate experiments. Statistical evaluation of the differences between test and control results was performed by analysis of variance (ANOVA) by Tukeyôs test. A *P*-value <0.01 was considered significant.

3. Results

In all experiments, PMN viability remained unchanged throughout the experiments.

The MIC of erythromycin for ERY^S *S. pyogenes* was found to be 0.25 μg/mL. Direct exposure of PMNs and ERY^S bacteria to an inhibitory concentration of erythromycin did not improve the phagocytosis until 90 min of incubation (Table 1). Conversely, in

the same experimental conditions a marked increase in PMN intracellular killing was achieved with the addition of erythromycin ($P < 0.01$) (Table 2).

The erythromycin MICs of ERY^R *S. pyogenes* were 128 μg/mL for the $cMLS_B$ phenotype, 16 μg/mL for the M phenotype, 128 μg/mL both for $iMLS_B$ A and B phenotypes and 8 μg/mL for the $iMLS_B$ C phenotype. Addition of erythromycin to human PMNs separately incubated with the different *S. pyogenes* resistant strains had no effect on the phagocytic capacity of PMNs throughout the observation period, as shown by phagocytosis percentages of ingested GAS comparable with that for controls without the drug (Table 1). In contrast, addition of the macrolide to PMNs after phagocytosis had occurred significantly enhanced phagocyte killing against ingested ERY^R streptococci resulting in an increased number of killed bacteria for all three incubation times compared with the antibiotic-free systems, in which PMNs were totally unable to kill ingested streptococci at 60 min and 90 min of incubation ($P < 0.01$) (Table 2).

Similar data were obtained by evaluating the effects of lower levels of erythromycin (0.25× MIC) on either PMN phagocytosis of radiolabelled streptococci (data not shown) or intracellular killing by incubating the bacteria and phagocytes for periods of 30, 60 or 90 min (Table 2).

4. Discussion

Streptococcus pyogenes is a bacterium that has evolved sophisticated mechanisms to disrupt many critical aspects of PMN function; GAS successfully evades PMN phagocytosis and killing to cause human infections including pharyngitis, impetigo, cellulitis and necrotizing fasciitis [32,33]. It is well known that the rate of phagocytosis and killing of GAS by PMNs is maximal during initial host cell/pathogen interaction (within 30 min) and fails to increase thereafter [24,34].

These findings are consistent with the ability of *S. pyogenes* to regulate actively the number of differentially expressed genes that modulate PMN functions [15,16,34-36]. Although *S. pyogenes* employs numerous mechanisms to block phagocytosis, the pathogen is only partially successful and is ingested by PMNs or in the presence of specific antibodies [33,34,36]. These data are consistent with the present results: in fact, in erythromycin-free systems, PMNs were able to engulf both ERY^S and ERY^R streptococci at values that remained constant during incubation and to kill them in only the first 30 min of observation (Tables 1 and 2).

The results of this study showed that addition of erythromycin resulted in PMN phagocytosis which remained at levels similar that of drug-free controls, underlying that the macrolide did not adversely affect PMN functionality (Table 1). In contrast, the results emphasised a significant high increase of intracellular killing by PMNs in the presence of erythromycin for all *S. pyogenes* strains during the observation period ($P < 0.01$) (Table 2). The most interesting data pertain to the ability of phagocytes to kill the intracellular ERY^R streptococci independently from their

different levels of resistance to MLS_B antibiotics. Since erythromycin even highly concentrated within phagocytes [19,20] has a lower antimicrobial activity compared with that of other antibiotics owing to its intracellular instability in the acid medium [21,22], these current results suggest that the enhanced intra-PMN streptococcal killing detected is mainly attributable to PMN bactericidal systems that tightly co-operate with intracellular erythromycin in eradicating the ERY^R streptococci. In fact, PMNs were able to synergise with erythromycin for the intracellular bacterial killing of highly resistant streptococci of phenotypes $cMLS_B$, $iMLS_B$ A and $iMLS_B$ B. During the entire time of exposure, the internalised constitutive and inducible resistant streptococci were more susceptible to the microbicidal intracellular mechanisms of human PMNs compared with drug-free controls, where the phagocytes were unable to counteract bacterial growth at 60 min and 90 min of incubation ($P < 0.01$) (Table 2). In the presence of erythromycin, also M phenotype and $iMLS_B$ C phenotype streptococci, characterised by a moderate resistance level, were killed at a rate higher than that observed for highly resistant phenotypes, highlighting a pattern quite similar to that of the ERY^S strain (Table 2).

Interestingly, a similar picture was detected even at lower levels of erythromycin (0.25× MIC) where the synergism between PMNs and erythromycin resulted in SI values that overlapped with those observed in presence of an erythromycin level equal to the MIC (Table 2), without affecting phagocytosis (data not shown). The hypothesis of a tight bactericidal co-operation between PMNs and erythromycin is corroborated from our previous studies [21,22], where it emerged that pre-exposure of human phagocytes to erythromycin before phagocytosis had no effect on

subsequent intracellular bacterial killing. Once highly accumulated within acid lysosomes, erythromycin, being a weak base, showed a reduced limited antibacterial activity.

In conclusion, according to the recent literature trend, the current methods of predicting whether or not an antibiotic will be effective against a bacterial pathogen in vivo and determining the optimal usage of an antimicrobial are still crude. Until we take account of inherent host defences and how antibiotics react with pathogens at the site of infection, the way we choose an antimicrobial agent over another will continue to be flawed. Our results, along with those from clinical trial studies [13], provide further evidence of the clinical success of erythromycin and indirectly confirm that the antibiotic resistance detected in vitro does not always imply in vivo treatment failure inasmuch as favourable pharmacokinetic and pharmacodynamic parameters and the complex interactions among antibiotic-resistant pathogens, administered drug and host defences play a key role as predictors of clinical outcome.

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Competing interests

None declared.

Ethical approval

Not required.

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Table 1

Effect of erythromycin (ERY) at the MIC on human polymorphonuclear cell phagocytosis of erythromycin-sensitive (ERY^S) and erythromycin-resistant (ERY^R) *Streptococcus pyogenes* determined by phagocytosis assay

S. pyogenes phenotype	Time (min)	Phagocytosis (mean ± S.E.M.) (%)	
		Drug-free control	1× MIC ERY
ERY^S (MIC = 0.25 μg/mL)	30	51.2 ± 3.7	45.9 ± 4.16
	60	42.5 ± 1.64	46.6 ± 4.05
	90	44.9 ± 1.39	45.3 ± 4.1
ERY^R [a]			
$cMLS_B$ (MIC = 128 μg/mL)	30	41.8 ± 3.8	40.0 ± 1.25
	60	46.8 ± 4.2	45.2 ± 4.2
	90	47.4 ± 4.5	46.9 ± 4.3
M (MIC = 16 μg/mL)	30	58.9 ± 1.9	55.1 ± 4.1
	60	48.3 ± 4.4	44.6 ± 4.4
	90	37.1 ± 3.7	35.1 ± 3.4
$iMLS_B$ A (MIC = 128 μg/mL)	30	31.8 ± 1.8	29.9 ± 2.7
	60	33.2 ± 2.4	33.0 ± 2.0
	90	33.9 ± 3.3	33.4 ± 2.8
$iMLS_B$ B (MIC = 128 μg/mL)	30	47.9 ± 0.6	41.1 ± 3.8
	60	38.2 ± 1.4	39.0 ± 1.9
	90	36.2 ± 3.6	37.7 ± 0.9
$iMLS_B$ C (MIC = 8 μg/mL)	30	76.0 ± 7.6	73.6 ± 5.6
	60	72.3 ± 4.01	70.8 ± 6.11
	90	69.9 ± 0.9	76.2 ± 3.7

MIC, minimum inhibitory concentration; S.E.M., standard error of the mean; MLS_B, macrolideï lincosamideï streptogramin B.

[a] Erythromycin resistance phenotypes include: constitutive ($cMLS_B$) and inducible ($iMLS_B$) resistance due to a methylase target site modification; and resistance due to active efflux（M phenotype).

Table 2

Effect of erythromycin (ERY) at the MIC and 0.25× MIC on human polymorphonuclear cell intracellular killing of erythromycin-sensitive (ERY^S) and erythromycin-resistant (ERY^R) *Streptococcus pyogenes* determined by intracellular killing assay

S. pyogenes phenotype	Time (min)	Survival index (mean ± S.E.M.)		
		Drug-free control	1×MIC ERY	0.25× MIC ERY
ERY^S (MIC = 0.25 μg/mL)	30	1.42 ± 0.09	1.18 ± 0.04 *	1.20 ± 0.02 *
	60	>2	1.29 ± 0.06 *	1.26 ± 0.05 *
	90	>2	1.30 ± 0.05 *	1.32 ± 0.04 *
ERY^R [a]				
$cMLS_B$ (MIC = 128 μg/mL)	30	1.76 ± 0.06	1.38 ± 0.11 *	1.37 ± 0.13 *
	60	>2	1.75 ± 0.14 *	1.79 ± 0.10 *
	90	>2	1.84 ± 0.17 *	1.81 ± 0.15 *
M (MIC = 16 μg/mL)	30	1.69 ± 0.04	1.29 ± 0.10 *	1.32 ± 0.09 *
	60	>2	1.32 ± 0.07 *	1.30 ± 0.05 *
	90	>2	1.43 ± 0.12 *	1.42 ± 0.13 *
$iMLS_B$ A (MIC = 128 μg/mL)	30	1.55 ± 0.11	1.45 ± 0.07	1.44 ± 0.04
	60	>2	1.67 ± 0.06 *	1.62 ± 0.08 *
	90	>2	1.82 ± 0.04 *	1.79 ± 0.07 *
$iMLS_B$ B (MIC = 128 μg/mL)	30	1.85 ± 0.15	1.40 ± 0.09 *	1.43 ± 0.10 *
	60	>2	1.71 ± 0.03 *	1.70 ± 0.05 *
	90	>2	1.72 ± 0.07 *	1.74 ± 0.04 *
$iMLS_B$ C (MIC = 8 μg/mL)	30	1.75 ± 0.06	1.27 ± 0.04 *	1.29 ± 0.06 *
	60	>2	1.36 ± 0.07 *	1.38 ± 0.08*
	90	>2	1.42 ± 0.01 *	1.42 ± 0.03 *

* Significantly different from the drug-free controls ($P < 0.01$).

[a] Erythromycin resistance phenotypes include: constitutive ($cMLS_B$) and inducible ($iMLS_B$) resistance due to a methylase target site modification; and resistance due to active efflux（M phenotype).