International Journal of Antimicrobial Agents 36 (2010) 319–323

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

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

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ARTICLE INFO

Article history:
Received 7 May 2010
Accepted 18 June 2010

Keywords:
Streptococcus pyogenes
Erythromycin
Polymorphonuclear cells
Phagocytosis
Phagocytic killing

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 [3–6]; 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 macrolide–lincosamide–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 [11–13]. 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 [14–16]. 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 host's defence mechanisms. The literature reports much evidence regarding the impact of erythromycin on the primary functions of phagocytes, namely human PMNs and macrophages [17–20]. 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*,

☆ This study was presented at the European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), 10–13 April 2010, Vienna, Austria.

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doi:10.1016/j.ijantimicag.2010.06.041

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 [17–22]. 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 (18–24 h) were picked up to ca. 3–4 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 −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 Mueller–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 15–20 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 Todd–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 Todd–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 Todd–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