

Influences of clarithromycin on the primary functions of human polymorphnuclear leucocytes against *Streptococcus pneumoniae*

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Abstract:

Current antibiotic therapy encourages the use of antibiotics that may potentiate the host's immune defense. Antibiotics that can interact positively with the immune response and that also possess microbicidal properties might significantly contribute to improving the outcome of *St.pneumoniae* infections .Therefore, in the present study we investigated the effect of clarithromycin,an extended spectrum macrolide currently used in the treatment of respiratory tract infections ,on the in vitro interaction between human polymorphnuclear granulocytes (PMN) and strains of *St.pneumoniae* with different resistance patterns to penicillin .At a concentration of one –half the minimal inhibitory concentration(MIC),clarithromycin, an significantly enhanced human PMN functions particularly intracellular bactericidal activity, against all the *St.pneumoniae* strains ,our findings support the use of clarithromycin in the treatment of respiratory tract infections caused by *St.pneumoniae* as it acts directly against the pathogen as well as in cooperation with PMNs by eliciting their intracellular killing.

تأثيرات الكلاريثرومايسين على الوظائف الاولية لكريات الدم البيضاء متعددة اشكال
النواة في الانسان ضد المكورات السبحية الرئوية

المستخلص:

إن العلاج بالمضادات المستخدمة حاليا يشجع استخدام مضادات لها القدرة على التفاعل ايجابيا مع الاستجابة المناعية والتي تمتلك بنفس الوقت خصائص قاتلة للبكتريا وتساهم بفاعلية في التقليل من الاصابات بالمكورات السبحية الرئوية. في الدراسة الحالية تم مناقشة تأثير الكلاريثرومايسين (مضاد ذو طيف واسع المدى يستعمل حاليا لمعالجة إصابات القناة التنفسية) في التفاعل خارج خلوي بين كريات الدم الحبيبية متعددة اشكال النوى مع سلالات من المكورات السبحية الرئوية المقاومة للبنسلين. وفي تركيز مثبط أدنى معين فإن الكلاريثرومايسين يساهم بفاعلية في تعزيز وظائف كريات الدم البيضاء متعددة اشكال النوى خاصة ذات الفاعلية القاتلة للبكتريا داخل خلوية ضد كل سلالات بكتريا المكورات الرئوية السبحية. لقد وجد ان استخدام الكلاريثرومايسين في علاج إصابات القناة التنفسية الناتجة عن الإصابة بالمكورات السبحية الرئوية بالإضافة إلى تأثيره المباشر على الكائن الممرض فإنه يتداخل مع عمل كريات الدم البيضاء المتعددة اشكال النوى الداخل خلوي.

Introduction:-

Successful management of infections requires a multidisciplinary approach in which account is taken of the kinetics and microbiological features of the drugs employed, The type of microorganism causing the infection and the host's immune system .the differences in the way an antibiotic affects bacterial virulence and adhesiveness, as well as certain physiological activities of phagocytes (Phagocytosis, intracellular killing, release of cytokines, apoptosis, etc.) must be of fundamental importance when choosing appropriate empirical therapy⁽¹⁾. Among the respiratory tract infections ,*Streptococcus pneumoniae* infections ,such as pneumonia, meningitis and otitis media result in significant mortality and morbidity in both adults and children worldwide⁽²⁾. Their treatment is becoming more complex and difficult because of the recent dramatic increase in the number of penicillin –resistant strains of *St.pneumoniae* that has started to limit the choice of available agents⁽³⁾. Therefore ,diversification in the use of antimicrobial agents to treat pneumococcal infections will help to reduce selective pressure in part of this strategy may include therapeutic alternatives. Antibiotics that can interact positively with host defenses and that also possess microbicidal properties might also significantly contribute to improving the outcome of *St.pneumoniae* infections.

This paper describes the in vitro evaluation of the effect of clarithromycin on the primary functions of human PMNs against a recently isolated clinical *St.pneumoniae* strains.

Materials and methods:-

-Polymorphnuclear granulocytes:

Blood was drawn from healthy , patients with coetaneous lymphoma who gave their informed consent .Peripheral venous blood was collected in sterile evacuated blood collection tubes containing lithium heparin (15u/ml blood)and settled at room temperature by gravity for 30 min in 2.5% dextran (500.000 mol wt:pharmacia S.P.A.,Milan,Italy) in normal saline (1:1 ratio).the leukocyte-rich plasma supernant was carefully layered on ficol –paque and was then centrifuged twice at 160 × g for 15 min to obtain pure PMNs ,residual erythrocytes were

lysed by hypotonic shock for 30 sec. in sterile distilled water and then the PMNs were further centrifuged after being counted in a Bürker cell counting chamber ,the PMN density was adjusted to 10⁶ cells / ml in phosphate buffered saline supplied with 1% glucose and 1% human albumin (Sigma,St.Louis.Mo,USA).The PMNs were placed in sterile plastic tubes ,treated with RPMI 1640(Gibco laboratories, Grand island ,NY,USA),supplemented with 10% fetal calf serum (Gibco)and incubated at 37°c in a shaking water bath (150 rpm). Viability was assayed by trypan blue exclusion and was greater than 95%.Aviability test was performed before and after each experiment .the time between blood collection and the beginning of the experiment did not exceed 3h.the interval between PMN harvest and the start of experiments was less than 30min⁽⁴⁾.

- Antibiotic:

Clarithromycin was dissolved in ethanol diluted in phosphate buffered saline (1:1), the solutions were freshly prepared for each batch of experiments and they were shown to be free of endotoxin by a standard limulus amebocyte lysate assay. Antibiotics susceptibility testing was performed by standard dilution method in Muller Hinton broth (unipath, Milan, Italy).⁽⁵⁾

- Bacteria:

St.pneumoniae strains obtained from human infections were used: penicillin – resistant strain (MIC of penicillin G, 2 µg/ml for 10⁵ cfu/ml).⁽⁴⁾

-Phagocytosis assay:

Bacteria : PMN ratio was 10:1 ,aliquots of 1.0 ml of serum opsonized or non-opsonized bacteria (10⁷ cfu) in RPMI 1640 with 10% fetal calf serum were added to 10 ml PMNs in sterile plastic tubes (10⁶ cells)and the tubes were then incubated at 37°c in a shaking water bath ,after incubation for periods of 30,60,90,and 120 min .The tubes were centrifuged at 160 ×g for 5min.The pellet was then resuspended in phosphate saline and the mixture was centrifuged at 160xg for 5 min.To remove the free bacteria. The cells were then resuspended in 1 ml of sterile distilled water for 5 min, and 100 ml samples of this suspension were placed in scintillation fluid and counted by liquid scintillation spectrophotometry –radioactivity

was expressed as the cpm/sample .The percentage of Phagocytosis at a given sampling time was calculated as: % Phagocytosis =cpm in PMN pellet/cpm in total bacterial pelletx100⁽⁵⁾.

-Measurement of antimicrobial activity of PMNs:

In experiment the bacteria :PMN ratio was 10:1 ,aliquots of 1ml of serum opsonized or non-opsonized bacteria (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 30min to allow Phagocytosis to proceed .The PMN-bacterium mixtures were centrifuged at 160xg for 5 min and washed with phosphate saline to remove the free extracellular bacteria .An aliquots of the cells containing bacterium was taken ,the cells were lysed by adding sterile water and a viable count of intracellular bacteria was performed (t^0).The cells were then incubated further and at intervals (t^x)the viable counts of the surviving intracellular bacteria were measured in the same way .The PMN killing values were expressed as the survival index (SI),which was calculated by adding the number of surviving bacteria at t^0 to the number of survivors at t^x and dividing by the number of survivors at t^0 .

According to this formula if bacterial killing were 100% effective, the SI would be 1.⁽⁷⁾

- Influence of clarithromycin on Phagocytosis and intracellular killing:

The effects of clarithromycin on the Phagocytosis and intracellular killing of different strain of *St.pneumoniae* by PMNs were investigated by incubating the bacteria and the phagocytes at 37°C in a shaking water bath for periods of 30,60,90,120 min.in the presence of one-half the MIC of clarithromycin .Antibiotic-free controls were also included .The PMN killing values were expressed as the survival index (SI),which was calculated by adding the number of surviving bacteria at time zero to the number of survivors at time x ,and dividing by the number of survivors at time zero⁽⁸⁾.According to this formula ,if bacterial killing was 100% effective, the SI would be 1.

Results:-

Table (1) illustrate the differentiation between the effects of clarithromycin on

St.pneumoniae from those on granulocytes ,the phagocytic and bactericidal activities of PMNs against *St.pneumoniae* were assessed following pre exposure of PMNs and Streptococci individually to one –half the MIC of the antibiotic .Brief prior exposure of penicillin – resistant *St.pneumoniae* to one-half the MIC of clarithromycin modified the interaction between the bacteria and the PMNs:the rate of Phagocytosis of these Streptococci was approximately twice that of the controls after 60 min. and also showed greater susceptibility to microbicidal PMN mechanisms($p<0.01$).pre incubation of PMNs with one –half the MIC of clarithromycin had no effect upon the activities of human PMNs throughout the observation period :penicillin-resistant *St.pneumoniae* treated with clarithromycin was both phagocytosed and killed within 120 min at the same percentage as that registered for untreated penicillin – resistant *St.pneumoniae*.

Table (1): Effect of pre exposure of penicillin – resistant *St.pneumoniae* (A) or human polymorphnuclear granulocytes (B) to one – half the MIC of clarithromycin for one hour. On Phagocytosis and intracellular killing

Time	Phagocytosis %			Survival index		
	Controls	A	B	Controls	A	B
30 min	12.18±1.8	13.5 ^b ±0.5	11.9±1.0	1.49±0.1	1.43 ^a ±0.2	1.51±0.1
60 min	7.3±1.4	15.1 ^a ±0.2	7.7±0.4	1.75±0.15	1.44 ^a ±0.1	1.81±0.3
90 min	5.6±0.2	9.3 ^a ±0.7	5.6±0.1	>2	1.5 ^a ±0.13	>2
120 min	4.1±0.2	9.2 ^a ±0.8	4.3±0.3	>2	1.84 ^a ±0.1	>2

A: significantly different ($p < 0.01$ from controls) B: significantly different ($p < 0.05$ from controls)

The MIC of clarithromycin for the penicillin –resistant strains of *St.pneumoniae* was 0.06 µg/ml .The presence of one-half the MIC of clarithromycin elicited human PMN Phagocytosis, resulting in a significantly increased percentage of engulfed penicillin – resistant streptococci for up to 2h.compared with that for controls (table 2).In the drug –free cultures ,the intracellular survival (SI) of the penicillin – resistant strain of *St. pneumoniae* shifted from 1.48 at 30 min to 1.85 at 60 min and to >2 at 90 min and 120 min ,this corresponded with a progressive increase in the number of intracellular bacteria . The presence of clarithromycin on the other hand ,produced a significantly higher killing effect($p < 0.01$),compared with that of the control systems .the number of the intracellular penicillin-resistant *St.pneumoniae* that survived after 120 min fell to 35% of the initial population (Table 2).

Discussion:-

Several antibiotics (fluoroquinolons, macrolides and some β-lactam drugs)in addition to their antimicrobial effects ,have the capability of modulating the host immune responses in different ways :some are concentrated in the phagocytes where they enhance intracellular killing , whereas others become inert following

intracellular uptake ;some lead to alteration in bacteria that render them more susceptible to Phagocytosis; some directly enhance the phagocytic and antimicrobial activities of the host cells; some depress the bacterial up take by phagocytes ;and others modulate T and B cell responses .Recent investigations have demonstrated that some antibiotics can also interfere with the cytokine production, some inhibiting the production of IL-1 or TNF-α in LPS-stimulated human monocyte cultures ,others inducing hyper production of IL-2 and interferon gamma (IFN-γ)in cultures of lymphocytes^(9,10,11).The mechanism of *St.pneumoniae* resistance to penicillin is by alterations in the targets of penicillin activity ,specifically penicillin-binding proteins, which are also important in manifesting the effect of other β-lactam antimicrobials such as cephalosporin's also by production of β-lactamase ⁽¹²⁾.With the increase in penicillin – resistance in *St. pneumoniae* ,empirical therapy for pneumococcal infections has shifted from penicillin to amoxicillin ,the second and third generation cephalosporin's and the macrolides or azolides ⁽¹³⁾.Consequently we evaluated the effect of clarithromycin a macrolide commonly used for the treatment of respiratory tract infections .

azolides ⁽¹³⁾.Consequently we evaluated the effect of clarithromycin a macrolide commonly

used for the treatment of respiratory tract infections .

Table (2): Effect of one-half the MICof clarithromycin on Phagocytosis and intracellular killing of penicillin-resistant *St.pneumoniae* by human polymorphnuclear granulocytes.

Time	Phagocytosis(%)		Survival index	
	Controls	clarithromycin	Controls	Clarithromycin
30 min	11.2±1.9	16.3 ^b ±1.2	1.48±0.1(41%) ^c	1.31 ^a ±0.15 (59%)
60 min	7.3±1.3	10.9 ^b ±0.6	1.85±0.15(16%)	1.64 ^a ±0.16 (36%)
90 min	5.6±0.2	9.9 ^a ±0.9	>2(0%)	1.65 ^a ±0.15 (34%)
120 min	4.2±0.5	8.7 ^a ±0.2	>2(0%)	1.65 ^a ±0.12 (35%)

a: significantly different (p<0.01 from controls)

b: significantly different (p<0.05 from controls)

c: percentages of the initial bacterial population killed by polymorphnuclear granulocytes in presence of the antibiotic.

On the interaction between human PMNs and isolates of *St.pneumoniae* taking of immunomodulation by antibiotics. Our results suggest that clarithromycin has some interesting immunomodifying properties: it was able to influence the host –bacteria interaction resulting in a considerable enhancement of human PMN functions against all the strains of *St.pneumoniae* tested. The mechanism by which clarithromycin synergizes with PMNs for Phagocytosis and killing of *St.pneumoniae* is unknown, although direct damage to the bacterium by the drug might, at least impart ,be responsible (table 1).Pre exposure of *St.pneumoniae* to one-half the MIC of clarithromycin caused streptococci to be more efficiently Phagocytosis and killed ,compared with the untreated streptococci .Clarithromycin interferes with bacterial protein synthesis: possibly ,one-half the MIC of the drug alters superficial components and affects products .Consequently ,it may in part reduce the virulence of *St.pneumoniae* on the other hand ,pre exposure of human PMNs to one-half the MIC of clarithromycin had no effect on the phagocytic capacity and

intracellular killing of human granulocytes ,thus ruling out a direct action by clarithromycin on the phagocyte itself .Although clarithromycin was found to be highly concentrated in phagocytic cell⁽¹⁴⁾.From our results it emerged that the macrolide, once accumulated did not affect the intracellular killing of *St.pneumoniae* .These data could be explained by the lower activity of clarithromycin in the acid medium ,clarithromycin ,like other macrolides is a weak base that probably accumulates within acid lysosomes where its activity may be reduce.

Clarithromycin was able to synergize with human PMNs for Phagocytosis of the penicillin – resistant strains *St.pneumoniae* ,resulting in an increase number of engulfed streptococci compared with that for the control (table 2).In the drug –free controls , the number of intracellular penicillin-resistant *St.pneumoniae* progressively increased ,indicating the inability of PMNs to stop bacterial growth .Indeed ,in the clarithromycin –containing cultures the number of intracellular penicillin –resistant *St.pneumoniae* that survived fell to 35% of the initial population (p<0.01;table 2).

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