ORIGINAL RESEARCH

EFFECT OF CLARITHROMYCIN ON THE CELL PROFILE OF BRONCHOALVEOLAR LAVAGE FLUID IN MICE WITH NEUTROPHIL-PREDOMINANT LUNG DISEASE

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PINTO LA et al. Effect of clarithromycin on the cell profile of bronchoalveolar lavage fluid in mice with neutrophil-predominant lung disease. **Rev. Hosp. Clín. Fac. Med. S. Paulo 59**(3):99-103, 2004.

OBJECTIVE: Macrolide antibiotics have anti-inflammatory properties in lung diseases. The aim of this study was to investigate the effect of clarithromycin in pulmonary cellular inflammatory response in mice.

METHOD: Eight adult Swiss mice were studied. All animals received an intranasal challenge (80 μL) with dead *Pseudomonas aeruginosa* (1.0 x 10^{12} CFU/mL). Bronchoalveolar lavage was performed 2 days later, with total cell count and differential cell analysis. The study group (n = 4) received clarithromycin treatment (50 mg/kg/day, intraperitoneal) for 5 days. Treatment was initiated 2 days before intranasal challenge.

RESULTS: There was no significant difference in total cell count between the groups (mean: 2.0 x 10^6 and 1.3 x 10^6, respectively). In both groups, there was a predominance of neutrophils. However, the study group had a higher percentage of lymphocytes in the bronchoalveolar lavage than the control group (median of 19% vs 2.5%, P = .029).

CONCLUSION: Clarithromycin alters the cytological pattern of bronchoalveolar lavage of Swiss mice with neutrophil pulmonary inflammation, significantly increasing the percentage of lymphocytes.

KEY WORDS: **Clarithromycin. Lung inflammation. Lung. Mice. Neutrophil.**

Macrolides have been used for the treatment of different bacterial infections. They are frequently prescribed in clinical practice, particularly for respiratory infections. Erythromycin, clarithromycin, and azithromycin are the most common macrolides used, and they are recognized as the first line treatment for infections caused by *Mycoplasma sp., Chlamydia sp., Ureaplasma sp*., and other bacteria.[1]

Recently, studies have been emerging demonstrating an anti-inflammatory effect of these drugs in lung diseases.[2] Clinical trials have shown a benefit of macrolide therapy in some pulmonary disorders, such as diffuse panbronchiolitis (DPB), asthma, and cystic fibrosis.[6-8]

Macrolides may inhibit neutrophil recruitment and interleukin (IL)-8 production.[3-5] Other studies have shown that macrolides may inhibit corticosteroid metabolism and may increase the treatment effect in asthmatic patients.[7,9] However, the anti-inflammatory mechanisms of macrolides in pulmonary diseases are not fully understood.

Little data has been published about the effect of macrolides on cellular inflammation in neutrophil-predominant lung disease in animal models. One previous study using mice with chronic infection by *Pseudomonas aeruginosa* (*P. aeruginosa*) and treated with clarithromycin has demonstrated a reduction on lymphocyte cell counts in bronchoalveolar lavage (BAL).[10] This study, however, used live *P.*

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aeruginosa for inducing lung disease. On the other hand, Sugiyama et al. found no significant changes in differential cell counts of *in vitro* lipopolysaccharide (LPS)-stimulated BAL in rats treated with erythromycin.[11]

The aim of this study is to analyze the effect of clarithromycin on the pattern of pulmonary cellular response in BAL of mice with neutrophil-predominant lung disease that is induced using dead *P. aeruginosa*.

METHOD

Animals

Eight adult (6 to 8 weeks old) male Swiss mice from our University were used. Animals were provided by the University and were kept at Institute during the study period.

Protocol for induction of neutrophil pulmonary disease

The Microbiology Department provided the *P. aeruginosa* sample in a culture plate. After being scraped from the plate, the sample was diluted in phosphate-buffered saline (PBS), to a concentration of 1 x 10^{12} CFU/mL. All *P. aeruginosa* were frozen to –20°C.

While under sedation, the animals from both the study group (n = 4) and control group (n = 4) received 1 intranasal challenge (80 mL) with *P. aeruginosa* solution.[14,15] Sedation, performed to allow pulmonary aspiration of the instilled solution by suppressing upper airway reflexes, consisted of intraperitoneal administration of 0.1 mL of a solution of ketamine (0.4 mL), xylazine (0.1 mL), and normal saline (0.5 mL).

There was no control group without *P. aeruginosa* in this study. The authors of this study have previously shown a significant induction of neutrophil-predominant lung disease in mice using this protocol.[13]

Clarithromycin treatment

A dose of 15 mg/kg/day is used for clinical treatment of infections in humans. Based on previous study protocols[14,15] in experimental models, we used a dosage of 50 mg/kg/day. The treatment was initiated 2 days before the intranasal challenge with *P. aeruginosa*.

The study group received clarithromycin treatment over a 5-day period, with a single daily intraperitoneal (i.p.) dose. The protocol used in the study is illustrated in figure 1.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed 2 days after intranasal challenge. Mice were anesthetized with an i.p. injection of 0.2 mL of the same ketamine and xylazine solution that was used at the intranasal challenge. After anesthesia, a tracheotomy was performed, with trachea cannulation and tube fixation. One mL of normal saline was instilled. After 5 seconds, the material was aspirated. This procedure was repeated 3 times with the same solution.

Total cell count and differential cytological test

The BAL was weighed and centrifuged (2,000 rpm for 2 minutes). The precipitate was diluted in 1 mL of PBS. Determination of TCC and cell viability was performed in a Neubauer chamber (Boeco, Germany), with trypan blue staining.

For differential cytology, 40mL of the sample was cytocentrifuged (FANEM, São Paulo, Mod. 218), at 500 rpm, for 5 minutes. Slides were fixed with methyl alcohol and stained with May-Grunwald-Giemsa stain. Cells were analyzed according to their morphology. The cell types at light microscopy were expressed as a percentage after counting 200 cells.

Statistical analysis

Values are described as mean and median, and the statistical difference was calculated using the Mann-Whitney or *t* test, depending on the sample distribution. Differences were considered significant when $P < .05$.

Ethics

The study was approved by the Ethics Committee for Animal Research

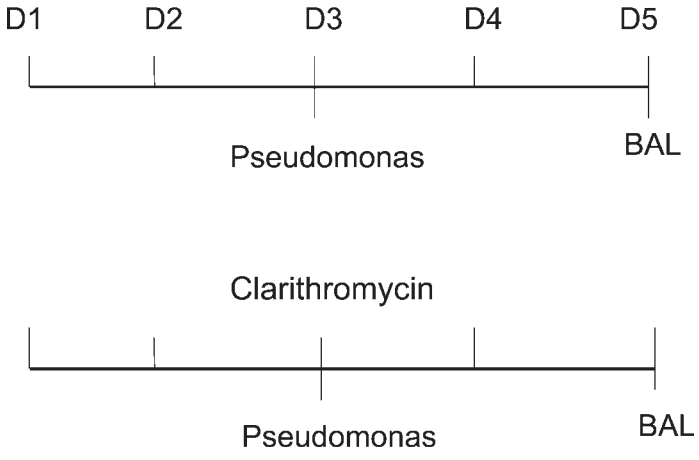

Figure 1 - Study protocol.

of the Institution and was based on animal models research guidelines.

RESULTS

The BAL was performed successfully in all studied mice. The mean of returned volume of BAL was 66%. Mice did not present adverse reactions associated with clarithromycin treatment.

The mean TCC in all mice studied was 1.65 x 10^6 cells/mL. The mean TCC of the study and the control group were 2.01 x 10^6 cells/mL and 1.3 x 10^6 cells/mL, respectively. There was no significant difference between the groups.

All mice had increased neutrophil counts in the BAL. The differential cell count results are presented in table 1. The study group had a significantly higher percentage of lymphocytes in the BAL compared with the control group, with medians of 19% and 2.5% (P = .029), respectively (Fig. 2). Neutrophil counts were not different between the groups studied (P = .097) (Fig. 3).

DISCUSSION

In the present study, the BAL of mice having a neutrophilic pulmonary disorder that was induced by dead *P. aeruginosa* and treated with clarithromycin had a significant increase in the percentage of lymphocytes compared with control group.

We used a small number of mice. Since the groups are homogeneous and the bias factors are smaller compared with human studies, the practice of using small numbers of animals is usually sufficient.

The pulmonary inflammatory abnormalities in the BAL of mice following aspiration of *P. aeruginosa* have been previously described. Pinto et

Table 1 - Comparison of differential cell counts in the bronchoalveolar lavage (BAL) between the groups studied.

	All		Control group		Macrolide group	
	x 10^6 cells/mL	%	x 10^6 cells/mL	%	x 10^6 cells/mL	%
Total cell count	1.30	100	1.21	100	1.29	100
Neutrophils	1.00	74.6	1.00	81.9	0.87	67.2
Lymphocytes	0.10	8.30	0.03	2.90	0.29	22.5
Macrophages	0.20	12.8	0.18	15.2	0.13	10.2

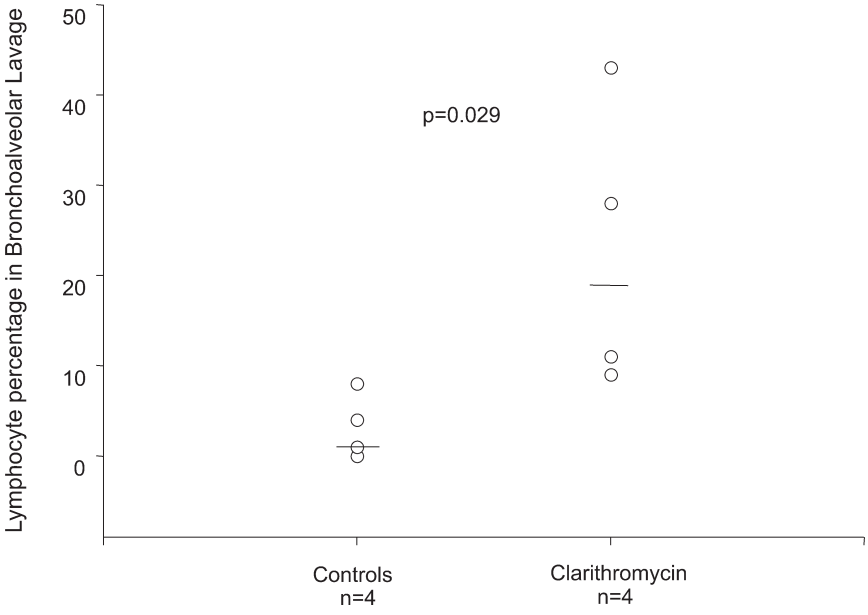

Figure 2 - Comparison of lymphocyte percentage in the bronchoalveolar lavage (BAL)between the groups studied.

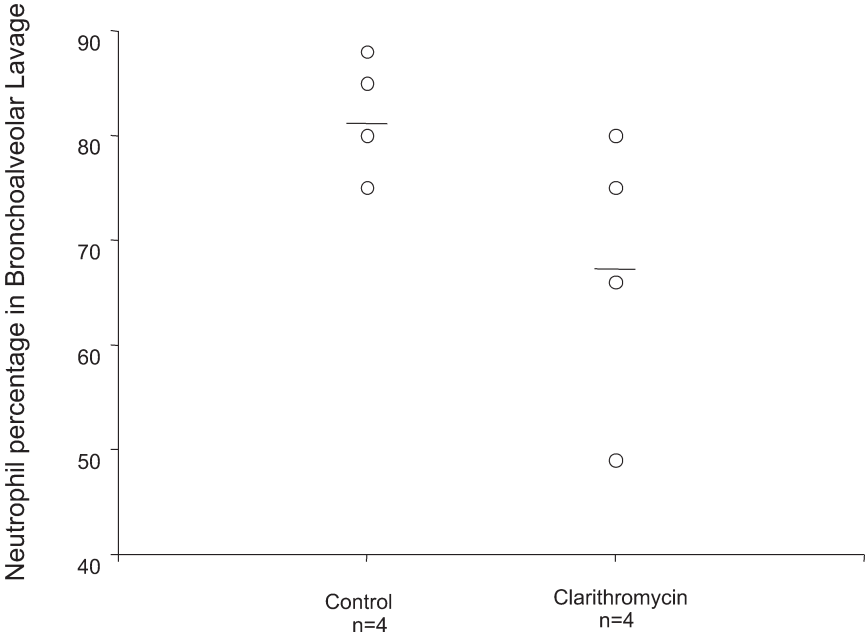

Figure 3 - Comparison of neutrophil percentage in the bronchoalveolar lavage (BAL) between the groups studied.

al.[13] showed that mice with pulmonary inflammation induced by dead *P. aeruginosa* had an increase in TCC and in the percentage of neutrophils in the BAL. Thus, this experimental model may be used for studies analyzing the macrolide anti-inflammatory effect in neutrophilic pulmonary diseases.

There are a few mechanisms that may explain the anti-inflammatory effect of macrolides. Some studies have shown a decreased neutrophil recruitment to the lungs following treatment with erythromycin. Kadota et al. demonstrated this effect in mice that received an intratracheal injection of lipopolysaccharide.[3] Furthermore, a decrease in neutrophil count, IL-8, and IL-1 was observed in the BAL of patients with DPB, who received treatment with erythromycin or roxythromycin.[5-12] Therefore, these drugs may inhibit neutrophil recruitment.[4]

Yanagihara et al. showed that mice chronically infected by *P. aeruginosa*, which have similar pathologic changes to DPB, had a progressive reduction of the lymphocyte count in the BAL when treated with clarithromycin.[10] However, in 1 previous study analyzing cellular response in BAL following lipopolysaccaride challenge in rats treated with erythromycin, no difference in differential cell percentage was detected.[11] The present study has shown an increased lymphocyte percentage in the BAL of mice with neutrophilic pulmonary disease and treated with clarithromycin. Therefore, our results demonstrate that macrolides alter the differential cell count in the BAL in this illness. However, our results should not be considered particularly an anti-inflammatory effect of macrolides and should be interpreted with caution. In spite of this limitation, we speculate that macrolide treatment could both stimulate an earlier onset of an adaptive cellular response or inhibit an acute neutrophilic response (with a consequent increase in lymphocyte percentage), resulting in a quicker resolution of the airway inflammation. On the other hand, the increased number of lymphocytes in the airways of the treated group could be interpreted as a marker of increased pulmonary inflammatory response.

Studies with a larger number of animals may reveal more significant alterations both in lymphocytic and neutrophilic response. Moreover, the bacterial concentration used for intranasal challenge was very high ($1x10^{12}$ CFU/mL). Studies with lower concentrations of *P. aeruginosa* in the intranasal challenge may show greater effects of macrolides on the BAL.

Therefore, additional studies using larger numbers of mice, lower concentrations of *P. Aeruginosa,* and including the measurement of some interleukin levels (IL-8, IL-10, or alpha-tumor necrosis factor) in the BAL are essential for a better understanding of the anti-inflammatory mechanisms of these medications. Furthermore, the present experimental model using dead intranasal *P. aeruginosa* for inducing neutrophil lung inflammation have been shown to be low-cost and effective and consequently should be used for the development of further studies with macrolides.

In conclusion, our study demonstrates that macrolide treatment affects lymphocyte response in the BAL of mice with neutrophilic lung disease. Further studies are required to better investigate the mechanisms of the anti-inflammatory action of macrolides in neutrophilic pulmonary diseases, which will be useful in treatment of many prevalent diseases with high morbidity.

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RESUMO

PINTO AL e col. Efeito da claritromicina na celularidade do lavado broncoalveolar em camundongos com doença pulmonar neutrofílica induzida. **Rev. Hosp. Clin. Fac. Med. S. Paulo 59**(3):99-103, 2004.

OBJETIVO: Os antibióticos macrolídeos podem apresentar um efeito antiinflamatório em doenças pulmonares. O objetivo deste estudo é investigar o efeito da claritromicina na resposta inflamatória celular pulmonar em camundongos Swiss. **MÉTODO:** Foram utilizados 8 camundongos Swiss adultos (6-8 semanas). Todos os animais receberam um desafio intranasal (80 µL) com *Pseudomonas aeruginosa* mortas (1×10^{12} UFC/mL). Dois dias após o desafio, foi realizado lavado broncoalveolar (LBA) com contagem total de células (CTC) e exame citológico diferencial. O grupo em estudo (n=4) recebeu tratamento com claritromicina (50mg/kg/dia, intraperitoneal) por 5 dias, sendo iniciado o tratamento 2 dias antes do desafio intranasal. O grupo controle (n=4) não recebeu tratamento com claritromicina. **RESULTADOS:** Não houve diferença significativa na CTC entre os grupos (média de $2x10^6$ e $1,3x10^6$, respectivamente). Em ambos os grupos, houve predomínio absoluto de neutrófilos. Contudo, o grupo tratado com claritromicina, apresentou um número percentual significativamente

maior de linfócitos no LBA (mediana de 2,5% vs 19%, p=0,029). **CONCLUSÃO:** O uso de claritromicina altera o exame citológico diferencial do lavado bronco-alveolar de camundongos Swiss com inflamação pulmonar neutrofílica, aumentando significativamente o número percentual de linfócitos.

UNITERMOS: **Claritromicina, Inflamação. Doenças pulmonares. Modelos animais. Neutrófilo.**

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