Combined effect of monoclonal antilipopolysaccharide antibody and ceftazidime in intranasal mice model of Klebsiella pneumoniae infection

Vasiljev Marchesi, Vanja; Frančišković, Ivica; Bastiančić, Luka; Rukavina, Tomislav

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Combined effect of monoclonal antilipopolysaccharide antibody and ceftazidime in intranasal mice model of *Klebsiella pneumoniae* infection

VANJA VASILJEV MARCHESI[1]
IVICA FRANCIŠKOVIĆ[2]
LUKA BASTIANČIĆ[2]
TOMISLAV RUKAVINA[1,3]

[1] Department of Social Medicine and Epidemiology
School of Medicine
University of Rijeka
Brace Branchetta 20
HR-51000 Rijeka, Croatia

[2] Clinical Hospital Center Rijeka
Krešimirova 42
HR-51000 Rijeka, Croatia

[3] Department of Microbiology
Teaching Institute of Public Health
Kresimirova 52
HR-51000 Rijeka, Croatia

Correspondence:
Vanja Vasiljev Marchesi
Department of Social Medicine and Epidemiology
School of Medicine
University of Rijeka
Braće Branchetta 20
HR-51000 Rijeka, Croatia
E-mail: vvanja@medri.hr

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Abstract

Background and Purpose: *The evolution of microorganisms in the sense of antimicrobial resistance is an emerging problem, especially in hospital settings. It seriously complicates the ability to combat severe infections. Bacteria of the genus Klebsiella are well known bacterial pathogens that cause a wide variety of infections. Numerous recent articles have reported the growing trend of antimicrobial resistance among clinical isolates of Klebsiella. Besides capsular antigen the most important virulence factor of genus Klebsiella is lipopolysaccharide (LPS). Therefore, previously generated antilipopolysaccharide monoclonal antibody for O1 antigen of Klebsiella pneumoniae was used in this study. The purpose of this study was to examine the existence of synergy between antilipopolysaccharide monoclonal antibody Ru-O1 (mAb Ru-O1) and ceftazidime in a model of lung Klebsiella infection.*

Materials and Methods: *The study was conducted using Klebsiella pneumoniae strain Caroli (O1:K2) to inoculate BALB/c mice by intranasal route. Mice were lethally infected with the bacteria. The effects of mAb Ru-O1 and ceftazidime, as a single treatment protocol or in combination, were monitored on the survival of experimental animals.*

Results: *The overall survival rates in groups pretreated only with mAb Ru-O1 prior to infection or treated only with ceftazidime 24 hours after infection were 33%. The outcome of infection was best in a group of mice that received both mAb and single dose of ceftazidime with overall survival rate of 66%.*

Conclusions: *The study shows that the combination treatment with anti-LPS mAb Ru-O1 and ceftazidime in a mice model of lethal Klebsiella pneumoniae pneumonia exerts synergistic effect and enhances the survival of experimental animals compared to animals treated with mAb Ru-O1 or ceftazidime alone.*

INTRODUCTION

Antimicrobial resistance is an ever increasing problem in everyday medical practice. This issue is especially emphasized in hospital settings where it may represent significant problem regarding the selection of efficient antimicrobial therapy for the treatment of severe and sometimes life threatening infections such as bacterial pneumonia and sepsis. Literature data suggest that improper and exaggerated administration of antimicrobials contributes to the development of bacterial re-

sistance *(1, 2)*. On the other hand, considerable amount of data suggest that health care environments with limited and more rational approach to antibiotic therapy are characterized with lower rates of antibiotic resistant bacteria. Therefore, the limitation in antibiotic consumption, according to some recommendations, may be beneficial for slowing down antibiotic resistance development (*3, 4*).

Pneumonia caused with *Klebsiella pneumoniae* (*K. pneumoniae*) is a common infection in hospitalized patients. The clinical course of infection is often rapidly progressive and may be complicated with the development of sepsis and septic shock. These infections, even with administration of proper antimicrobial agent, sometimes do not solve efficiently and are associated with high mortality rates *(5, 6)*. Situation is nowadays even more serious because of the emergence of multidrug-resistant *Klebsiella* strains. In many patients the delay in resolution of infection, especially in immunocompromised patients, results in even higher mortality.

The monoclonal antibody Ru-O1 (mAb Ru-O1) specific for O1 lipopolysaccharide (LPS) antigen of *K. pneumoniae* was previously described *(7)*. The antibody was shown to be protective in a model of experimental lethal *Klebsiella* sepsis, resulting in considerable survival of animals that were protected prior to lethal intraperitoneal infection.

Some literature data suggest that the combination of antibiotics with immunological preparations may contribute to the better resolution and increased survival of patients with severe bacterial infections *(8, 9)*.

This study was performed to test the efficacy of mAb Ru-O1 in a mouse model of lethal lung *Klebsiella* infection. Besides that, the goal of this study was to examine whether the effect of mAb, that was expected, may be enhanced by administration of a single dose of antibiotic.

MATERIALS AND METHODS

Mice

BALB/c male mice were used in this study which were obtained from the breeding colony of the Medical Faculty, University of Rijeka. Eight to ten weeks old specific pathogen free mice were kept in plastic cages and given standard laboratory food and water ad libitum. The experiments were conducted according to the laws and principles found in the International Guiding Principles of Biomedical Research Involving Animals by the Council of International Organisations of Medical Science *(10)* and the corresponding legislation of the Republic of Croatia.

Bacteria

Experimental infections were preformed using a highly virulent strain *K. pneumoniae* Caroli (O1:K2) that has been used and described previously *(7, 11, 12)*.

Bacteria were grown on blood agar plates for 18 hours harvested and suspended in sterile saline solution. The bacterial number was determined by spectrophotometric analysis using measurement in the absorbance mode at wavelength of 600 nm, which was confirmed by colony counts on blood agar plates after serial 10-fold dilution. The original bacterial suspension was adjusted spectrophotometrically to a final concentration of $5x10^7$ CFU/mL. Bacterial inoculum was prepared from the original suspension by the addition of the appropriate volume of sterile saline and adjusted to contain 250 CFU of *K. pneumoniae* Caroli in 25 μL.

Monoclonal antibody Ru-O1

Experiments were performed by using mAb Ru-O1 that was described previously (7). The antibody has shown to be highly specific for the O1 antigen of *Klebsiella* and to be protective in a model of lethal *Klebsiella* systemic infection. The antibody was given in a dose of 1 mg per mouse and was administered four hours prior to infection by intraperitoneal injection.

Antibiotic

For the treatment of experimental pneumonia, the third generation cephalosporin ceftazidime (Mirocef, Pliva, Zagreb, Croatia) was used, in a single dose of 80 mg/kg according to standard therapeutic regimens. The antibiotic was administered by intraperitoneal injection.

Survival study – Determination of Ru-O1 mAb efficiency alone or in combination with ceftazidime

Before the proposed experiments, the 50% lethal dose (LD_{50}) for the intranasal route of lung infection with *K. pneumoniae* Caroli, was determined using the Reed and Muench method *(13)*. The animals were infected by intranasal route of infection with 25 μL of bacterial suspension. For inoculation, mice were briefly anesthetized with ether. The bacterial inoculum was applied to the nose tip of mouse with a pipette tip and involuntarily inhaled by animals. Mice were held vertically for 2 minutes after the inoculation. The animals were observed for 14 days, and mortality was recorded daily. The LD_{50} of *K. pneumoniae* Caroli for the intranasal route of infection was determined to be 50 CFU of bacteria per mouse (data not shown).

To determine the efficiency of Ru-O1 mAb alone or in combination with ceftazidime, a series of experiments were conducted. The total number of 48 animals were divided in 4 groups. Each group contained 12 animals.

The first group was designated as untreated, which means that animals received neither antibody nor antibiotic. Four hours prior to infection those animals received sterile saline solution intraperitoneally.

The pretreated group received antibody Ru-O1 by intraperitoneal injection four hours prior to infection.

The third group (antibiotic) received intraperitoneally single therapeutic dose (80 mg/kg) of ceftazidime 24 hours after the infection.

The last, fourth group (combination), was pretreated with antibody four hours before the infection and was treated with a single dose of ceftazidime 24 hours post infection.

Bacterial counts in organs

To confirm the effectiveness of intranasal route of infection and the establishment of lung infection, another experiment was performed in order to determine bacterial counts in different organs. The untreated experimental group of ten animals was infected as described above. Mice were divided in two subgroups of five animals. The first subgroup was sacrificed 24, and the second 48 hours after the infection by inhalation of CO_2 and the blood was immediately obtained by cardiac puncture. Livers, spleens, tracheas and lungs were aseptically removed, dissected and homogenized in 5 ml of sterile saline. Serial ten-fold dilutions of organ homogenates were plated on the surface of blood agar plates, incubated for 24 hours, colonies were counted and bacterial counts in organs were calculated. It should be mentioned that two of five animals in 48 hour subgroup succumbed to infection. Therefore, the results that are presented for that time point represent values obtained from the remaining three mice.

Statistical analysis

Survival of different groups was presented using Kaplan-Meier survival curves. Statistical comparison of survival curves between groups was performed by Logrank test using MedCalc Statistical Software, version 9.2.1.0 (MedCalc Software, Belgium). Differences were considered statistically significant at $p<0.05$.

RESULTS

Survival study

After the infection of animals with 100% lethal dose of *K. pneumoniae* strain Caroli, all mice in untreated group died until the forth day post infection. The development of lethal effect in these animals was very rapid in spite of relatively low infectious dose, suggesting highly virulent properties of the used bacterial strain. The overall survival rates in groups pretreated only with mAb Ru-O1 prior to infection or treated only with ceftazidime 24 hours after infection were 33%. However, there are minor differences between these groups since the animals in the pretreated group died from infection between the second and fourth day while animals in antibiotic group succumbed to infection between the second and sixth day after the infection. The outcome of infection was best in the group of mice that received both mAb and ceftazidime with the overall survival rate of 66%. The statistical comparison of survival curves revealed significant differences of survival between animals from combination vs. untreated group ($p<0.0001$), combination vs. pretreated group ($p=0.0243$), combination vs. antibiotic group ($p=0.0243$), and antibiotic vs. untreated group ($p=0.0079$). The survival was not statistically different between pretreated vs. antibiotic group and between pretreated vs. untreated group of animals (Figure 1).

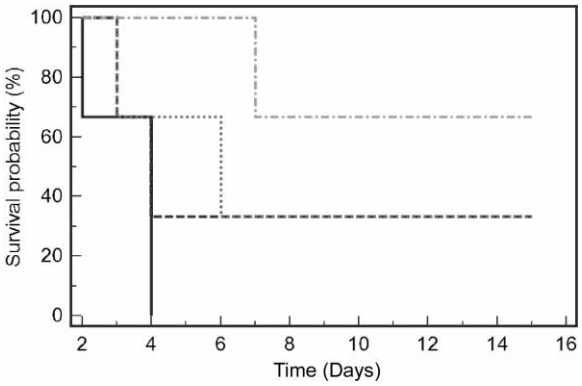

Figure 1. *Survival of untreated group (——), pretreated group with mAb Ru-O1 (- - - -), antibiotic group (·····) and a combination group of animals (- · - · -) lethally infected with K. pneumoniae Caroli.*

Bacterial counts in organs

The aim of this part of study was to confirm the effectiveness of intranasal route of infection and the establishment of lung infection. Therefore, the bacterial counts in lungs and several other organs of infected animals that

TABLE 1

The bacterial counts in organs from untreated animals 24 hours post infection expressed as CFU per 1 mL of organ homogenates.

Organ	Median value	Range
Trachea	8×10^2	$6.9 \times 10^2 – 8.8 \times 10^2$
Lung	4.3×10^3	$1.1 \times 10^3 – 4.9 \times 10^3$
Liver	4.4×10^2	$3.9 \times 10^1 – 4.6 \times 10^2$
Spleen	1.8×10^2	$1.1 \times 10^2 – 2.1 \times 10^2$
Blood	1.1×10^4	$8.4 \times 10^3 – 1.4 \times 10^4$

TABLE 2

The bacterial counts in organs from untreated animals 48 hours post infection expressed as CFU per 1 mL of organ homogenates.

Organ	Median value	Range
Trachea	1.5×10^5	$7.1 \times 10^4 – 1.8 \times 10^5$
Lung	6.5×10^4	$2.1 \times 10^4 – 7 \times 10^4$
Liver	9.7×10^4	$4.1 \times 10^4 – 1.2 \times 10^5$
Spleen	1.7×10^5	$8.2 \times 10^4 – 2.4 \times 10^5$
Blood	8×10^5	$5.5 \times 10^5 – 9.2 \times 10^5$

were treated neither with mAb Ru-O1 nor with ceftazidime were determined. The effectiveness of experimental infection was obvious already 24 hours after the infection, when in all analyzed organs and blood, bacterial counts reached considerably higher values compared with a relatively low number of bacteria that were used for infection, e.g. 250 CFU per mice (Table 1). The results indicate that bacteria started to disseminate very early during the course of infection resulting in high bacterial contents in all analyzed organs. During the following 24 hours further increase in bacterial counts was detected (Table 2). The continuous growth of bacterial count during that period was associated with the beginning of mortality in experimental animals.

DISCUSSION

Although significant advances in the therapy of bacterial infections have been achieved in the last few decades, some of these infections have still remained a significant therapeutic problem, especially in hospitalized patients. Infections like nosocomial pneumonia and sepsis are still associated with high mortality rates *(14, 15)*. Explanations for such undesired outcomes may include rapidly progressive nature of these infections, especially in immunocompromised patients, that often results in the late onset of antimicrobial therapy. Additional important reason may be the emerging bacterial resistance against antimicrobial agents. Moreover, numerous recently published data support the thesis that antimicrobial resistance represents one of the major problems of modern medicine *(16, 17)*.

The problem of antimicrobial resistance attracts attention of researchers worldwide. Literature data suggest the existence of a strong correlation between bacterial resistance properties and antibiotic consumption *(3, 4, 18)*. On the other hand, the amount of newly developed antimicrobial agents that have recently been introduced as therapeutic options for the treatment of bacterial infections is limited. Therefore, alternative strategies for the management of bacterial infections are inevitable. Some of described strategies include changes in antibiotic administration policies, while some other include alternative approaches to their management *(19)*.

Bacteria of the genus *Klebsiella* are well known bacterial pathogens that cause a wide variety of infections. Some of these infections are life threatening, especially in hospital settings. Numerous recent articles have reported the growing trend of antimicrobial resistance among clinical isolates of *Klebsiella (20, 21)*. The emergence of broad spectrum β-lactamases (AmpC) and extended spectrum β-lactamases (ESBL) among clinical isolates of *Klebsiella* has resulted in limiting the number of therapeutic agents available. Therefore, alternative approaches to prevention and/or therapy of *Klebsiella* infections seem to be inevitable. In that regard, an anti-LPS monoclonal antibody Ru-O1 was described previously to be protective in a model of lethal *Klebsiella* sepsis *(7)*. The present research was undertaken in order to reveal whether such beneficial effect can be achieved in another experimental model of lethal lung infection. Therefore, this study was undertaken to establish a model of mice pneumonia caused by intranasal bacterial challenge with *K. pneumoniae* Caroli. Similar models were described earlier with several other bacterial pathogens *(22, 23)*. This first goal of the research was successfully reached and a model of infection with LD_{50} value of only 50 CFU per mice was established. Five times greater bacterial challenge resulted in rapidly evolving infection with 100% lethality and rapid bacterial dissemination from lungs throughout the body within 24 hours after the infection. When animals were pretreated with anti-LPS mAb Ru-O1 four hours prior to infection, 33% of animals survived the infection with a lethal bacterial challenge, suggesting beneficial effect of administered antibody. The explanation of a lower degree of protection compared with the previous results in a model of sepsis where it reaches 70%, may be the fact that during the early stages of pulmonary infection, bacteria multiplied in lung tissue and then suddenly in a large number reached the blood and disseminated through animal organism. The mechanisms of antibody action could not counteract such a large number of bacteria suddenly reaching bloodstream, which resulted in lower survival rate. On the other hand, it may be presumed that the lower survival rate may also be a consequence of irreparable lung tissue damage. In conclusion, pretreatment of animals with anti-LPS mAb was shown, although to a lesser extent to exert the protection in a mouse model of lethal *Klebsiella* lung infection.

The available literature data suggest the synergistic activity of antibiotics and immunological preparations in the treatment of various bacterial infections *(24, 25)*. In that regard, it was decided to test whether previously described beneficial effect of mAb Ru-O1 in experimental model of mice pneumonia caused by a highly virulent strain of *Klebsiella* may be enhanced by administration of a single dose of antibiotic. Therefore, a single therapeutic dose of ceftazidime was applied 24 hours post infection. The treatment of animals that were not pretreated with mAb prior to infection resulted in survival of 33% of mice. On the other hand, when antibiotic was administered to animals that were pretreated with mAb, survival rate rose to 66%. According to these results it can be concluded that the administration of anti-LPS monoclonal antibody in combination with a single dose of antibiotic significantly enhances survival of lethally infected animals with pneumonia.

In conclusion, the results of the present study clearly demonstrated the existence of synergism between antibiotic treatment and the monoclonal antibody used. Such combined treatment of severe infections like pneumonia, which was described in this paper, has two possible beneficial effects. The first one is enhanced survival compared to the treatment with each agent alone. The second possible beneficial effect is, however, indirect. As described before, the administration of a single dose of antibiotic in animals pretreated with mAb resulted in significantly increased survival of infected animals. The-

refore, such combined treatment may influence the needs for antibiotic consumption. As a consequence, this may result in slowing down the negative trend of antibiotic resistance development, but this assumption needs to be supported by more extensive future studies.

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