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Effect of cytostatic agents on the number of alveolar phagocytes and the efficacy of ceftriaxone in an experimental murine lung infection

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ABSTRACT: Mice made monocytopenic and granulocytopenic by cyclophosphamide or monocytopenic by etoposide were infected by exposure to an aerosol containing *Klebsiella pneumoniae*. Eighteen hours later ceftriaxone was administered and three hours after that the experiment was ended. At the time of infection and at 18 and 21 h the numbers of alveolar macrophages and granulocytes in bronchoalveolar lavage (BAL) fluid were significantly lower in the cyclophosphamide-pretreated animals than in the controls. Furthermore, outgrowth of *K. pneumoniae* in the lungs was significantly stronger in cyclophosphamide-pretreated mice and a fourfold higher dose of ceftriaxone was needed to obtain the same antibacterial effect as in the controls.

In the etoposide-pretreated mice the number of alveolar macrophages in BAL was not significantly lower than that in the controls, but the number of granulocytes was. Compared with the controls, there was no significant difference in the number of *K. pneumoniae* in the lungs, and the efficacy of ceftriaxone did not differ either.
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A close inverse relationship has been found between the number of granulocytes in blood and the proliferation of bacteria in an experimental thigh infection model in mice, in which the number of granulocytes was decreased by irradiation or by cytostatic treatment [1–4]. Furthermore, in granulocytopenic mice higher doses of antibiotics were needed to reduce the number of bacteria at the site of infection to the same level as in control animals. However, in the short-term thigh infection that was used, the importance of other phagocytic cells, *i.e.* macrophages, could not be established, because they were not present at the site of infection [5]. In a pulmonary infection model it was expected that macrophages play a crucial role. Alveolar macrophages are present in normal lungs while granulocytes are lacking [6]. Therefore, alveolar macrophages might be an important barrier against the development of bacterial infections in the lung. Furthermore, a lung infection is also a well-established experimental model to investigate the efficacy of antibiotic treatment against microorganisms [7–9].

Most studies regarding the role of phagocytes in lung infections are concerned with the clearance of microorganisms from the site of infection [10–13]. There are suggestions in the literature that macrophages are predominantly responsible for the clearance of Gram-positive organisms and granulocytes for that of Gram-negative organisms [10, 14, 15].

The present study was undertaken to determine the impact of both types of phagocytic cells during the early phase of a lung infection in which the number of bacteria increases. Since both alveolar macrophages and granulocytes are derived from blood leucocytes [16, 17], a reduction of the number of leucocytes by treatment with cytostatic drugs might reduce the number of phagocytes at the site of infection. In the present study etoposide is used because it decreases the number of blood monocytes [4] and cyclophosphamide because it decreases the numbers of both blood monocytes and blood granulocytes [4]. The infection in leucocytopenic mice mimics the situation in immunocompromised patients with pulmonary infections and makes it possible to elucidate the efficacy of antibiotic treatment.

Klebsiella pneumoniae was chosen as the infective agent because this pathogen is commonly encountered in infections in immunocompromised patients [18–20]. Ceftriaxone was chosen because this antibiotic

shows excellent activity against most Gram-negative rods [21–24].

Material and methods

Drugs

Etoposide (VP 16-213, kindly donated by Bristol Myers, Weesp, The Netherlands) was dissolved in a specific vehicle to 20 $mg \cdot ml^{-1}$. The vehicle without etoposide was prepared according to the manufacturer's prescription. Both etoposide and the vehicle were diluted in pyrogen-free phosphate-buffered saline (PBS) (pH 7.5). Cyclophosphamide, (Montedison, Rotterdam, The Netherlands) was dissolved in PBS to final concentrations of 10 and 15 $mg \cdot ml^{-1}$.

Ceftriaxone (disodium salt, 81.5% activity, kindly donated by Hoffmann-LaRoche, Mijdrecht, The Netherlands) was dissolved in PBS to a final concentration of 1 $mg \cdot ml^{-1}$.

Microorganism

A Klebsiella pneumoniae strain (ATCC 43816, capsular serotype 2, kindly donated by Dr I. Bakker-Woudenberg, Dept of Microbiology, Erasmus University, Rotterdam, The Netherlands) was used. This strain is serum resistant. The minimal inhibitory concentration (MIC) of ceftriaxone for this microorganism was 0.032 $mg \cdot l^{-1}$. After overnight incubation at 37°C, the cells were stored in brain-heart infusion broth (Oxoid, Basingstoke, England) at -70°C in a suspension of about 5×10^9 colony forming units (CFU)$\cdot ml^{-1}$. Just before the start of each experiment, one vial of this suspension was rapidly thawed in a waterbath at 37°C.

Animals

Male specific pathogen free Swiss mice weighing 20–30 g (Broekman Institute, Someren, The Netherlands), were used in this study and housed for one week before the experiments were performed. Food and water were given *ad libitum.*

Short-term growth of Klebsiella pneumoniae in vitro

For short-term growth experiments, a 1:4000 dilution of an overnight culture of *Klebsiella pneumoniae* with approximately 10^6 CFU$\cdot ml^{-1}$ was incubated for 60 min in a shaking waterbath at 37°C. Next, 20 ml aliquots of the suspension were brought into 50 ml flasks to which ceftriaxone was added at various concentrations before reincubation for 180 min at 37°C. Samples were taken at 45 min intervals and plated in appropriate dilutions on diagnostic sensitivity test agar (DST, Oxoid, Basingstoke, England). After overnight incubation of the plates at 37°C, the bacteria were counted as colony forming units.

In vivo experiments

Induction of leucocytopenia

Monocytopenia was induced by injecting 16 $mg \cdot kg^{-1}$ etoposide in a volume of 100 µl subcutaneously into the nuchal region on three consecutive days before infection; the control animals received the same volume of the vehicle diluted in PBS. Granulocytopenia and monocytopenia were induced by injecting 150 $mg \cdot kg^{-1}$ and 100 $mg \cdot kg^{-1}$ cyclophosphamide intraperitoneally in a volume of up to 300 µl four and one days, respectively, before infection [25]. Control animals received the same volume of PBS.

The numbers of granulocytes and monocytes in the blood were determined in samples taken from the retro-orbital plexus by puncture with a heparinized 20-µl capillary and diluted with 40 µl saline containing heparin (400 $U \cdot ml^{-1}$) [26].

Isolation of macrophages and granulocytes from the alveoli

The numbers of alveolar macrophages and granulocytes were determined after the mice had been killed by exposure to CO_2. Bronchoalveolar lavage (BAL) was performed after the pulmonary arteries had been flushed with 2 ml 0.6 mM edetic acid (EDTA) (Merck, Darmstadt, FRG) dissolved in PBS to eliminate blood leucocytes [27]. The bronchial tree was lavaged 15 times with 1 ml doses of the same solution of EDTA to collect alveolar phagocytes quantitatively. The BAL fluid was kept on ice and centrifuged at 300×g for 10 min. The pellet was resuspended in 1 ml PBS and the numbers of alveolar macrophages and granulocytes were counted in a Bürker haemocytometer; their viability was established by trypan blue (0.2%) exclusion. Differential counts of up to 600 cells per mouse were performed on cytocentrifuge smears stained with Giemsa stain.

Experimental infection

Mice pretreated with either etoposide or cyclophosphamide and the respective controls were infected by exposure to an aerosol of *Klebsiella pneumoniae* generated by a three-jet Collison nebulizer [28] in a Henderson-type apparatus [29]. The nebulizer contained a suspension of about 3×10^8 bacteria in a volume of 40 ml PBS. The animals were exposed to the spray for 30 min and to clean air for another 30 min. The infection was allowed to develop for 18 h before ceftriaxone was administered subcutaneously in various dosages from 0–0.5 $mg \cdot kg^{-1}$. Three hours later, the animals were killed by exposure to CO_2.

Determination of the numbers of Klebsiella pneumoniae

Numbers of bacteria were determined at 0, 18 and 21 h after infection, the animals having been killed by exposure to CO_2. The thoracic cage was opened and the lungs were detached from the trachea and homogenized in 3 ml PBS with a tissue homogenizer (type X-1020, Ystral GmbH, Dottingen, FRG) at 0°C. Appropriate dilutions of the homogenate were plated on diagnostic sensitivity test (DST) agar (Oxoid) and colonies were counted after overnight incubation at 37°C as colony forming units (CFU).

Statistical analysis

Significance of differences between the cytostatic-treated and control animals with respect to blood cell counts, numbers of alveolar macrophages and granulocytes in BAL fluid, and the log number of bacteria at the time of administration of ceftriaxone were assessed by Student's t-test. The results concerning effects of the cytostatic agents, the dosage of ceftriaxone, and the interaction between the cytostatics and the antibiotic (independent variables) on bacterial outgrowth (dependent variable), were analysed by multiple regression analysis [30].

Results

Short-term growth of Klebsiella pneumoniae in vitro

The effect of ceftriaxone at various concentrations on numbers of *K. pneumoniae in vitro* is shown in figure 1. Ceftriaxone had a bactericidal effect at concentrations of 0.04 $\mu g \cdot ml^{-1}$ or higher. Concentrations higher than 0.05 $\mu g \cdot ml^{-1}$ did not lead to a more rapid decrease of bacterial numbers.

Effect of cytostatic treatment on the numbers of leucocytes in blood and phagocytes in BAL fluid

The effect of cytostatic treatment on the numbers of blood leucocytes and alveolar phagocytes is shown in table 1. In cyclophosphamide-treated animals the number of monocytes in blood at the start of the infection experiment was significantly reduced to 23% of that in the controls ($p<0.01$), whereas the number of granulocytes was reduced to 1% ($p<0.001$). Etoposide reduced the monocyte population to 10% ($p<0.01$), whereas the number of granulocytes was only reduced to 52% ($p<0.05$) of that in the controls.

The number of alveolar macrophages at the time of infection was also lower ($p<0.01$) in the animals treated with cyclophosphamide than in the control mice (table 1), and both numbers did not change much during the infection. In etoposide-treated animals the number of alveolar macrophages at the start of the infection was not significantly lower ($p>0.05$) than that in the control mice. In both groups 18 h later this number had decreased, but in the controls it had increased again markedly 21 h after infection, ending up with the same number as at the start of the infection, whereas this was not the case in the etoposide-treated animals. The difference at 21 h between the etoposide-treated and control animals was significant ($p<0.05$).

All infected animals showed an influx of granulocytes (table 1). In the cytostatic-treated mice at 18 and 21 h there were fewer granulocytes than in the controls, with cyclophosphamide ($p<0.001$) as well as with etoposide ($p<0.01$), but the decrease was more pronounced with cyclophosphamide than with etoposide. The numbers of granulocytes in the saline and the vehicle controls were very similar throughout the experiment.

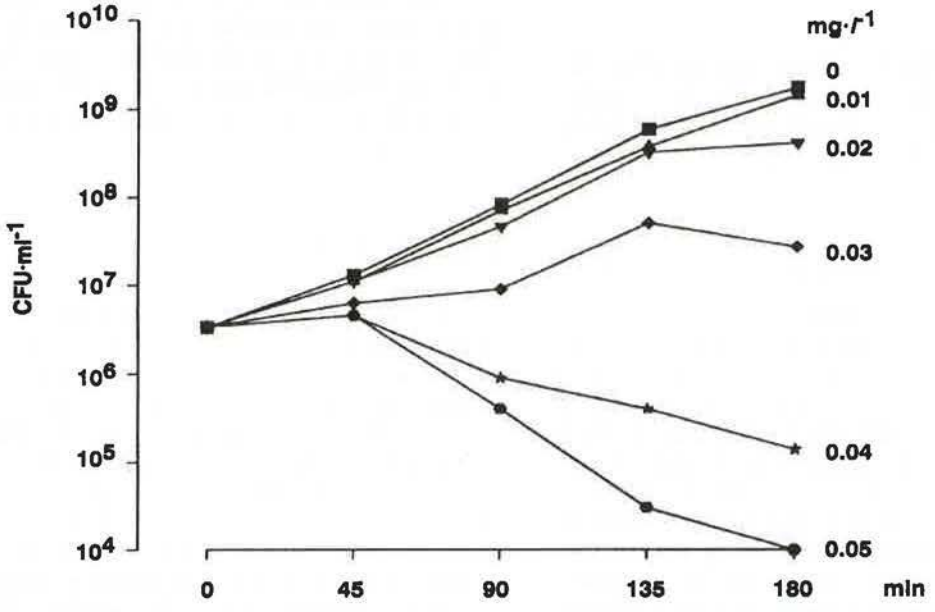

Fig. 1. – Short-term growth curves of *Klebsiella pneumoniae* ATCC 43816 in the presence of various concentrations of ceftriaxone. CFU: colony forming units.

Table 1. – Mean numbers of phagocytic cells at the beginning of and during a *Klebsiella pneumoniae* infection, in relation to pretreatment with cyclophosphamide and etoposide

Treatment	Time	Blood		BAL fluid	
	h	Monocytes n·mm^{-3}	Granulocytes n·mm^{-3}	Macrophages	Granulocytes
Saline*	0	120	1443	1.5×10^6	0
	18			1.0×10^6	1.3×10^5
	21			1.3×10^6	2.1×10^5
Cyclophos-phamide**	0	28	15	0.5×10^6	0
	18			0.7×10^6	2.0×10^4
	21			0.4×10^6	2.0×10^4
Vehicle*	0	125	1558	1.5×10^6	0
	18			1.0×10^6	2.0×10^5
	21			1.6×10^6	1.0×10^5
Etoposide†	0	12	814	1.0×10^6	0
	18			0.8×10^6	5.0×10^4
	21			0.7×10^6	5.0×10^4

Data given are the means of at least six mice. *: saline served as control for cyclophosphamide and vehicle for etoposide; **: 150 mg·kg^{-1} four days before infection and 100 mg·kg^{-1} one day before infection; †: 16 mg·kg^{-1} on three consecutive days before infection; BAL: bronchoalveolar lavage.

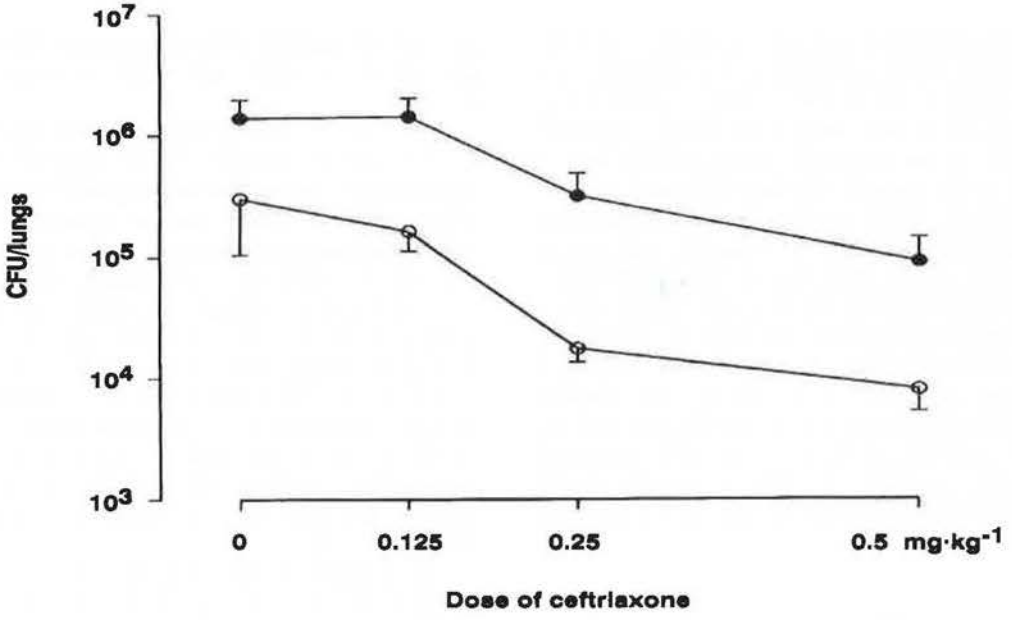

Fig. 2. – Numbers of colony forming units (CFU) of *Klebsiella pneumoniae* in the lungs of cyclophosphamide-treated (closed symbols) and control (open symbols) mice three hours after the administration of ceftriaxone. Each symbol represents the mean and SEM of six mice.

Effect of cytostatic treatment on the numbers of Klebsiella pneumoniae

Immediately after exposure to the aerosol the total number of *Klebsiella pneumoniae* in the lungs was approximately 5×10^3; this number increased in all mice in all treatment groups. At 18 h the cyclophosphamide-treated mice showed stronger outgrowth than the control mice did (1.0×10^6 *versus* 1.5×10^5 CFU, $p<0.001$). Three hours later, without antibiotic treatment, this number had increased to 1.3×10^6 CFU in the cyclophosphamide-treated mice and to 3.2×10^5 CFU in the control animals. When ceftriaxone was given at 18 h, a dose-dependent effect on the numbers of CFU was found in the dose range of 0.125–0.5 mg·kg^{-1} (fig. 2). Within this dose range the number of CFU three hours after antibiotic administration was always higher in the cytostatic-treated mice than in the controls ($p<0.001$), but there was no difference in the slope of the dose-effect curves of these two groups. Multiple regression analysis showed no interaction between the dose of the antibiotic and the cyclophosphamide treatment in inhibiting the outgrowth of *Klebsiella pneumoniae.*

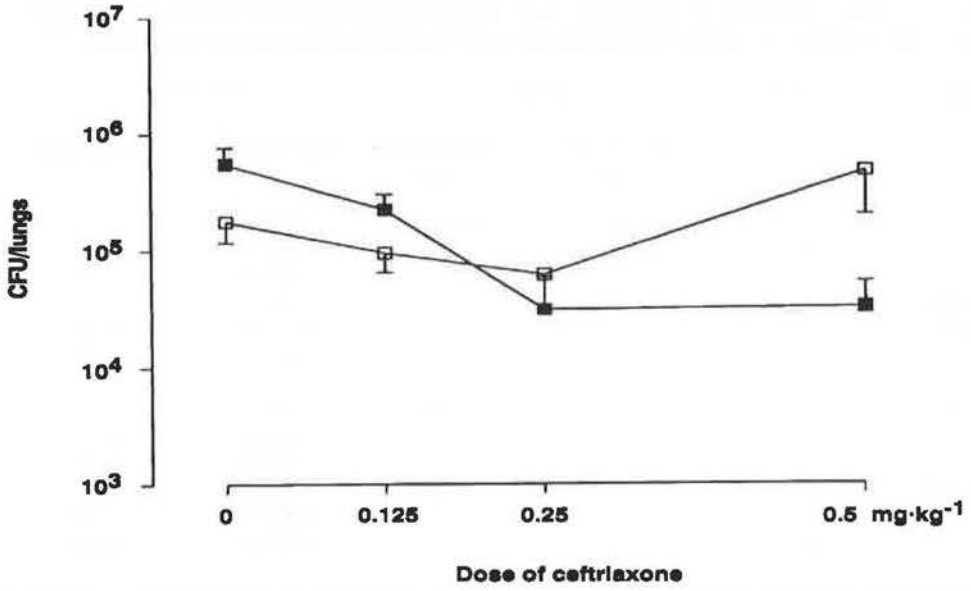

Fig. 3. – Numbers of colony forming units (CFU) of *Klebsiella pneumoniae* in the lungs of etoposide-treated (closed symbols) and control (open symbols) mice three hours after the administration of ceftriaxone. Each symbol represents the mean and SEM of six mice.

In the etoposide-treated animals the number of CFU 18 h after infection was not significantly different from that in the controls (8×10^4 CFU *versus* 1.2×10^5 CFU, $p>0.05$). Three hours later, these numbers had increased to 5.6×10^5 CFU in the etoposide-treated animals and to 1.8×10^5 CFU in the controls, but this difference was not significant ($p>0.05$). When ceftriaxone was administered, a dose-dependent effect up to 0.25 mg·kg^{-1} was seen in both groups (fig. 3) and there was no significant difference between the slopes of the two dose-effect curves. Again, no interaction between the dose of ceftriaxone and the cytostatic treatment in inhibiting the outgrowth of bacteria was observed. At 0.5 mg·kg^{-1} the numbers in the etoposide-treated animals did not decrease further, whereas the controls showed a markedly increased outgrowth, the number of CFU remaining at the same level at even higher doses of ceftriaxone (data not shown).

Discussion

The results of this study show that a reduction of the number of blood monocytes and granulocytes by cytostatic treatment with etoposide or cyclophosphamide leads to a reduction of the number of alveolar phagocytes in the bronchoalveolar lavage (BAL) fluid of uninfected lungs and of lungs infected with *Klebsiella pneumoniae*. In cyclophosphamide-treated mice the decrease in numbers of alveolar granulocytes was more pronounced than in the etoposide-treated animals, and only in the cyclophosphamide-treated animals was the proliferation of bacteria significantly higher than in the control animals. The efficacy of ceftriaxone treatment was also reduced in the cyclophosphamide-treated animals. These results indicate that during the phase of infection in which the number of bacteria increases the granulocytes are the most important cells to slow down this process.

In animals not treated with cytostatic agents we found no substantial increase in the number of alveolar macrophages during the development of the infection. Other authors found similar results in infections with *Pseudomonas aeruginosa* or *Klebsiella pneumoniae* [12, 14, 31, 32]. Several authors found an increase of alveolar macrophages in *Staphylococcus aureus* infections [6, 11, 14]. Possible explanations for the divergent results may be the difference in the route of inoculation, the composition of the bacterial cell wall between Gram-positive and Gram-negative bacteria or in the mouse strain used. In contrast to the lack of macrophage response the increase in the number of alveolar granulocytes was large, as found by others [6, 11, 14, 33].

The reduction in the numbers of both alveolar macrophages and alveolar granulocytes by cyclophosphamide has been reported previously [13, 15, 34], but the effect of etoposide was not investigated before. The reduction in numbers of alveolar macrophages by both etoposide and cyclophosphamide is probably caused by the decrease in numbers of blood leucocytes. The small effect on the number of alveolar macrophages, although the number of blood monocytes was markedly reduced, may be explained by the short period that the infection was studied in this model and the relatively long turnover time of the alveolar macrophages [17]. Granulocytes enter the site of infection much earlier than monocytes [35], and have a relatively fast turnover [16]. During granulocytopenia the number of granulocytes will be markedly reduced and, thus, will affect the local antibacterial defence more profoundly than during monocytopenia. The larger decrease in the number of alveolar

granulocytes by cyclophosphamide, compared with etoposide, is consistent with the profound granulocytopenia found after administration of the drug.

Ceftriaxone was highly effective for the treatment of this experimental lung infection; it reached its maximal effect already at the relatively low dose of about 0.5 $mg \cdot kg^{-1}$. At this dose plasma concentrations were even below the level of detection. Therefore, the response *in vivo* reflects the high antibacterial efficacy in short-term growth curves *in vitro,* as illustrated by the maximal effect of 0.05 $mg \cdot l^{-1}$. Because of this sensitivity of *Klebsiella pneumoniae* a direct bactericidal activity is likely, the more so, since the lungs are highly vascularized and, therefore, the tissue concentration will follow the plasma concentration directly. Indeed after administration of 1 $mg \cdot kg^{-1}$ ceftriaxone we detected concentrations of 0.6 $mg \cdot l^{-1}$ in exsanguinated lung tissue homogenate (unpublished results). Therefore, it is very likely that the difference in bacterial numbers between animals with and without antibiotic treatment is explained by the presence of the drug at the site of infection.

The effect of cyclophosphamide treatment on the efficacy of ceftriaxone *in vivo* can be expressed quantitatively by calculating the increase in the dose of the antibiotic required to obtain the same final number of bacteria in the cyclophosphamide-treated and the control mice. In the present study this amounted to an approximately fourfold increase of the dose of ceftriaxone. With respect to antibiotic efficacy, the vehicle-treated mice showed an unusual pattern of bacterial growth: up to a dose of 0.25 $mg \cdot kg^{-1}$, the number of bacteria decreased with the dose of ceftriaxone, but at 0.5 $mg \cdot kg^{-1}$ there was an increase relative to the bacterial numbers at 0.25 $mg \cdot kg^{-1}$. This phenomenon was reproducible, at higher doses as well, but we cannot offer any explanation for it.

With respect to the clinical situation, the present results indicate that treatment with ceftriaxone for a pulmonary infection with *Klebsiella pneumoniae* might be less succesful if the patient is both granulocytopenic and monocytopenic, because it can be expected that the number of phagocytes in the inflammatory exudate will be low.

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Effets des agents cytostatiques sur le nombre de phagocytes alvéolaires et l'efficacité du ceftriaxone au cours d'une infection pulmonaire expérimentale chez la souris. W. Calame, et H. Mattie.

RÉSUMÉ: Des souris rendues monocytopéniques et granulocytopéniques par le cyclophosphamide ou monocytopéniques par l'étoposide ont été infectées par exposition à un aérosol contenant *Klebsiella pneumoniae.* Dix huit heures plus tard, la ceftriaxone a été administrée et 3 heures après l'expérimentation a été clôturée. Au moment de l'infection et aux heures 18 et 21, les nombres de macrophages alvéolaires et de granulocytes du liquide de lavage broncho-alvéolaire (BAL), s'avère significativement plus bas chez les animaux pré-traités au cyclophosphamide que chez les contrôles. De plus, la culture de *Klebsiella pneumoniae* dans les poumons est significativement plus abondante chez les souris prétraitées au cyclophosphamide et des doses quatre fois supérieures de ceftriaxone sont nécessaires pour y obtenir le même effet antibactérien que chez les souris-contrôle. Chez les souris prétraitées à l'étoposide, le nombre de macrophages alvéolaires du BAL n'est pas significativement plus bas que chez les contrôles, alors que le nombre de granulocytes l'est. Par comparaison avec les contrôles, il n'y a pas de différence significative dans le nombre de *Klebsiella pneumoniae* présents dans les poumons et l'efficacité du ceftriaxone n'est pas différente non plus.
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