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Effect of Cefepime (BMY-28142) on the Hemolytic and Bactericidal Activity of Serum *in vivo* and *in vitro*

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The present study was undertaken to determine whether cefepime (BMY-28142) could influence the hemolytic and bactericidal activity of serum both *in vivo* and *in vitro*. For the *in vivo* studies, mice (Swiss aged 15 ± 2 weeks) were studied before and 1, 2, 4, 8, and 12 h after an intramuscular injection of 200 mg/kg of cefepime. Likewise, another group of mice were injected for 7 days with a daily injection of the same dose as before, and sacrificed one hour after the last injection. For the *in vitro* studies, samples were incubated in the presence and absence of cefepime (1/2, 1/4 and 1/8 MIC against *Staphylococcus aureus*) (2.5, 1.25 and 0.625 mg/l respectively). The results indicate that cefepime at sublethal concentrations increases the bactericidal activity of serum both *in vivo* and *in vitro* against *S. aureus*, but does not significantly modify the hemolytic activity of the complement.

Key words: Cefepime, Complement system, Hemolytic activity, Bactericidal capacity.

Cefepime (BMY-28142), 7-[α-(2-Aminothiazol-4-yl)-α-(Z)-methoxymino-acetamido]-3-(1-methyl-pyrrolidinio)-methyl-3-cephem-4 -carboxylate, is a new extended-spectrum cephalosporin for parenteral use. The compound is structurally similar to cefotaxime but has a 1-methyl-pyrrolidine instead of the acetoxy group at position 3 of the dihydrothiazine ring. The third-generation cephalosporins combine a high ß-lactamase stability with a broad antibacterial spectrum and potent activities against gram-negative bacteria (18). Cefepime in particular has a broad spectrum of activity against gram-negative and gram-positive bacteria (11), has poor affinities for ß-lactamases and high resistance to enzymatic hydrolysis (8, 15). It is

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active *in vitro* against many gram-positive and gram-negative bacteria which are responsible for serious infections (2, 5), and has shown significantly greater bactericidal activity in serum and blister fluid against *Escherichia cloacae* than other drugs. Preliminary studies have demonstrated that cefepime presents excellent activity against Enterobacteriaceae, *Pseudomonas aeruginosa*, staphylococci, and streptococcal species other than enterococci (3, 12), and is now regarded as a promising antimicrobial agent for the treatment of serious infections (10).

Failure of the immune response is often the background for the establishment of infections. This is true not only for patients who are immuno-suppressed, but also for normal subjects in whom the immune response fails to prevent infection due to factors such as bacterial inoculum, virulence and toxic properties of microorganisms, local condictions at the focus of infection, etc. The main bacterial serum killing mechanism other than opsonization/phagocytosis by phagocytic cells such as macrophages or polymorphonuclear neutrophil leukocytes (PMNL) is complement-mediated lysis (9). The frequency of reports describing enhanced complement-mediated bacterial lysis in bacteria grown with sublethal concentrations of antibiotics has increased over the past several years (14, 16, 17). Although these reports differ in methodology, a consensus is emerging that certain ß-lactam-type antibiotics can increase the susceptibility of some bacterial strains to complement-mediated lysis (4).

Hence, the purpose of this study was to compare the hemolytic activity of complement and the bactericidal activity of serum obtained from mice treated *in vivo* with cefepime, as well as of serum *in vitro* incubated with different doses of this antibiotic.

Materials and Methods

Antibiotics.– Cefepime was obtained from Bristol-Myers Research Institute (Madrid, Spain). It was diluted in Isogever medium for the in vitro studies of the hemolytic activity, in Mueller-Hinton Broth (MHB) (Difco) for the *in vitro* study of bactericidal activity, and in saline solution for both *in vivo* studies.

Cefepime was given to mice in a dose of 200 mg/kg, about seven times higher on a weight basis than recommended for man (1). For the *in vitro* study, serum was incubated with 1/2, 1/4, and 1/8 MIC's (*Staphylococcus aureus*).

Bacteria.– One strain of *Staphylococcus aureus* was used: ATCC 9144 is a strain resistant to cefepime (MIC 5 mg/l).

The MIC's were determined by the broth microdilution method with cation-supplemented Mueller-Hilton Broth (Difco, Detroit, Michigan, USA) in accordance with the procedures outlined by the National Committee for Clinical Laboratory Standards (13).

Treatment with cefepime.– In vivo studies: Swiss mice of 15 ± 2 weeks of age were used. One group was injected with a single intramuscular dose of 200 mg cefepime per kg animal weight, after which blood was obtained from these and control animals (injected with saline solution) at the times 1, 2, 4, 8, and 12 hours. Another group (together with the corresponding controls) was administered a once-daily intramuscular injection of 200 mg/kg (or saline solution, controls) for 7 days, and one hour after the end of the

treatment, the animals were sacrificed to obtain blood. In addition, there was another group, denominated basal, which underwent no treatment.

In vitro *studies.*– The blood was obtained from healthy volunteers and allowed to clot at room temperature for 1 h. After 24 h at 4 °C, the blood was centrifuged for 15 min at 300 *g*. Serum was removed, pooled, and stored at –70 °C in small amounts.

Isogever preparation.– Gelatine (10 %): 10 g gelatine (Sigma) plus 0.425 g sodium azide (Sigma) in 100 ml of distilled water was heated to 56 °C until dissolved.

The Isogever was prepared by mixing 1 ml of the gelatine solution and 100 ml of the dissolved Complement Fixation test (CFT) (Flow, Virginia, USA). The mixture was kept at 4 °C protected from light, being ready for use over a period of two weeks.

Serum preparation.– For the study of the hemolytic activity of complement, the serum was diluted in Isogever to dilutions of 1/200, 1/300, 1/400 and 1/500. For the study of serum bactericidal activity, the serum was diluted serially in MHB to a dilution of 1:64.

Hemolysin.– Hemolysin (Lab. Limited Hornby, Ontario, Canada), a specific antibody for sheep erythrocytes, was diluted to 1/1000 in Isogever.

Erythrocyte treatment.– The sheep erythrocytes (Biomedics, Madrid, Spain) were first washed with PBS (phosphate buffered saline solution) and then with Isogever, then incubated for 30 min at 37 °C with hemolysin. At the end of this time, the cells were adjusted to a concentration in distilled water that gave 100 % absorbance at 415 nm (total hemolysis value).

Serum hemolytic activity.– The serum hemolytic activity was evaluated using a modification of the CH-50 technique of GAITHER and FRANK (7). Thus, 100 µl of erythrocytes were incubated for 30 min at 37 °C with the various dilutions of serum at a final volume of 400 µl (in the *in vitro* studies, 100 µl of cefepime at 1/2, 1/4, and 1/8 MIC were added, and 100 µl of Isogever in the control samples), then adding 1.5 ml of Isogever to each sample followed by centrifugation for 2 min at 500 *g*. The supernatants from each sample were read spectrophotometrically at 415 nm, using Isogever as the blank tube. Finally, the values thus obtained were transformed into a Probit index by means of the following equation: Probit = Y/100 – Y × 100, where Y = Sample absorbance – Blank absorbance/Total hemolysis absorbance × 100.

The CH-50 is obtained when Probit is equal to unity.

Serum bactericidal activity.– The serum was diluted in MHB to a titre of 1:64, with a final tube volume of 100 µl. Then 10 µl of 1 x 105 CFU/ml *S. aureus* was added, including a tube with MHB and bacteria inoculum but without serum. For the *in vitro* samples, 10 µl of 1/2, 1/4, 1/8 MIC cefepime diluted in MHB was added to the samples, and 10 µl MHB alone to the controls (without antibiotic). The content of the tubes was seeded onto Mueller-Hinton agar plates and incubated at 37 °C, 100 % humidity, and 5 % CO_2. After 24 h, CFU's were determined, expressing the results as a growth percentage (CFU %) by means of the expression: CFU (%) = CFU samples with serum/CFU samples

without serum × 100, where a 100 % value represents the number of CFU surviving in a control sample where the bacteria had been incubated in the absence of serum, representing therefore the maximum growth.

Statistical analysis.– Differences in the hemolytic and bactericidal activity of serum were compared for statistical significance. All data are expressed as mean ± standard deviation of the number of experiments performed. In the statistical study, the normality of samples was confirmed by the Kolmogorov-Smirnov test. Student's *t* test was used for comparison between parametric samples, $p < 0.05$ being taken as the minimum significance level.

Results and Discussion

To demonstrate the correct functioning of the totality of the classical pathway (CCP) C1-C9, the test of hemolytic activity of the complement, based on the capacity of the intact complement system to lyse sheep erythrocytes when they are covered by specific antibodies has been used. The results obtained *in vivo* indicate that there are no significant differences between the values obtained at the different times after the administration of the antibiotic (142 ± 26, 137 ± 10, 123 ± 15, 121 ± 24, 138 ± 13, 150 ± 15, 137 ± 10 at 0, 1, 2, 4, 8, 12 hours and 7 days after antibiotic administration, respectively). Nor were there any differences between the cefepime-treated samples and the controls injected only with saline solution at any of the measurement times, or between the different times for the controls alone. Also, *in vitro*, no significant differences ($p > 0.05$) were found between the results (CH-50 units) obtained in the absence of cefepime (215 ± 20) and in the presence of 1/8, 1/4, and 1/2 MIC of the antibiotic for *S. aureus* (178 ± 17, 188 ± 15 and 192 ± 15, respectively).

The bactericidal mechanism, in the absence of agglutinating antibodies, presented a marked variation when the serum was treated both *in vivo* and *in vitro* with sublethal concentrations of cefepime against *S. aureus*. Thus, in the *in vivo* determinations (fig. 1), there was generally a significant increase in the bactericidal activity after 7 days of the antibiotic administration with all the dilutions of serum. In addition, in the *in vitro* determinations (fig 2), the highest values of bactericidal activity of serum against *S. aureus* were observed with 1/2 MIC. In relation to this, other authors in preliminary studies (6) have reported the following: Some antibiotics, such as streptomycin, at subinhibitory concentrations seem to be able to produce a strong enhancement of the bactericidal activity of

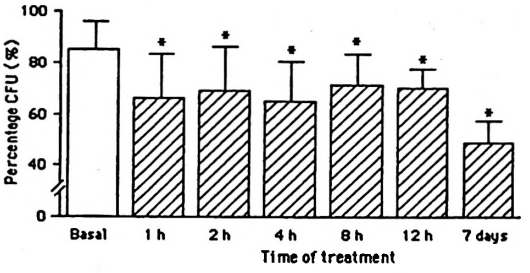

Fig. 1. In vivo *bactericidal activity of serum (1:8 dilution) against* S. aureus *in the presence of cefepime.* A group of mice were administered 200 mg/kg intramuscularly and studied before (white column), and 1, 2, 4, 8, and 12 h after treatment. Another group was injected daily with 200 mg/kg of cefepime for 7 days, the study being performed on the seventh day one hour after the last injection (dotted column). Each column represents the mean ± S. D. of 10 determinations performed in duplicate. * $p < 0.05$ with respect to the results for basal sera from animals that had received no treatment.

normal human serum; two cephalosporin derivatives, cefotaxime and cefazolin, show a synergistic activity with the bactericidal action of normal human serum against enterobacteriaceae; there seems to be a synergistic effect with complement-dependent bactericidal activity in normal human serum for all tetracycline derivatives; sulphonamides and penicillins, however, at therapeutic doses, have no effect on the serum bactericidal activity.

In summary, cefepime (BMY-28142), both *in vivo* and *in vitro* at the doses used here and at all the times studied, does not significantly modify the hemolytic activity of the complement. On the contrary, this antibiotic at sublethal concentrations increases the bactericidal activity of serum against S. aureus. The killing of *S. aureus* appears to be dependent on both serum dilution and antibiotic dose as well as on time. In addition to the effect of cytolysis produced by the serum complement on the cell membranes, the joint presence of serum and cefepime (an antibiotic which acts by inhibiting the synthesis of the peptidoglicane of the bacterial cell-wall) would explain the increase obtained in serum bactericidal activity in the presence of this antibiotic. This effect of the antibiotic could be due to an increase of complement consumption on more iC3b bound to the surface of the bacteria but further work will be needed to clarify this point. Cefepime could be a promising antimicrobial agent for the treatment of infections due to *S. aureus.*

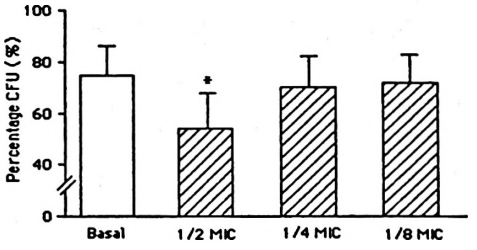

Fig. 2. In vitro *bactericidal activity of serum (1:8 dilution) against* S. aureus *in the presence of cefepime.* Sera were incubated without (white column) and with 1/2, 1/4 and 1/8 MIC antibiotic (dotted columns). Each column represents the mean ± S.D. of 10 determinations performed in duplicate. * $p < 0.05$ with respect to the results for basal sera (without antibiotic).

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Se evalúa en ratones Swiss (15 ± 2 semanas), si la cefepime (BMY-28142) tiene algún efecto sobre la actividad hemolítica y bactericida del suero, tanto *in vivo* como *in vitro*. Para el estudio *in vivo*, se realizan las determinaciones antes y después de 1, 2, 4, 8 y 12 h de una inyección intramuscular (200 mg/Kg) del antibiótico y a otro grupo de ratones se trata durante 7 días con una inyección i. m. diaria de la misma dosis, sacrificándose 1 h después de la última inyección. Para los estudios *in vitro*, las muestras se incuban en presencia y ausencia de cefepime (1/2, 1/4 y 1/8 CMI para *Staphylococcus aureus*) (2,5, 1,25 y 0.625 mg/l, respectivamente). Los resultados indican que la cefepime, a concentraciones subletales, incrementa la actividad bactericida del suero, tanto *in vivo* como *in vitro* frente a *S. aureus*, sin modificar significativamente la actividad hemolítica del complemento.

Palabras clave: Cefepime, Sistema del complemento, Actividad hemolítica, Capacidad bactericida.

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