CEFODIZIME, AN AMINOTHIAZOLYL CEPHALOSPORIN

IV. INFLUENCE ON THE IMMUNE SYSTEM

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Studies concerning the activity of cefodizime (HR 221), on certain aspects of the immune response, were conducted. It was found that lymphocytes from Balb/c mice treated with 3 and 30 mg/kg/day of cefodizime display increased responsiveness to B-cell mitogens and specific antigens. Also, the amount of antigen specific antibody producing plaque forming cells was increased in these mice and was accompanied by a rise in the specific IgG haemagglutinin titer. These effects were not observed in lymphocytes obtained from NMRI mice that had been treated with cefodizime.

Peritoneal macrophages from NMRI mice, treated with cefodizime prior to harvesting of the cells, contained increased levels of lysosomal enzymes, developed enhanced chemiluminescent reaction to stimuli and showed elevated pinocytosis rates. Furthermore, NMRI mice treated with cefodizime during the immunization, developed enhanced DTH-reaction, when challenged with the antigen (SRBC).

The prophylactic treatment of Balb/c mice with cefodizime (2×30 mg/kg/day ip for 4 days) significantly prolonged the mean survival time of the animals after intravenous infection with *Candida albicans* 200/175 (16.7 days as against 3.5 days in the case of the controls). This stimulatory effect of cefodizime on the host defence system was not observed for NMRI mice.

Treatment with latamoxef or cefoperazone under the same experimental conditions did not reduce the susceptibility of mice to *C. albicans*.

The protective activity of cefodizime against *C. albicans* in Balb/c mice, may be due to the immuno-stimulatory activity of this agent.

Cefodizime (HR 221) is a β-lactamase resistant cephalosporin with a broad antibacterial spectrum, including staphylococci, streptococci, and the most important Gram-negative species (*Neisseria*, *Haemophilus*, Enterobacteriaceae). Compared with other newer cephalosporins, the minimal inhibitory concentrations (MIC) of cefodizime are somewhat inferior[1~4]. Surprisingly, the efficacy of cefodizime in experimental infections is at least as good or even better than that of the compounds as to predict from the *in vitro* data[5,6].

The high *in vivo*-activity of cefodizime may be explained by pharmacokinetic profile (serum half life, tissue penetration[7]). This explanation, though, is not true in cases in which cefodizime was protecting mice from infections with bacterial strains resistant to cefodizime *in vitro*. In these cases other properties of cefodizime may contribute to the *in vivo* effect.

During recent years there has been increased interest in the impact of antimicrobial chemotherapy on the host defence systems. It has been found that various antibiotics interfere with specific and unspecific immune functions[8~11]. We, therefore, investigated if the *in vivo* effect of cefodizime may be related to potentiation of the host defence mechanism.

Materials and Methods

Antibiotics and Reagents

Cefodizime was synthesized by Hoechst AG (Frankfurt, FRG). Latamoxef (Lilly), cefoperazone (Pfizer), benzylpenicillin (Hoechst) and streptomycin (Flow) are commercially available.

Phytohaemagglutinin-A (PHA) was purchased from Gibco, concanavalin A (Con-A), luminol and zymosan was bought from Serva, lipopolysaccharide (LPS) from *Escherichia coli* was supplied by Calbiochem, and dextran sulfate (DXS) was obtained from Pharmacia.

Heparin, preservative free and L-glutamin were obtained from Seromed. Triton X-100, phenolphthalein glucuronic acid, *p*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-2-acetamido-β-D-deoxyglucopyranoside were from Sigma. Luminol was from EGA-Chemie.

Mice

Female NMRI mice were obtained from our own breeding station. Balb/c mice were purchased from Charles River, Wiga. At the beginning of the experiments the average weight of the mice was approx 20 g.

In Vitro Experiments

Influence on Lymphocytes and Antibody Titers: On day 1 the mice were injected ip with 5×10^8 sheep red blood cells (SRBC). Starting on day 1, the animals were treated twice daily with 60, 30 or 3 mg/kg/day cefodizime (split in 2 doses/day). On day 5, one hour after drug administration, the animals were bled, and the spleens removed and pooled (6 animals/group). Cell suspensions were prepared in Clicks/RPMI 1640 tissue culture medium. As a positive control one group was injected with SRBC and administered with solvent alone (normal saline). The negative control did not receive SRBC.

Lymphocyte Transformation: 0.2 ml of a mixture of Clicks and RPMI 1640 tissue culture medium (50/50 Seromed) supplemented with 1% mouse serum, 10 mM HEPES buffer, containing 3×10^5 spleen cells and various concentrations (suboptimal to optimal) of either Con A, PHA, LPS, DXS or SRBC was placed in flat-bottomed wells of tissue-culture microtiter plates (Greiner). For each group 6 replicates were set up. After 48 hours of incubation at 37°C in a 5% CO_2-atmosphere, the cultures were given 0.25 μCi/ml of [^{3}H]thymidine (New England Nuclear). 24-hour later the cells were harvested (Cell harvester, Flow Laboratories) and the amount of radioactivity incorporated in the DNA determined (Packard Tricarb-460 c).

Assay for Direct Plaque Forming Cells (PFC) Anti SRBC: Modified version of the Jerne PFC assay was employed as described[12].

Haemagglutination-test: The detection of the haemagglutinin titer to SRBC was determined for each group of animals as discribed[13].

Influence on Peritoneal Mouse Macrophages: Mouse macrophages were obtained by peritoneal lavage of the mice with 5 ml of TCM 199 containing penicillin and streptomycin and 10 IU/ml heparin as described[14].

Macrophages were recovered either from normal untreated mice or from mice after ip or iv administration of 0.5 ml phosphate buffered saline containing cefodizime and cultured in 2 ml fresh serum-free TCM 199. Macrophages and culture supernatants were tested after 24 hours of incubation for biological activities.

Enzyme Assays: All assays were conducted under conditions giving linear release of product in relation to the amount of sample used and the time of incubation.

β-Glucuronidase was assayed by the method of TALALAY *et al.*[15]

β-Galactosidase was assayed by the method of CONCHIE *et al.*[16] using *p*-nitrophenyl-β-D-galactopyranoside as substrate.

N-Acetyl-β-D-glucosaminidase was assayed by the method of WOOLEN *et al.*[17] using *p*-nitrophenyl-2-acetamido-2-β-D-glucopyranoside as substrate dissolved in 0.1 M citrate - phosphate buffer pH 4.5. Lactate dehydrogenase was assayed by determining the rate of oxidation of reduced nicotinamide adenine dinucleotide at 340 nm.

Measurement of Chemiluminescence: The production of oxygen-derived radicals by activated

macrophages was measured by a luminol-chemiluminescence assay. Our chemiluminescence measurements were done in a Biolumat (model LB9505, Berthold Co., D-7547 Wildbad, W. Germany) which allows the simultaneously measurements of 6 samples. Mononuclear phagocytic cells (1×10^7/ml) in 100 μl TCM 199 without serum were placed in round-bottomed polystyrene vials, preincubated for 1~2 hours at 37°C and washed. The cell samples were then placed into the biolumat counting chambers, mixed with 50 μl luminol (100 μg/ml) and light emission was continously monitored, calculated and corrected by using an Apple II computer with a MX-82F/T Epson dot matrix printer (Cupertino, California, U.S.A.).

Quantitative Estimation of Endocytic Activity: Radioactive colloidal gold has been used as a quantitative indicator of the pinocytic activity of mononuclear phagocytes as described by DAVIES *et al.*[18]. Radioactive colloidal gold (^{198}Au), particle up to 20 nm, specific activity 4~12 mCi/mg. The radioactive gold was obtained from the Radiochemical Centre, Amersham, Bucks., GB. All experiments were carried out with amounts of radioactivity ranging from 0.1 to 1.0 μCi/ml of culture medium.

Statistical Test: Means and standard deviations were calculated after samples were shown to be homogeneous by calculation of coefficients of variance. The significance of differences was established by the Student's t-test.

In Vivo Experiments

DTH-reaction: Measurement of DTH-reaction was performed according to COLLINS and MACKANESS[19]. Female NMRI mice were immunized intravenously with 10^6 SRBC. Five days later the animals were restimulated with 2×10^8 SRBC ip. Increase in footpad swelling was measured 24 hours later.

Candida albicans Septicaemia: Different groups of 15 animals were treated intraperitoneally with cefodizime, latamoxef or cefoperazone. The animals received 30 mg/kg twice a day for four consecutive days. 24 hours after the last administration of the antibiotics the mice were infected intravenously with *C. albicans* 200/175. The infective dose was 5×10^6 cfu/Balb/c mouse.

As a control one group of animals received physiological saline instead of the test compounds.

After infection the mice were observed for two weeks. The number of surviving animals was recorded each day and the mean survival times calculated.

Results

In Vitro Studies

Table 1 shows the influence of cefodizime on antibody titers and lymphocyte subsets. Spleen cells from animals treated with cefodizime show very little or no suppressive effects on the lymphocyte proliferation to mitogens or specific antigen (SRBC). The lymphocytes from Balb/c mice treated with 3 and 30 mg/kg/day of this substance do, though, display increased responsiveness to B-cell mitogens and SRBC's. Such an effect was not observed in NMRI mice. Furthermore, the amount of PFC anti SRBC was increased in Balb/c mice, accompanied by a rise in the specific IgG haemagglutinin titer. This was also not found in NMRI mice. With the exception of the small, yet significant, inhibition of PFC anti SRBC at 3 mg/kg/day, we could not detect any modulating effect of cefodizime on the immune response of NMRI mice.

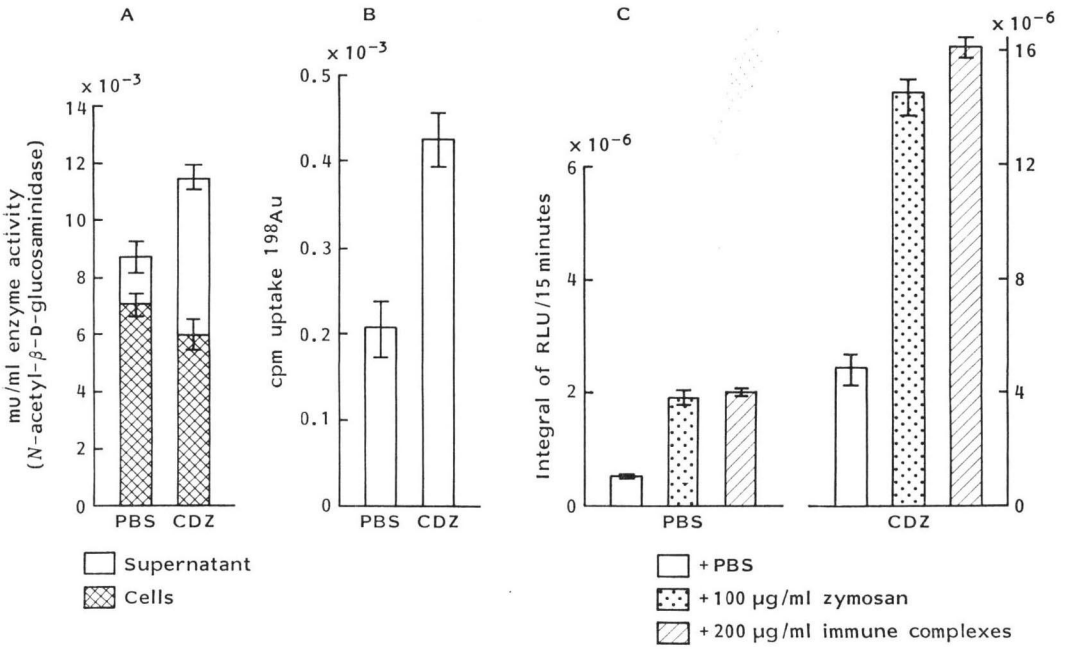
The effect of cefodizime on macrophage activation generating the secretion of lysosomal enzymes (β-glucuronidase, β-galactosidase and *N*-acetyl-β-D-glucosaminidase), or generating O_2-radicals was evaluated by testing the chemiluminescence.

Another parameter of macrophage activation was determined by measuring the pinocytic uptake of radioactive colloidal gold. When NMRI mice were treated with the drug parenterally with two injections of 30 mg/kg/day on days 0~3 and the macrophages from those mice were collected 3 days later, the total number of peritoneal cells recovered from treated mice did not differ significantly from that re-

Table 1. Influence of cefodizime on the immune response of different strains of mice.

	PFC anti SRBC (direct) % change	Haemaggutinin titer (SRBC) (anti-SRBC)		Lymphocyte proliferation (cpm±SD)				
		1gM	1gG	Con-A (0.5 μg/ml)	PHA (0.5%)	DXS (30 μg/ml)	LPS (1 μg/ml)	SRBC (10^5 cells)
Balb/c								
60 mg/kg/day	−19	2,048	64	29,600±1,400	11,000± 920	1,600± 400	2,600± 84	2,600± 310
30 mg/kg/day	+29	4,096	128	30,700± 540	12,900± 330	6,000± 220	3,100±410	8,500± 170
3 mg/kg/day	+95	1,024	256	24,900± 850	7,900± 740	13,300±1,100	14,800±210	16,300±1,700
0 mg/kg/day	±0	2,048	4	32,100±1,200	12,300± 910	2,000± 160	1,900±310	3,200± 260
NMRI								
60 mg/kg/day	+3	512	256	18,600±1,400	12,400±1,900	1,300± 300	290±110	260± 120
30 mg/kg/day	−16	512	128	32,600±1,800	17,100±2,800	2,000± 160	520± 20	890± 7
3 mg/kg/day	−32	512	128	26,200± 920	16,400± 620	2,400± 130	920± 60	1,400± 60
0 mg/kg/day	±0	512	128	30,400±1,000	21,700±1,800	1,300± 300	500± 50	1,100± 240

Fig. 1. The effect of cefodizime (CDZ) (2×30 mg/kg/day) on mouse peritoneal macrophages.
A: Release of lysosomal enzymes; B: pinocytosis of ^{198}Au; C: chemiluminescence reaction to zymosan or immune complexes.



covered from normal mice. Cefodizime interacts with macrophages *in vivo* by increasing their secretory activity of acid hydrolases. Marked changes in the levels and distribution of the activities of lysosomal enzymes were induced. There was an increase of secreted enzyme activity after intravenous and subcutaneous administration. This stimulation did not cause any morphologically detectable cell death and no significant increase in the amount of the cytoplasmic enzyme lactate dehydrogenase in the culture supernatant.

Table 2. Influence of cefodizime on DTH-reaction against SRBC in NMRI mice (n=5).
Treatment of the animals ip twice a day for four days prior to the challenge.

Dose (mg/kg)	% Footpad swelling
Control (saline)	21.6±9.2
30	26.9±6.4
40	30.5±9.6

Table 3. Effect of cefodizime and other cephalosporins (2×30 mg/kg/day for 4 days before infection) on the *Candida albicans* septicaemia of Balb/c* and NMRI mice*.

	Mean survival times post infection (days)					
	Balb/c			NMRI		
	Mean value	95%**	99%**	Mean value	95%	99%
Control	3.5	3.1~ 3.9	2.9~ 4.1	1.9	Estimated	
Latamoxef	2.8	Estimated		1.8	Estimated	
Cefoperazone	2.7	2.4~ 3.1	2.2~ 3.2	2.1	1.9~2.3	1.8~2.4
Cefodizime	16.7	13.5~25.6	12.8~34.6	1.9	Estimated	

* Infective dose: Balb/c: 6×10^8 cfu/mouse.
NMRI: 5×10^8 cfu/mouse.
** Limits of confidence.

Fig. 2. Effect of cefodizime and other cephalosporins (2×30 mg/kg/day for 4 days before infection) on the *C. albicans* septicaemia of the Balb/c mouse (infective dose 6×10^6 cfu/mouse iv).

□ Control, △ cefodizime, ○ latamoxef, ● cefoperazone.

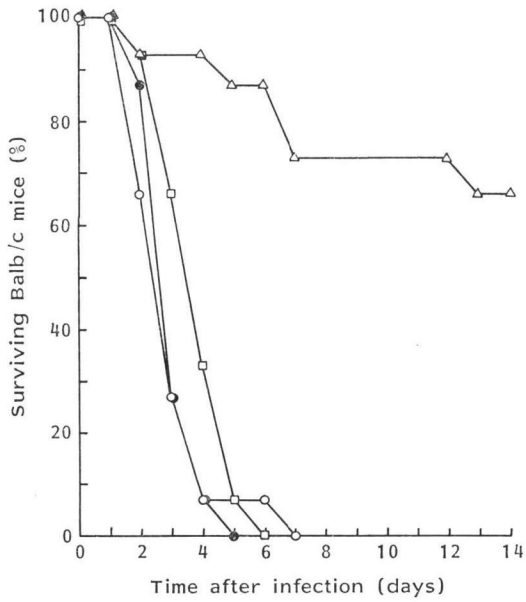
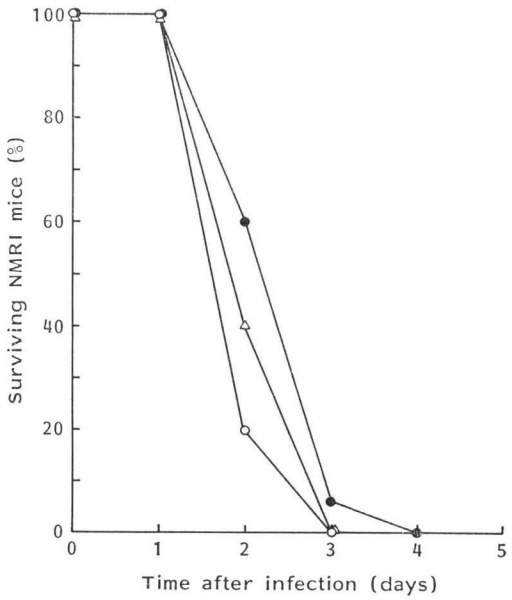


Fig. 3. Effect of cefodizime and other cephalosporins (2×30 mg/kg/day for 4 days before infection) on the *C. albicans* septicaemia of the NMRI mouse (infective dose 5×10^6 cfu/mouse iv).

△ Control and cefodizime, ○ latamoxef, ● cefoperazone.



When mouse peritoneal macrophages from mice treated *in vivo* with cefodizime intraperitoneally (2×30 mg/kg/day) on days 0~3 were isolated 72 hours after the last injection and investigated for their pinocytic activity, cefodizime induced *in vivo* a significant increase in pinocytic uptake of radioactive colloidal gold compared to controls.

We next performed several experiments to compare the chemiluminescence production by macrophages from mice treated with cefodizime to that of control animals. After base-line chemiluminescence of unstimulated resting macrophages had been measured, the chemiluminescent response after ip or iv application (2×30 mg/kg/day) on day 0 to 3 showed a significant increase over background chemiluminescence. In contrast, macrophages from PBS-treated mice displayed a negligible chemiluminescence. In another experiment we explored the question of whether macrophages from cefodizime treated mice can be restimulated *in vitro* to produce additional amounts of chemiluminescence on exposure to a second stimulus like zymosan (100 μg/ml) or immune complexes (200 μg/ml). In those experiments macrophage cultures from initially ip treated mice induced an increase of chemiluminescence. When zymosan or immune complexes were added to the reaction vial an even more striking enhancement of macrophage mediated chemiluminescence was noted. Macrophages from cefodizime-treated animals demonstrated a greater capacity to respond to *in vitro* stimulation when compared to control macrophages.

In Vivo Study

The influence of cefodizime on the DTH-reaction against SRBC is shown in Table 2. A dose-dependent stimulation of DTH-reaction was observed when the substance was administered 2 times/

day in the concentration of 30 and 40 mg/kg for 4 days prior to the immunization with SRBC.

Figs. 2 and 3 show the mortality curves of Balb/c and NMRI mice after pretreatment and iv infection with *C. albicans*. Table 3 contains the mean survival times.

It can be seen from the slope of the appropriate curve in Fig. 2 that in the Balb/c control group, the first animals died two days after the infection. By the 6th day, after infection, all the animals in this group were dead. The mean survival time of the control mice was calculated to be 3.5 days. The Balb/c mice, pretreated with latamoxef or cefoperazone, died in the same period (mean survival times 2.8 and 2.7 days respectively).

A totally different mortality kinetic was found with Balb/c mice pretreated with cefodizime. The administration of cefodizime significantly increased the resistance of the Balb/c mice to *C. albicans* infection. The mean survival time of these animals was 16.7 days. Ten of the animals receiving cefodizime prior to infection survived the whole observation period.

NMRI mice (Fig. 3) were more susceptible to *C. albicans* than Balb/c mice. NMRI mice from the control group infected intravenously with 5×10^6 cfu/animal died within 3 days of infection. NMRI mice pretreated with cefodizime or the two other cephalosporins showed the same susceptibility to *C. albicans* as the controls. The mean survival times of both control and antibiotic-treated animals were between 1.8 and 2.1 days post infection.

Discussion

In a recent study we found that cefodizime, even at very high concentrations, produces *in vitro* neither negative nor positive effects on guinea pig granulocytes chemiluminescence reaction to zymosan (unpublished data).

In the present study we investigated the *in vivo* effects of cefodizime on certain aspects of the immune response and the host defence system.

As *in vivo* test model we used the *C. albicans* septicaemia of two different mouse strains (Balb/c, NMRI). *C. albicans* served as the pathogen because cefodizime, like other cephalosporins, shows no direct inhibitory effects on this microorganism.

In our investigation cefodizime and the comparative compounds latamoxef and cefoperazone, were administered ip to the experimental animals in a dosage of 2×30 mg/kg/day for a period of four days. 24 hours after the final injection of the antibiotics the mice were infected iv with *C. albicans* 200/175. We demonstrated that the administration of cefodizime, latamoxef or cefoperazone in this application scheme induced no measurable alterations in the host defence systems of NMRI mice (Fig. 2). Like the mice of the control group all the animals pretreated with the antibiotics were dead three days after infection.

In Balb/c mice latamoxef and cefoperazone likewise did not bring about changes in the sensitivity of the animals to *C. albicans*.

In contrast to these findings, cefodizime-pretreated Balb/c mice showed a significant increase in host resistance to this pathogen. Ten out of 15 animals receiving cefodizime prior to infection survived for more than 14 days.

Resistance to fungal infection is mediated primarily by T-lymphocytes and the entire cellular arm of specific immunity. Antibodies often play a secondary role in the defence of fungal infections. Considering that cefodizime had protective properties in Balb/c mice, but not in NMRI mice, we investigated the effects of cefodizime on certain aspects of the immune response in healthy mice of each strain.

Different subsets of lymphocytes can be selectively stimulated by various substances of plant and bacterial origin. T-Cells can be stimulated by phytohaemagglutinin (PHA), and concanavalin A (Con-A), whereas T-cell independent B-cells are responsive to lipopolysaccharide (LPS). Immature B-cells proliferate in the presence of dextran sulfate (DXS). Stimulation of lymphocytes with specific antigen,

to which the animal has already been exposed, results in the proliferation of both T- and B-cells.

We found that lymphocytes from cefodizime-pretreated Balb/c mice unlike those of NMRI mice showed enhanced proliferation rates to B-cell mitogens and SRBC. Peritoneal macrophages from NMRI mice, treated with cefodizime showed an increase of secreted lysosomal enzymes. Moreover, these macrophages exhibited a significant increase in pinocytosis of radioactive colloidal gold. The enhanced metabolic activity of mouse macrophages under the influence of cefodizime was paralleled by an elevated chemiluminescent reaction to opsonized zymosan or immune complexes.

As could be shown by the increase of DTH-reaction cefodizime could also stimulate the T-helper lymphocyte activity in NMRI mice.

Taking the results obtained in our studies together, protective effects against *C. albicans* displayed by cefodizime in Balb/c mice, may be due to immuno-stimulating activity of this agent on B-lymphocytes and macrophages.

Genetic differences between the NMRI and Balb/c mice may be responsible for the lack of influence of this substance on the lymphocyte response of NMRI mice, whereas the macrophages of this strain were stimulated *in vivo* by cefodizime. Our investigations suggest that the activity of cefodizime in experimental bacterial infections, which is higher than expected from its *in vitro* effect, might not only be explained by its pharmacokinetic behavior, but also through its immuno-modulatory effect.

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