Modification of Natural Immunity in Mice by Imipenem/Cilastatin

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The imipenem/cilastatin constitutes a broad spectrum β-lactam antibiotic formulation, especially used in pre and post-operatory treatments for transplanted or drug-immunosuppresed patients. The effect of the dose and the duration of the treatment with imipenem/cilastatin on some parameters of natural immunity in BALB/c mice were examined. The treatment by intraperitoneal route with 1 or 2 g/70 kg/day during 7 days did not alter significantly the parameters tested, whereas the greater dose used (4 g/70 kg/day) had an inhibitory effect on peritoneal cell counts and phagocytic activity, as well as it caused an increase on IL-1 production and natural killer activity. The greater stimulating effect of innate immunity was obtained with the lowest imipenem/cilastatin dose used (0.5 g/70 kg/day). Since this antibiotic apparently does not impair the studied innate immune responses at 1 or 2 g/70 kg/day, it seems to be especially suited for the therapy of systemic bacterial infections in immunocompromised patients.

Antibiotics have biological properties other than direct antimicrobial activity. These include interactions and alterations in the cellular and/or humoral immune functions of the patients[1~7]. The concept of immune response modifiers applied to antibacterial agents has arised recently, and it is a subject of growing research[8~11]. Current investigations attempt to describe the immunological profiles of the new antimicrobial agents[12~14], as well as avoiding the use of those having immunosuppresing activity on patients with any immunological deficiency[15,16].

The state of the nonspecific host defense system is important for the outcome of antimicrobial chemotherapy. Moreover, the effect of antibiotics on phagocytic function is of potential importance since these agents are often administered to patients who are susceptible to infection because of defects in phagocytic function or granulocytopenia. It has been established that the phagocytic function is impaired by a wide variety of antimicrobial agents, as tobramycin and azlocillin[17], tetracyclines, sulphonamides and trimethoprim[18].

Metronidazole[19], clindamycin and cloramphenicol[20], and some β-lactam antibiotics[21,22] do not affect this function.

Imipenem (*N*-formimidoyl-thienamycin) is the first member of a new family of β-lactam antibiotics (carbapenems) produced by *Streptomyces cattleya*. The imipenem/cilastatin association constitutes a broad spectrum β-lactam antibiotic for intravenous administration[23], with well characterized pharmacokinetics and activity[24~26]. The chemical structure of imipenem makes it very active against proteins involved in the biosynthesis of the bacterial cell wall and so, very powerful in its bactericidal effect over a wide range of Gram-positive and Gram-negative bacteria[27]. Furthermore, because of its stability against β-lactamases, it is effective against a great range of microorganisms that are resistant to other β-lactam antibiotics. We have selected this antibiotic because of its great use in human therapy, and especially its use in pre and post-operative treatments of transplanted or drug-immunosuppresed patients.

The effects of imipenem/cilastatin on various parameters of the phagocytic function have been reported. The preincubation of human granulocytes with imipenem induces an increase of adherence[28] and chemotaxis[28,22], but it does not influence the level of cellular activation measured by chemiluminescence[28,29], opsonization, phagocytosis and the candidicide index[22]. The effects of intramuscular administration of imipenem and cilastatin on the phagocytic activity of murine peritoneal macrophages have also been examined by NUÑEZ *et al.*[30]. They described that, in imipenem/cilastatin-treated mice, peritoneal macrophages show a significant increase both in adherence to substrate and nylon fiber, as well as in spontaneous motility and chemotaxis index; Moreover, imipenem produces an increase in the

nitroblue tetrazolium reduction test in macrophages from treated mice stimulated with latex beads, but it does not influence opsonization, phagocytosis and the candidicide index.

In this study, we have investigated the influence of the dose and the duration of the treatment with imipenem/cilastatin on several parameters of nonspecific immunity in mice.

Materials and Methods

Animals

Six- to eight-week-old male BALB/c mice were used for the experiments. They were maintained under pathogen-free conditions, with free access to food and water.

Antibiotic Regimen

The combination imipenem/cilastatin (I/C) was provided by Merck Sharp & Dohme (Madrid). The antibiotic combination was given by i.p. injection to mice (5 animals per group) in 24 hours intervals for 7 (or 14) consecutive days. The doses were calculated on a per kg body weight basis, according to therapy in human medicine (4, 2, 1 and 0.5 g/70 kg/day for 7-days therapy, and 2 g/70 kg/day for 14-days therapy), and the antibiotic was diluted in sterile phosphate buffered saline (PBS; Sigma). Control mice received equal volumes of PBS, during 7 or 14 days. Immune parameters were tested the day after the last injection.

Preparation of Peritoneal Macrophage Suspensions

Mice were sacrificed and injected i.p. with 3 ml of HANK's balanced salt solution without phenol red (HBSS; Sigma). The peritoneal fluids were aspirated, washed twice by centrifugation at $200 \times g$ and 4°C for 5 minutes with HBSS, and finally resuspended in HBSS for chemiluminescence assays, or in complete medium RPMI 1640 (Sigma) supplemented with 10% heat-inactivated calf serum (Flow Laboratories), 50 μM 2-Mercaptoethanol (Sigma), 1% Penicillin G/Streptomycin solution (Sigma), 1% L-Glutamine (Sigma), 1% Sodium Pyruvate (Sigma), 5% Sodium Bicarbonate (Sigma) and 1% Hepes (Flow Laboratories), for IL-1-producing cultures. The peritoneal cells were counted by the trypan blue exclusion test.

Chemiluminescence (CL) Assay

This assay was performed according to the technique described by MOELLER-LARSEN *et al.* (1989)[31], slightly modified by us. Peritoneal cells were adjusted to a concentration of 10^5 viable cells/ml with HBSS without phenol red. The assay mixtures were prepared in Eppendorf tubes of 1.5 ml and consisted of 100 μl of adjusted cellular suspension, activated by adding 20 μl of an opsonized dark-adapted zymosan A suspension at a concentration of 50 mg/ml. Finally, 70 μl of luminol (5-amino-2,3-dehydro-1,4-phthalazinedione) in PBS at a concentration of 100 μg/ml were added. The chemiluminescence mixtures were incubated for 2 minutes at 37°C and disposed in scintillation vials. The phagocytic activity of peritoneal cells was measured by photon emission (LS-1801, Beckman Instruments, Irvine, Calif.) for 0.1 minute sequentially, till 60 minutes. For each four analyzed samples we disposed of two vials containing 100 μl of cellular suspension, 20 μl of zymosan and 70 μl of HBSS without phenol red. The values obtained without luminol expressed as counts per minute (cpm), were considered as background counts. These values were subtracted to those obtained for the remaining vials.

Interleukin-1 (IL-1) Assay

The thymocyte co-stimulation bioassay was used for the measurement of soluble IL-1 according to the technique described by MUEGGE and DURUM[32]. Briefly, the peritoneal cells were adjusted to a concentration of 2×10^6 cells/ml with complete medium. One ml of the cell suspensions was added to 35-mm-diameter plastic Petri dishes, as well as 1 ml of *Escherichia coli* lipopolysaccharide (LPS; Sigma) at a concentration of 20 μg/ml. Thus, the final concentrations in the dishes were 10^6 cells/ml and 10 μg/ml LPS. The mixtures were incubated for 24 hours at 37°C in 5% CO_2, and finally supernatants were collected and stored at −80°C until quantitative assay. Thymus from 6- to 8-week-old mice were aseptically removed and homogenized in sterile HBSS. Thymocytes were washed twice by centrifugation at $200 \times g$ and 4°C for 10 minutes with HBSS, and finally resuspended in complete medium with 10 μg/ml Phytohemagglutinin (PHA; Sigma). Cell suspensions were adjusted to a concentration of 10^7 cells/ml. IL-1 test samples were serially diluted 5-fold in complete medium without PHA. 96-well flat-bottom microtiter plates received 100 μl of thymocyte suspension and 100 μl of diluted samples per well. The plates were incubated for 48 hours at 37°C in 5% CO_2. Cellular proliferation was measured by colorimetric reading of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction as described by MOSMANN[33].

Natural Killer (NK) Activity Assay

Mice were sacrificed and spleens were aseptically removed and homogenized in sterile HBSS. Mononuclear splenic cells used for this assay were isolated according to BOYUM[34], in a Ficoll-Hypaque density gradient. The mononuclear cell band obtained was collected, washed twice with HBSS by centrifugation at $200 \times g$ and 4°C for 5 minutes and finally resuspended and adjusted to a concentration of 6×10^5 viable cells/ml with complete medium. Natural Killer activity was tested according to the technique described by DINOTA *et al.*[35], based on the inhibition of target cell clone growth in plasma clot semisolid medium. A suspension of the NK-susceptible K562 cell line, adjusted at a concentration of 10^5 viable cells/ml in complete medium, was used as target cells. 200 μl of effector cell suspension were incubated with 200 μl of target cell suspension at 37°C for 4 hours in the presence of 5% CO_2. Control experiments were carried out by using K562 cells alone. Next, 20 μl of cell mixture were plated in 35-mm-diameter petri dishes, along with 1 ml of plasma clot medium (25% pool of human plasma +75% complete medium). Finally, 40 μl of calcium chloride (55 mg/ml) were added directly to the petri dishes in order to obtain the plasma clot. After ascertaining the homogeneous dispersion of the cells, the dishes were incubated at 37°C in a 5% CO_2 atmosphere. Four days later, clonal scoring was performed using an inverted microscope. Only clones with more than eight cells were counted, and the percentage of cloning inhibition was calculated, taking control experiments as maximum cell proliferation (100%).

Statistics

The differences between treated and control groups were analyzed by using Student's t-test. A P value of less than 0.05 was considered significant.

Results

Effect of I/C on Peritoneal Cell Counts

We checked the peritoneal cell (PC) counts to determine the possible presence of an inflammatory reaction caused by the I/C treatment that may explain an alteration on the cellular activity of peritoneal leukocytic cells. As shown in Table 1, two different behaviours were obtained in mice treated with I/C depending on the dispensed dose. Thus, treatment with 4 g/70 kg/day induced a decrease in PC counts with regard to that obtained in untreated control mice ($P<0.05$). On the other hand, treatment with 0.5 g/70 kg/day induced a remarkable increase ($P<0.05$) of peritoneal cell counts.

Table 1. Effect of imipenem/cilastatin treatment on peritoneal cells (PC) counts.

Dosage of I/C (g/70 kg/day)[a]	Treatment duration (days)	No. of PC/mouse (10^6)[b]
Control mice	7	4.02±1.79
4	7	1.43±0.25[c]
2	7	4.40±1.85
1	7	3.25±0.35
0.5	7	8.58±3.42[c]
Control mice	14	3.10±0.25
2	14	4.30±1.48

[a] Mice in the control group received sterile phosphate buffered saline. The assay was performed at 24 hours after the last I/C or PBS injection.
[b] Data represent the means±standard deviations for five mice.
[c] $P<0.05$.

Table 2. Effect of imipenem/cilastatin treatment on phagocytic activity measured by chemiluminescence (CL).

Dosage of I/C (g/70 kg/day)[a]	Treatment duration (days)	CL values expressed as cpm (10^3)[b]
Control mice	7	131.48± 28.41
4	7	69.57± 15.08[c]
2	7	173.69± 61.57
1	7	167.31± 83.13
0.5	7	258.31± 91.02[c]
Control mice	14	121.19± 16.23
2	14	330.01±175.96[d]

[a] Mice in the control group received sterile phosphate buffered saline. The assay was performed at 24 hours after the last I/C or PBS injection. Data represent the means±standard deviations for five mice.
[b] Values represent peaks of the chemiluminescence responses. Data represent the means±standard deviations for five mice.
[c] $P<0.05$.
[d] $P<0.01$.

Effect of I/C on Phagocytic Activity

Because phagocytic activity is an important antibacterial mechanism of the natural immune response, we examined the effect of I/C on the phagocytic cell activity, determined by CL. The results are shown in Table 2; the higher dose tested (4 g/70 kg/day) induced a pronounced suppression of peritoneal cell CL ($P<0.05$). In mice receiving 0.5 g/70 kg/day for 7 days, or with prolonged therapy at 2 g, CL values were increased by more than 100% with regards to the control group ($P<0.05$ and $P<0.01$, respectively).

Effect of I/C on IL-1 Production

In order to examine the effect of I/C treatment on the

Table 3. Effect of imipenem/cilastatin treatment on interleukin-1 (IL-1) production measured by the thymocyte costimulation bioassay.

Dosage of I/C (g/70 kg/day)[a]	Treatment duration (days)	Optical density at 570 to 630 nm (10^3)[b]
Control mice	7	279.00±12.72
4	7	296.40± 8.34[c]
2	7	277.77±17.84
1	7	265.00±38.28
0.5	7	315.15±28.92[c]
Control mice	14	280.94± 5.20
2	14	310.33±23.85[c]

[a] Mice in the control group received sterile phosphate buffered saline. The assay was performed at 24 hours after the last I/C or PBS injection. Data represent the means±standard deviations for five mice.
[b] Values represent the IL-1-induced thymocyte proliferation. Results represent the means±standard deviations for five mice, and of at least 3 replicates of each sample.
[c] $P<0.05$.

Table 4. Effect of imipenem/cilastatin treatment on natural killer activity of mononuclear splenic cells.

Dosage (g/70 kg/day)[a]	Treatment duration (days)	Percentage of cloning inhibition[b]
Control mice	7	30.15±9.00
4	7	61.16±5.20[c]
2	7	19.77±3.32
1	7	19.13±7.34
0.5	7	58.58±8.17[c]
Control mice	14	30.36±8.47
2	14	42.60±8.02[d]

[a] Mice in the control group received sterile phosphate buffered saline. The assay was performed at 24 hours after the last I/C or PBS injection. Data represent the means±standard deviations for five mice.
[b] Values represent the percentage of cloning inhibition of the NK-susceptible K562 cell line. Results represent the means±standard deviations for five mice, and in duplicate for each sample.
[c] $P<0.001$.
[d] $P<0.05$.

cytokine production by peritoneal cells, we tested the production of IL-1 by peritoneal leukocytes. In previous assays, it was established that the greater proliferative capacity of thymocytes was obtained with the 1:4 dilution of samples from peritoneal macrophage cultures. Thus, the results summarized in Table 3 show the proliferation of thymocytes co-cultured with 1:4 dilutions of samples obtained from supernatants of LPS-stimulated peritoneal macrophages. The dose of I/C influenced the effect of this drug on the IL-1 production. Thus, treatment of mice with 2 or 1 g/70 kg/day, did not modify significantly the IL-1 production in relation to that observed in untreated control mice. However, treatment with 4 or 0.5 g/70 kg/day, or therapy prolonged for 14 days, induced a significant enhancement in the production of IL-1 by peritoneal macrophages from antibiotic-treated mice, in relation to untreated control mice ($P<0.05$).

Effect of I/C Treatment on Natural Killer Activity

In order to examine the NK activity of mononuclear splenic cells that, together with phagocytes, play a fundamental role in the cellular mechanisms of the natural immune response, we tested the cytotoxic activity from splenic mononuclear leukocytes against K562 cell line. As shown in Table 4, mice treated with different doses of the antimicrobial agent show different behaviours in the natural killer activity of mononuclear splenic cells. Thus, this immune parameter was significantly increased ($P<0.05$) in mice treated with a prolonged therapy for 14 days, and with 4 or 0.5 g/70 kg/day during 7 days ($P<0.001$).

Discussion

Similar to other therapeutic drugs, antimicrobial agents and/or their metabolites may modify the interaction between bacterial pathogens and the immune response of the host. In recent years it has been described that the effector cells of the innate immune defense, as phagocytes and NK cells, and their mediators, have an important role, both in the clearance of bacterial agents and in the improvement of some clinical aspects of the infectious diseases including shock, respiratory distress syndrome and multiple organ failure[36,37].

The data presented here demonstrated the modification of several parameters of the natural immunity by I/C in a murine experimental model. The dose and time of administration of this antibacterial combination influenced its effect on the tested parameters. Obviously, this regime of treatment cannot completely imitate therapeutic conditions; particularly since a faster metabolism of the drug can be expected in mice[29]. Thus, it seems that the pharmacological exposure of the mice to the antibiotic used in these experiments was even less than under maximal therapeutic conditions.

Our results showed a dose-dependent modification of the phagocytic function, so that a high dose induced depression and low or long-term treatment caused a stimulation in the level of cellular activation of murine peritoneal phagocytic cells induced by zymosan and measured by CL assay. These results are contradictory

with those described by GROCHLA *et al.*[29] and GNARPE *et al.*[28], which showed no influence on the phagocytic activity determined by CL assay, using an experimental model of *in vitro* I/C treatment. The reason for this discrepancy between our results and those of these authors could be attributed to the fact that they used an *in vitro* experimental model with a phagocytic cell population isolated from untreated-human peripheral blood, mainly polymorphonuclear cells, that were pre-incubated with different concentrations of I/C, whereas in our *ex vivo* experimental model, the population was constituted of murine peritoneal phagocytes, macrophages (75%) and polymorphonuclear cells (15%). Moreover, the stimulatory effect of I/C on phagocytic activity of peritoneal leukocytes is in agreement with previous studies made by NUÑEZ *et al.*[30] in a murine experimental model with I/C-treatment by intramuscular route, that showed a significant increase in nitroblue tetrazolium reduction in macrophages stimulated with latex beads from treated mice. Since cilastatin is a potent inhibitor of dehydropeptidase I, thus reducing imipenem metabolism in the kidney, some metabolites of this antibiotic may exert certain biological activities that are not detectable by an *in vitro* assay. According to our data, the I/C treatment by i.p. route was a modifier of the phagocytic response: At a high dose, it induced a significative inhibition of phagocytic activity, stimulated by zymosan and measured by CL assay. At low and at human therapeutic dose administered during 14 days, I/C acted as a potent adjuvant agent of peritoneal phagocytic cell activity in an *ex vivo* murine experimental model.

The IL-1 is a cytokine produced by macrophages and epithelial cells, that induces the activation of macrophages and T-lymphocytes. Both at the highest and lowest doses, and in long-term administration, i.p. treatment with I/C induced a significant increase of IL-1 production by murine peritoneal macrophages. In the case of 0.5 g/70 kg/day during 7 days and 2 g/70 kg/day during 14 days, this increase correlated with the effect of the same treatment on the phagocytic activity, but the behaviour of the highest dose employed was contradictory with respect to both of the tested parameters. The dose-dependent modification of IL-1 production by antimicrobial agents has been described by ROCHE *et al.*[38] for quinolones with a *in vitro* model of human monocytes treatment.

The IL-1 production and the NK activity of murine cells have a similar behaviour in I/C treatment by i.p. route. In both cases high dose and long-term I/C administration induced a significant increase in such cellular activities, which in the case of NK activity was very strong. Since IL-2 is known to be a potent inducer of both proliferation and citolytic activity of NK cells, the effect of the antibiotic therapy as far as increase in NK cell activity could be due to the concurrent increase in IL-2 production by T cells, which is also promoted by high dose and long-term I/C administration to mice (data not shown).

The absence of a possible toxic effect of the highest I/C concentration on these cellular activities is remarkable, as opposed to that exerted on the phagocytic function as shown previously.

The duration of treatment and dose of I/C play a decisive role in the effect on immune functions. The immune parameters analyzed in treated mice do not undergo any statistically significant modification in the presence of I/C treatment at middling doses (1 and 2 g/70 kg/day) during 7 days, whereas the greater I/C dose used (4 g/70 kg/day) has an inhibitory effect on PC counts and phagocytic activity of peritoneal leukocytes, but it causes an increase on IL-1 production and NK activity of splenic cells from treated mice. The maximal effect on the stimulation of immune system was obtained at the lowest I/C dose (0.5 g/70 kg/day) used, which is compatible with the idea that this dose causes the maximal release of active bacterial factors that has been described by us in the case of the quinolone ciprofloxacin[6]. Based on these investigations, imipenem/cilastatin may be considered to be a potent broad-spectrum beta-lactam antibiotic for clinical application. Since it apparently does not impair the studied innate immune responses, at 1 or 2 g/70 kg/day, it should be especially suited for the therapy of systemic bacterial infections in immunocompromised patients. However, further studies should be initiated to determine the effect of a widespread use of this broad-spectrum antibiotic on the human immune responses.

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