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Modification of Immune Response in Mice by Ciprofloxacin

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Some studies have suggested that the addition of ciprofloxacin to in vitro cultures of mitogen-stimulated lymphocytes exerts inhibitory effects on cell cycle progression and immunoglobulin (Ig) secretion. We tested the effects of this drug on some immunity parameters in BALB/c mice. Mice treated intraperitoneally with ciprofloxacin (10 mg/kg of body weight per day) for 3 consecutive days and immunized with sheep erythrocytes 24 h after the last injection showed significant suppression of hemolytic IgG-forming cells, whereas the response of IgM-forming cells remained unchanged. When treatment lasted 7 days the response of antibody-forming cells was not modified. When the 3-day treatment was started at 24 h after immunization with sheep erythrocytes, the response of IgM-forming cells was increased, whereas the response of IgG-forming cells was suppressed. Delayed-type hypersensitivity to sheep erythrocytes was significatively suppressed in animals that received the 3-day treatment with ciprofloxacin and were immunized subcutaneously 24 h after the last injection. In vitro proliferation of lymphocytes from ciprofloxacin-treated mice in response to either lipopolysaccharide or concanavalin A was also suppressed. Leukopenia and an increase in the level of granulocyte-macrophage colony-forming cells in bone marrow were also observed in ciprofloxacin-treated mice. These results, together with those from other reports, suggest that modification of the biological responses by ciprofloxacin is a complex phenomenon that may be influenced by several factors.

The knowledge of possible influences of antibiotics on the immune response seems to be of great importance for the clinical approach to the process of therapy. This is specially interesting when antibiotics are given to immunosuppressed patients. The capacity to modify the immune response to unrelated antigens has been observed with numerous antibiotics, including β-lactams (8, 19), aminoglycosides (19), tetracyclines (10), rifamycins (2), and others.

The fluoroquinolones are antimicrobial agents with a broad antimicrobial spectrum (21). The primary target of fluoroquinolones is the bacterial DNA gyrase (topoisomerase II) (21). However, some inhibitory effects of these agents on the eucaryotic enzymes involved with DNA replication have been observed (9). A number of quinolones have been shown to possess genotoxicity in vitro, and this has been considered a possible explanation for some of the abnormal eucaryotic cellular responses obtained after treatment with these agents (9). Some of these effects have been observed on leukocytes. It has been found that ciprofloxacin penetrates leukocytes and reaches intracellular levels with bactericidal activity (4, 20). The incorporation of [^{3}H]thymidine into T lymphocytes incubated with phytohemagglutinin was increased in the presence of fluoroquinolones, but the progression of mitogen-stimulated lymphocytes through the S and G_2/M stages of the cell cycle and the secretion of immunoglobulins (Ig's) by pokeweed mitogen-stimulated B lymphocytes were inhibited (7).

Since some of the effects of fluoroquinolones on immunocompetent cells were obtained at pharmacological concentrations, it is desirable to examine the influences of these agents on the in vivo immunity functions. The effects of subcutaneous (s.c.) administration of ciprofloxacin on the immune response of mice has been examined by Roszkowski et al. (18). They observed a dose-dependent increase in the IgM- and IgG-specific responses of ciprofloxacin-treated mice. There is a discrepancy between these in vivo results and those of the in vitro assays mentioned above. In the study described in this report, we investigated the modification of the immune response in mouse by ciprofloxacin given by the intraperitoneal (i.p.) route. We describe several factors that influence the direction of the immune response modification by ciprofloxacin.

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MATERIALS AND METHODS

Animals. Six- to eight-week-old male BALB/c mice were provided by the Technical Services of the University of Granada (Granada, Spain). They were maintained under pathogen-free conditions with free access to sterile food and water.

Antibiotic regimen. Ciprofloxacin was kindly provided by Bayer S.A. (Barcelona, Spain). The dosages (ciprofloxacin, in milligrams per kilogram of body weight) were similar to those used in human therapy. Control mice received injections of sterile water.

Splenic response to SRBCs. Mice were immunized with sheep erythrocytes (SRBCs) by i.p. administration of 0.5 ml of 2% (vol/vol) SRBCs in phosphate-buffered saline (PBS). Five days after immunization, mice were sacrificed and weighed, and the spleens were also weighed for determination of the splenic index, which was the spleen weight corresponding to 25 g of body weight. Spleens were homogenized in Hanks' balanced salt solution (HBSS; Sigma Chemical Co., St. Louis, Mo.), and the number of cells secreting anti-SRBC antibodies was determined in hemolytic plaque assays (11). For the direct assay for the detection of cells producing anti-SRBC IgM antibodies, plastic petri dishes with bottom layers (1.4% agarose in PBS) received 2 ml of 0.6% agarose (type VII; Sigma) in HBSS, 0.1 ml of 20% SRBC in HBSS, and 0.1 ml of an appropriate dilution of splenocyte suspension. After 1 h of incubation at 37°C, each dish received 1.5 ml of guinea pig serum (Sigma), diluted 1:10 in HBSS, as a source of complement. Dishes were incubated at 37°C for 1 h and hemolytic plaques were counted. After determination of direct plaque-forming cells (PFCs), each dish received 1.5 ml of an appropriate dilution of rabbit antisera against mouse IgG (Sigma) in complement. An incubation at 37°C for 1 h produced new hemolytic plaques because of the cells that secreted anti-SRBC IgG antibodies, which were counted as indirect PFCs.

DTH response to SRBCs. To study the delayed-type hypersensitivity (DTH) response, mice were immunized by s.c. injection of 0.1 ml of 1% SRBCs in PBS. Six days after immunization, mice were lightly anesthetized with ether, and 0.01 ml of 10% SRBCs was injected intracutaneously into the ear with a 30-gauge needle as described by Athanassiades (1). The contralateral ear received an equal volume of PBS. The thickness of the ears was measured before injection and again at 24 h with a dial caliper gauge. Any increase in thickness of the saline-injected ear was subtracted from that of the ear injected with SRBCs.

Mitogen-induced proliferation of splenocytes. Ciprofloxacin-treated and control mice were sacrificed, and their spleens were removed aseptically and homogenized in sterile HBSS. Splenocytes were sedimented by centrifugation,

TABLE 1. Splenic response to SRBCs of mice treated i.p. with ciprofloxacin before immunization

Expt no. and dosage of ciprofloxacin (mg/kg of body wt/day)[a]	Splenic index[b]	No. of PFCs (10^3) per spleen	
		Direct (IgM)	Indirect (IgG)
Expt 1			
0	0.142 ± 0.0088[c]	43.7 ± 15.29	73.7 ± 16.53
5	0.141 ± 0.0129	40.0 ± 16.83	64.1 ± 14.05
Expt 2			
0	0.140 ± 0.0144	66.7 ± 15.60	83.5 ± 14.84
10	0.154 ± 0.0190	61.7 ± 13.62	47.7 ± 15.72[d]

[a] Treatment was continued for 3 days. Mice in the control groups (0 mg/kg) were given sterile water. At 24 h after the last ciprofloxacin or water injection, the mice were immunized with SRBCs.
[b] Values represent the splenic weight corresponding to 25 g of body weight.
[c] Data are given as the means ± standard deviations for eight animals.
[d] $P < 0.001$ with regard to the respective control group.

resuspended in erythrocyte lysing buffer (Sigma) for 10 min, washed in HBSS, and resuspended finally in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 μM 2-mercaptoethanol, penicillin G (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), 1 mM sodium pyruvate, and 2 mM L-glutamine (Sigma). Cell suspensions were adjusted to 10^7 viable cells per ml and distributed (100 μl per well) into 96-well tissue culture clusters with flat-bottom wells (Costar, Cambridge, Mass.). *Salmonella typhi* lipopolysaccharide (LPS; Sigma) was used at 10 μg/ml as the B-cell mitogen, and concanavalin A (ConA; Sigma) was used at 1 μg/ml as the T-cell mitogen. After incubation at 37°C in 5% CO_2 for 3 days, proliferation of cells was measured by colorimetric reading of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction as described by Mosmann (13).

Culture of granulocyte-macrophage progenitor cells. In vitro proliferation of granulocyte-macrophage progenitor cells was measured by using the clonal culture of mouse bone marrow cells in semisolid agarose medium (12). The serum of mice injected with LPS by the intravenous route (200 μg/kg) was used as a source of colony-stimulating factor (CSF). Bone marrow cells from ciprofloxacin-treated and control mice were obtained by flushing the femurs with RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum, penicillin G (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), 1 mM sodium pyruvate, and 2 mM L-glutamine (Sigma). Cell suspensions were adjusted to 10^6 viable cells per ml and were distributed into 35-mm-diameter tissue culture dishes (100 μl per dish). Each dish received CSF (100 μl) and 1 ml of supplemented medium containing 0.3% agarose. The cells were cultivated for 7 days at 37°C in 5% CO_2, and aggregates containing 40 or more cells were counted as colonies under an inverted microscope.

Cell counts. For peritoneal cell (PC) counts, mice were sacrificed and injected i.p. with 3 ml of HBSS. The peritoneal fluids were aspirated and viable cells were counted by trypan blue exclusion. The results were expressed as the number of viable PCs per mouse. For peripheral blood leukocyte (PBL) counts, blood samples were obtained from the retro-orbital plexus. Blood was immediately diluted 1:10 with Türk solution (0.1% crystal violet in 1% acetic acid), and nucleated cells were counted. The results were expressed as the number of PBLs per mm^3 of blood. For bone marrow nucleated cell counts, cells were obtained by flushing the femurs with HBSS and were counted by trypan blue exclusion. The results were expressed as the number of viable bone marrow nucleated cells per femur.

Statistical analysis. 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

Effects of ciprofloxacin on splenic response to SRBCs. We tested the splenic response to SRBCs in mice treated with ciprofloxacin by the i.p. route. Since Roszkowski et al. (18) described a dose-dependent effect of ciprofloxacin on the immune response when it is given by the s.c. route, we assayed two different doses (5 and 10 mg/kg of body weight in a single dose per day) given by the i.p. route. Treatment was continued for 3 consecutive days. Immunization with SRBCs was performed 24 h after the last ciprofloxacin injection, and the PFC response was measured at 5 days postimmunization. The results are presented in Table 1. Neither the splenic index nor direct (IgM) PFC counts were significantly modified by either ciprofloxacin dose. However, treatment with a daily dose of 10 mg/kg suppressed nearly 43% ($P < 0.001$) of the indirect (IgG) PFC response, whereas the direct PFC response was not modified. The total number of PFCs (direct plus indirect) was suppressed by 27% in animals treated with 10 mg/kg/day.

Because the route of administration is known to affect the direction of the effects of immunomodulating agents, we next examined the effects on both direct and indirect PFC responses when ciprofloxacin was given by the s.c. route as described by Roszkowski et al. (18). Mice were injected twice a day at 10-h intervals for 7 consecutive days with 10 mg/kg/day. SRBCs were given 24 h after the last injection. There were no significant differences between the PFC responses in ciprofloxacin-treated mice and untreated controls (data not shown). To facilitate the comparison of both routes of administration, we studied PFC responses in mice that received 10 mg of ciprofloxacin per kg/day by the i.p. route for 7 consecutive days. The effects of s.c. and i.p. administrations were studied in separate experiments because the respective control mice were given sterile water by the s.c. or the i.p. route. In this experiment we repeated the i.p. treatment with 10 mg/kg for 3 days; both groups of mice were immunized with SRBCs 24 h after the last ciprofloxacin injection. To determine the effects of ciprofloxacin given after immunization we also included another group of mice that were immunized with SRBCs 24 h before the ciprofloxacin treatment was started. The results are presented in Table 2. Ciprofloxacin given before the administration of SRBCs did not significantly modify either the splenic index or the direct PFC response, but the 3-day treatment suppressed 48% ($P < 0.005$) of the indirect PFC response. These results indicate that the duration of the treatment is important in determining the modification of the PFC response by

TABLE 2. Effects of duration and time of i.p. treatment with ciprofloxacin on splenic response to SRBCs of mice

Treatment duration (days)	Treatment time[a]	Splenic index[b]	No. of PFCs (10^3) per spleen	
			Direct (IgM)	Indirect (IgG)
None	None	0.146 ± 0.0121[c]	35.9 ± 13.82	53.0 ± 11.02
3	After SRBCs	0.169 ± 0.0250[d]	60.2 ± 14.62[e]	43.0 ± 8.41[d]
3	Before SRBCs	0.163 ± 0.0229	31.4 ± 18.53	27.5 ± 13.07[e]
7	Before SRBCs	0.156 ± 0.0141	39.6 ± 11.80	45.4 ± 14.45

[a] The treatment time is in relation to the day of immunization with SRBCs (day 0). The treatment with ciprofloxacin (10 mg/kg) started on day +1 (after administration of SRBCs), on day −3 (before administration of SRBCs), and on day −7 (before administration of SRBCs). Mice in the control groups were given sterile water.
[b] See footnote *b* of Table 1.
[c] Data are given as the means ± standard deviations for 7 animals.
[d] $P < 0.05$.
[e] $P < 0.005$.

TABLE 3. DTH response to SRBCs of mice treated i.p. with ciprofloxacin before immunization

Expt no. and dosage of ciprofloxacin (mg/kg of body wt/day)[a]	Increase in ear thickness (0.01 mm)[b]
Expt 1	
0	13.9 ± 2.26
5	12.0 ± 3.02
Expt 2	
0	14.8 ± 2.96
10	11.4 ± 4.29[c]

[a] See footnote *a* of Table 1.
[b] Results represent the means ± standard deviations for 10 mice.
[c] $P < 0.05$ with regards to the respective control group.

ciprofloxacin. Ciprofloxacin given after the administration of SRBCs increased the splenic index by 15% ($P < 0.05$), increased the direct PFC response by 67% ($P < 0.005$), and suppressed the indirect PFC response by 19% ($P < 0.05$).

Effects of ciprofloxacin on DTH response to SRBCs. Because the greatest suppression of the PFC response was obtained when ciprofloxacin was given by the i.p. route, before the administration of SRBCs, and for 3 consecutive days, we examined the effect of this treatment on the DTH response to SRBCs. The results in Table 3 show that a dosage of 5 mg of ciprofloxacin per kg/day did not change the DTH response, whereas a dosage of 10 mg/kg/day suppressed this response by 23% ($P < 0.05$).

Effects of ciprofloxacin on splenocyte response to mitogens. We examined the effect of the 3-day treatment with ciprofloxacin by the i.p. route on the response of lymphocytes to mitogens. The proliferation of splenocytes from ciprofloxacin-treated mice in response to LPS and ConA was significantly suppressed compared with that of splenocytes from untreated control mice (Table 4). Treatment of mice with 5 mg of ciprofloxacin per kg/day suppressed the response of splenocytes to LPS by 25% ($P < 0.005$) and the response to ConA by 27% ($P < 0.0005$). Treatment with a dosage of 10 mg of ciprofloxacin per kg/day suppressed the response to each mitogen by greater than 55% ($P < 0.0005$).

Modification of myelopoiesis by ciprofloxacin. The colony count obtained from bone marrow cells cultured in the presence of CSF represents the number of granulocyte-macrophage progenitor cells, and changes in this parameter caused by a treatment reflect the effects on bone marrow function. The number of colonies formed by myeloid precursors in mice that received 5 mg of ciprofloxacin per kg/day by the i.p. route was increased by 50% ($P < 0.005$) in relation to that in untreated control mice (Table 5). When the ciprofloxacin dose was increased to 10 mg/kg, the colony number was increased by 37% ($P < 0.05$) with regard to that for untreated control mice. The difference in colony numbers between both ciprofloxacin-treated groups was not significant.

TABLE 4. Effects of i.p. treatment with ciprofloxacin on splenocyte response to mitogens

Dosage of ciprofloxacin (mg/kg of body wt/day)[a]	Optical density at 570 to 630 nm in cultures stimulated with[b]:	
	LPS (10 μg/ml)	ConA (1 μg/ml)
0	0.249 ± 0.0667	0.254 ± 0.0603
5	0.187 ± 0.0383[c]	0.185 ± 0.0429[d]
10	0.112 ± 0.0541[d]	0.113 ± 0.0660[d]

[a] Treatment was continued for 3 days. Mice in the control groups (0 mg/kg) were given sterile water. The assay was performed at 24 h after the last ciprofloxacin or water injection.
[b] Background values, obtained from wells with cells with no mitogen, were taken as blank. Results represent the means ± standard deviations of at least 12 replicates.
[c] $P < 0.005$ (in relation to the control group).
[d] $P < 0.0005$ (in relation to the control group).

TABLE 5. Effect of i.p. treatment with ciprofloxacin on colony formation by granulocyte-macrophage PCs

Dosage of ciprofloxacin (mg/kg of body wt/day)[a]	Colony no./10^5 bone marrow cells[b]
0	156 ± 21.8
5	234 ± 50.4[c]
10	214 ± 60.2[d]

[a] See footnote *a* of Table 4.
[b] Data represent means ± standard deviations for six mice.
[c] $P < 0.005$ (in relation to the control group).
[d] $P < 0.05$ (in relation to the control group).

Effects of ciprofloxacin on PC, PBL, and BMC populations. The finding that i.p. treatment with ciprofloxacin enhanced myelopoiesis raised the possibility that this drug has a cytotoxic effect on leukocytes or their progenitors. To examine this possibility we checked PBL and bone marrow cell (BMC) counts at 24 h after the last ciprofloxacin injection. We also checked PC counts, since information about the possibility of an inflammatory reaction caused by ciprofloxacin may be useful for interpreting data from PFC assays. The results in Table 6 show that the 3-day treatment with 10 mg/kg/day caused significant modifications in these three parameters. The PC count increased by 36% ($P < 0.05$), the PBL count decreased by 21% ($P < 0.05$), and the BMC count increased by more than 65% ($P < 0.05$).

DISCUSSION

The data presented here demonstrate the modification of the immune response by ciprofloxacin in a murine experimental model. The route of administration, the duration of the treatment, the time of administration with regard to antigen administration, and the dose of ciprofloxacin influenced the effect of this drug on the immune functions.

A 3-day treatment with ciprofloxacin by the i.p. route before SRBC administration suppressed both the PFC (IgG) and DTH responses to SRBCs. Splenocyte proliferation in response to the mitogens LPS and ConA was also suppressed. These findings are in agreement with the results of experiments in which immunocompetent cells were exposed to ciprofloxacin in vitro. Forsgren et al. (7) demonstrated that the addition of a clinically achievable concentration of ciprofloxacin to lymphocyte cultures caused a 50% decrease in Ig pro-

TABLE 6. Effect of i.p. treatment with ciprofloxacin on PC, PBL, and BMC counts

Dosage of ciprofloxacin (mg/kg of body wt/day)[a]	No. of PCs/mouse (10^6)	No. of PBLs/mm^3 (10^3)	No. of BMCs/femur (10^6)
0	2.2 ± 0.60[b]	8.1 ± 1.79	3.2 ± 0.90
10	3.0 ± 0.81[c]	6.3 ± 1.46[c]	5.3 ± 2.41[c]

[a] See footnote *a* of Table 4.
[b] Data represent the means ± standard deviations for six mice.
[c] $P < 0.05$ (in relation to the control group).

duction by B cells in T-cell-dependent and -independent in vitro assays. Although it has been shown that the addition of ciprofloxacin to culture medium increased the incorporation of [^{3}H]thymidine into the DNA of mitogen-stimulated lymphocytes (5, 6), this has been explained as a consequence of the inhibition of de novo pyrimidine synthesis in ciprofloxacin-treated cells (6). Thus, the addition of ciprofloxacin in vitro inhibited the progression of mitogen-stimulated lymphocytes through the cell cycle (7). This is in agreement with our findings that splenocytes from ciprofloxacin-treated mice had decreased responses to mitogens, since the colorimetric method that we used did not measure the incorporation of nucleotides into DNA but the activity of dehydrogenase enzymes in the active mitochondria of living cells (13).

There is a discrepancy between our results, which showed a supression of the indirect PFC response in mice treated with ciprofloxacin by the i.p. route and those of Roszkowski et al. (18), which showed a potentiation of both the direct and the indirect PFC responses in mice treated by the s.c. route. In an attempt to explain these disparities, we reproduced the protocol of Roszkowski et al. (18), but in our hands ciprofloxacin given by the s.c. route for 7 days did not modify either PFC response. The reasons for these disagreements between the results obtained with the same protocol could relate to possible differences between the indigenous microflora of BALB/c mice used in each one of the two studies, because ciprofloxacin is bactericidal with a broad spectrum of activity (3), and the ciprofloxacin-mediated killing of indigenous bacteria in the intestinal tract may result in the release of bacterial fractions with immunomodulating properties. However, differences between the results of our assays performed by different experimental protocols could be attributed to other factors such as the route of administration or the treatment duration. Our data showing that a 7-day treatment by the i.p. route also had no effect on the PFC responses suggest that the choice between the i.p. or the s.c. route does not have a significant influence on immunomodulation by ciprofloxacin. However, the duration of i.p. treatment was a decisive factor: the 3-day treatment suppressed the PFC (IgG) response, but the 7-day treatment had no effect. It is possible that the suppressive activity of ciprofloxacin induces some compensatory mechanisms that abrogate suppression in longer treatments, in the same way that ciprofloxacin-mediated leukopenia was linked to an increase in myelopoiesis. Also, if bacterial fractions play a role in immunosuppression by ciprofloxacin, changes in the indigenous microflora because of a longer treatment may limit this role.

Ciprofloxacin given by the i.p. route induced a moderate influx of inflammatory cells into the peritoneal cavity. Changes in the peritoneal cell populations before i.p. administration of antigen may be a cause for modifications of the PFC responses. However, suppression of the DTH response to SRBC given by the s.c. route demonstrates that the peritoneal inflammation does not explain the modification of the immune response by ciprofloxacin. Moreover, PFC responses were also modified by ciprofloxacin when it was given after SRBC administration. In this case there was a modulation of the response, because the direct PFC response (IgM) increased, whereas the indirect PFC response (IgG) decreased and the total number of PFCs (direct plus indirect) was slightly increased.

Since the switch of the Ig heavy chain is driven by helper T cells (15), it appears that ciprofloxacin treatment resulted in the suppression of T-cell functions such as ConA-induced lymphoproliferation, the DTH response, and help for the IgG response.

The stimulation of granulocyte-macrophage progenitor cells in ciprofloxacin-treated mice may be interpreted as a compensatory mechanism against a possible cytotoxic effect of the drug on leukocytes or their progenitors. Although leukopenia has been reported as a rare side effect of ciprofloxacin treatment in humans (16), our data showed that mice developed leukopenia as a consequence of the i.p. treatment with ciprofloxacin. When leukopenia was observed, the number of nuclear BMCs increased. This may be a result of the stimulation of progenitor cells that we measured by the CFU assay. A similar compensatory mechanism was observed when mice were treated with myelotoxic agents (17). In disagreement with our results, Pallavicini et al. (14) found that human myelopoiesis was not affected by the presence in vitro of the quinolones ofloxacin and pefloxacin. Besides the possibility of differences between the side effects caused by different quinolones, the discrepancy may be due to differences between in vitro and in vivo studies. Thus, up to 20% of each ciprofloxacin dose is metabolized in vivo (22), and ciprofloxacin metabolites may exert some biological activities that are not detectable by in vitro assays. On the other hand, the possible release of bacterial factors, from indigenous bacteria killed by the drug, with activity on myelopoiesis should not be neglected. Our data suggest that the maximal effect on granulocyte-macrophage progenitor cells is attained at the lower ciprofloxacin dose, which is compatible with the idea that this dose causes the maximal release of active bacterial factors. In any event, we believe that our experimental design represents a more suitable approximation of the clinical situation than the in vitro assays.

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