**N-Formyl-Methionyl-Leucyl-Phenylalanine Inhibits both Gamma Interferon- and Interleukin-10-Induced Expression of FcγRI on Human Monocytes**

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Three different classes of receptors for the Fc portion of immunoglobulin G (FcγRs), FcγRI, FcγRII, and FcγRIII, have been identified on human leukocytes. One of them, FcγRI, is a high-affinity receptor capable of induction of functions that include phagocytosis, respiratory burst, antibody-dependent cell-mediated cytotoxicity (ADCC), and secretion of cytokines. This receptor is expressed on mononuclear phagocytes, and this expression is regulated by cytokines and hormones such as gamma interferon (IFN-γ), IFN-β, interleukin-10 (IL-10), and glucocorticoids. We have recently demonstrated that the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) is capable of inducing a time-dependent downregulation of both FcγRIIIB and FcγRII in human neutrophils, altering FcγR-dependent functions. Considering the biological relevance of the regulation of FcγRI, we investigated the effect of FMLP on the overexpression of FcγRI induced by both IFN-γ and IL-10 on human monocytes. We demonstrate that FMLP significantly abrogated IFN-γ- and IL-10-induced FcγRI expression, although its basal level of expression was not altered. However, other IFN-γ-mediated effects such as the overexpression of the major histocompatibility complex class II antigens and the enhancement of lipopolysaccharide-induced secretion of tumor necrosis factor alpha were not affected by FMLP treatment. The formyl peptide completely inhibited the IFN-γ- and IL-10-induced enhancement of ADCC and phagocytosis carried out by adherent cells. The inhibitory effect of FMLP on FcγRI upregulation could exert an important regulatory effect during the evolution of bacterial infections.

The receptors for the Fc portion of immunoglobulin G (IgG) (FcγRs) are widely distributed in cells of the immune system and have been considered a link between cellular and humoral immunity by serving as a bridge between antibody specificity and effector cell functions. They also enable monocytes/macrophages and neutrophils to exert regulatory functions, as well as to trigger a variety of cytotoxic mechanisms (8, 9).

Three different classes of FcγRs, FcγRI, FcγRII, and FcγRIII, have been identified on human leukocytes through the use of monoclonal antibodies (MAbs), functional analysis, and the molecular characterization of the primary structure. One of them, FcγRI, is a high-affinity receptor capable of induction of phagocytosis, clearance of immune complexes, respiratory burst, antibody-dependent cell-mediated cytotoxicity (ADCC), enhancement of antigen presentation, and secretion of inflammatory cytokines (28). This receptor is expressed primarily on mononuclear phagocytes, and this expression is regulated by cytokines and hormones such as gamma interferon (IFN-γ) (24), IFN-β (29), interleukin-10 (IL-10) (30), granulocyte colony-stimulating factor (5), and glucocorticoids (13).

The monocyte/macrophage activation by IFN-γ is characterized by a pronounced increase in FcγRI expression that frequently results in a concomitant enhancement of several FcγR-dependent functions (7, 14, 24). This effect has also been shown in vivo on circulating monocytes of cancer patients who received IFN-γ treatment (20). Surprisingly, a similar upregulation can be induced by IL-10 (30), a cytokine known for its anti-inflammatory activity. On the other hand, IL-1β and IFN-β downregulate IFN-γ-induced FcγRI expression (3, 29), while corticoids can either enhance or inhibit this FcγRI expression, depending on whether cells have been primed with IFN-γ (21).

We have recently demonstrated that the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP), a prototype of N-formyl peptides, is capable of inducing a time-dependent downregulation of both FcγRIIIB and FcγRIII in human neutrophils, altering different FcγR-dependent functions (1). These N-formyl peptides are released at the site of infection as a consequence of bacterial destruction by the immune system or by autolysis.

Considering the biological relevance of FcγRI, the regulation of this receptor would be of fundamental importance in different immunological mechanisms. Therefore, taking into account our previous results and the pleiotropic and proinflammatory activities of the formyl peptides on the immune system (26, 27), we investigated the possible effect of FMLP on the induction of FcγRI by both IFN-γ and IL-10 on human monocytes. In addition, we studied other important effects induced by IFN-γ such as the overexpression of molecules of the major histocompatibility complex (MHC) class II and the...
enhancement of lipopolysaccharide (LPS)-induced secretion of tumor necrosis factor alpha (TNF-α).

MATERIALS AND METHODS

Reagents. FMLP, Ficol 400, tissue culture medium (RPMI 1640 medium), LPS from Escherichia coli O111:B4, anti-sheep red blood cell (anti-SRBC) antibody, human recombinant IFN-γ, human recombinant IL-10, anti-IL-1β polyclonal antibody, and catalase were obtained from Sigma, St. Louis, Mo. Fluorescein isothiocyanate-labeled anti-FcγRII (clone 10.1) and FcγRII (clone 3G8), phycoerythrin-labeled anti-FcγRII (clone 1C155) MAbs, and mouse IgG1 (clone MOPC21) isotype control antibody were obtained from CALTAG Laboratories, Burlingame, Calif. Phycoerythrin-labeled anti-CD14 (clone RMO52) and mouse IgG2a (clone U7.27) isotypes were from Immunotech, Marseille, France. Fluorescein isothiocyanate-labeled anti-HLA-DR (clone L243) was purchased from Becton Dickinson, San Jose, Calif.

Preparation of human mononuclear cells. Fresh human blood was obtained by venipuncture from healthy adult volunteers and was placed in citrate (84%)–dextrose (84%)–adenine (0.003%). Blood was diluted 1:2 with saline, layered on a Ficoll-Hypaque cushion, and centrifuged at 400 × g for 25 min as described previously (6). Peripheral blood mononuclear cells (PBMCs) were collected, washed, and resuspended in RPMI 1640 medium with 10% heat-inactivated fetal calf serum and 50 μg of gentamicin per ml. The viability of the mononuclear cells was always more than 98%, as measured by the trypan blue exclusion test.

FMLP treatment. FMLP was diluted in dimethyl sulfoxide at a concentration of 0.1 M. The subsequent dilutions of FMLP were made in saline. PBMCs were incubated with FMLP at a concentration of 1 μM in polypropylene tubes unless stated otherwise. After 1 h of incubation at 37°C in 5% CO₂, the cells were washed and incubated with either 240 U of IFN-γ per ml or 100 U of IL-10 per ml for 24 h. After this period the cells were used as effector cells in ADCC and phagocytosis assays or were used for flow cytometry studies.

ADCC assay. When total PBMCs at 4 × 10⁶ cells/ml (100 μl) were used as effector cells, ADCC assay was performed in 96-well polyurethane plates. The adherent PBMC population was obtained from 4 × 10⁶ PBMCs/ml that had been left to adhere in 96-well round-bottom plates for 1 h at 37°C. After that, the nonadherent cells were removed and ADCC assay was performed. In both cases the cells were incubated with 10⁷ 51Cr-labeled chicken red blood cells (51CRBCs) and a suboptimal concentration of rabbit IgG anti-CRBCs as described previously (12). After 18 h of incubation at 37°C in 5% CO₂, the culture plate was centrifuged and the percentage of cytotoxicity was calculated as follows: percent ADCC = (amount of 51Cr released into the supernatant × 100)/total amount of radioactivity. This value was corrected by subtracting the percentage of 31Cr released in the absence of antibody (spontaneous release). Quadruplicates were set up for each sample.

Phagocytosis assay. One hundred microliters of human mononuclear cells at a concentration of 2 × 10⁶ cells/ml were seeded onto slides and allowed to adhere for 1 h at 37°C. This procedure was repeated once under the same conditions. Then, the cells were rinsed with RPMI 1640 medium to remove nonadherent cells and incubated with 100 μl of a suspension of IgG-sensitized 1% SRBCs for 30 min at 37°C. After this period, nonphagocytosed SRBCs were removed by hypotonic lysis and the cells were stained with May-Grünwald and Giemsa stains. At least 100 cells per donor were counted. The phagocytic index represents the percentage of monocytes/macrophages containing erythrocytes (percent phagocytosis) (18). The ingestion of unsensitized SRBCs (control group) was less than 2% in all cases.

To evaluate the percentage of phagocytosis, 0.5 ml of mononuclear cells at 2 × 10⁶ cells/ml were allowed to adhere to flat-bottom tissue culture chambers (Lab-Tek, Naperville, Ill.). This procedure was repeated once under the same conditions. Then, a suspension of 100 μl of IgG-coated 51Cr-labeled 1% SRBCs was added and the mixture was incubated for 30 min at 37°C. After this period, the cells were washed with RPMI 1640 medium to remove the nonphagocytosed SRBCs, and membrane-adherent but nonphagocytosed SRBCs were eliminated by hypotonic lysis. The cells were washed and removed from the chambers with 0.5% deoxycholate, and then phagocytosis was evaluated by counting the amount of radioactivity in the pellet. This value was corrected by subtracting the percentage of uptake of unsensitized 51Cr-labeled SRBCs by phagocytic cells (spontaneous phagocytosis).

Measurement of TNF-α. TNF-α-sensitive actinomycin D-treated murine L-929 fibroblasts were used to quantify TNF-α activity by the method of Wang et al. (31). One milliliter of mononuclear cells at a concentration of 10⁶ cells/ml was incubated with different agonists and LPS (1 μg/ml) for 24 h at 37°C in 5% CO₂. After this period, the cells were centrifuged and supernatants were removed for TNF-α evaluation. The amount of TNF-α secreted by cells not treated with LPS was negligible. The results are expressed as 50% lytic units.

Flow cytometry. After different treatments, 10⁶ mononuclear cells were washed and incubated with the MAbs indicated above. The cells were washed and resuspended in ISOFLOW, and flow cytometry was performed on a fluorescence-activated cell sorter analyzer (Becton-Dickinson Immunocytometry Systems, San Jose, Calif.). The results were expressed as the percentage of the median fluorescence intensity (MFI) for CD14-positive cells (CD14⁺) compared with that for nontreated groups (controls). In some experiments permeabilization of PBMCs for staining of intracellular FcγRII was performed. Briefly, PBMCs were treated with FMLP or IFN-γ, or both. After 24 h of incubation, the cells were fixed for 30 min with cold 0.5% paraformaldehyde. Aliquots of these cells were permeabilized in a buffer containing 0.04% saponin, 0.1% ovalbumin, phosphate-buffered saline, and glycine and were then stained with the MABs. Parallel aliquots of fixed PBMCs were treated identically but without saponin for nonpermeabilized controls. Background fluorescence intensity was obtained by using mouse IgG1 isotype-matched control antibody.

Statistical analysis. All experiments were repeated at least five times, and the results of a representative experiment are presented unless stated otherwise. The statistical significance of the results was calculated by the nonparametric Mann-Whitney or Wilcoxon test (two tailed).

RESULTS

FMLP downregulates IFN-γ-induced expression of FcγRI. As described previously (24), FcγRI, which is constitutively expressed on human monocytes, was significantly upregulated by 240 U of human recombinant IFN-γ per ml after 24 h of incubation. However, incubation of the cells with 1 μM FMLP for 1 h, which did not modify the basal level of expression of FcγRI, exerted a drastic inhibition of the FcγRI upregulation induced by IFN-γ (Fig. 1). The untreated cells (controls) were incubated overnight under the same conditions used for the experimental groups. The magnitude of the FcγRI expression after FMLP treatment was different with PBMCs from various cell donors, as follows. After treatment with FMLP, IFN-γ, or both FMLP and IFN-γ, the magnitudes of FcγRI expression were 96 ± 2, 238 ± 32 (significantly different [P < 0.0002] from that for the control), and 130 ± 12 (significantly different [P < 0.05] from those for the control and IFN-γ treatment), respectively (the data are percent MFI relative to the control ± standard errors for the CD14⁺ population of PBMCs [n = 12]). In this experiment PBMCs (10⁶/ml) were incubated with medium or 1 μM FMLP for 1 h. Then, the cells were washed and incubated with medium or IFN-γ (240 U/ml) for 24 h. After this period, the PBMCs were stained with anti-FcγRI and anti-CD14 antibodies. Statistical significance was calculated by Mann-Whitney test (two tailed). This variability can be attributed to the susceptibilities of the individual donors to FMLP or IFN-γ and/or to the heterogeneity of monocyte subpopulations. These results were observed by using concentrations of FMLP as low as 0.01 μM (percent MFIs of that for the control were as follows: IFN-γ, 284%; 0.01 μM FMLP plus IFN-γ, 173%; 0.01 μM FMLP, 109% [the data are representative of four experiments]). Concentrations of 1 nM had no effect (data not shown). It is noteworthy that in these experiments PBMCs were exposed for 1 h to FMLP, and after this period the cells were washed and incubated with IFN-γ for 24 h in the absence of FMLP. Similar results were achieved by incubation of human mononuclear cells with 1 μM FMLP for 5 min instead of 1 h (n = 3). Dimethyl sulfoxide alone at the same concentration used for the dilution of FMLP (see Mate-
The maximal expression of FcγRI was previously shown in human neutrophils for FcγRIIa (1, 2). Regarding the intracellular pool of FcγRI, we found that FMLP did not significantly modify the amount of intracellular FcγRI compared to that in IFN-γ-treated cells, indicating that transcription and translation of FcγRI may not be affected and suggesting a posttranslational event (percent MFIs of that for the control corresponding to the intracellular pool were as follows: IFN-γ, 97%; 1 μM FMLP plus IFN-γ, 91% [data are representative of three experiments]).

LPS, a frequent contaminant in cell cultures, is also able to induce the downregulation of FcγRI through the secretion of IL-1β (3). However, experiments carried out in the presence of anti-IL-1β antibody at a concentration capable of inhibiting 20 U of IL-1β per ml (7.5 ng/ml) did not modify the effect of 1 μM FMLP on FcγRI downregulation, discarding any effect due to IL-1β.

**FMLP downregulates IL-10-induced expression of FcγRI.** It has previously been reported that IL-10 is also capable of inducing an increase in the level of expression of FcγRI on human monocytes (30). As shown in Fig. 2 and below, preincubation of PBMCs with 1 μM FMLP for 1 h was also able to inhibit the FcγRI upregulation induced by 100 U/ml of IL-10. After treatment with 1 μM FMLP, IL-10, 1 μM FMLP plus IL-10, 0.01 μM FMLP, and 0.01 μM FMLP plus IL-10, the magnitudes of FcγRI expression were 107 ± 5 (n = 7), 190 ± 17 (n = 9), 142 ± 9 (n = 9), 116 ± 2 (n = 9), and 135 ± 8 (n = 9) (the data are percent MFI relative to the control ± standard errors for the indicated numbers of experiments). Data for the IL-10 treatment were significantly different (P < 0.0002) from those for the control. Data for the 1 μM FMLP plus IL-10 and
TABLE 1. Effect of FMLP on the IFN-γ-induced HLA-DR expression and enhancement of TNF-α secretion

| Treatment          | HLA-DR expressiona (% MFI of control ± SEM) | Enhancement of TNF-α secretionb (LU50/ml) |
|--------------------|---------------------------------------------|------------------------------------------|
| FMLP               | 136 ± 29                                    | 169 ± 72                                 |
| IFN-γ              | 439 ± 42                                    | 804 ± 268                                 |
| FMLP and IFN-γ     | 488 ± 53                                    | 960 ± 535                                 |

a PBMCs (10⁶ cells/ml) were treated with medium or 1 μM FMLP for 1 h. Then, the cells were washed and incubated with medium or IFN-γ (240 U/ml) for 24 h. After this period, the PBMCs were stained with anti-HLA-DR and anti-CD14 antibodies. Statistical significance was calculated by the Mann-Whitney test (two tailed). Data are medians of nine experiments. LU50, 50% lytic units.

b PBMCs (10⁶ cells/ml) were incubated with medium or 1 μM FMLP for 1 h. Then, the cells were washed and incubated with LPS (1 μg/ml) plus medium or LPS (1 μg/ml) plus IFN-γ (240 U/ml) for 24 h. After this period, supernatants were collected for TNF-α evaluation (see Materials and Methods). Statistical significance was calculated by the Mann-Whitney test (two tailed). Data are medians of six experiments.

| Treatment          | Enhancement of TNF-α secretionb (LU50/ml) |
|--------------------|------------------------------------------|
| FMLP               | 439 ± 42                                 |
| IFN-γ              | 804 ± 268                                 |
| FMLP and IFN-γ     | 960 ± 535                                 |

0.01 μM FMLP plus IL-10 treatments were significantly different (P < 0.05) from those for the control and were significantly different (P < 0.05) from those for the IFN-γ treatment. PBMCs (10⁶ cells/ml) were incubated with medium, 1 μM FMLP, or 0.01 μM FMLP for 1 h. Then, the cells were washed and incubated with medium or IL-10 (100 U/ml) for 24 h. After this period, PBMC were stained with anti-FcγRI and anti-CD14 antibodies. Statistical significance was calculated by the Mann-Whitney test (two tailed). Similar results were obtained with 200 or 400 U of IL-10 per ml at either 24 or 48 h of incubation (data not shown).

FMLP does not alter other immunoregulatory actions of IFN-γ. It is well known that IFN-γ also enhances the expression of MHC class II antigens and the release of TNF-α from LPS-stimulated mononuclear phagocytes (10). With the pose of investigating whether FMLP was also capable of modifying these IFN-γ-induced effects, PBMCs were incubated with 1 μM FMLP for 1 h and were then washed and treated with LPS (1 μg/ml) plus 240 U of IFN-γ per ml for 24 h at 37°C. After that, the cells were centrifuged and the supernatants were collected for TNF-α evaluation. On the other hand, after FMLP treatment, PBMCs were incubated with IFN-γ for 24 h and MHC class II antigen expression was analyzed by flow cytometry. As depicted in Table 1, neither the overexpression of MHC class II antigens nor the enhancement of TNF-α secretion induced by IFN-γ was modulated by FMLP.

Effect of FMLP on ADCC and phagocytosis. The upregulation of FcγRII induced by IFN-γ could correlate with FcγR-dependent functions such as ADCC and phagocytosis (24). As shown in Figure 3, the enhancement of ADCC by IFN-γ 240 U/ml was completely inhibited by 1 μM FMLP in total PBMCs. However, when ADCC was carried out with adherent cells, an enriched monocyte preparation, FMLP inhibited the enhancement of cytotoxicity only in the presence of catalase. This means that the adherence of monocytes to the plates activates these cells, which exert part of their cytotoxic effect by oxygen radicals, masking the conventional ADCC (essentially independent of oxygen radicals), as shown previously (17).

Figure 4 also indicates that, as in IFN-γ-treated cells, FMLP inhibited the ADCC enhancement induced by 100 U of IL-10 per ml in the adherent cell population only in the presence of catalase.

Figure 5 shows that while phagocytosis by mononuclear cells was increased by incubation with 240 U of IFN-γ per ml, the uptake of sensitized 51Cr-labeled SRBCs was reduced when these cells were previously treated with 1 μM FMLP for 1 h. In addition, similar results were obtained when the phagocytic index, a method that allows analysis of individual cells, was considered (the phagocytic indices were as follows: for untreated PBMCs, 52%; for IFN-γ-treated PBMCs, 78%; for FMLP plus IFN-γ-treated PBMCs, 46%; for IFN-γ versus...
FMLP plus IFN-γ treatment, $P < 0.05$ ($n = 4$) by the Mann-Whitney test (two-tailed).

**DISCUSSION**

When bacteria are destroyed either by autolysis or by cytotoxic mechanisms, formyl peptides (26, 27) are released into the milieu, establishing a close contact with neutrophils and monocytes, the most representative cells in acute and chronic inflammation, respectively (15).

The aim of the study described here was to examine the effect of FMLP, a prototype of $N$-formyl peptides, on the expression of the high-affinity Fcγ receptor (FcγRI), which is a key effector molecule in monocyte/macrophage function. We were able to demonstrate that FMLP induced a significant and dose-dependent downregulation of IFN-γ-induced FcγRI expression. As shown in Fig. 1, although this formyl peptide strongly inhibited the IFN-γ-dependent FcγRI expression on human monocytes, the basal level of expression of this receptor was not altered by FMLP treatment.

The FMLP downregulatory effect was not observed when naive PBMCs were incubated with supernatants from FMLP-stimulated monocytes, suggesting that the mechanism responsible for FcγRI downregulation in these cells is different from those described for FcγRII and FcγRIII in human neutrophils (1). In fact, we have previously demonstrated that FMLP induces the release of a serine protease(s) (2), which in turn downregulates the basal levels of FcγRII and FcγRIII expressed on these cells.

Our results demonstrate that FMLP induces a rather specific action on FcγRI expression since the enhancement of LPS-dependent TNF-α secretion and the MHC class II antigen upregulation induced by IFN-γ were not altered by FMLP treatment. These results suggest that the effect of FMLP would not be exerted at the first steps of the IFN-γ transduction signal cascade, in which the activation pathway is common for different signals (11, 29). Thus, central molecules from the IFN-γ signaling pathway such as the receptor for IFN-γ; JAK1 and JAK2 kinases, or the transcription factor STAT1 cannot be altered (4, 16, 19, 25). Whether FMLP acts on a specific step in the pathway of FcγRI upregulation is not known since this is a complex mechanism that depends on the IFN-γ response region localized in the promoter of the FcγRI gene and multiple transcription factors that have not been yet elucidated (22, 23). It has recently been reported that, similar to FMLP, IFN-β has an antagonistic action on the IFN-γ-induced FcγRI expression (29). However, although this effect seems to be due to a posttranscriptional event, the mechanism of action is not known. In our experiments, the pool of FcγRI was not modified by FMLP on IFN-γ-treated cells, suggesting a posttranscriptional event.

The FMLP-induced FcγRI downregulation is an early event since incubation of cells with FMLP for 5 min was sufficient for inhibition of IFN-γ-induced FcγRI upregulation. In addition, we observed that this effect was reversible, discarding any toxic effects, and that the presence of FMLP throughout the exper-
ment was not necessary. Taking into account the fact that FMLP also inhibited the FcγRI upregulation induced by IL-10 (Fig. 2), we can speculate that FMLP acts on a common step in the pathway of FcγRI upregulation shared by IFN-γ and IL-10.

It has also been reported that LPS is capable of inducing FcγRI downregulation through the secretion of IL-1β (3). However, in our experiments, the effect of FMLP cannot be ascribed to this cytokine since the incubation with the formyl peptide in the presence of anti-IL-1β antibody did not modify the FMLP downregulatory effect.

To investigate whether the observed differences in FcγRI expression could have any physiological relevance, FcγR-dependent cytotoxic mechanisms such as phagocytosis and ADCC were assayed. The formyl peptide inhibited the IFN-γ enhancement of ADCC carried out by total and adherent PBMCs. Moreover, the inhibition of IFN-γ-dependent enhancement of phagocytosis by FMLP confirms the monocyte/macrophage lineage of the cells and indicates that the upregulation of FcγRI and FcγR-dependent functions were tightly linked. Since the basal levels of phagocytosis and ADCC were not modified by FMLP treatment, it indicates that FMLP acts only on the overexpression of FcγRI, without modifying the functional ability of this receptor.

Meanwhile, although FMLP completely inhibited the IFN-γ- and IL-10-induced enhancement of ADCC and phagocytosis, the overexpression of FcγRI was only partially inhibited. This indicates that even when the level of expression of FcγRI does not return to basal levels upon FMLP treatment, the remaining receptors are not enough to increase effector functions significantly.

As far as we know, this is the first description of the inhibitory effect exerted by FMLP on either IFN-γ- or IL-10-induced FcγRI upregulation. This is a paradox in which a prototype proinflammatory molecule (FMLP) exerts a dramatic anti-inflammatory effect. Another paradox has been observed with glucocorticoids, a typical anti-inflammatory drug, which can nevertheless behave as a proinflammatory agent (32). In the pathophysiology of bacterial infections, in which bacteria multiply and die at the site of infection and phagocytic cells could be exposed to high concentrations of N-formyl peptides, we can speculate that the inhibitory effect of FMLP on IFN-γ and IL-10-induced FcγRI upregulation can exert an important regulatory and/or anti-inflammatory effect during the evolution of bacterial infections.

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