

HYPOTHESIS: AN ALTERNATIVE PATHWAY FOR THE REGULATION OF INFLAMMATION

MARTIN A. ISTURIZ, MACARENA BEIGIER-BOMPADRE, PAULA BARRIONUEVO, FERNANDA ALVES-ROSA, MARINA S. PALERMO, MARISA VULCANO

*División Inmunología, Instituto de Investigaciones Hematológicas,
Academia Nacional de Medicina, Buenos Aires*

Abstract Regulation of inflammation is a crucial event since its alteration, such as in sepsis and chronic autoimmune (i.e. rheumatoid arthritis, lupus erythematosus) or infectious diseases (i.e. tuberculosis, leprosy), determines severe tissue damage. Although there is a general consensus that regulation of inflammation results from a balance between proinflammatory and antiinflammatory pathways, we arrived at the conclusion that well known chemoattractants/proinflammatory molecules such as bacterial formyl peptides or immune complexes (IC), could induce, paradoxically, strong antiinflammatory effects. Thus, we demonstrated that N-formyl-methionyl-leucyl-phenylalanine (FMLP) exerted a drastic antiinflammatory effect, inhibiting the secretion of tumor necrosis alpha (TNF- α) induced by lipopolysaccharides, a potent TNF- α inducer. We also determined that in human neutrophils FMLP and IC induced the downregulation of receptors for the Fc portion of IgG (Fc γ RII and Fc γ RIIIB). Moreover, FMLP inhibited interferon gamma (IFN- γ)-induced Fc γ RI expression and IC downregulate class II molecules of the major histocompatibility complex on monocytes. Part of these effects were mediated by the release of aspartic-, serin-, or metalloproteases. All these results favor the postulation of a new concept on the regulation of inflammation carried out through an alternative and non conventional pathway, in which a chemoattractant/proinflammatory agent could, under certain circumstances, act as an antiinflammatory molecule.

Key words: chemoattractants, immune complexes, formyl peptides, Fc γ Rs

Resumen *Hipótesis: una vía alternativa de regulación de procesos inflamatorios.* La regulación de mecanismos inflamatorios es un evento crucial debido a que una alteración de los mismos, como sucede por ejemplo, en la sepsis, en enfermedades autoinmunes crónicas (artritis reumatoidea, lupus eritematoso) o en enfermedades infecciosas (tuberculosis, lepra), genera daños tisulares severos. Aunque hay un consenso general de que la regulación de procesos inflamatorios resulta de un balance entre vías proinflamatorias y antiinflamatorias, nosotros arribamos a la conclusión de que moléculas quimioattractantes / proinflamatorias como, por ejemplo, péptidos formilados bacterianos o complejos inmunes (CI), pueden también inducir, paradójicamente, potentes efectos antiinflamatorios. Así, demostramos que el péptido formilado prototipo N-formil-metionil-leucil-fenilalanina (FMLP), ejerce un drástico efecto antiinflamatorio, inhibiendo la secreción de factor de necrosis tumoral alfa (TNF- α) inducido por lipopolisacáridos, un potente inductor de la secreción de TNF- α . También determinamos que el FMLP y los CI inducen la disminución de la expresión de receptores para el fragmento Fc de IgG (Fc γ RII and Fc γ RIIIB) en neutrófilos humanos. Más aún, el FMLP inhibe la inducción de la expresión de los Fc γ RI por interferón gamma (IFN- γ) y los CI disminuyen la expresión de moléculas de clase II del complejo mayor de histocompatibilidad en monocitos humanos. Parte de esos efectos fueron mediados por la liberación de aspártico-, serino-, o metaloproteasas. Todos estos resultados nos permiten especular sobre un nuevo concepto en el cual la regulación de los procesos inflamatorios también puede llevarse a cabo por una vía alternativa, no convencional, en la cual un agente quimioattractante / proinflamatorio, bajo determinadas circunstancias, puede actuar como una molécula antiinflamatoria.

Palabras clave: quimioattractantes, complejos inmunes, formil péptidos, o Fc γ Rs

Inflammation is a response to insults with accumulation of leukocytes and fluid at sites of infection or injury and it is important both for the defense against infections

and in the repair of injured tissues. This recruitment of leukocytes is accomplished by the unidirectional migration of inflammatory cells (mainly neutrophils and monocytes) through a gradient of concentration of chemotactic factors¹. A number of these chemoattractants have been identified in the last 30 years, including the classical formyl peptides, activated complement component 5 (C5a), leukotriene B₄, platelet activating factor (PAF), and a superfamily of chemokines¹.

Received: 2-III-2004

Accepted: 24-III-2004

Postal address: Dr. Martín A. Isturiz, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Pacheco de Melo 3081, 1425 Buenos Aires, Argentina
Fax: (54-11) 4803-9475 e-mail: isturiz@hematologia.anm.edu.ar

The regulation of inflammation carried out by a series of complex and versatile homeostatic mechanisms is a crucial event since its disruption as observed, for instance, in sepsis, in chronic autoimmune (i.e. rheumatoid arthritis, lupus erythematosus), or infectious diseases (tuberculosis and leprosy), determines tissue damage by cells, or their products, belonging to the immune system².

There is a general consensus that vertebrates achieve internal homeostasis during infection or injury by balancing the activities of proinflammatory and antiinflammatory pathways², which are considered to be independent. Thus, the inflammatory reaction with their typical signs of heat, pain, redness, and edema, is induced by the release of products such as reactive oxygen species (ROS), complement components, tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), IL-12, IL-18, interferon gamma (IFN- γ), prostaglandins, leukotrienes, bacterial lipopolysaccharides (LPS), N-formyl peptides, and chemokines. On the other hand, substances like IL-4, IL-10, IL-13, transformig growth factor beta (TGF- β) and corticoids, exert anti-inflammatory effects contributing to the resolution of inflammation.

However, studying the inflammatory phenomenon we arrived at the conclusion that, paradoxically, well known chemoattractants / proinflammatory molecules such as bacterial formyl peptides or immune complexes, could induce strong antiinflammatory effects³⁻¹⁰. All these results allow us to postulate that the regulation of inflammation could be also carried out through an alternative and non conventional pathway, in which an inflammatory agent could, under certain circumstances, act as antiinflammatory.

Before discussing this hypothesis, we will describe briefly the basic characteristics of formyl peptides as well as the results obtained in our laboratory.

The proinflammatory agent

While eukaryotes initiate protein synthesis with methionine (with the exception of mitochondria)¹, bacteria initiate this process with the aminoacid N-formylmethionine. Thus, when bacteria are destroyed at the site of infection, either by autolysis or by the immune system, formyl peptides are released to the external milieu.

Most of these molecules share the ability to induce chemotactic and secretory events which have been thoroughly studied since they were described by Schiffman et al.¹. Many leukocyte populations express specific receptors that recognize these formyl peptides, allowing the leukocyte recruitment at the site of infection. Formyl peptides have chemotactic activity even at a concentration as low as 10 pM. However, at higher concentrations (1 μ M) they can also induce the secretion of enzymes,

cytokines, and the generation of reactive oxygen species¹¹.

In our experiments we used a prototype of formyl peptides, the N-formyl-methionyl-leucyl-phenylalanine (FMLP). This formyl peptide acts through high (FPR) and low affinity receptors (FPRL1) present on the surface of inflammatory cells¹¹.

Experimental protocol

Our experimental protocol consists on the evaluation of neutrophil (PMN) and monocyte responses to inflammatory agents (i.e. LPS, cytokines) after exposure to FMLP. This effect was evaluated through different parameters such as TNF- α secretion, expression of the receptors for the Fc portion of IgG (Fc γ R), or Fc γ R-dependent functional activities. These Fc γ R have an active role in proinflammatory mechanisms since they are responsible for phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), and the respiratory burst, which leads to the generation of reactive oxygen species^{3, 5, 10}.

Experimental results

Effect of FMLP on human PMN

In the first studies carried out in our lab, we demonstrated that FMLP exerted, paradoxically, a drastic antiinflammatory effect at concentrations compatible with those present at the infectious focus (1 μ M). In fact, the treatment of PMN with FMLP 1 μ M inhibits the secretion of TNF- α by PMN when these cells were later exposed to LPS, a potent TNF- α inducer⁴. Although the mechanism of this antiinflammatory activity still remains unclear, the lower expression of TNF- α mRNA suggest that the secretion of TNF- α was impaired.

We also demonstrated that in human PMN, FMLP 1 μ M induced the downregulation of surface Fc γ RII and Fc γ RIIIB³. This effect, mediated by serin proteases released in the extracellular milieu⁵, was accompanied by the inhibition of effector functions carried out by these receptors such as phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC) and respiratory burst triggered by immune complexes³.

The inhibitory effects induced by FMLP were specific since they could be reversed by incubation of the cells with N-t-BOC-phen-leu-phen-leu-phen (N-t-BOC), a competitive antagonist⁴. In addition, while N-t-BOC inhibits FMLP-induced superoxide anion production, this molecule does not alter the free radical generation induced by phorbol myristic acid ester (PMA 50 ng/ml), a potent protein kinase C stimulator, indicating that the formyl peptide neither induces a toxic effect nor cell anergy.

Effect of FMLP on human monocytes

Our initial studies were carried out on PMN, which represent the first cells arriving at the inflammatory site. However, these short-living effector cells are only part of the inflammatory phenomenon, being monocytes the most representative ones in chronic inflammatory processes.

Thus, taking into account the biological relevance of type I receptor of Fc γ R in monocytes (Fc γ RI)⁶, we demonstrated that treatment of these cells with relatively low amounts of FMLP (10 to 100 nM), inhibited the upregulation of Fc γ RI induced by IFN- γ and interleukin-10 (IL-10) without modifying the basal expression of the receptor. Since these concentrations of FMLP do not induce secretory events in monocytes, FMLP could be affecting the metabolic pathway leading to the overexpression of Fc γ RI in IFN- γ and IL-10-treated cells⁶.

The expression of other molecules overexpressed in monocytes after IFN- γ treatment such as the major histocompatibility complex class II (MHC-II) was not modified by FMLP treatment, indicating that the effect of the formyl peptide is not widespread.

On the other hand, when monocytes were pretreated with IFN- γ for long periods of time, and then exposed to FMLP at concentrations compatible with those present in the inflammatory focus (1 μ M), a drastic downregulation of Fc γ RI was observed. This effect, due to the release of serine and metalloproteases⁹ is not unique for monocytes, since supernatants obtained from FMLP-treated PMN also induced downregulation of Fc γ RI on the surface of naive monocytes, demonstrating that different cell populations could cooperate to resolve the inflammatory focus.

In conclusion, and depending on its concentration, FMLP is capable of either inhibiting the overexpression of Fc γ RI or downregulating the already expressed Fc γ RI. Similar results were obtained using C5a instead of FMLP (Beigier Bompadre et al, unpublished).

Effects of other chemoattractant and proinflammatory agents

We also demonstrated that other chemoattractant / proinflammatory agents such as IgG immune complexes (IC), present in almost every infectious process, also induce a clear antiinflammatory activity. In fact, IC induce downregulation of the basal and IFN- γ -induced expression of MHC-II molecules on human monocytes⁷. Other molecules such as MHC-I, adhesion molecules (ICAM-1) and LPS receptors (CD14), were also susceptible to downregulation by IC^{7,9}. These effects are due to the release of aspartic and metalloproteases inducing the cleavage of membrane molecules.

Taken together, all these experiments show that proinflammatory/chemoattractants agents can behave as antiinflammatory under certain circumstances.

Antiinflammatory effects of FMLP and IC *in vivo*

With the purpose of investigating the antiinflammatory effect of FMLP and IC *in vivo*, we introduced subcutaneously an open-ended glass cylinder into adult mice⁹. The cylinder caused a chronic inflammation and, after 20 days of implantation, the open ends of the cylinder are "closed" by fibrotic tissue, generating a chamber containing infiltrating macrophages inside it.

Results obtained by the injection of FMLP or IC into this chamber allowed us to conclude that the downregulation of Fc γ Rs and MHC-II (I-A^d) observed in murine macrophages^{8,9}, is in agreement with the antiinflammatory effects observed *in vitro*.

Hypothesis

That a chemoattractant / proinflammatory agonist (i.e. FMLP, IC) may, under appropriate circumstances, behave as antiinflammatory is a novel concept that led us to put forward an alternative and non conventional interpretation on how an inflammatory process could be regulated.

Firstly, we must keep in mind that although by definition, all chemoattractants stimulate directed migration, at higher doses (about > 20 fold) they may also activate mechanisms such as the opening of calcium channels and enzymes (i.e. phospholipase D), correlating with the onset of cytotoxic responses such as exocytosis and respiratory burst¹².

Surrounding an inflammatory / infectious focus, a gradient of chemoattractants is generated, and we can speculate that cells recognize these products at the periphery of this gradient. In this way, cells that express high affinity specific formyl peptide receptors (FPR), which are activated by picomolar to low nanomolar concentrations¹¹, receive the chemotactic signal and could start the unidirectional migration towards the inflammatory focus, without stimulating secretion^{11,13} (Figure 1, step 1). Along its way across the concentration gradient and towards the inflammatory focus, the cells could be exposed to different cytokines (i.e. IFN- γ , IL-10) and to higher concentrations of the chemoattractant. Then, the regulatory effects of the chemoattractant can inhibit the overexpression of membrane molecules with proinflammatory activity, such as Fc γ RI in monocytes, without modifying the already expressed receptors (Figure 1, step 2).

Finally, when cells arrive at the site of infection, chemotaxis is stopped (there is no gradient at this point) and cells are exposed to the highest concentration of chemoattractant. Then, at this point, FMLP elicits the secretion of different cell products (e.g. enzymes, ROS) through the low affinity formyl peptide receptor like 1 (FPRL1), (Figure 1, step 3)¹¹, causing bacterial lysis and inducing the antiinflammatory process through downregulation.

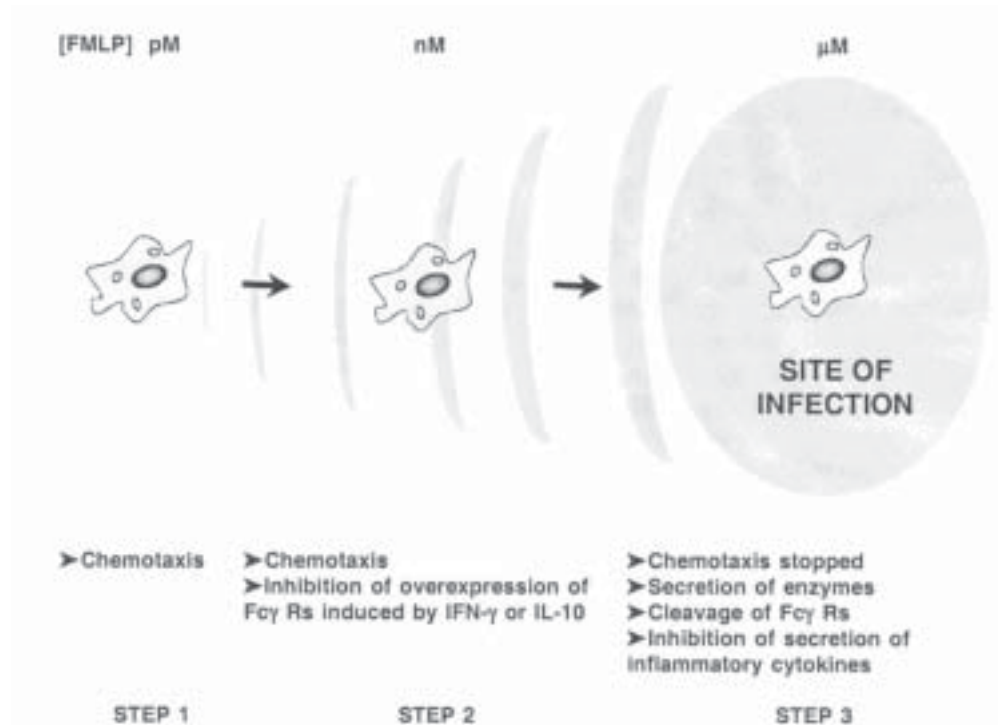


Figure 1

lation of membrane proteins or receptors which, at least in part, could be responsible for proinflammatory effects.

Interestingly, we observed that the antiinflammatory effect of FMLP is not observed in PMN and monocytes from patients with active pulmonary tuberculosis¹⁴. This is in agreement with the uncontrolled inflammatory reactions present in this disease which is the major cause of pulmonary lesions. In addition, cells from tuberculosis patients release ROS at a low concentration of FMLP (1 nM), suggesting that in their route to the site of infection, these cells can generate toxic products damaging normal surrounding tissues which are out of the infectious focus.

We consider that the established dogma that defines substances as pro- or anti-inflammatory should be revised since proinflammatory / chemoattractant molecules such as FMLP or IC, could exert antiinflammatory effects, depending on the concentration, on cells they encounter in their route to inflammatory sites.

Similar anti-inflammatory effects have been reported for the proinflammatory chemokine, MIP-3α/CCL20¹⁵. In addition, it has been shown recently that exposure of neutrophils to the chemoattractant C5a exerts an antiinflammatory effect by the decrease of gene transcription of TNF-α as well as the LPS-induced TNF-α production¹⁶.

Our results are in a conceptual agreement with Dinarello's opinion about cytokines when he said: "...listing cytokines in various categories should be done with

an open mind, in that, depending on the biological process, any cytokine may function differentially"².

Whether the antiinflammatory behavior of FMLP, IC, MIP-3α/CCL20 and C5a are isolated phenomena, or could be extended to other chemoattractants /proinflammatory molecules is not known and further studies will be needed. If this is the case, the cross-regulation of chemoattractant-mediated biological responses such as adhesion, chemotaxis, Ca⁺⁺ mobilization, degranulation, and phospholipase A₂ and C activation should also be taken into account¹².

Acknowledgements: This work was supported by grants from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) and Agencia Nacional de Promoción Científica y Tecnológica.

References

1. Murphy PM. The molecular biology of leukocyte chemoattractant receptors. *Ann Rev Immunol* 1994; 12: 593-633.
2. Dinarello CA. Proinflammatory cytokines. *Chest* 2000; 118: 503-8.
3. Alves-Rosa F, Vulcano M, Minnucci F, Di Gianni P, Isturiz MA. Inhibition of FcγR-dependent functions by N-formyl-methionyl-leucyl-phenylalanine in human neutrophils. *Clin Immunol Immunopathol* 1997; 83: 147-55.
4. Vulcano M, Alves-Rosa F, Minnucci F, Cherňavsky A, Isturiz MA. fMLP inhibits oxygen radical-dependent functions and TNF production on lipopolysaccharides (LPS) stimulated human neutrophils. *Clin Exp Immunol* 1998; 113: 39-47.

5. Alves-Rosa F., Vulcano M, Beigier M, Breyer I, Isturiz MA. The down-regulation of FcγRII and FcγRIIIb by N-formyl-methionyl-leucyl-phenylalanine (FMLP) depends on secretory events in human neutrophils. *Immunol Letters* 1999; 70: 119-26.
6. Beigier-Bompadre M, Barrionuevo P, Alves-Rosa F, Rubel C, Palermo M, Isturiz MA. N-Formyl-methionyl-leucyl-phenylalanine (FMLP) Inhibits the Interferon-γ and Interleukin-10 Induced expression of FcγRI on human monocytes. *Clin Diag Lab Immunol* 2001; 8: 402-8.
7. Barrionuevo P, Beigier-Bompadre M, De la Barrera S, et al. Immune complexes (IC) down regulate the basal and interferon-γ induced expression of MHC class II on human monocytes. *Clin Exp Immunol* 2001; 125: 251-7.
8. Beigier-Bompadre M, Barrionuevo P, Alves-Rosa F, Rubel C, Palermo MS, Isturiz MA. The formyl peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) downregulates the expression of FcγRs on activated monocytes/macrophages *in vitro* and *in vivo*. *Scand J Immunol* 2003; 57: 221-8.
9. Barrionuevo P, Beigier-Bompadre M, Fernández G, et al. Immune complex-FcγR interaction modulates monocyte/macrophage molecules involved in inflammation and immune-response. *Clin Exp Immunol* 2003; 133: 200-7.
10. Trevani A, Fontán P, Andonegui G, Isturiz MA, Geffner J. Neutrophil chemotaxis induced by immune complexes. *Clin Immunol Immunopathol* 1995; 74:107-11.
11. Le Y, Oppenheim JJ, Wang JM. Pleiotropic roles of formyl peptide receptors. *Cytokine and Growth Factor Reviews* 2001; 12: 91-105.
12. Ali H, Richardson RM, Bodduluri H, Snyderman R. Chemoattractant receptor. Cross desensitization. *J Biol Chem* 1999; 274: 6027-30.
13. Durstin M, Gao JL, Tiffany HL, McDermott D, Murphy, PM. Differential expression of members of the N-formyl-peptide receptor gene cluster in human monocytes. *Biochem Biophys Res Commun* 1994; 201: 174-9.
14. Beigier-Bompadre M, Alemán M, Barrionuevo P, et al. Monocytes and neutrophils from tuberculosis patients are insensitive to anti-inflammatory effects triggered by the prototypic formyl peptide FMLP. *Clin Exp Immunol* 2003; 133: 267-74.
15. Yang D, Chen O, Hoover D M, et al. Many chemokines including CCL20/MIP-3 alpha display antimicrobial activity. *J Leukoc Biol* 2003; 7: 448-55.
16. Riedemann NC, Guo RF, Bernacki KD, et al. Regulation by C5a of neutrophil activation during sepsis. *Immunity* 2003; 19: 193 -202.

In 1967, Niels K. Jerne concluded his talk in a Cold Spring Harbor Symposium with these words "As this younger generation of professionals is pressing rapidly toward the definitive solution of the antibody problem, we older amateurs had better sit back, waiting for the End". Nine years later at the same Symposium Jerne was forced to observe that the "younger generation of professionals" who had aged somewhat in the meantime, had not only failed to reach the predicted End of immunology but had even failed to provide the "definitive solution to the antibody problem". ...new avenues had opened up for which he received the Nobel Prize with Milstein in 1984.

En 1967, Niels K. Jerne terminó su conferencia en un *Symposium* de *Cold Spring Harbor* con estas palabras: "Mientras esta generación más joven de profesionales está presionando rápidamente hacia la solución definitiva para el problema de los anticuerpos, sería mejor que nosotros, más viejos aficionados nos sentáramos, esperando el Final". Nueve años después, en el mismo *Symposium*, Jerne tuvo que admitir que la "más joven generación de profesionales", que en ese lapso ya habían envejecido algo, no sólo había fracasado en alcanzar el Final predicho para la inmunología, sino que además había fracasado en proveer la "solución definitiva para el problema de los anticuerpos". ...nuevos caminos se habían abierto por los que él recibió el Premio Nobel de 1984 junto con Milstein.

Jan Klein

Immunology. The Science of Self-Nonself Discrimination. New York: Wiley e Sons, 1982