Brief Definitive Report

Interleukin 10 (IL-10) Upregulates IL-1 Receptor Antagonist Production from Lipopolysaccharide-stimulated Human Polymorphonuclear Leukocytes by Delaying mRNA Degradation

By Marco A. Cassatella,* Lucia Meda,‡ Sara Gasperini,* Federica Calzetti,* and Stefano Bonora§

From the Institute of General Pathology, University of Verona, 37134, Verona; the ‡Department of Neurology, Dino Ferrari Center, University of Milan, 20100 Milan; and the §Institute of Immunology and Infectious Diseases, University of Verona, 37134 Verona, Italy

Summary

Polymorphonuclear leukocytes (PMN) have been identified as cells capable of producing a number of pro- and antiinflammatory cytokines in response to specific agonists. Previously, we showed that tumor necrosis factor (TNF), interleukin-1β (IL-1β), and IL-8, are produced by PMN after stimulation with agonists, such as lipopolysaccharide (LPS). In this study, we demonstrate that LPS is also a potent stimulus for the mRNA expression and release of the IL-1 receptor antagonist (IL-1ra). In addition, we show that the release of IL-1ra from LPS-stimulated PMN is markedly potentiated in the presence of IL-10 (from two to threefold after 18 h of stimulation). Moreover, we observed that this upregulation of IL-1ra production by IL-10 in LPS-stimulated PMN took place through IL-1ra mRNA stabilization. Indeed, the half-life of IL-1ra mRNA was prolonged in PMN stimulated in the presence of IL-10 and LPS, as compared with cells stimulated with LPS alone. That IL-10 selectively upregulates IL-1ra production in LPS-activated PMN, while it inhibits the production of IL-1β, TNF, and IL-8 under the same conditions, suggests that IL-10 may be an important physiologic regulator of cytokine production from PMN, and emphasizes the potential role of IL-10 in inflammatory responses.

PMN are specialized effector cells involved in acute inflammatory responses. For instance, PMN mediate antimicrobial defense and antitumoral activity through the release of toxic oxygen intermediates or lytic enzymes (1). In addition, several studies have shown that PMN have the ability to produce many different cytokines known to play an important role in inflammatory and immune responses, including TNF, IL-1, and IL-8 (reviewed in reference 2). More recently, it has been reported that the gene expression and production of the IL-1 receptor antagonist (IL-1ra) can be stimulated in human neutrophils (3–5). IL-1ra is a 23- to 25-kD glycosylated protein, originally purified from supernatants of human monocytes cultured on immune complex–coated surfaces (6), or from the urine of patients with monocytic leukemia (7), which has been recently cloned (8, 9). While IL-1ra competes with both IL-1α and IL-1β for binding to the IL-1 receptor (7), it does not initiate internalization of the receptor–ligand complex, or transduce an activation signal (10).

Although IL-1ra has generated considerable interest based on its ability to block the proinflammatory activities of IL-1 in vivo (11, 12), the regulation of IL-1ra expression in human PMN remains poorly understood. Since IL-10, a molecule known for its immunosuppressive properties (13), was previously shown to modulate TNF, IL-1β, and IL-8 cytokine gene expression and production in PMN (14), we examined the effect of IL-10 on neutrophil-derived IL-1ra release. We now report that in human PMN, the release of IL-1ra was potently induced by LPS, and that IL-10 significantly upregulated LPS-elicited IL-1ra production. These findings suggest that IL-10, by favoring the production of IL-1ra by PMN over that of IL-1 (14), may exert potent antiinflammatory effects on host responses to gram-negative infections.

Materials and Methods

Cell Purification and Culture. Highly purified (>99.5%) PMN were isolated under endotoxin-free conditions from buffy coats of healthy donors, as previously described (14, 15). Immediately after purification, PMN were suspended in RPMI 1640 medium supplemented with 10% low endotoxin FCS (<0.006 ng/ml; Irvine Scientific, Santa Ana, CA), in the presence or absence of purified recombinant human IL-10 (protein concentration, 50 μg/ml; sp act, 2×10^7 U/mg; kindly provided by Dr. K. Moore, DNAX and

Schering-Plough Corporation, Palo Alto, CA). Cells were then stimulated with 1 μg/ml LPS (from *Escherichia coli* 026:B6, purchased from Sigma Chemical Co., St. Louis, MO) and cultured at 37°C, either in 24-well tissue culture plates (Nunc, Roskilde, Denmark) at 10^6 cells/300 μl, or in polystyrene flasks (Greiner, Nurtingen, Germany) at 5×10^6 cells/ml (14). Cell-free supernatants were harvested at various time points, spun at 2,000 rpm for 30 s, and stored at −70°C before IL-1ra determination. All reagents were of highest grade and were dissolved in pyrogen-free water for clinical use (15).

RNA Isolation and Northern Blot Analyses. Extraction and Northern blot analysis of total RNA from PMN were performed as already described (14, 15). The cDNA fragment for IL-1ra (which was a gift from Dr. A. Mantovani, Mario Negri Institute, Milano, Italy), as well as the cDNA fragments for IL-8, IL-1β, heavy chain subunit of cytochrome b_{558} (gp91-phox), and actin, were ^{32}P-labeled using a Ready-to-go DNA labeling kit (Pharmacia LKB, Uppsala, Sweden), before hybridization of nylon filters and autoradiography.

Detection of IL-1ra. Extracellular IL-1ra was determined using a specific ELISA kit (50 pg/ml detection limit) purchased from Amersham International (Little Chalfort, UK).

Statistical Analyses. Where mentioned, data were assessed for statistical significance using Student's *t* test for paired data. Data are expressed as means ± SEM.

Results

Effect of IL-10 on IL-1ra Production in LPS-stimulated PMN. Purified populations of PMN were preincubated for 30 min with optimal doses of IL-10 (100 U/ml) (14), and cultured for up to 18 h in the presence or absence of LPS. Fig. 1 shows that the release of IL-1ra protein into the culture supernatants of unstimulated PMN (4, 5) was not significantly affected by the presence of IL-10. In contrast, PMN stimulation with LPS resulted in a substantial release of IL-1ra protein that began as early as after 2 h and increased progressively with time (Fig. 1). Moreover, IL-10 treatment greatly potentiated the secretion of IL-1ra protein in LPS-stimulated PMN (Fig. 1). This enhancing effect of IL-10 was significant after 6 h of LPS stimulation, and a maximal effect of IL-10 was observed at 18 h. Similar results were obtained when PMN were exposed to as little as 10 ng/ml LPS (not shown).

Effect of IL-10 on IL-1ra mRNA Expression in PMN Stimulated with LPS. We next determined whether IL-10 exerted its enhancing effect on LPS-stimulated IL-1ra release at the level of mRNA. Northern blot analyses were performed on total RNA isolated from PMN preincubated with IL-10, and stimulated for different times in the presence or the absence of LPS. As shown in Fig. 2, resting PMN expressed low levels of IL-1ra transcripts, which were not influenced by the presence of IL-10. Stimulation of PMN with LPS increased the mRNA steady-state levels of IL-1ra, as well as those of IL-8 and of NADPH oxidase component gp91-phox (16), in keeping with previous observations (4, 14, 15). The LPS-induced increase in IL-1ra mRNA steady-state levels was time dependent, peaked at 1.5–2 h, and gradually returned to near-baseline levels by 20 h (not shown). Pretreatment of PMN with IL-10 had no significant effect on the LPS-elicited enhancement of IL-1ra, IL-8, and gp91-phox mRNA accumulation after 2 h. At later time points, however, the amounts of IL-1ra mRNA transcripts were maintained at high levels in IL-10–treated PMN, whereas under the same conditions, the LPS-induced accumulation of IL-8 mRNA was suppressed by IL-10 pretreatment (14), while that of gp91-phox mRNA was not influenced by the cytokine (Fig. 2).

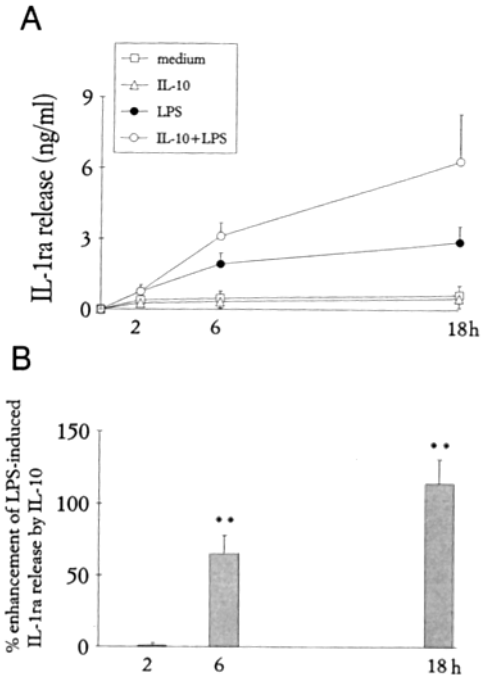

Figure 1. Effect of IL-10 on LPS-induced release of IL-1ra from PMN. PMN (10^6/300 μl) were preincubated for 30 min with or without 100 U/ml IL-10, and then cultured for up to 18 h after the addition of LPS. Released IL-1ra in the cell-free supernatants were measured by ELISA. *A* shows the mean values ± SEM of duplicate assays for each time point, obtained from four experiments performed under the same conditions. *B* shows the mean values ± SEM of the percentage of enhancement of LPS-induced IL-1ra release determined by IL-10. Percent values were calculated from the difference in the amount of cytokine produced in the absence and presence of IL-10. The values of the constitutive cytokine secretion were not subtracted. The asterisks represent significant differences between IL-10–treated and untreated PMN. $*p < 0.01$.

IL-10 Decreases Turnover of Message for IL-1ra, but not that of IL-1β. In an attempt to elucidate the mechanism whereby

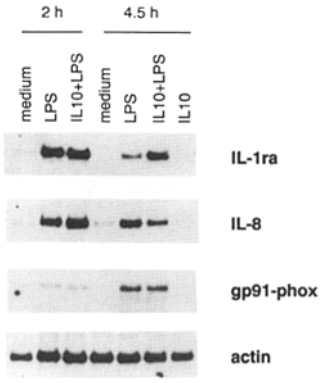

Figure 2. Effect of IL-10 on the modulation of neutrophil IL-1ra, IL-8, and gp91-phox gene expression induced by LPS. PMN were preincubated with or without IL-10 for 15 min before addition of LPS for the times indicated. Total RNA was extracted and analyzed by Northern analysis. The figure shows one experiment representative of four, with similar results.

IL-10 could affect the accumulation of IL-1ra transcripts in LPS-treated PMN, we examined the influence of IL-10 on cytokine mRNA stability. PMN were stimulated with LPS for 3.5 h, in the presence or absence of IL-10, and then treated with actinomycin D to block the formation of additional transcripts. At increasing intervals thereafter, the cultures were processed for Northern blot analysis, and changes in the amount of cytokine mRNA were quantitated by laser densitometric scanning. Whereas preliminary experiments had established that LPS stimulation (3 h) does not significantly alter IL-1ra and IL-1β mRNA stability (not shown), Fig. 3 shows that IL-10 pretreatment markedly prolonged IL-1ra mRNA half-life relative to diluent-treated cells, after 3.5 h of LPS stimulation (180 vs. 54 min, respectively). A similar effect of IL-10 on the turnover rate of IL-1ra transcripts was exerted in cells stimulated with LPS for 5 h, a time point at which the differences in IL-1ra mRNA levels were more evident (not shown). IL-10 did not significantly affect the stability of IL-1β and actin mRNAs isolated from LPS-treated PMN (Fig. 3).

Discussion

IL-1 is widely viewed as an important proinflammatory mediator and is thought to play a crucial role in a number of pathophysiological conditions (17). The actions of IL-1 can be controlled in several ways, such as by the regulation of its synthesis (18), by the release of soluble IL-1 receptors (19, 20), or by the production of IL-1ra, an antagonistic inhibitor that blocks IL-1 binding to its receptor (6, 7), thereby effectively preventing the biological actions of IL-1 (17). To date, studies addressing the expression of IL-1ra in human PMN remain scarce. It has been reported, for instance, that the expression of IL-1ra occurred only after neutrophil treatment with GM-CSF or TNF, but not with LPS, even if used over a 24-h period and at concentrations ranging from sub- to superoptimal levels (5). In contrast, other investigators have found that PMN have the ability to express IL-1ra mRNA and protein in response to LPS (100 μg/ml) (4). In the same study, it was also demonstrated that, after intratracheal injection of LPS in the rat, substantial amounts of IL-1ra mRNA were present in PMN-rich bronchoalveolar lavage (4). More recently, Re et al. (21) showed that concentrations of 1–10 ng/ml LPS were sufficient to induce IL-1ra expression in PMN. Our data are therefore consistent with the findings of the latter two reports, as well as with preliminary observations form our laboratory, that indicate that LPS represents an important stimulus for the expression of IL-1ra in PMN, as it is more potent than IgG-opsonized yeast particles, or TNF-α (Cassatella, M. A., unpublished observations).

IL-10 is a product of various cell types including monocytes and lymphocytes (13), that potently inhibits monocyte/macrophage function including proinflammatory cytokine release, oxidative burst, and nitric oxide production (22–24), and that contributes to the regulation of lymphocyte proliferation and differentiation (13). We have recently demonstrated that IL-10 can also act upon PMN function by specifically suppressing the LPS-induced release of TNF-α, IL-1β, and IL-8 by LPS-stimulated neutrophils (14). In the present report we show that IL-10 markedly enhances the production of IL-1ra in PMN stimulated with LPS. Further investigation of this effect of IL-10 revealed that whereas the cytokine substantially increased the half-life of IL-1ra transcripts, it failed to modulate IL-1β mRNA stability, thereby demonstrating that the posttranscriptional actions of IL-10 are restricted to selected, LPS-inducible cytokine mRNA species. Consistent with our findings, it has been recently reported that the suppression by IL-10 of TNF and IL-1 mRNA accumulation in LPS-stimulated murine macrophages depended on de novo protein synthesis, and was not attributable to inhibition of TNF or IL-1 gene transcription rate (25), a conclusion that supports an action of IL-10 on posttranscriptional events. Although many cytokine mRNA species (e.g., TNF, IL-1β, IL-8) contain all AU-rich sequences in their 3′-untranslated regions that are believed to be involved in the regulation of mRNA stability (26), these AU-rich motifs are

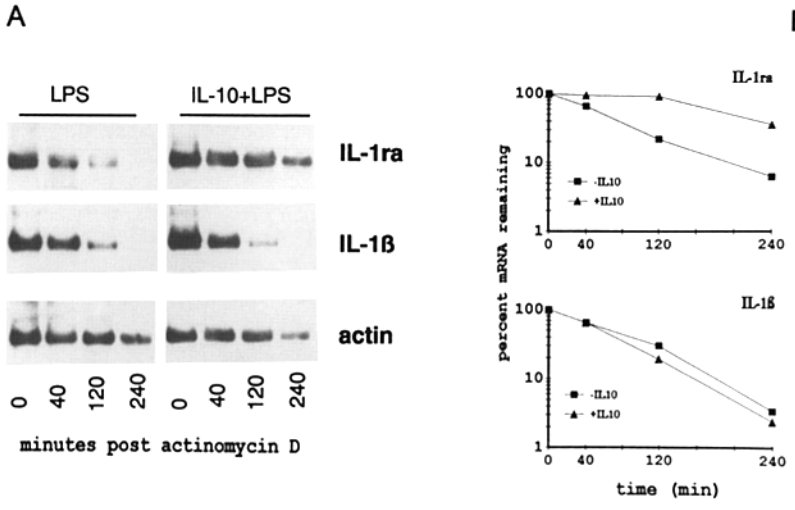

Figure 3. Effect of IL-10 on the turnover rate of IL-1ra and IL-1β mRNAs in LPS-stimulated PMN. PMN were cultured with 1 μg/ml LPS, in the presence or absence of IL-10. After 3.5 h, the cells were treated with actinomycin D (5 μg/ml) for an additional 4 h. (*A*) PMN were harvested at the indicated times, and total RNA was prepared and analyzed by Northern blotting using IL-1ra, IL-1β, and actin cDNA probes. (*B*) RNA bands were analyzed densitometrically, the data were plotted on semilogarithmic graphs as percent remaining mRNA vs. time decays in min, and the resulting values plotted against time. Half-lives were calculated by regression analysis. One representative experiment out of four performed is shown.

not present in IL-1ra mRNA (8, 9). Thus, it appears that IL-1ra mRNA stability may be regulated by IL-10 in a unique manner. It is, for example, possible that IL-10 reduces the expression of a nuclease that selectively degrades IL-1ra mRNA, or that induces/increases the expression of a factor that decreases the susceptibility of IL-1ra mRNA to the action of such a nuclease. In this respect, Bogdan et al. (25) postulated the possible existence of nucleolytic activities controlling TNF and IL-1 mRNA expression, which IL-10 could modulate. In any case, other effects of IL-10 at the level of PMN transcription, translation or secretion cannot be excluded.

The ability of IL-10 to upregulate IL-1ra production in PMN may reflect one of the mechanisms underlying the immunosuppressive actions of IL-10 (13), and in particular could contribute to the efficacy of IL-10 in downregulating LPS toxicity in vivo (27, 28). Very recently, mouse mutants were generated in which the IL-10 gene was inactivated by targeted mutation (29). As these IL-10–deficient mice suffered predominantly from chronic enterocolitis (29), it was proposed that their primary defect was a failure to control normal intestinal immune responses against undefined enteric antigens, which led to an overproduction of proinflammatory cytokines, presumably by macrophages (29). On the basis of the present data, and of our previous study (14), it appears that one of the net effects of IL-10 toward neutrophils is to neutralize IL-1, both by inhibiting IL-1 production and by promoting the synthesis and secretion of its antagonist. Considering that in their initial phases, bowel inflammation diseases are also characterized by a massive infiltration of neutrophils (30), the importance of the PMN as a source of cytokines under those circumstances must not be underestimated.

We thank Professor Filippo Rossi for his comments and continuous encouragement; Giorgio Berton, Patrick McDonald, Alberto Mantovani, and Giorgio Trinchieri for their critical reading of the manuscript; and Mara Giardini for her excellent technical assistance.

This work was supported by grants from Ministero dell'Universitá e della Ricerca Scientificae Tecnologia (fondi 40% e 60%), Consiglio Nazionale delle Ricerche (93.04629.CT04), and the Associazione Italiana per la Ricerca sul Cancro.

Address correspondence to Dr. Marco A. Cassatella, Istituto di Patologia Generale, Strada Le Grazie 4, 37134 Verona, Italy.

Received for publication 8 December 1993 and in revised form 24 February 1994.

References

1. Gallin, J.I., I.M. Goldstein, and R. Snyderman. 1992. Inflammation: Basic Principles and Clinical Correlates. 2nd edition. Raven Press Ltd., New York. 1186 pp.
2. Loyd, A.R., and J.J. Oppenheim. 1992. Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. *Immunol. Today.* 13:169.
3. Tiku, K., M.L. Tiku, S. Liu, and J.L. Skosey. 1986. Normal human neutrophil are a source of a specific interleukin 1 inhibitor. *J. Immunol.* 136:3686.
4. Ulich, T.R., K. Guo, S. Yin, J. del Castillo, E.S. Yi, R.C. Thompson, and S.P. Eisenberg. 1992. Endotoxin-induced cytokine gene expression in vivo. IV. Expression of interleukin 1-α/β and interleukin 1 receptor antagonist mRNA during endotoxemia and during endotoxin-initiated local acute inflammation. *Am. J. Pathol.* 141:61.
5. McColl, S.R., R. Paquin, C. Ménard, and A.D. Beaulieu. 1992. Human neutrophils produce high levels of the interleukin 1 receptor antagonist in response to granulocyte/macrophage colony-stimulating factor and tumor necrosis factor. *J. Exp. Med.* 176:593.
6. Arend, W.P., F.G. Goslin, and R.J. Massoni. 1985. Effects of immune complexes on production by human monocytes of interleukin 1 or an interleukin 1 inhibitor. *J. Immunol.* 134:3868.
7. Seckinger, P.J., W. Lowenthal, K. Williamson, J.M. Dayer, and H.R. MacDonald. 1987. A urine inhibitor of interleukin 1 activity that blocks ligand binding. *J. Immunol.* 139:1546.
8. Eisenberg, S.P., R.J. Evans, W.P. Arend, E. Verderber, M.T. Brewer, C.H. Hannum, and R.C. Thompson. 1990. Primary structure and functional expression from complementary DNA of a human interleukin 1 receptor antagonist. *Nature (Lond.).* 343:341.
9. Carter, D.B., M.R. Deibel, Jr., C.J. Dunn, C.S. Tomich, A.L. Laborde, J.L. Slightom, A.E. Berger, M.J. Bienkowski, F.F. Sun, R.N. McEwan, et al. 1990. Purification, cloning, expression and biological characterization of an interleukin-1 receptor antagonist protein. *Nature (Lond.).* 344:633.
10. Dripps, D.J., B.J. Brandhuber, R.C. Thompson, and S.P. Eisenberg. 1991. Interleukin 1 (IL-1) receptor antagonist binds to the 80-kDa IL-1 receptor but does not initiate signal transduction. *J. Biol. Chem.* 266:10331.
11. Ohlsson, K., P. Bjork, M. Bergenfeldt, R. Hageman, and R.C. Thompson. 1990. An interleukin 1 receptor antagonist reduces mortality in endotoxin shock. *Nature (Lond.).* 348:550.
12. Cominelli, F., C.C. Nast, B.D. Clark, R. Schindler, R. Lierena, V.E. Eysselein, R.C. Thompson, and C.A. Dinarello. 1990. Interleukin 1 (IL-1) gene expression, synthesis, and effect of specific IL-1 receptor blockade in rabbit immune complex colitis. *J. Clin. Invest.* 86:972.
13. Moore, K.W., A. O'Garra, R. de Waal Malefyt, P. Vieira, and T.R. Mosmann. 1993. Interleukin 10. *Annu. Rev. Immunol.* 11:165.
14. Cassatella, M.A., L. Meda, S. Bonora, M. Ceska, and G. Con-

statin. 1993. Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1β in mediating the production of IL-8 triggered by lipopolysaccharide. *J. Exp. Med.* 178:2207.
15. Cassatella, M.A., F. Bazzoni, R.M. Flynn, S. Dusi, G. Trinchieri, and F. Rossi. 1990. Molecular basis of Interferon-γ and lypopolysaccharide enhancement of phagocyte respiratory burst capability. Studies on the gene expression of several NADPH oxidase components. *J. Biol. Chem.* 265:20241.
16. Malech, H.L. 1993. Phagocyte oxidative mechanisms. *Curr. Opin. Hematol.* 1:123.
17. Dinarello, C.A., and S.M. Wolff. 1993. The role of Interleukin-1 in disease. *N. Engl. J. Med.* 328:106.
18. Kern, J.A., R.J. Lamb, J.C. Reed, R.P. Daniele, and P.C. Nowell. 1988. Dexamethasone inhibition of Interleukin 1 Beta production by human monocytes. Posttranscriptional mechanisms. *J. Clin. Invest.* 81:237.
19. Novic, D., H. Engelmann, D. Wallach, and M. Rubinstein. 1989. Soluble cytokine receptors are present in normal human urine. *J. Exp. Med.* 170:1409.
20. Colotta, F., F. Re, M. Muzio, R. Bertini, N. Polentarutti, M. Sironi, J.G. Giri, S.K. Dower, J.E. Sims, and A. Mantovani. 1993. Interleukin-1 type II receptor: a decoy target for iL-1 that is regulated by IL-4. *Science (Wash. DC).* 261:472.
21. Re, F., M. Mengozzi, M. Muzio, C. Dinarello, A. Mantovani, and F. Colotta. 1992. Expression of interleukin 1 receptor antagonist by human circulating polymorphonuclear cells. *Eur. J. Immunol.* 23:570.
22. de Waal Malefyt, R., J. Abrams, B. Bennett, C.G. Figdor, and J.E. de Vries. 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* 174:1209.
23. Fiorentino, D.F., A. Zlotnik, T.R. Mosmann, M. Howard, and A. O'Garra. 1991. Interleukin 10 inhibits cytokine production by activated macrophages. *J. Immunol.* 147:3815.
24. Bogdan, C., Y. Vodovotz, and C. Nathan. 1991. Macrophage deactivation by interleukin 10. *J. Exp. Med.* 174:1549.
25. Bogdan, C., J. Paik, Y. Vodovotz, and C. Nathan. 1992. Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-β and interleukin-10. *J. Biol. Chem.* 267:23301.
26. Caput, D., B. Keutler, K. Hartog, R. Thayer, S. Brown-Shimer, and A. Cerami. 1986. Identification of a common nucleotide-sequence in the 3'-untranslated region of mRNA molecule specifying inflammatory mediators. *Proc. Natl. Acad. Sci. USA.* 83:1670.
27. Gérard, C., C. Bruyns, A. Marchant, D. Abramowicz, P. Vandenabeele, A. Delvaux, W. Fiers, M. Goldman, and T. Velu. 1993. Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. *J. Exp. Med.* 177:547.
28. Howard, M., T. Muchamuel, S. Andrade, and S. Menon. 1993. Interleukin 10 protects mice from lethal endotoxemia. *J. Exp. Med.* 177:1205.
29. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell.* 75:263.
30. Podolsky, D.K. 1991. Inflammatory bowel disease. *N. Engl. J. Med.* 325:928.