

## **Human Neutrophils Produce High Levels of the Interleukin 1 Receptor Antagonist in Response to Granulocyte/Macrophage Colony-stimulating Factor and Tumor Necrosis Factor $\alpha$**

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### **Summary**

Neutrophils, an abundant cell type at sites of inflammation, have the ability to produce a number of cytokines, including interleukin 1 (IL-1), IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ). In this study, we have examined the ability of human neutrophils to produce the IL-1 receptor antagonist (IL-1Ra), a 17–23-kD protein recently isolated and cloned from macrophages. Since IL-1Ra has been shown to inhibit both the *in vitro* and *in vivo* effects of IL-1, its production by large numbers of tissue-invading neutrophils might provide a mechanism by which the effects of IL-1 are regulated in inflammation. Using antibodies that are specific for IL-1Ra and a cDNA probe encoding for this protein, we were able to show that neutrophils constitutively produce IL-1Ra. However, after activation by GM-CSF and TNF- $\alpha$ , IL-1Ra was secreted into the extracellular milieu where it constituted the major *de novo* synthesized product of activated neutrophils. None of a large array of other potent neutrophil agonists were found to affect the production of IL-1Ra by neutrophils. Quantitative measurements by enzyme-linked immunosorbent assay revealed that intracellular IL-1Ra is in eightfold excess of the amount secreted in supernatants when studying nonactivated neutrophils. However, in GM-CSF- and TNF- $\alpha$ -activated cells, this difference was reduced to values between four- and fivefold, as virtually all of the *de novo* synthesized IL-1Ra was secreted. In activated cells, the intracellular content of IL-1Ra was found to be in the 2–2.5-ng/ml range per  $10^6$  neutrophils, whereas levels reached the 0.5-ng/ml range in supernatants. This would imply that IL-1Ra is produced in excess of IL-1 by a factor of at least 100, an observation that is in agreement with the reported amounts of IL-1Ra needed to inhibit the proinflammatory effects of IL-1. Neutrophils isolated from an inflammatory milieu, the synovial fluid of patients with rheumatoid arthritis, were found to respond to GM-CSF and TNF- $\alpha$  in terms of IL-1Ra synthesis, indicating that the *in vitro* observations made in this study are likely to occur in an inflammatory setting *in vivo*.

**A** monocyte product with interleukin 1 receptor antagonist (IL-1Ra) activity has recently been isolated, cloned, and sequenced (1). Considerable interest has been generated by this molecule in view of its ability to block the proinflammatory activities of IL-1 both *in vitro* and *in vivo* (2, 3). Most studies have shown that monocytes, along with leukemia monocytic cell lines, produce this molecule after activation by a variety of stimuli. Although one report has suggested that neutrophils may also secrete products with IL-1 inhibitory activity (4), no molecular characterization of this activity has yet been performed. It therefore remains unknown if neutrophils produce an IL-1 inhibitor similar to that made by monocytes.

In recent years, studies from our laboratory have focused on investigating *de novo* protein synthesis and secretion by neutrophils after activation by proinflammatory molecules (5–7). In the context of these studies, we have observed that a limited number of neutrophil agonists can mediate the nuclear-signaling events necessary to induce *de novo* RNA synthesis in this cell type (8). Of a wide range of stimuli investigated, only three were found to induce significant RNA synthesis in neutrophils. These are GM-CSF, TNF- $\alpha$ , and the formylated oligopeptide FMLP. However, only the former two agonists were capable of inducing the synthesis and secretion of a 23-kD protein, the nature of which was not identified at the time of our initial studies (9). We now report that

this protein has the same molecular characteristics as the IL-1Ra produced by monocytes. We also report that of de novo synthesized and secreted proteins by neutrophils, IL-1Ra is the major product in response to stimulation by GM-CSF and TNF- $\alpha$ .

## Materials and Methods

**Reagents.** The following molecules used in this study were generous gifts from the Genetics Institute (Boston, MA): recombinant human (rh)GM-CSF, rhIL-3, rhIL-4, and rhIL-6. rhTGF $\beta$  and rh platelet-derived growth factor<sub>bb</sub> (PDGF<sub>bb</sub>) were purchased from Collaborative Research (Bedford, MA) and Bachem Fine Chemicals (Torrance, CA), respectively, whereas rhG-CSF was purchased from R & D Systems, Inc. (Minneapolis, MN). rhTNF- $\alpha$  was a gift from Knoll Pharmaceuticals (Whippany, NJ) and rhIFN- $\gamma$  was purchased from Chemicon International, Inc. (Temecula, CA). rhIL-1 $\beta$  was a generous gift from Genentech Inc. (San Francisco, CA) and rhIL-8 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The formylated oligopeptide FMLP, rhC5a, and platelet-activating factor (PAF) were purchased from Sigma Chemical Co. (St. Louis, MO). Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) was generously provided by Merck-Frosst Canada (Dorval, Quebec, Canada). The endotoxin LPS was purchased from Gibco Laboratories (Burlington, Ontario, Canada). [<sup>35</sup>S]Methionine, [<sup>35</sup>S]cysteine, and [<sup>32</sup>P]deoxycytidine triphosphate were purchased from Amersham (Oakville, ON, Canada). All other reagents were of molecular biological grade and were purchased from the Sigma Chemical Co.

**Neutrophil Isolation.** Neutrophils were isolated from venous blood obtained from normal healthy volunteers and synovial fluid of patients with rheumatoid arthritis as previously described (5, 6). All neutrophil preparations contained <0.2% monocytes as determined by nonspecific monoesterase staining. Viability was estimated by the trypan blue dye exclusion procedure and found to be >99% in all preparations.

**Metabolic Labeling of Neutrophils.** The neutrophils were suspended at a density of 10<sup>8</sup>/ml in methionine- and cysteine-free RPMI 1640. Labeling was performed in the presence of 0.1% FCS with [<sup>35</sup>S]methionine (1,000 Ci/mmol) and [<sup>35</sup>S]cysteine (1,300 Ci/mmol), at a concentration of 125  $\mu$ Ci/ml for each label incubated at 37°C for various time periods as specified in the relevant figure legends.

**Immunoisolation of IL-1Ra.** Cell culture supernatants were processed for immunoisolation using two different sources of antibodies directed against IL-1Ra: (a) a cocktail of mAbs to IL-1Ra, a generous gift from Dr. Ann Berger (The Upjohn Co., Kalamazoo, MI); and (b) a polyclonal rabbit antibody to IL-1Ra, a generous gift from Dr. William Arend (University of Colorado School of Medicine, Denver, CO). Immunoisolation was carried out according to the procedure of Davis et al. (10).

**RNA Isolation and Northern Blotting.** Isolation of total cellular RNA and Northern blots were performed as previously described (5). The IL-1Ra cDNA probe used for these studies, a 1.8-kb fragment cloned into the EcoRI site of a PGEM<sub>2</sub> plasmid, was a generous gift of Dr. Daniel E. Tracey (Upjohn Co.) (11).

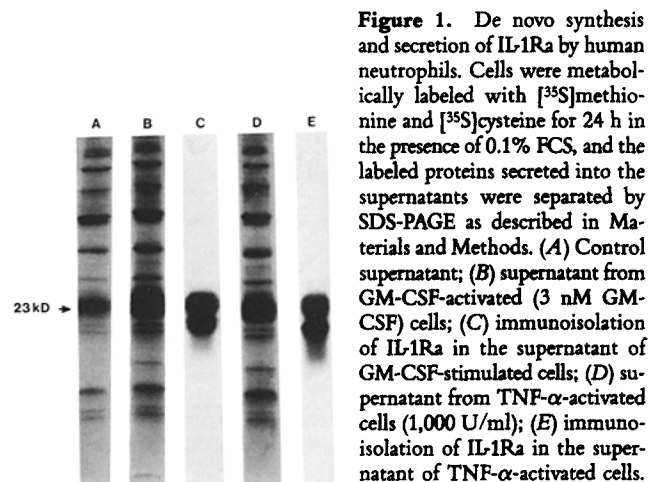
**Quantitative Measurement of IL-1Ra.** The measurement of IL-1Ra was performed using an ELISA purchased from R & D Systems, Inc. This immunoassay was performed as specified by the manufacturer. Statistical analyses were performed using the student's *t* test.

## Results

**Production of IL-1Ra by Neutrophils.** Neutrophils were stimulated with a number of known neutrophil agonists in the

presence of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine in order to study the effects of each agonist on the induction of de novo protein synthesis and secretion. SDS-PAGE was then performed on the incubation media obtained from treated and untreated neutrophils. It was observed that of all the agonists used in this study, only GM-CSF and TNF- $\alpha$  induced the synthesis and secretion of a 23-kD molecule by neutrophils. A fluorogram obtained from a typical experiment in which neutrophils were stimulated with either GM-CSF or TNF- $\alpha$  is shown in Fig. 1. The proteins secreted from nonactivated neutrophils are shown in Fig. 1, lane A, whereas those obtained from GM-CSF- and TNF- $\alpha$ -stimulated neutrophils are shown in Fig. 1, lanes B and D, respectively. Although neither GM-CSF nor TNF- $\alpha$  significantly altered general protein synthesis in neutrophils, it can be clearly seen that the intensity of the 23-kD band, along with two slightly lower molecular mass bands, was significantly enhanced. The optimal concentrations for the induction of this synthesis were 3 nM for GM-CSF and 1,000 U/ml for TNF- $\alpha$ . Optimal stimulating and labeling periods were 4 h. It was also observed that the use of a minimum of 0.1% FCS in the incubation media was sufficient for optimal induction of synthesis by GM-CSF and TNF- $\alpha$  (results not shown). Using a polyclonal antibody to IL-1Ra, the 23-kD band along with the two lower molecular mass bands were immunostained both from the GM-CSF, and the TNF- $\alpha$ -stimulated cells (Fig. 1, lanes C and E, respectively). Similar results were obtained when using a cocktail of mAbs to IL-1Ra for immunoisolation. However, under the same conditions, neither IL-1 $\alpha$  nor IL-1 $\beta$  was detected using specific mAbs directed against these two antigens (data not shown). The various stimuli used in this study are shown in Table 1. For each neutrophil agonist, a dose-response course was performed using concentrations ranging from sub- to supraoptimal levels as defined in other systems. Furthermore, time course studies over a 24-h period were also performed with each agonist.

Northern blot hybridization studies were also performed using a cDNA probe for IL-1Ra in order to measure levels



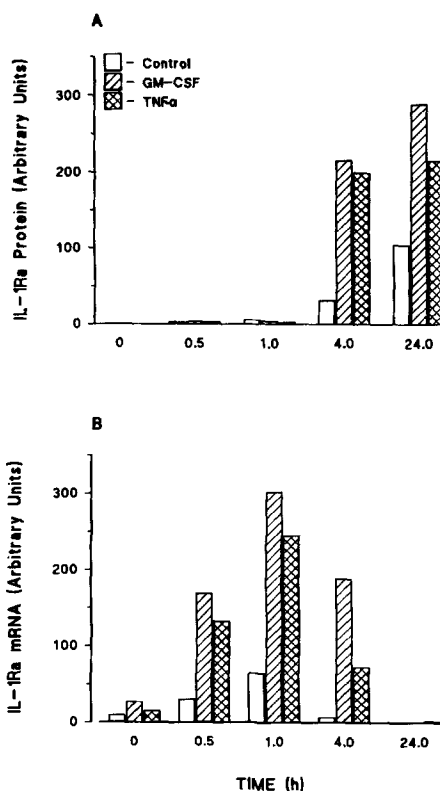
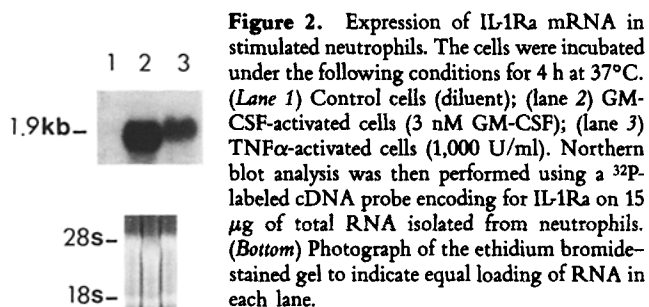
**Table 1. Induction of IL-1Ra Synthesis by Neutrophil Agonists**

Agonists	Induction of synthesis
GM-CSF	Positive
TNF- $\alpha$	"
G-CSF	Negative
IL-1 $\beta$	"
IL-3	"
IL-4	"
IL-6	"
IL-8	"
TGF $\beta$	"
PDGF $\beta$	"
IFN- $\gamma$	"
PAF	"
C5a	"
LTB $_4$	"
LPS	"
fMet-Leu-Phe	"

Neutrophils were activated with the agonists and metabolically labeled as described (see Materials and Methods). Cell supernatants were then analyzed by SDS-PAGE for the presence of de novo synthesized IL-1Ra. Experiments were repeated with cells isolated from the peripheral blood of at least six normal individuals.

of IL-1Ra mRNA in neutrophils. Results are shown in Fig. 2, where it can be seen that the levels of mRNA were considerably increased in GM-CSF- and TNF- $\alpha$ -activated cells (Fig. 2, lanes 2 and 3, respectively) over control cells (Fig. 2, lane 1), with a more pronounced effect after GM-CSF activation. With a longer exposure time, a 1.9-kb band was also observed in unstimulated cells. Fig. 2, *bottom*, shows the photograph of the ethidium bromide-stained gel. It reveals the integrity of the RNA preparations along with a comparison of the total amounts loaded on the gel.

**Kinetics of Induction of IL-1Ra mRNA Accumulation, and IL-1Ra Synthesis and Secretion by Neutrophils after GM-CSF and TNF- $\alpha$  Stimulation.** Time course studies on the influence of GM-CSF and TNF- $\alpha$  on IL-1Ra mRNA levels in neutrophils and on the induction of synthesis and secretion of IL-1Ra



were performed. Densitometric scanning of SDS-PAGE performed with cell lysates revealed that the level de novo synthesized intracellular IL-1Ra was maximal at 1 h (data not shown), while secretion was maximal at 4 h (Fig 3 A). Similar analyses performed on Northern blots (Fig. 3 B) showed increased levels of IL-1Ra mRNA as early as 0.5 h, reaching a peak at 1 h. At 24 h, no IL-1Ra mRNA was detectable in neutrophils, indicating significant degradation at this point in time.

**Measurement of IL-1Ra Production by Neutrophils.** We next measured the intra- and extracellular levels of IL-1Ra using nonactivated and activated neutrophils. IL-1Ra was measured by ELISA using cells isolated from three normal individuals, and the results are expressed in nanograms per milliliter/ $10^6$  neutrophils. As can be seen from Table 2, there was an eight-fold excess of intracellular over extracellular IL-1Ra in nonactivated cells and a four- to fivefold excess in activated cells, implying that the great majority of newly synthesized IL-1Ra was being secreted. The differences between nonactivated and activated neutrophils with respect to intracellular IL-1Ra levels were not statistically significant. In contrast, activated cells

**Table 2.** Measurement of IL-1Ra Production by Neutrophils

	Cell lysate		Cell supernatant	
	cycloheximide		cycloheximide	
	-	+	-	+
Control	1.6 ± 0.2	1.50 ± 0.06	0.2 ± 0.02	0.09 ± 0.01
GM-CSF	2.5 ± 0.4	1.90 ± 0.11	0.5 ± 0.06	0.1 ± 0.01
TNF-α	2.0 ± 0.2	1.40 ± 0.17	0.5 ± 0.07	0.08 ± 0.01

IL-1Ra was measured by ELISA from neutrophil cell lysates and supernatants. Neutrophils were isolated from normal human blood and the values represent the mean ± SEM of experiments performed in duplicate on cells from three different normal donors. Statistics were performed using the student's *t* test.

\* Not statistically different.

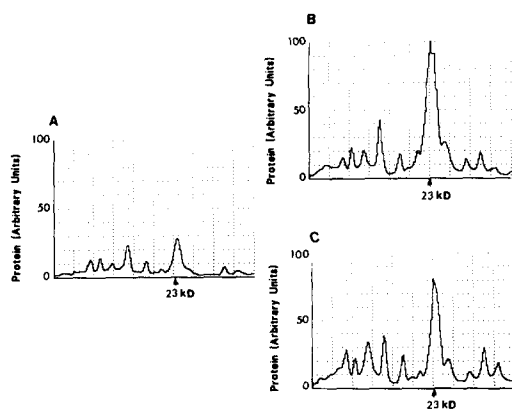
† Statistically significant at *p* < 0.007.

§ Statistically significant at *p* < 0.001.

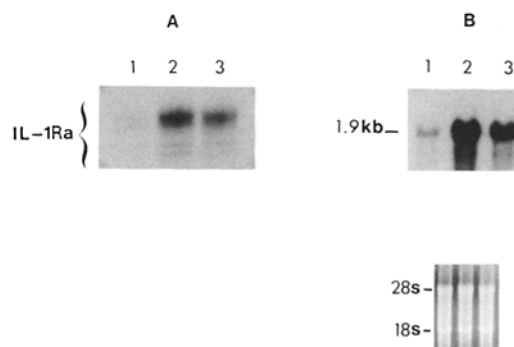
|| Statistically significant at *p* < 0.006.

secreted significantly higher levels of IL-1Ra than did nonactivated cells (*p* < 0.007 for GM-CSF and *p* < 0.001 for TNF-α). We next investigated whether the increased extracellular levels of IL-1Ra observed after activation were due to increased de novo synthesis of this molecule as opposed to increased secretion of intracellular IL-1Ra content. To address this question, we activated neutrophils with GM-CSF and TNF-α in the presence or absence of the protein synthesis inhibitor, cycloheximide. The results presented in Table 2 show that extracellular IL-1Ra, after activation with either GM-CSF or TNF-α, was markedly decreased by cycloheximide (*p* < 0.006), whereas intracellular levels were found not to be significantly affected.

*Level of IL-1Ra Production by Neutrophils Relative to Other De Novo Synthesized and Secreted Proteins.* Upon visual inspection of the fluorograms presented in Fig. 1, it can be observed that relative to the other products represented by the various bands seen on the fluorogram, IL-1Ra appears as the major de novo synthesized and secreted neutrophil product in response to either GM-CSF or TNF-α. To further illustrate this, densitometric scanning studies were performed on the fluorograms shown in Fig. 1, lanes A, B, and D. The results are presented in Fig. 4. Intensity levels for all the bands obtained from nonactivated cells are shown in Fig. 4, A, whereas Fig. 4, B and C, indicate the levels obtained from GM-CSF- and TNF-α-activated cells, respectively. It can be



**Figure 4.** Relative levels of de novo IL-1Ra synthesis and secretion compared with total protein synthesis and secretion by human neutrophils. Neutrophils were labeled and treated as described in the legend to Fig. 1, and the proteins secreted into the supernatants were separated by SDS-PAGE. Densitometric scanning data were then performed on the fluorograms obtained after SDS-PAGE. The 23-kD marker represents IL-1Ra. (A) Control cells; (B) GM-CSF-stimulated cells; (C) TNF-α-stimulated cells.



**Figure 5.** IL-1Ra protein synthesis and secretion, and mRNA expression by neutrophils isolated from the synovial fluid of a patient with rheumatoid arthritis. (A) The cells were treated as described in the legend to Fig. 1 and the supernatants were processed for immunoisolation to detect IL-1Ra. (B) The cells were treated as described in the legend to Fig. 2, and the level of IL-1Ra mRNA expression was determined by Northern blot analysis. (Bottom) Photograph of the ethidium bromide-stained gel used for the Northern blot indicating equal loading of RNA in each lane. (Lane 1) Control cells; (lane 2) GM-CSF-activated cells; (lane 3) TNF-α-activated cells.

clearly seen that IL-1Ra (the 23-kD band) is by far the major component of all de novo synthesized and secreted products of activated neutrophils. This is particularly evident in GM-CSF-activated cells.

*Production of IL-1Ra by Neutrophils Isolated from the Synovial Fluid of Patients with Rheumatoid Arthritis.* We next wanted to determine whether neutrophils isolated from an inflammatory milieu retained their ability to synthesize and secrete IL-1Ra in response to stimulation by GM-CSF and TNF- $\alpha$ . To perform this study, we used neutrophils isolated from the synovial fluid of patients with rheumatoid arthritis. Neutrophils were stimulated and metabolically labeled as described above. Immunoprecipitation of IL-1Ra from cell supernatants was then performed using the polyclonal antibody to IL-1Ra. Results are presented in Fig. 5 A, where it can be seen that there is an induction of de novo synthesis and secretion of IL-1Ra after GM-CSF (Fig. 5 A, lane 2) and TNF- $\alpha$  (Fig. 5 A, lane 3) stimulation compared with nonactivated cells (Fig. 5 A, lane 1). Northern blots were also performed using RNA isolated from these cells and hybridized with the IL-1Ra cDNA probe. The results presented in Fig. 5 B show that levels of mRNA in neutrophils are increased after GM-CSF (Fig. 5 B, lane 2) and TNF- $\alpha$  (Fig. 5 B, lane 3) stimulation when compared with nonactivated cells (Fig. 5 B, lane 1). Fig. 5 B, bottom, shows a photograph of the ethidium bromide-stained gel used for the Northern blot.

## Discussion

We have previously shown that GM-CSF and TNF- $\alpha$  induce the de novo synthesis and secretion of a 23-kD protein by neutrophils in a highly selective manner (9). In the present study, we have characterized this product as the IL-1Ra.

Measurement of the amounts of IL-1Ra associated with cell lysates and supernatants of neutrophils leads us to suggest that the contribution of neutrophils to the production of IL-1Ra may be substantial when compared with that by macrophages. A recent study quantified IL-1Ra production induced by GM-CSF in in vitro derived macrophages using ELISA (12). Measurements were reported as the quantity (ng/ml) product by  $0.83 \times 10^6$  macrophages. Under these conditions,  $\sim 40$  ng/ml of IL-1Ra was associated with the cell lysates and 10 ng/ml was detected in the cell supernatants. Although it may be inappropriate to compare our results (4-h incubation of neutrophils) with those of that study using 7-d macrophage cultures, our measurements would indicate that macrophages produce  $\sim 20$  times more IL-1Ra. If one is to take the example of synovial fluid from patients with

rheumatoid arthritis, where neutrophils can outnumber macrophages by a factor of at least 20, it can be concluded that in the presence of GM-CSF (or TNF- $\alpha$ ), production of IL-1Ra by neutrophils may equal that by macrophages.

Studies on de novo protein synthesis by neutrophils must always consider the possibility of a contribution by small numbers of contaminating monocytes. We have estimated that monocytes produce  $\sim 20$  times more IL-1Ra than neutrophils. Our neutrophil preparations would therefore have to be contaminated by a minimum of 5% monocytes to account for all of the IL-1Ra that was measured. By nonspecific monoesterase staining, contaminating monocytes never exceeded 0.2%.

It has been shown that IL-1Ra must be in excess of IL-1 to inhibit the stimulatory effects of this cytokine (13). It therefore makes physiological sense that neutrophils would produce the amounts of IL-1Ra that they do. When analyzing the results of a recent study that measured IL-1 production by neutrophils using conditions that are highly similar to those used in this study (14), comparisons between IL-1 $\beta$  and IL-1Ra production by neutrophils can be made. After 4 h of stimulation by TNF- $\alpha$ , the cell-associated IL-1 $\beta$  per  $10^6$  cells was  $\sim 15$  pg/ml, whereas we measured 2 ng/ml of IL-1Ra, a 130-fold excess. Again under the same conditions, the levels of secreted IL-1 $\beta$  was  $\sim 5$  vs. 500 pg/ml for IL-1Ra, a 100-fold excess.

We also observed that of the de novo protein synthesis and secretion by neutrophils in response to activation by GM-CSF and TNF- $\alpha$ , IL-1Ra constitutes by far the major protein. Therefore, in terms of quantity alone, production of IL-1Ra by neutrophils is a major event in response to this type of activation. This obviously raises the issue of the biological significance for the need of neutrophils to mount this relatively massive response in terms of IL-1Ra production. Studies performed in vitro on the stimulatory effects of IL-1 on neutrophils have yielded conflicting results (15–17) and will have to await further investigation before any firm conclusions can be made. Interestingly, however, IL-1 injected into animals leads to the accumulation of neutrophils at the site of injection (18), and in vivo studies on the use of IL-1Ra have shown that it reduces the number of tissue-invading neutrophils in septic shock (3). This latter finding would be in agreement with the observation that IL-1Ra reduces IL-1-induced adhesion of neutrophils to endothelial cells (11). In this context, production of relatively large amounts of IL-1Ra by neutrophils at inflammatory sites could be of major biological significance.

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