Differential Regulation of the Stability of Cytokine mRNAs in Lipopolysaccharide-activated Blood Monocytes in Response to Interleukin-10*

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Adenosine-uridine (AU) instability elements, found in the 3'-untranslated regions of numerous mRNAs, target these mRNAs for rapid degradation. In addition, the degradation rate of some mRNAs that contain AU instability elements can change. This modulation of mRNA stability is an important component in the regulation of expression of many of the cytokines that control the production and function of blood cells. However, it has not been clear whether the stabilities of individual cytokine mRNAs that contain AU instability elements are coordinately regulated or whether different mRNAs can be independently regulated. We have investigated the influence of the cytokine synthesis inhibitory factor interleukin (IL)-10 on the turnover of granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), and IL-10 mRNAs in human blood monocytes stimulated with lipopolysaccharide. We find that all three mRNAs are destabilized in response to IL-10 but at different times. The G-CSF and GM-CSF mRNAs respond similarly, being rapidly destabilized, consistent with a direct influence of IL-10 receptor-mediated signals on the stability of these mRNAs. In contrast the IL-10 mRNA became unstable only after several hours of treatment with IL-10, suggesting that the IL-10 mRNA, although it also contains AU instability elements, is not co-regulated with the G-CSF and GM-CSF mRNAs but is regulated by a secondary factor produced in response to IL-10.

Many other cytokine mRNAs, including the IL-10 mRNA, also contain AUIEs, making it likely that these mRNAs too are unstable in unactivated cells.

Cells activated in vitro typically produce cytokines transiently, with cytokine mRNA levels and cytokine synthesis peaking several hours after stimulation of the cells and subsequently declining. The stability of the cytokine mRNA also varies with time, facilitating the initial accumulation and subsequent removal of the mRNA. However, the modulation of mRNA stability is poorly understood, and it is not clear whether all the AUIE-containing cytokine mRNAs produced in a cell respond identically or whether the stabilities of some cytokine mRNAs can be independently regulated.

Various agents that induce cytokine mRNA stabilization have been identified (such as IL-1 (5), TNFα (1, 4, 5), LPS (8), and anti-CD28 (2, 3)), but few mRNA destabilizing agents have been identified. We chose to investigate the influence of IL-10 on cytokine mRNA stability because it is known to act on some cells to suppress cytokine synthesis (9–13), suggesting that it may promote destabilization of cytokine mRNAs. Of further interest was the fact that the 3'-UTR of the IL-10 mRNA contains multiple copies of the AUIE, suggesting that the stability of the IL-10 mRNA might be regulated also.

IL-10 can serve as a stimulatory factor for B cells and mast cells, but its most well documented effects are its inhibitory activities (reviewed in Ref. 14). IL-10 inhibits proliferation and reduces cytokine production from T-cells (9), particularly affecting interferon-γ and IL-2 production. It deactivates macrophages (15) and reduces their synthesis of IL-1, IL-6, and TNFα (10) following stimulation with LPS and reduces synthesis of IL-8, MIP-1α, MIP-1β (16), IL-1, and TNFα (11, 17) in neutrophils stimulated with LPS. In polymorphonuclear leukocytes IL-10 up-regulates production of IL-1 receptor antagonist, a potent anti-inflammatory agent (18, 19). It inhibits LPS-induced survival of eosinophils and their synthesis of GM-CSF, TNFα, and IL-8 (12) and in monocytes activated with LPS IL-10 reduces synthesis of IL-1α, IL-1β, IL-6, IL-8, TNFα, GM-CSF, and G-CSF (13).

In this report we show that in monocytes activated by LPS, IL-10 decreases the stability of the mRNAs for G-CSF, GM-CSF, and IL-10. The effect on the G-CSF and GM-CSF mRNAs occurs relatively rapidly, whereas the IL-10 mRNA becomes unstable several hours later, indicating that the stability of IL-10 mRNA is regulated independently from that of G-CSF and GM-CSF mRNAs.

EXPERIMENTAL PROCEDURES

Reagents—Human IL-10 and anti-IL-10 mAb 19F1 were generous gifts from Dr. Anne O’Garra and Dr. Rene de Waal Malefyt (DNAX Research Institute, Palo Alto, CA) and were used at concentrations of 100 units/ml and 5 μg/ml, respectively. LPS (Difco) was used at a concentration of 1 μg/ml, and actinomycin D (Boehringer Mannheim)
was used at 5 μg/ml.

Isolation and Culture of Human Monocytes—All experiments were performed using freshly isolated cells. Human peripheral blood monocytes were isolated from 500 ml of blood from normal donors (gift from Red Cross Transfusion Service, Adelaide, Australia). Mononuclear cells were isolated on lymphoprep (Nycomed) density gradients followed by fractionation into lymphocytes and monocytes by centrifugal elutriation. The monocyte fraction (7 × 10^7 to 2 × 10^8 cells) was collected and judged 94–99% pure by Giemsas staining of cytocentrifuged preparations. Monocytes were cultured in RPMI 1640 (endotoxin free) supplemented with 10% fetal calf serum in 60-mm tissue culture dishes at a concentration of 2–8 × 10^6/ml. All data in any one figure are from a single monocyte preparation.

RNA Isolation and RNase Protection Assay—Total RNA was isolated by the guanidine thiocyanate lysis procedure of Chomczynski and Sacchi (20). To maximize RNA yield, we added 10 μg of tRNA before the initial phenol extraction and a further 10 μg of tRNA prior to the first precipitation. The yield of cellular RNA was usually about 5 μg/dish, and the entire sample was assayed, using GAPDH to correct for variations in yield. G-CSF, GM-CSF, and IL-10 mRNA levels were measured by RNase protection assay using complementary RNA probes synthesized as described previously (21) using SP6 RNA polymerase with 400 Ci/mmol [α-32P]UTP. GAPDH probe was synthesized using T7 RNA polymerase with 100 Ci/mmol [α-32P]UTP. RNase protection assays were performed as described previously (21) using Rnase A at a concentration of 10 μg/ml at 26°C. Undigested probe and tRNA controls were included in all experiments to verify band identity and digestion efficiency. Specific mRNA abundance was quantitated by phosphor image analysis using Imagequant version 3.21 (Molecular Dynamics, Sunnyvale, CA) and normalized with respect to the GAPDH internal standard.

Calculation of Degradation Rates—Relative mRNA levels were measured at various times after inhibition of transcription with 5 μg/ml actinomycin D. Rate constants were calculated by nonlinear regression analysis assuming single phase exponential decay to zero using GraphPad Prism data analysis software (GraphPad Software, Inc., San Diego, CA). Half-lives were derived from the degradation rate constants (t½ = 0.69/k).

RESULTS

IL-10 Acts Rapidly to Destabilize and Inhibit the Accumulation of GM-CSF and G-CSF mRNAs—GM-CSF mRNA is not detectable by RNase protection assay in freshly isolated unstimulated monocytes (Fig. 1). To establish conditions under which the influence of IL-10 on GM-CSF mRNA stability could be measured, we first determined the time course of GM-CSF mRNA expression in response to LPS (Fig. 1). GM-CSF mRNA accumulated for at least 8 h following stimulation with LPS, but by 24 h poststimulation the level of GM-CSF mRNA had declined to be almost undetectable. When monocytes were incubated with LPS in the presence of 100 units/ml IL-10, GM-CSF mRNA was not detected at any of the time points, indicating that IL-10 prevents accumulation of GM-CSF mRNA. Because monocytes can themselves produce IL-10 in response to LPS, activated monocytes were also incubated in the presence of anti-IL-10 monoclonal antibody 19F1. This IL-10-neutralizing mAb has previously been shown to enhance the production of GM-CSF protein by LPS-activated human monocytes (13). By 8 h poststimulation, much higher (5-fold) levels of GM-CSF mRNA had accumulated than in cells incubated with LPS alone, suggesting that endogenously synthesized IL-10 was capable of down-regulating GM-CSF mRNA.

Because IL-10 completely inhibited the accumulation of GM-CSF mRNA when added simultaneously with LPS, we investigated whether it could inhibit G-CSF and GM-CSF mRNA accumulation if added some time after stimulation by LPS. IL-10 was added 2 or 3.5 h after stimulation of monocytes with LPS, following which mRNA levels were measured by RNase protection assay. At either time of addition IL-10 rapidly blocked subsequent accumulation of both G-CSF and GM-CSF mRNAs (Fig. 2). In the case of addition of IL-10 at 3.5 h, the mRNA levels actually declined rapidly, indicating the mRNAs were unstable. We chose this time of addition to measure the influence of IL-10 on mRNA stability.

Freshly isolated monocytes were stimulated with LPS for 3.5 h, at which time IL-10 was added for a further 0.5 h. Actinomycin D was then added to inhibit transcription, and RNA was isolated at this and subsequent times. Exposure to actinomycin D was limited to no more than 2.5 h to minimize secondary effects that can result from long term inhibition of transcription. In initial experiments we found that the effects of IL-10 on G-CSF and GM-CSF mRNA degradation were dose-dependent and were maximal at IL-10 concentrations greater than 25 units/ml (data not shown). IL-10 was used at 100 units/ml in all subsequent experiments.

We found that both G-CSF and GM-CSF mRNAs were very stable in cells treated with LPS alone, with no loss of mRNA over 2 h, whereas in IL-10-treated cells both mRNAs were unstable (Fig. 3). In experiments with monocytes from six different donors, we consistently found no significant degradation of GM-CSF or G-CSF mRNAs in the absence of IL-10, whereas in the presence of IL-10 both mRNAs were consistently unstable, with mean half-lives of 1.8 ± 0.4 h (n = 6) and 2.5 ± 0.4 h (n = 6), respectively (Table I).

IL-10 Negatively Regulates Its Own mRNA in LPS-activated Monocytes—The IL-10 mRNA contains several perfect or nearly perfect copies of the AU instability element in the 3’-UTR, suggesting that the IL-10 mRNA could be unstable. Furthermore, because IL-10 destabilizes the mRNAs for G-CSF and GM-CSF, it is also possible that IL-10 may influence the stability of its own mRNA. To investigate these possibilities, we first measured IL-10 mRNA levels in monocytes following activation by LPS, either alone or in the presence of exogenously
added IL-10 or anti-IL-10 mAb.

In LPS-activated monocytes the level of IL-10 mRNA increased for at least 8 h but had substantially declined by 24 h (Fig. 1). When IL-10 was added to the culture medium simultaneously with the LPS, there was no effect after 4 h, but the amount of IL-10 mRNA at 8 and 24 h poststimulation was markedly reduced. The addition of anti-IL-10 mAb with the LPS resulted in a modest enhancement of the IL-10 mRNA level at 8 h and a much slower rate of decline in mRNA level subsequently. Thus activated monocytes respond to both exogenously added and endogenous IL-10 by down-regulating IL-10 mRNA. However, the influence on IL-10 mRNA was delayed, compared with the effects on G-CSF and GM-CSF mRNAs (Fig. 1, compare B and C). For example, the addition of IL-10 at the time of exposure to LPS completely eliminated accumulation of GM-CSF, but inhibition of IL-10 accumulation was not seen until the 8 h time point. The delay was also evident when the down-regulation due to endogenously synthesized IL-10 was monitored using anti-IL-10 monoclonal antibody. There was a large effect of the monoclonal antibody on GM-CSF mRNA accumulation within 8 h, whereas the effect on IL-10 mRNA levels was slight at 8 h but large at 24 h, indicating that the endogenously synthesized IL-10 was affecting GM-CSF mRNA levels several hours earlier than it was affecting IL-10 mRNA.

IL-10 Acts Late to Destabilize Its Own mRNA—To investigate whether IL-10 affects the stability of its own mRNA, we initially used the same conditions that revealed a destabilization of the G-CSF and GM-CSF mRNAs; we added IL-10 to

### Table I

|                        | G-CSF                  | GM-CSF                  |
|------------------------|------------------------|-------------------------|
| IL-10                  |                        |                         |
| K value                | 0.038 ± 0.043          | 0.133 ± 0.074           |
| P value                | 0.003                  | 0.010                   |
| Half-life (h)          | 2.54 ± 0.40            | 1.83 ± 0.44             |

* First order rate constants assuming exponential decay.

* Two tailed Student's t test, paired two sample for means.

* No degradation was observed.
monocytes 3.5 h after stimulation with LPS and inhibited transcrip-
tion with actinomycin D 0.5 h later. Under these condi-
tions we found no effect on the stability of the IL-10 mRNA (Fig. 4 and Table II), consistent with the observation of a lag before any inhibition of accumulation of IL-10 mRNA (Fig. 1). We therefore chose to expose cells to IL-10 for a longer period before measuring IL-10 mRNA degradation rates. Freshly iso-
lated monocytes were incubated with LPS for 2 h, after which IL-
10 or carrier was added to the culture medium for a further
4 h. Actinomycin D was then added, and RNA was harvested at
intervals thereafter. With this longer incubation in the pres-
ence of IL-10, there was a significant effect on the IL-10 mRNA
stability (p = 0.026; Fig. 4 and Table II). Thus the stability of
the IL-10 mRNA is regulated but not contemporaneously with
the G-CSF and GM-CSF mRNAs.

**DISCUSSION**

We have found that IL-10 causes destabilization of the G-
CSF, GM-CSF, and IL-10 mRNAs and inhibits their accumu-
lation in LPS-activated monocytes. Because cytokine secretion
by monocytes generally parallels mRNA levels (13), the
changes in mRNA are probably reflected at the protein level.
However, whereas IL-10 acts rapidly to destabilize G-CSF and
GM-CSF mRNAs, being effective within half an hour of expo-
sure of cells to IL-10, the action of IL-10 on its own mRNA is
delayed, not being evident until after several hours of exposure.
The rapidity of the effect on the stability of the G-CSF and
GM-CSF mRNAs suggests that this is a direct effect; the IL-10
receptor activates signaling pathways that influence the de-
gradation of the G-CSF and GM-CSF mRNAs. On the other
hand, the lack of a similar response in the degradation of IL-10
mRNA indicates that different or additional signaling path-
ways control the degradation of IL-10 mRNA. The fact that
IL-10 mRNA responds after a delay of several hours suggests
that the effect is indirect. The IL-10 could be inducing the
synthesis of a factor that promotes IL-10 mRNA degradation,
or it could be inhibiting the production of a factor that causes
stabilization of the IL-10 mRNA. If the latter were the case,
the putative stabilizing factor, whether intracellular or secreted,
would have to be labile (because the medium the cells were
incubated in was not changed during these experiments).

Many types of cell, especially those of the immune system,
can produce multiple cytokine mRNAs that contain AU insta-
bility elements and are subject to stability regulation. An im-
portant implication of our findings is that the stability of these
cytokine mRNAs can be independently regulated. This sug-
gests there may be regulatory elements in these mRNAs in
addition to the AUIE. The possibility that sequences in addi-
tion to the AUIE participate in the regulation of cytokine
mRNA stability is also suggested by the observation that a
region of the GM-CSF 3′-UTR about 160 bases upstream of the
AU-rich region is required for regulation in response to phorbol
myristate acetate (22). In the case of IL-11 mRNA, which also
contains AUIEs in the 3′-UTR, sequences outside of the 3′-UTR
appear to be necessary for stabilization in response to IL-1 (23).
In addition, we have recently identified a novel stem loop
instability element in the G-CSF mRNA.2 Regulatory elements
such as these could provide a mechanism for differentially
regulating the stabilities of different cytokine mRNAs.

Although we find that IL-10 down-regulates expression of
G-CSF, GM-CSF, and IL-10 mRNAs by increasing the turnover
rate of their mRNAs, this does not preclude the possibility that
other mechanisms may contribute to the regulation of produc-
tion of these cytokines. For example, Wang et al., (24) found
that in human peripheral blood mononuclear cells IL-10 down-regu-
lates TNFα and IL-1β by acting mainly at the level of gene
transcription. On the other hand, Bogdan et al. (25) found no
evidence for modulation by IL-10 of TNFα gene transcription in
macrophages and proposed that IL-10 promoted mRNA degra-

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**Table II**

| Degradation rates of IL-10 mRNA in LPS-activated monocytes in the presence and the absence of IL-10 |
|---------------------------------|----------------|----------------|----------------|
|                                 | -IL-10 for 0.5 h | +IL-10         | -IL-10 for 4 h  | +IL-10         |
| K value                         | 0.101 ± 0.038   | 0.092 ± 0.107  | 0.279 ± 0.092  | 0.652 ± 0.156  |
| P                               | 0.92            | 0.92           | 0.026          | 0.026          |
| Half-life (h)                   | 11.3 ± 4.5      | 8.3 ± 5.9      | 1.74 ± 0.78    |

1 C. Y. Brown and G. J. Goodall, unpublished observation.
2 Two tailed Student’s t test, paired two sample for means.
3 No degradation was observed in some experiments, so an average half-life cannot be calculated. However, converting the average rate constant to an “average” half-life gives 7.5 h.
Our results as well as recent reports of destabilization by IL-10 of IL-8 (16, 17), MIP-1
transcriptional mechanisms play a role in the regulation of neutrophils provide further support for the proposal that post-
activation the cells produce cytokines such as G-CSF and GM-CSF. Several hours later another factor induced by the IL-10 causes the IL-10 mRNA to be destabilized, down-regulating the synthesis of IL-10.

Monocyte/macrophages play a crucial role in defense responses to infection and trauma, releasing a variety of products including a number of cytokines, some of which act as pro-inflammatory mediators. Their uncontrolled activation, however, is a major contributing factor to chronic inflammatory diseases. Negative regulators of monocyte activation therefore play an essential part in moderating and inhibiting an inflammatory response. The delayed down-regulation of IL-10 mRNA compared with G-CSF and GM-CSF mRNAs may be an important factor in the generation and control of inflammatory responses. The delay this imposes before IL-10 down-regulation occurs before its own synthesis is down-regulated (Fig. 5). This presumably allows the initial development of inflammatory responses and the recruitment of a first wave of leukocytes to the site of inflammation but then dampens the response unless cells at the inflammatory site are further stimulated, for example by antigen-mediated activation. The low but detectable level of IL-10 mRNA in freshly isolated monocytes may indicate a low level constitutive production of IL-10 by resting monocytes. This might help in vivo to suppress synthesis of activating cytokines such as G-CSF and GM-CSF and ensure that monocytes remain unactivated in the absence of exogenous stimulation.

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