Regulation of Cytokine Release From Mononuclear Cells by the Iron-Binding Protein Lactoferrin

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The iron-binding protein lactoferrin (Lf) is a constituent of neutrophil secondary granules and is discharged into the surrounding medium when neutrophils are activated. Lf released from neutrophils phagocytosing opsonized particles inhibits proliferation of mixed lymphocyte cultures (MLC) and has also been shown to inhibit granulopoiesis, suppress antibody production, and regulate natural killer cell activity. All of these processes are controlled by cytokines, suggesting that Lf may modulate immune responses by inhibiting cytokine activity. When MLC were cultured in round-bottomed wells to crowd the cells together, Lf, 50% saturated with iron, inhibited both proliferation and interleukin-2 (IL-2) release into the supernatants. Inhibition was concentration-dependent and lost at concentrations of Lf greater than 10⁻¹² mol/L. Lf at 10⁻¹⁰ mol/L inhibited release of tumor necrosis factor-α (TNF) and interleukin-1β (IL-1) into MLC supernatants, as well as inhibiting IL-2 release. TNF in the supernatant was significantly reduced at 5 and 24 hours, becoming less and losing significance by 72 hours. IL-1 in the supernatant was not significantly reduced at 5 and 24 hours, becoming significant at 48 and 72 hours. IL-2 was significantly reduced at 48 and 72 hours and followed the same time course as proliferation. Inhibition was blocked by specific antisera to Lf, but not by a preimmune serum. Lf, 10⁻¹⁰ mol/L, also inhibited the production of TNF (49.15% ± 7.98%; n = 10, P = .032) and IL-1 (42.67% ± 6.72%; n = 6, P = .032) from endotoxin-stimulated mononuclear cells. As with MLC, inhibition was dose-dependent and abrogated by specific antisera. Lf did not block the biological action of TNF, IL-1, or IL-2 in specific assays using cytokine-sensitive cell lines. These data suggest that Lf, released from activated neutrophils, acts as a negative feedback mechanism to prevent recruitment and activation of leukocytes in sites of inflammation.

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and incubated together for 30 minutes at 37°C. The protein was then dialyzed against Tris/HCl for 24 hours at 4°C. Successful 50% Fe saturation was determined by detection of an absorbancy peak at 460 nm.

Isolation of MNC. Venous blood was taken from normal healthy volunteers into EDTA at a final concentration of 3 mmol/L. The blood was used immediately after venesection. The MNC were obtained using a rapid single-step technique. The blood was layered onto Ficoll-hypaque solution of 1.114 g mL⁻¹ density and incubated together for 30 minutes at 37°C. The protein was then centrifuged at 400 x g for 10 minutes, and the resulting cell-free supernatant was filtered through a 0.2-µm sterile filter before storage at -70°C until required for assay. The platelet-rich plasma was discarded and the MNC collected and washed twice in RPMI-1640 medium (Flow Laboratories). This process routinely produced a MNC fraction of greater than 98% viability, with a purity of approximately 97%. The MNC collected contained an average of 30% monocytes as determined by Diff-Quick- (Merz & Dade, Dübtingen, Switzerland) stained cytopsin preparations.

Mixed lymphocyte cultures. MNC from three individual donors were suspended at 1.1 x 10⁶ mL⁻¹ in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L glutamine, 100 U mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin (all Flow Laboratories), and 10⁻⁶ mol/L indomethacin (Sigma Chemical). Equal volumes of each donor cell suspension were mixed together and 180 µL added to triplicate wells in a 96 round-bottomed well tissue culture plate (Costar, Northumbria Biologicals Ltd, Cramlington, Northumberland, UK). Fifty percent Fe-saturated Lf was added where required at the appropriate concentrations at the beginning of the culture period. In some experiments, wells were set up with the addition of a rabbit polyclonal antibody to human Lf (Dakopatts Ltd, High Wycombe, Bucks, UK) at a dilution of 1:500. In control wells, the same concentration of preimmune rabbit serum (Dakopatts) was used. Both of these antibody preparations were dialyzed free of sodium azide. The cells were then incubated at 37°C plus 5% CO₂ with 100% humidity for 5, 24, 48, and 72 hours. At the end of the culture period, cell proliferation was determined by measuring tritiated thymidine (Amersham, UK) incorporation following a 6-hour pulse. The samples were then counted for 3 minutes each in a Packard Tri Carb Series 4000 scintillation counter (Packard, Pagnobbe, Berks, UK) with window settings of 2 to 19 MeV. The means of triplicates were taken to represent the proliferation for a particular sample. Duplicate plates were set up for determining cytokine production. After incubation, the plates were spun down at 400 g for 10 minutes and the cell-free supernatant was harvested and stored at -70°C until required for assay.

Mononuclear cell cultures. The effect of Lf on cytokine production from MNC stimulated with 1 µg · mL⁻¹ bacterial LPS (Escherichia coli 0111:B4, Sigma Chemical) was also investigated. MNC were isolated as for MLC, separate donor MNC were cultured at 3 x 10⁶ mL⁻¹ in complete medium. These cells were incubated under the same conditions as described for MLC in 12-well tissue culture plates (Costar). Again, where appropriate, 50% Fe-saturated Lf was added in the presence or absence of either the anti-Lf antibody or control serum. TNF production has been shown to be maximal at 5 to 6 hours, with IL-1 peak production occurring slightly later. Therefore, culture supernatants were collected at 5 hours for measurement of TNF and at 18 hours for detection of IL-1β. The conditioned media (MNCM) were centrifuged at 400 g for 10 minutes, and the resulting cell-free supernatants filtered through a 0.2-µm sterile filter before storage at -70°C until required for assay.

Cytokine assays. IL-2 was measured using the method of Gillis et al with the mouse CTLL-2 cell line; the IL-2 standard was obtained from Boehringer Mannheim (Lewes, East Sussex, UK) and 1 bioactive unit was equivalent to 500 pg of protein. Immunoreactive IL-2 was determined using an enzyme-linked immunoabsorbent assay (ELISA; R & D Systems, Minneapolis, MN; lot no. 91 47 149). IL-1 was assayed by the technique of Gearing et al using the EL-4.NOB-1 and CTLL-2 cell lines. Immunoreactive IL-1β was determined using an enzyme-linked immunoabsorbent assay (ELISA; R & D Systems, Minneapolis, MN; lot no. 90 46 056). Immunoreactive TNF was measured using a sensitive ELISA according to the method of Meager et al with antibodies kindly supplied by Dr A. Meager, National Institute of Biological Standards and Control (NIBSC), Potters Bar, UK. The recombinant human IL-1β and TNF were also supplied by NIBSC.

All reagents were tested for possible endotoxin contamination.

![Fig 1. The dose-dependent action by Lf on tritiated thymidine uptake and IL-2 production by MLC. The results represent the means ± SEM of six separate experiments.](image-url)
using the limulus amebocyte lysate (LAL) assay E-Toxate (Sigma Chemical). Reagents were determined to contain less than 20 pg · mL⁻¹ contaminating endotoxin.

Statistics. The results are expressed as the means ± SE. The Mann-Whitney U test was used to determine statistical significance, with P < .05 considered to be significant.

RESULTS

We have previously shown that Lf inhibits the production of a growth factor in MLC with cells crowded in a round-bottomed well during culture. Cell proliferation in MLC is considered to be a T-cell-mediated phenomenon driven by IL-2; therefore, this lymphokine was thought to be the likely identity of the growth factor. MLC prepared in round-bottomed wells were incubated with increasing concentrations of Lf, and proliferation was measured at 72 hours. Our previous studies demonstrated that Lf inhibits the MLC at concentrations down to 10⁻¹² mol/L Lf. Figure 1 shows that the dose-response curves for IL-2 production correlated with the dose-dependent inhibition of thymidine uptake into the MLC by Lf. In addition, Lf was not exerting its effect on the actions of IL-2; when CTLL-2 cells were exposed to increasing concentrations of IL-2, the presence of Lf in the culture medium did not affect cell proliferation (data not shown).

The production of IL-2 from T lymphocytes is dependent on IL-1 secretion from monocytes, and TNF is at least partially responsible for stimulating monocyte production of IL-1. Therefore, the inhibition of IL-2 may be an indirect effect of Lf acting via the modulation of IL-1 production and perhaps TNF from monocytes. This was investigated by the effect of 10⁻¹⁰ mol/L Lf on MLCs measuring proliferation by tritiated thymidine incorporation and assay of immunoreactive IL-2, IL-1, and TNF in the supernatants by ELISAs. The results showed inhibition of all three cytokines (Fig 2). Proliferation was significantly
reduced at 24 hours, but more so by 48 and 72 hours; the reduction of proliferation followed the same pattern as that seen with IL-2 secretion. The effect on IL-1 was similar, being detectable, although not significant, at 5 and 24 hours, and becoming significant at 48 and 72 hours. TNF was already significantly inhibited at 5 and 24 hours, becoming less and losing significance by 72 hours. The addition of rabbit anti-human Lf polyclonal antibody, but not preimmune serum, blocked the inhibitory activity of the Lf on both proliferation and cytokine production. Control experiments in which MLC were incubated with antisera or preimmune serum alone showed that neither had any stimulatory activity with respect to proliferation or cytokine production (data not shown).

The production of IL-1 and TNF from MNC cultured in the presence of LPS was used as an alternative system to confirm the inhibitory action of Lf on cytokine production. As with MLC, $10^{-10}$ mol/L Lf significantly reduced both IL-1 and TNF in the supernatant (Fig 3). Inhibition was abrogated by a specific antibody, but not by preimmune serum, and neither antibody preparation alone had any effect on stimulating cytokine production. The effect of Lf was dose-dependent (Fig 4), although at high concentrations ($\geq 10^{-5}$ mol/L), Lf is known to polymerize and this accounts for loss of inhibitory activity. Lf at $10^{-10}$ mol/L had no effect on the activity of IL-1 when measured by the bioassay, suggesting that Lf was acting by inhibiting production of the cytokine and not by affecting its bioactivity.

**DISCUSSION**

These data suggest that Lf acts to inhibit release of a number of inflammatory mediators from activated MNC. We previously described the inhibition of a transferrable growth factor present in 72-hour MLC and this is almost
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