Lactoferrin Derived From Neutrophils Inhibits the Mixed Lymphocyte Reaction

By Kevin Slater and John Fletcher

The effects of the products of phagocytosing neutrophils on lymphocyte transcriptional activity have been investigated using allogeneic mixed lymphocyte cultures (MLC). When MLCs are grown crowded in round-bottomed wells, uptake of tritiated uridine is inhibited by the presence of neutrophil products. Similarly, inhibitory activity is shown by purified lactoferrin. The inhibitory factor released by neutrophils is positively identified as lactoferrin, since its activity is lost in the presence of specific antibody. In addition, inhibitory activity is lost when lactoferrin is removed by a monoclonal antibody (MoAb) column and is recovered with lactoferrin from the column. The active form of lactoferrin carries iron and the apoprotein is inactive. MLCs crowded in round-bottomed wells take up considerably more uridine than when spread in flat-bottomed wells, and it is this enhanced transcriptional activity that is inhibited by neutrophil-derived lactoferrin. Enhanced uridine uptake by crowded cultures is due to factors that can be transferred in the supernatants to promote uridine uptake by spread cultures. Neutrophil lactoferrin inhibits both production and effect of the transferable factor(s).

A NUMBER OF ROLES have been suggested for the iron binding protein lactoferrin (Lf), usually in relation to host defenses, since the binding of iron to apo-Lf makes it unavailable to invading organisms and limits their growth. This is probably its function in the many secretions in which it is found in high concentrations, including milk, tears, gastric and bronchial secretions, and seminal fluid. Its role in plasma is less clear, as the plasma protein transferrin is quantitatively much more important in binding iron and limiting its availability to microorganisms. However, Lf binds iron at lower pH than transferrin and may take over a bacteriostatic function in the acidic conditions of pus.

Plasma lactoferrin is derived from the secondary granules of neutrophils and is discharged from the cell when neutrophils are activated during inflammation. A number of extracellular functions influencing the inflammatory response have been described, including aggregation of neutrophils, inhibition of the complement system, suppression of antibody production, modulation of natural killer (NK) cell activity, and inhibition of colony-stimulating-factor (CSF) production. The inhibition of CSF production may be a direct action of Lf on monocytes and macrophages or an indirect action by inhibition of a monokine required for CSF production from T lymphocytes, fibroblasts, and vascular endothelial cells. Thus both the effects upon CSF production and on antibody production indicate an interaction between Lf and mononuclear cells involving control of protein synthesis. There is some evidence for a similar action of neutrophil products in vivo, as patients with peripheral blood neutrophil counts greater than $15 \times 10^9$ L$^{-1}$ showed impaired skin responses to various T and B mitogen; normal responses return when neutrophil counts fall. The hypothesis that lactoferrin released from neutrophils may have a role in control of lymphocyte responses has been investigated using an allogeneic mixed lymphocyte culture (MLC) that allows measurement of factors in the culture supernatants in the absence of stimulatory substances such as lectins and exogenous endotoxins. We have shown that a factor released from phagocytosing neutrophils inhibits transcriptional activity in lymphocyte cultures, identified the factor as lactoferrin, and demonstrated a requirement for iron to activate the molecule.

MATERIALS AND METHODS

Subjects. Blood was obtained from normal, healthy volunteers among the laboratory staff.

Neutrophils. Peripheral blood was taken into EDTA and the neutrophils separated by centrifugation over a Mono-Poly Resolving Medium (Flow Laboratories, Rickmansworth, UK). The cells were washed three times in phosphate-buffered saline (PBS) free of Ca$^{2+}$ and Mg$^{2+}$. The cells were then resuspended in PBS containing 1 mmol/L CaCl$_2$, 0.7 mmol/L MgCl$_2$, and 0.7 mmol/L CaCl$_2$ at a final cell concentration of $2 \times 10^7$ mL$^{-1}$.

Phagocytosis. Candida guilliermondii were cultured overnight in glucose broth and then heat killed at 100°C for 15 minutes. The cells were washed and opsonized in pooled human serum for 45 minutes at 37°C with continuous mixing. The C. guilliermondii were resuspended at $10^7$ mL$^{-1}$ in PBS containing Ca$^{2+}$ and Mg$^{2+}$.

Equal volumes of the neutrophil and C. guilliermondii preparations were mixed together and incubated with continuous mixing at 37°C for 30 minutes. The cells were removed by centrifugation and the supernatant sterilized by passage through a micropore filter (Flow Laboratories). Aliquots of the “phagocytosing neutrophil-conditioned medium” (PCNMC) were stored at $-20°C$ until required.

Lactoferrin. Lactoferrin (Lf) from human breast milk was obtained from Sigma Chemical Co, St. Louis. This was prepared in Tris/HCl buffer and used either iron free (Apo-Lf) or 50% iron saturated (Fe-Lf). Fifty percent iron saturation was achieved according to the method of Gutteridge et al to avoid nonspecific binding. Briefly, Lf was prepared in 0.2 mol/L Tris/HCl buffer containing 10 mmol/L NaHCO$_3$, and iron was added as Fe$_3$(NH)$_4$(SO$_4$)$_2$ taking 1.4 μg Fe per mg of protein to give 100% saturation. The iron and protein were incubated at 37°C for 30 minutes. Fifty percent saturation was confirmed by detection of an absorbance peak at 460 nm. Sigma Lf is stated to be 0.1% iron saturated, but no absorbance could be detected at 460 nm. The Lf preparations were filtered sterilized and aliquots stored at $-20°C$ until

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required. The Lf concentration of PNCM was kindly measured by Dr. J. Porter of University College Hospital, London using an enzyme-linked immunosorbent assay (ELISA) technique.

**Antibodies.** Rabbit anti-human breast milk Lf was obtained from Dako-Patts, High Wycombe, UK. Monoclonal anti-Lf against human neutrophil Lf was a generous gift from Dr. J. Brock (Glasgow University).

The antibodies were bound to CNBr-activated Sepharose 4B according to the method of van Eijk.1,2 The efficacy of the antibody columns was determined by passage of 59Fe-labeled Lf down the columns. This could be completely retained and subsequently recovered using Glycine/HCl buffer at pH 2.8. Detection of an absorbance peak at 460 nm indicated that this treatment did not cause dissociation of the bound iron.

**Mixed lymphocyte cultures.** Peripheral blood from three volunteers was taken into heparin and the mononuclear cells obtained by centrifugation over lymphocyte separation medium (LSM) (Flow Laboratories). The cells were washed three times in RPMI 1640 and resuspended at 1.1 x 10^6 cells/ml in RPMI containing 15% fetal calf serum, nonessential amino acids, penicillin, streptomycin, and 10^-6 mol/L indomethacin (c-RPMI). The cells from the three donors were kept separate throughout and were only mixed prior to use. One hundred eighty microliters of the mixed lymphocyte culture (MLC) was added to the wells of 96-well tissue culture plates (Costar, Cramlington, UK). Twenty microliters of Lf or PNCM dilution was added in quadruplicate; thus standard conditions consisted of 2 x 10^5 cells in 200 µL. Spread cultures and crowded cultures were produced in flat-bottomed and round-bottomed wells respectively. The cultures were incubated at 37°C at 100% relative humidity with 5% CO2 in air for 72 hours unless otherwise stated.

To determine the direct effect of Lf and PNCM on the MLC, 0.2 µCi tritiated uridine (3H-UdR) in 20 µL was added to the cultures for the last six hours of the culture period. The cells were then harvested onto glass fiber mats using a Dynatech Automash 2000. Incorporation of uridine was assessed by liquid scintillation counting in a Packard Tri-carb counter. For the determination of growth factor production, supernatants were prepared by resuspending the pellet followed by centrifugation to remove the cells. Aliquots of spread and crowded cell supernatants were stored at -20°C until required.

**RESULTS**

Both PNCM and 50% iron-saturated Lf (Fe-Lf) were able to significantly reduce the incorporation of 3H-UdR into a 72-hour MLC. However, as can be seen from Fig 1, significant inhibition could only be achieved if the cells of the MLC were crowded together in round-bottomed wells of tissue culture plates. When compared with cultures spread in flat-bottomed wells, the crowded cultures resulted in considerably greater stimulation. It was this stimulation, resulting from cell crowding, that was inhibited by both PNCM and Lf.

Figure 2 shows the effect of removal of Lf from PNCM by monoclonal antilactoferrin bound to CNBr-activated Sepharose columns. It can be seen that this procedure almost completely abrogates the inhibitory activity of PNCM. Previous experiments from 59Fe-labeled Lf had shown that Lf could be recovered from the column with glycine HCl buffer pH 2.8. Lf could therefore be recovered from PNCM, and Fig 2 shows that this Lf retained its inhibitory activity against the MLC.
Figure 3 shows that polyclonal antilactoferrin in the liquid phase was able to abrogate the inhibitory activity of PNCM. Polyclonal antilactoferrin (Dako-Patts) was extensively dialyzed against PBS at 4°C to remove all sodium azide; serial dilutions of the azide-free antibody were mixed with PNCM and incubated at 37°C for one hour. This procedure resulted in the total abrogation of the inhibitory activity of PNCM.

Figure 4 shows that 50% Fe-Lf inhibits the MLC in crowded cultures at concentrations between $10^{-8}$ and $10^{-12}$ mol/L, but the effect is lost at $10^{-14}$ mol/L. Apo-Lf showed no significant inhibitory activity at any concentration. The dose-response curve for PNCM when expressed in terms of its Lf concentration was the same as purified 50% Fe-Lf. The data illustrated in Fig 4 suggests that the Lf in PNCM, being inhibitory to the MLC, is carrying iron. This was confirmed by the formation of a pink band on the affinity column during the separation of Lf from PNCM, thus indicating that the neutrophil-derived Lf was indeed carrying iron.

Having determined that the inhibitory factor in PNCM was Lf, we then investigated further the effects of Lf on the MLC. As discussed earlier, considerably more stimulation was achieved when the cells of the MLC were crowded together in round-bottomed wells of tissue culture plates than when spread in flat-bottomed wells. It was the extra stimulation induced in the crowded cultures that was inhibitable by Lf. It was therefore assumed that Lf acted on a factor that promotes transcriptional activity and is produced as a result of cell crowding. To test this hypothesis supernatants were collected from 72-hour crowded cultures grown in the presence or absence of PNCM or Fe-Lf. These supernatants were then added to a spread culture with an equivalent volume of cRPMI acting as a control. It can be seen from Fig 5 that crowded culture supernatants significantly increased the stimulation of the spread MLC above that of the control. When supernatants were produced in spread cultures and subsequently transferred to a second spread culture, no increase in stimulation was observed. Thus a factor that increases transcriptional activity was being produced as a result of cell crowding. Figure 5 also shows that both PNCM and Fe-Lf were able to reduce the apparent production of this factor. What is not clear from these data, however, is whether the Fe-Lf is inhibiting the production of the factor or whether the carry-over of Fe-Lf in the supernatant is affecting the factor concentration. Consequently Fe-Lf was added after completion of 72 hours crowded culture, and then supernatants were transferred to spread cultures. The results (Fig 6) show significant inhibition of $[^3]$H-UdR uptake in the spread culture but less inhibition than expected if carry-over was the whole explanation. Similarly, supernatant from which lactoferrin was removed by passage down an affinity column produced an intermediate level of inhibition (data not shown). Thus Lf appears to have an effect on both the production and the effect of the transcription-promoting factor.

Finally, the time course of Fe-Lf effects was investigated. Figure 7 shows no detectable inhibition by Fe-Lf of $[^3]$H-UdR uptake into crowded cultures until 72 hours. When the supernatants from these cultures were transferred to spread cultures the same result was obtained, as only supernatants from 72-hour cultures showed significantly impaired ability to increase $[^3]$H-UdR uptake.

**DISCUSSION**

During phagocytosis of opsonized particles, neutrophils discharge 60% to 70% of secondary granule proteins into the surrounding medium. Similarly, neutrophils obtained from...
patients undergoing surgical trauma show evidence of secondary granule discharge with reduced intracellular concentrations of granule proteins accompanied by rising levels in the plasma. Thus there is circumstantial evidence for an extracellular role for secondary granule constituents, including Lf released when neutrophils are activated. At least one possible extracellular action is negative feedback control of lymphocyte responses, which is in keeping with other evidence that Lf inhibits T cell function and antibody production.

The observations reported show that phagocytosing neutrophils release a factor that inhibits uptake of uridine in a MLC. The factor has been clearly identified as Lf, since inhibitory activity was lost when specific antibody was added or when Lf was removed on a monoclonal antibody (MoAb) column and restored when Lf was recovered from the column. The effects of the neutrophil-derived Lf could be reproduced by Lf purified from human breast milk. There was a clear requirement for iron, as the apoprotein lacked activity. This finding was unexpected, as Lf would bind any free iron in the growth medium; the explanation may be
binding of free iron by transferrin from fetal calf serum before Lf was added.

Lf binds to specific receptors identified on mononuclear phagocytes and B lymphocytes, where it presumably delivers iron that is subsequently involved in reactions limiting the production or action of factors influencing transcription and protein synthesis. There is little data about the iron saturation of Lf in neutrophil granules, but it is usually considered to be iron free, whereas both the pink color when neutrophil-derived Lf is retained on an antibody column and its inhibitory action against the MLC suggest that when released it carries iron. This raises the intriguing possibility that Lf released passively by cell death is iron free and inactive, while Lf released by activation of the neutrophil acquires iron and, with it, inhibitory activity. The sources of iron may be microbial ferritin, as superoxide radical generated by the neutrophils' respiratory burst and involved in microbial killing can certainly mobilize iron from ferritin.

During an inflammatory response there must be a balance between factors such as endotoxin and complement breakdown products, providing a positive stimulus to mononuclear cells and negative factors such as Fe-Lf released from neutrophils when invading organisms are phagocytosed and killed. Initially the reaction will be driven, but as it succeeds it must be inhibited.

An argument against a physiologic regulatory function for Lf is the concentration at 10⁻⁹ mol/L reported in normal plasma, as this is well within the concentration range at which Lf affects lymphocyte growth (Fig 4). One explanation may be the failure to distinguish between iron-free inactive molecules and the iron-carrying active form. A more likely explanation is the difficulty in avoiding neutrophil activation and secondary granule discharge during venesection, since when great care is taken the plasma Lf concentration may be only 10⁻¹³ mol/L and below the concentration effective on lymphocyte responses.

The present study has shown that a neutrophil product, namely Lf, inhibits RNA synthesis of H-UdR uptake in a MLC. It is possible to show the same, although less marked, effect upon DNA synthesis using labeled thymidine (data not shown). When the cells were crowded in a round-bottomed well, there was considerably more uridine uptake than when spread in a flat-bottomed well, and this effect appeared to be due to a soluble factor, since it was transferable in the supernatant from crowded cultures. The inhibitory action of Lf was on this factor, either reducing its production or its transcription-promoting effect or both. Experiments adding Lf to crowded culture supernatants or removal of Lf from supernatants produced in its presence appeared to show an effect upon both growth factor production and transcription-promoting activity. These results might be explained by inhibition of factor production in both the crowded cultures and spread cultures, as transcription-promoting factors are probably produced as a secondary event in the stimulated spread cultures. Specific assays are being performed to define the factors involved.

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