In Vitro Studies of Lactoferrin and Murine Granulopoiesis

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Human lactoferrin (LF) has been reported to inhibit in vitro granulopoiesis by means of decreasing colony-stimulating activity production by monocytes. We performed a series of experiments to determine if the reported experimental results could be replicated using highly purified murine LF and murine target cells. Three different types of experiments were performed. (1) Medium was conditioned by lung, femoral shaft, and adherent peritoneal cells in the presence and absence of LF, and the granulopoietic stimulating activity in the conditioned media was assayed by means of a 7-day agar colony assay and a 3-day liquid slide colony-forming units and endogenous marrow CSA production. Because murine LF is immunologically distinct from human LF, these experimental results may not be due to specific physiologic inhibition of granulopoiesis. Similar in vivo experiments employing purified murine LF would be desirable. Because the quantity of purified murine LF needed for such in vivo studies may be considerable, we initially conducted a series of in vitro experiments using murine LF and murine target cells to determine if the results seen in the human in vitro studies could be replicated in the mouse. The data from this series of experiments failed to demonstrate that purified iron-saturated murine LF had inhibitory activity on in vitro production of humoral granulopoietic stimulators by mouse peritoneal macrophages or cells associated with femoral shafts and lungs.

A NEGATIVE feedback mechanism by which neutrophilic granulopoiesis is regulated has been postulated by many investigators. At present, however, there has not been an unequivocal demonstration of the existence of such a mechanism or the identification of physiologically relevant biochemical mediators of granulopoietic inhibition. The mature neutrophil is a logical source of such an inhibitory mediator, and there are some in vitro experimental data that support this concept. Recently, Broxmeyer et al. have presented data that implicate lactoferrin (LF) as a possible inhibitory modulator of granulopoiesis, working to suppress the production of colony-stimulating activity (CSA) by monocytes and macrophages. LF is an attractive candidate for such a role because among cells in the bone marrow it is found only in the secondary granule of the neutrophil, making it both cell line specific and a product of the more mature cells. In the investigations published to date, purified iron-saturated human LF has been employed. Inhibition of spontaneous colony formation in vitro by light density human marrow cells has been demonstrated using as little as 10^-17 M iron-saturated LF. The only in vivo experiments that support LF’s inhibitory role in granulopoiesis employ human LF injected into mice during granulopoietic rebound after pretreatment with cyclophosphamide. In these experiments, administration of LF resulted in a decrease in granulocyte and macrophage colony-forming units and endogenous marrow CSA production. Because murine LF is immunologically distinct from human LF, these experimental results may not be due to specific physiologic inhibition of granulopoiesis. Similar in vivo experiments employing purified murine LF would be desirable. Because the quantity of purified murine LF needed for such in vivo studies may be considerable, we initially conducted a series of in vitro experiments using murine LF and murine target cells to determine if the results seen in the human in vitro studies could be replicated in the mouse. The data from this series of experiments failed to demonstrate that purified iron-saturated murine LF had inhibitory activity on in vitro production of humoral granulopoietic stimulators by mouse peritoneal macrophages or cells associated with femoral shafts and lungs.

MATERIALS AND METHODS

Experimental Animals

Female BALB/c mice, age 11-13 wk, were supplied from our own breeding colony.

Preparation of Iron-Saturated, Purified Murine LF

All experiments employed murine LF that had been purified to homogeneity as previously described. LF was fully saturated with iron using (III)-nitritolactacetate according to the method of Bates and Wernicke.

Measurement of Plasma LF

The concentration of LF in the plasma of normal BALB/c mice was measured by means of a radioimmunoassay. Details of this assay have been previously reported.

Preparation of Conditioned Media

One intact lung or one femoral shaft from which the marrow had been expressed were placed in 2 ml of RPMI-1640 (Grand Island Biological Company, Grand Island, N.Y.) containing 20% fetal bovine serum (FBS) (Flow Laboratories, Wattsville, Md.) and the media conditioned for 8 hr at 37°C. Contralateral lungs and femurs from individual mice were divided equally between control (no LF)
and experimental (LF added) media, and an equal number of right and left lungs or femurs were used in each instance. In experimental conditioned media, LF was added to a final concentration of $10^{-11} - 10^{-7} M$ at the initiation of media conditioning. After completion of conditioning, the media were sterilized by filtration, stored at 37°C, and assayed for granulopoietic stimulating activity the following day.

Conditioned media was also prepared from adherent peritoneal cells obtained from unstimulated mice. After removal of the cells from the peritoneum by means of a saline wash, the cells were washed once, and $10^6$ cells were placed in a Petri dish in 5 ml of RPMI-1640 with 20% FBS. After incubation for 2 hr at 37°C, the nonadherent cells were removed by gentle agitation, and the dishes were rinsed once with medium. The adherent cells were then overlaid with 5 ml of medium and incubated for 48 hr at 37°C in an atmosphere of 7.5% CO₂ in water-saturated air. The conditioned media were then removed, sterilized by filtration, and assayed for stimulatory activity. In the experimental conditioned media, LF was added to a final concentration of $10^{-7} M$ at the start of the media conditioning.

**Slide Chamber Assay of Granulopoiesis**

In specific experiments, a liquid culture method was used to assay the granulocyte proliferation stimulating activity by means of tritiated thymidine ($\text{H}^3$-TdR) incorporation into DNA after 3 days of culture. Light density bone marrow cells separated on Ficoll-Hypaque were placed in slide chambers (4-chamber slides, Lab Tek, Napierville, Ill.) in liquid medium at a final concentration of $10^6$ cells/ml/chamber. Conditioned media from lung and femur cultures were added at a final concentration of 25-100 μg/ml, and from adherent peritoneal cells at a concentration of 50-400 μg/ml. Each experimental point was assayed in quadruplicate. After 69 hr of culture, 2 μCi of TdR (New England Nuclear, Boston, Mass.), specific activity 0.67 Ci/mM) were added to each chamber. The cultures were terminated 3 hr later (72 hr total culture time) by the addition of 1 mg TdR/chamber, and the liquid medium was removed by automated aspiration. We have previously shown that with this technique, greater than 95% of the cells remain adherent to the glass slides. The cells on the slides were fixed and washed 3 times in absolute methanol, once in water, and dried. The cells on the glass slides were removed by digestion with 0.5 ml of a solution of pronase (0.3 mg/ml) (Calbiochem, LaJolla, Calif.) and Ribo nuclease A (0.01 mg/ml) (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M Trizma base buffer (Sigma Chemical) containing 0.1 M NaCl and 0.005 M CaCl₂, pH 7.5. A 1.9-cm square filter paper (Whatman 3M) was spotted with 0.4 ml of the digest. After drying, the filter paper was washed in 5% cold TCA for 5 min 3 times, washed in 70% ethanol once, and placed in the bottom of a counting vial. To solubilize the DNA, the paper was covered with 0.5 ml of 0.6 N solution of NCS tissue solubilizer (Amersham Corporation, Arlington Heights, Ill.) and incubated for 30 min at 50°C. A toluene-based scintillation cocktail containing 4% (v/v) Liquifluor (New England Nuclear) was added, and the $\text{H}^3$-TdR incorporated into DNA was counted using a Beckman Scintillation Spectrometer. Counting efficiency in this system as determined by a $\text{H}^3$-toluene standard was 41%. The humoral activity in the conditioned media, which stimulated $\text{H}^3$-TdR incorporation into DNA, will be referred to as thymidine incorporating activity (TIA). By means of autoradiography, we have previously demonstrated that in these 3-day slide chamber cultures stimulated by CSA from human leukemia urine, the $\text{H}^3$-TdR is incorporated into proliferating neutrophils, monocytes, and macrophages. Despite the different source of stimulator used in these studies, the same types of proliferating cells and clones were observed.

**Agar Colony Assay**

In addition to assaying for granulocyte proliferation in the 3-day liquid cultures, a conventional 2-layer, 7-day agar colony assay was employed. A 2-ml underlayer of 0.5% agar in RPMI-1640 with 20% FBS was placed in the 35-mm Petri dishes (Falcon Plastics, Oxnard, Calif.), and adherent peritoneal cell conditioned media were added to yield a final concentration of 50-400 μg/ml of total culture volume. One milliliter of 0.3% agar with 10³ light density marrow cells was then overlaid. After 7 days of culture, clusters (>15, >50 cells) and colonies (>50 cells) were counted using a dissecting microscope at a magnification of 20×. A minimum of 3 Petri dishes were used for each experimental point.

**Agar Colony Assay Using Endogenous CSA From Adherent Peritoneal Cell Underlayers**

Peritoneal cells were obtained from unstimulated mice, and 2 × 10⁶ cells were incubated 2 hr in 35-mm Petri dishes in 1 ml of media. Nonadherent cells were removed as described above, and the adherent cells were overlaid with 2 ml of medium with 0.5% agar. Marrow cells were then overlaid at a concentration of 10⁶ ml in 1 ml of 0.3% agar. In the experimental cultures, LF was added in the underlayer to give a final concentration of $10^{-7} M$. After 7 days of culture, the clusters and colonies were scored as described below.

**Statistical Considerations**

The slopes of regression lines from the TIA assays were computed by the method of least squares, and the significance of differences between the slopes of control and experimental data was determined by a t test. For the agar colony assays, the mean and standard error for each experimental point were determined, and the significance of differences between the experimental and control cultures was determined by a Student's t test. Each experimental result presented was confirmed in replicate experiments. A p value of 0.05 or less was considered significant.

**RESULTS**

**Concentration of LF Employed**

In this series of experiments, we employed concentrations of LF ranging from $10^{-12}$ to $10^{-7} M$. We saw no inhibitory effect of LF in four experiments using concentrations less than $10^{-7} M$; therefore, the data presented below will be from experiments in which the highest concentration ($10^{-7} M$) was employed.

To relate the concentration of LF used in these in vitro studies to in vivo levels, we determined the plasma LF concentration in normal BALB/c mice. A mean concentration of 0.96 μg/ml ±0.08 SE, n = 5) was observed. A molar concentration of $10^{-7}$ is equivalent to 8 μg/ml, or over eightfold higher than the normal plasma concentration.

**Effect of LF on TIA Production by Cells Associated with Femur Shafts and Lungs**

The relative amounts of stimulatory activity in the various conditioned media were determined in dose-response assays that related the amount of conditioned media added to the culture and the amount of $\text{H}^3$-TdR incorporated into DNA during the final 3 hr of the
72-hr cultures. The slopes of the calculated regression lines represent the TIA expressed as dpm/μl of conditioned media. As is demonstrated by the data in Table 1, the addition of LF at a concentration of 10^{-7} M did not influence the amount of TIA produced during media conditioning by empty femoral shafts or intact lungs.

**Effect of LF on TIA and CSA Production of Adherent Peritoneal Cells**

We reasoned that the failure of LF to inhibit TIA production in the above experiments might be due to the tissue source of the TIA producing cells or to the relatively short conditioning time (8 hr). In addition, there was the possibility that the 3-day liquid cultures measuring TIA might not be equivalent to the standard 7-day agar colony assay used by most investigators. To overcome these potential problems, we studied the effect of LF on both TIA and CSA produced during 48 hr of media conditioning by adherent peritoneal cells. In both the TIA and CSA assays, no significant inhibition was seen with 10^{-7} M LF present during the media conditioning (see Fig. 1).

**Effect of LF on Colony Formation in Cultures Stimulated by Adherent Peritoneal Cell Underlayers**

In the human system, the most sensitive assay of LF’s inhibitory effect has been marrow cultures where colony formation occurs secondary to endogenously derived CSA.\(^6\) A similar assay in mouse is hampered by the low levels of CSA produced by marrow cells.\(^7\) To simulate the endogenously stimulated human colony assay, we studied colony formation in mouse marrow cultures stimulated by adherent peritoneal cells in the underlayer. Again, the presence of 10^{-7} M LF had no influence on colony and cluster formation (see Table 2).

**Effect of LF Added to Marrow Culture Stimulated by Exogenous TIA**

In 10 experiments, LF in concentrations from 10^{-10} to 10^{-7} M was added to granulocyte cultures stimulated by exogenous stimulatory factor. In no instance was significant inhibition observed. Representative data from experiments in which lung and femoral shaft conditioned media were added to cultures with and without LF are shown in Table 3.

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**Table 1. The Effect of LF, 10^{-7} M, on TIA Production by Cells Associated With Mouse Femoral Shafts and Lungs**

<table>
<thead>
<tr>
<th>TIA Source</th>
<th>LF</th>
<th>Slope (dpm/μl)</th>
<th>SE</th>
<th>r</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td>Femoral shaft</td>
<td>-</td>
<td>33.0</td>
<td>3.1</td>
<td>0.987</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>31.7</td>
<td>1.0</td>
<td>0.998</td>
<td>NS</td>
</tr>
<tr>
<td>Femoral shaft</td>
<td>-</td>
<td>28.9</td>
<td>2.6</td>
<td>0.988</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>36.2</td>
<td>3.5</td>
<td>0.986</td>
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</tr>
<tr>
<td>Lung</td>
<td>-</td>
<td>26.4</td>
<td>1.0</td>
<td>0.997</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>27.8</td>
<td>1.4</td>
<td>0.996</td>
<td>NS</td>
</tr>
<tr>
<td>Lung</td>
<td>-</td>
<td>25.2</td>
<td>1.6</td>
<td>0.996</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>21.2</td>
<td>1.4</td>
<td>0.993</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Table 2. The Effect of LF, 10^{-7} M, on Colony and Cluster Formation in Mouse Marrow Cultures Stimulated by Adherent Peritoneal Cells**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>LF</th>
<th>Colonies and Clusters</th>
<th>Colonies</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>53.8 ± 2.8</td>
<td>27.6 ± 3.0</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>55.7 ± 0.9</td>
<td>30.3 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>51.0 ± 2.4</td>
<td>27.2 ± 1.3</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>46.3 ± 4.7</td>
<td>23.3 ± 2.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

*SEM.

**Table 3. The Effect of the Addition of LF, 10^{-7} M, on TIA in Cultures Stimulated by Lung or Femoral Shaft Conditioned Media**

<table>
<thead>
<tr>
<th>TIA Source</th>
<th>LF</th>
<th>Slope (dpm/μl)</th>
<th>SE</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femoral shaft</td>
<td>-</td>
<td>28.8</td>
<td>2.5</td>
<td>0.988</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>30.0</td>
<td>2.8</td>
<td>0.987</td>
<td>NS</td>
</tr>
<tr>
<td>Femoral shaft</td>
<td>-</td>
<td>21.3</td>
<td>0.9</td>
<td>0.997</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>20.5</td>
<td>1.2</td>
<td>0.995</td>
<td>NS</td>
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<tr>
<td>Lung</td>
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<td>23.9</td>
<td>1.0</td>
<td>0.997</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>27.8</td>
<td>1.9</td>
<td>0.993</td>
<td>NS</td>
</tr>
<tr>
<td>Lung</td>
<td>-</td>
<td>27.5</td>
<td>3.1</td>
<td>0.981</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>24.5</td>
<td>1.9</td>
<td>0.991</td>
<td>NS</td>
</tr>
</tbody>
</table>

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Fig. 1. Thymidine-incorporating activity (TIA) and colony-stimulating activity (CSA) produced during media conditioning by adherent peritoneal cells in the presence (A---A) and absence (B---B) of 10^{-7} M LF. The mean and standard error of each experimental point are presented. For the TIA assay, the calculated regression lines are displayed. There were no statistically significant differences between experimental and control values in either assay.
LACTOFERRIN AND GRANULOPOIESIS

DISCUSSION

In our studies using an all murine system, we found that LF added to cultures stimulated by exogenous granulopoietic stimulators had no inhibitory effect. This observation is in accord with other investigations using human LF in cultures stimulated with exogenous CSA. In contrast to results reported by others, we were unable to demonstrate inhibition by murine LF of the production of humoral granulopoietic stimulators by murine cells using a wide variety of experimental techniques.

There are a number of possible explanations for these disparate results. Our studies are the first to employ murine LF and murine target cells, while previous studies have used human LF and human or murine cells in the colony assays. Thus, there may be species differences in the role of LF as a regulator of granulopoiesis. Because of the similarities between other established regulatory mechanisms in different mammalian species, such an explanation would be unusual if LF were a major regulator.

Another possible explanation is that LF works only on a subset of macrophages that is responsible for the endogenous colony formation seen in human cultures using 3 x 10^5 light density marrow cells per milliliter. Because spontaneous colony formation does not occur in mouse cultures at this cell concentration, it was not possible to precisely duplicate the human endogenously stimulated marrow colony assay. The possibility of using higher cell densities in the mouse cultures was precluded by the inability to distinguish true clones from cell groupings resulting from aggregation under these conditions. In our studies we did use three different sources of granulopoietic stimulators (lung, femoral shaft, and peritoneal cells), and simulated to some extent the endogenously stimulated colony assay using adherent peritoneal cells in the underlayer.

Another possible explanation for the different result is that murine LF is inhibitory in a murine system at concentrations higher than those employed in these studies. We employed concentrations of LF from 10^{-13} M up to 10^{-7} M and did not use higher concentrations because of the quantity of LF that would be required for such experiments. We also demonstrated that 10^{-7} M LF exceeds normal mouse plasma concentrations by eight fold. It should be noted that human LF is reported to be effective at a concentration of 10^{-17} M, 10 orders of magnitude lower than the amount we employed.

Finally, there may be differences in the homogeneity of our murine LF preparation as compared to the preparation of the human LF used by others. Support for such an idea comes from the observation that the isoelectric point of both the human LF preparation and its equivalent colony-inhibiting activity (CIA) were reported to be pH 6.0–6.5. Recent investigations have shown that highly purified murine and human LF have isoelectric points in the range of pH 8.2–9.6. An earlier publication did report that the isoelectric point for human LF was pH 6.1. Subsequent studies have shown that LF strongly binds negatively charged molecules, which significantly influence its mobility in an electric field, accounting for the lower isoelectric points seen with some LF preparations. This also raises the possibility that the inhibitory effect seen with some LF preparations may be secondary to molecules bound to LF rather than the LF itself. In addition, it should be noted that the LF has a relatively high molecular weight, and low molar concentrations of the protein could present substantially higher molar concentrations of some associated small molecular weight inhibitor(s). This might explain why LF has been reported to be inhibitory at such extremely low concentrations.

In addition to the experimental data presented in this paper, there are several clinical observations that are not consistent with LF having a major role as an inhibitor of granulopoiesis. Most pertinent is the observation of normal peripheral blood granulocyte counts in several patients who have congenital absence of secondary granules and LF. Second are the observations that plasma LF levels are increased in situations where there is increased granulocyte turnover and augmented granulocyte production. This has been documented both in acute bacterial infections and in the granulocytosis of chronic granulocytic leukemia. A decreased responsiveness to LF by monocytes in the presence of endotoxin and by monocytes in chronic granulocytic leukemia has been offered as an explanation for these paradoxical observations.

In conclusion, we feel that if LF is to be accepted as a major inhibitory regulator of granulopoiesis, a number of issues remain to be resolved. Clearly our experimental data do not support this concept. At the present time we feel LF’s regulatory role is unestablished and should remain an open question.

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