Transferrin Synthesis by Mouse Lymph Node and Peritoneal Macrophages: Iron Content and Effect on Lymphocyte Proliferation

By A. Djeha, J.L. Pérez-Arellano, and J.H. Brock

Transferrin is an essential requirement for lymphocyte proliferation, because it supplies activated lymphocytes with iron needed for cell proliferation. However, during inflammation or an immune response, the iron content of circulating transferrin, which is of hepatic origin, decreases. It is hypothesized that activated lymphocytes may therefore obtain transferrin-iron from an alternative source, and we have investigated the possibility that transferrin is synthesized locally in lymphoid tissues. It was found that lymph node cells from mice stimulated in vivo with Freund's complete adjuvant were able to synthesize transferrin, and this was because of the macrophage rather than the lymphocyte population. Transferrin synthesized by mouse lymph node or peritoneal macrophages contained iron and was able to promote mouse lymphocyte proliferation. Peritoneal macrophages activated in vivo synthesized more transferrin, released more transferrin-bound iron, and were more effective than resident macrophages at enhancing lymphocyte proliferation, thus eliminating possible detrimental effect of hypoferremia on the immune system.

TRANSFERRIN (Tf) is the major plasma iron-binding glycoprotein. Its main function is to transport iron from sites of storage, absorption, and erythrocyte catabolism to cells that require iron for their metabolism. The vast majority of Tf is synthesized by the liver, but some extrahepatic tissues, such as brain and testes in which there is limited access by plasma Tf, can also synthesize Tf.

The immune system requires Tf for its proper function, in particular because iron is necessary for lymphocyte growth and proliferation. However, immune and inflammatory responses are accompanied by hypoferremia, characterized by a rapid decrease in the iron saturation of circulating Tf, but it is at this time that activation of the immune system requires an increased supply of iron to permit lymphocyte proliferation. Thus, there exists a paradoxical situation in which the hypoferremic response could lead to an impairment of lymphocyte proliferation at a time when this may be critically important. To overcome this, it is possible that during the immune response the main source of Tf for lymphocyte proliferation does not come from the circulating pool of hepatic origin but from Tf synthesized locally by cells in lymphoid tissues carrying iron derived from these cells.

To test this hypothesis, we have studied Tf synthesis by resting and activated mouse lymphocytes and macrophages. We have also examined the iron content of macrophage-derived Tf, and its ability to promote lymphocyte proliferation. Although Tf messenger RNA (mRNA) has been identified in mouse peritoneal macrophages, spleen, and thymus, and synthesis by activated human CD4+ T cells has also been reported, these studies did not investigate the iron content or possible role of Tf produced by lymphomyeloid cells.

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Supported by the Wellcome Trust.

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MATERIALS AND METHODS

Cell Cultures

Lymph node cells. Lymph node cells were obtained from unstimulated Balb/c mice 16 to 40 weeks of age or from the draining popliteal lymph nodes of mice that had been inoculated 3 weeks previously in the footpad with 50 μl of a 1:1 emulsion of phosphate-buffered saline (PBS)/Freund's complete adjuvant (Difco, Detroit, MI).

Cells from normal mice were stimulated in vitro by incubating for 48 hours with 4 μg/mL concanavalin A (ConA; Sigma, Dorset, UK) at 2 x 10⁶ cells/mL in RPMI-1640 (Flow, Ayshire, UK) containing 10% fetal calf serum (FCS; Sigma). Adherent and nonadherent cells from in vivo- stimulated mice were separated after incubating for 2 hours at 37°C in RPMI-1640 containing 10% FCS.

In vivo-stimulated lymph node cells were depleted of B and T lymphocytes by incubating with a mixture of 1:1,000 diluted rabbit antimouse IgG (Serotec, Oxford, UK), 1:2,000 diluted anti-Thy1 (Serotec), and 1:10 diluted low-Tox-M rabbit complement (Cederlane Laboratories, Ontario, Canada) for 1 hour. This process was repeated a second time.

Peritoneal cells. Resident peritoneal macrophages were obtained from normal mice and activated macrophages from mice that had been injected intraperitoneally 4 days before with 2 mg of Corynebacterium parvum. Adherent macrophage cultures were prepared as described previously.

Immunoprecipitation

Cells were washed three times in PBS and preincubated in 5 mL Tf-free medium consisting of RPMI-1640 (without cysteine, cystine, or methionine; Select-Amine Kit, GIBCO, Paisley, UK) for 1 hour at 37°C in an atmosphere of 5% CO₂ to allow exocytosis of any endogenous Tf to occur. Cells were again washed three times in PBS, resuspended at 5 x 10⁶/mL in 2 mL of Tf-free medium containing 100 μCi of [15S]cysteine (specific activity 1,021.8 Ci/mmol; DuPont NEN, Dreireich, Germany), and incubated at 37°C in a CO₂ incubator for 1 hour. The supernatant was harvested by centrifugation and phenylmethylsulphonyl fluoride (1 mM) (Sigma) was added to prevent proteolysis. An aliquot (500 μL) was then incubated for 1 hour at room temperature with 10 μL of rabbit antimouse Tf. Control aliquots contained either antiserum plus 20 μg of mouse Tf (competition control) or normal rabbit serum instead of antiserum. All samples were then incubated for a further 1 hour on an end-over-end rotator with 6 mg of IgGisorb (The Enzyme Center, Malden, MA) in 100 μl of 1% Triton X-100/0.5% deoxycholic acid/0.1% sodium dodecyl sulfate (SDS)/0.1 mol/L NaCl/1 mmol/L EDTA/10 mmol/L phosphate buffer, pH 7.5. The supernatants were dis-
Fig 1. Autoradiography of [35S]cysteine incorporated into transferrin by unstimulated, ConA-stimulated and in vivo-stimulated mouse lymph node cells. Test, +rabbit antimouse Tf (lane a); competition control, +mouse Tf and rabbit antimouse Tf (lane b); control, +normal rabbit serum (lane c).

carded, and the IgGsorb washed three times with the same buffer then resuspended in 7 mol/L urea/10% SDS/10 mmol/L Tris buffer, pH 7.0, and incubated for 1 hour at room temperature on the end-over-end rotator. The pellets were discarded and the supernatants analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions in 10% acrylamide gel. Autoradiography was performed after enhancement (EN'HANCE, New England Nuclear, Boston, MA) using Kodak XAR-5 film. 121I-labeled human Tf was used as standard.

Enzyme-Linked Immunosorbent Assay (ELISA) for Tf

Macrophages were cultured for 24 hours in serum-free RPMI-1640 culture medium containing 1 mg/mL Tf-free human serum albumin (HSA; Behringwerke, Hounslow, UK). Cell culture supernatants were assayed for Tf by an ELISA method. Plates were coated with a 1:5,000 diluted rabbit antibody to mouse Tf (the latter obtained from Chemicon International, London, UK) in 0.01 mol/L carbonate buffer, pH 9.6, and detection was performed using a 1:200 dilution of the same antibody conjugated to alkaline phosphatase (Sigma).

Effect of Tf in Culture Supernatants on Lymphocyte Proliferation

Lymph node cells from normal mice were washed three times in warm PBS and were then cultured at 2 × 10⁶/mL for 48 hours in conical test tubes in serum-free RPMI-1640 culture medium supplemented with 1 mg/mL HSA and 1 μmol/L 2-mercaptoethanol, in the presence of 1 μg/mL ConA and varying amounts of lymph node cell or macrophage culture supernatants. These were obtained by incubating the appropriate cells (5 × 10⁷/mL) for 36 hours in serum-free RPMI-1640 culture medium containing 1 mg/mL Tf-free HSA. When required, media were depleted of Tf by passage through a Sepharose-rabbit anti-mouse Tf column. Control media were passed through Sepharose-rabbit normal IgG to check for any possible nonspecific effect. Human Tf (50 μg/mL; Behringwerke, Germany) was added to some cultures as a positive control. Proliferation was assayed by ³H-thymidine incorporation.²

Iron Content of Newly Synthesized Tf

Cells were cultured at 2 × 10⁶/mL for 24 hours in RPMI-1640 containing 1 mg/mL HSA and ⁹⁹Fe-labeled human Tf (1 μCi/10⁷ cells), prepared as described previously.² The cells were then washed three times and incubated in unlabeled medium for 1 hour, after which they were incubated for a further 24 hours in RPMI-1640 containing 10 mg/mL iron-free hen ovo transferrin (ovoTf; provided by Dr R.W. Evans, Guy's Hospital, London, UK). The addition of ovoTf at this stage serves to prevent extracellular binding of iron leaking from moribund cells to newly secreted Tf. The culture supernatant was then chromatographed on an immunoabsorbent column containing 75 μL of Sepharose antimouse Tf and the bound radioactivity counted. Preliminary experiments showed that hen ovoTf did not cross-react immunologically with mammalian Tf and was not retained on the column. Nonspecific binding was corrected by passing a further aliquot through a column of immobilized normal rabbit IgG.

RESULTS

Tf Synthesis by Mouse Lymph Node Cells

Unstimulated mouse lymph node cells did not synthesize detectable amounts of Tf (Fig 1), and the stimulation of cells with the mitogen ConA also produced negligible synthesis of the protein. However, cells from animals stimulated in vivo by Freund's complete adjuvant showed clear evidence of synthesis of Tf. When adherent and nonadherent cells from the in vivo-stimulated lymph nodes were separated, synthesis

Fig 2. Autoradiography of [35S]cysteine incorporated into transferrin by adherent and nonadherent (A) and B- and T-lymphocyte-depleted (B) in vivo-stimulated mouse lymph node cells. Test, +rabbit antimouse Tf (lane a); competition control, +mouse Tf and rabbit antimouse Tf (lane b); control, +normal rabbit serum (lane c).
of Tf was markedly greater in the adherent population (Fig 2A), suggesting that it was the macrophages that were responsible for Tf synthesis. To confirm this, the in vivo-stimulated lymph node cells were depleted of B and T lymphocytes to obtain a macrophage-rich population. The supernatant of the lymphocyte-depleted population showed a much stronger radioactive Tf band than that of a similar number of non-depleted cells (Fig 2B). Thus, it was concluded that it was the macrophage population of the mouse lymph node that was responsible for Tf synthesis on in vivo stimulation.

**Effect of Tf Synthesized by Macrophages on Lymphocyte Proliferation**

To test whether Tf synthesized by macrophages was capable of promoting lymphocyte proliferation, experiments were initially performed using supernatants from mouse peritoneal macrophages, because these have been previously shown to synthesize Tf. Supernatants of normal (resident) macrophages diluted 1:2 produced a fivefold enhancement of lymphocyte proliferation compared with the control, which was lost when the supernatant was absorbed with an antimouse Tf immunoabsorbent column (Fig 3). However, at 1:8 dilution the effect was lost. In contrast, supernatants from C parvum-activated mouse macrophages gave Tf-specific enhancement of lymphocyte proliferation at both 1:2 and 1:8 dilutions, suggesting that increased synthesis of Tf occurs after in vivo stimulation by C parvum. This was confirmed by assaying Tf in the macrophage culture supernatants using an ELISA method. Resident cells produced 29 ± 6 ng/10^6 cells (n = 3), whereas C parvum-activated macrophages produced 138 ± 25 ng/10^6 (n = 3).

Similar experiments were performed using supernatants from adherent and nonadherent cells from in vivo-stimulated lymph nodes. Nonadherent cell supernatants produced only a slight enhancement of lymphocyte proliferation that was not reversed by passage through the antimouse Tf immunoabsorbent column (Fig 4). In contrast, supernatants of the adherent cells gave specific enhancement of lymphocyte proliferation at both 1:2 and 1:8 dilutions.

**Iron Content of Newly Synthesized Tf**

The previous experiments showed that Tf produced by macrophages can promote lymphocyte proliferation. Because enhancement of lymphocyte proliferation by Tf requires the presence of bound iron, and given that the present results were obtained in medium containing no added iron, it was of interest to know whether macrophages were releasing Tf with iron already attached. A significant proportion of iron released by macrophages was found to be bound to Tf, the proportion being greatest in the activated macrophages (Table 1). Very little iron was released from the nonadherent lymph node cells and of this, hardly any was bound to Tf.

**DISCUSSION**

The major site of Tf synthesis is the liver, but it is now clear that extrahepatic Tf synthesis may occur. Although it was reported many years ago that macrophages can synthesize Tf, and was confirmed more recently by Yang et al., who demonstrated the presence of mRNA in mouse peritoneal macrophages, there are few studies of Tf synthesis by cells of the immune system. Furthermore there is virtually no information concerning the physiologic function of this Tf, nor the regulation of its synthesis. In other nonhepatic tissues, such as the brain or testes, synthesis of Tf probably reflects the restricted physiologic environment of these sites, but this is less likely to explain why lymphomyeloid cells synthesize Tf.

During infection or inflammation the body responds with a redistribution of iron, resulting in a decrease in the iron saturation of circulating Tf by mechanisms that are not fully understood. The physiologic function of this response is not clear, but may represent an attempt to withhold iron from...
invading microorganisms. However, iron-containing Tf is required for the proliferation of lymphocytes, and thus hypoferremia could adversely affect lymphocyte function, whose requirements for iron are likely to increase during an inflammatory or immune response. It seems likely that, although Tf is undoubtedly present in the lymph node, under such conditions its iron content would be reduced to very low levels, which we have previously shown do not support optimal lymphocyte proliferation. Synthesis and release of iron-containing Tf by cells in lymphoid tissues could render activated lymphocytes independent of plasma Tf and enable them to function properly under these conditions. In this study we have found that mouse lymph node cells stimulated in vivo synthesize Tf, and that this seems to be a property of the macrophage rather than the lymphocyte, because depletion of the latter caused enrichment of the Tf-synthesizing cells. Macrophages are in close contact with lymphocytes within the lymph node and increase sharply in number after in vivo stimulation because of an influx of monocytes into the lymph node. This might explain the failure of in vitro-stimulated lymph node cells to show an active synthesis of the protein because the number of macrophages present would be much lower. It is also possible that only a specific macrophage subset, which infiltrates the lymph node after stimulation, is capable of Tf synthesis, although this seems unlikely because resident peritoneal macrophages were able to synthesize and secrete Tf, albeit at a lower level than activated cells. In support of the hypothesis that Tf synthesis by lymphomyeloid cells plays a role in the immune response, it was found that macrophage-derived Tf was able to promote proliferation of mitogen-stimulated mouse lymphocytes.

Table 1. Iron Content of Newly Synthesized Tf

<table>
<thead>
<tr>
<th>Cell Supernatant From</th>
<th>Total Fe Released (pmol/10⁶ cells)</th>
<th>% Released Fe Bound to Tf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peritoneal macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (resident)</td>
<td>0.87</td>
<td>18</td>
</tr>
<tr>
<td>Activated (C. parvum)</td>
<td>1.34</td>
<td>50</td>
</tr>
<tr>
<td>FCA-stimulated lymph node cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonadherent</td>
<td>0.03</td>
<td>2</td>
</tr>
<tr>
<td>Adherent</td>
<td>1.90</td>
<td>25</td>
</tr>
</tbody>
</table>

Results are mean of two separate experiments.

Abbreviation: FCA, Freund's complete adjuvant.

The fact that activation of mouse peritoneal macrophages in vivo with C. parvum increased both Tf synthesis and the amount of iron released bound to Tf suggests that macrophage-derived Tf can be regulated to function as a paracrine growth factor during the immune response. The data reported here suggest that Tf secreted by both resident and activated macrophages has an iron saturation of around 20%, which is perfectly adequate to support good lymphocyte proliferation. Lum et al. found that activated CD4⁺ human lymphocytes synthesize Tf and suggested that Tf could act in an autocrine manner; a similar proposal was made by Vostrejs et al. for lung carcinoma cells. The findings of Lum et al. differ from those reported here, in which macrophages rather
than lymphocytes were clearly the source of Tf. The reason for this discrepancy is unknown but might be caused by a species difference, because in preliminary studies we have been unable to detect synthesis of Tf by activated mouse lymphocytes or human macrophages.23

The results reported here and by Lum et al5 suggest that Tf synthesis by lymphoid tissues is regulated differently from hepatic synthesis. Evidence for multiple regulatory mechanisms is provided by Guillou et al,24 who found that the nuclear regulatory factors operating in testicular cells differ from those in the liver. In the case of Tf synthesis in lymphoid tissues, other immunoregulatory factors may be of importance, because we have preliminary evidence that certain cytokines can upregulate Tf synthesis by macrophages.25

REFERENCES
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