Prooxidant Activity of Transferrin and Lactoferrin

By Seymour J. Klebanoff and Ann M. Waltersdorph

From the Department of Medicine, SJ-10, University of Washington, Seattle, Washington 98195

Summary

Acceleration of the autoxidation of Fe$^{2+}$ by apotransferrin or apolactoferrin at acid pH is indicated by the disappearance of Fe$^{2+}$, the uptake of oxygen, and the binding of iron to transferrin or lactoferrin. The product(s) formed oxidize iodide to an iodinating species and are bactericidal to *Escherichia coli*. Toxicity to *E. coli* by FeSO$_4$ (10$^{-5}$ M) and human apotransferrin (100 µg/ml) or human apolactoferrin (25 µg/ml) was optimal at acid pH (4.5–5.0) and with logarithmic phase organisms. Both the iodinating and bactericidal activities were inhibited by catalase and the hydroxyl radical (OH·) scavenger mannitol, whereas superoxide dismutase was ineffective. NaCl at 0.1 M inhibited bactericidal activity, but had little or no effect on iodination. Iodide increased the bactericidal activity of Fe$^{2+}$ and apotransferrin or apolactoferrin. The formation of OH· was suggested by the formation of the OH· spin-trap adduct (5,5-dimethyl-1-pyrroline N-oxide [DMPO]/OH·), with the spin trap DMPO and the formation of the methyl radical adduct on the further addition of dimethyl sulfoxide (DMPO/OH·) formation was inhibited by catalase, whereas superoxide dismutase had little or no effect. These findings suggest that Fe$^{2+}$ and apotransferrin or apolactoferrin can generate OH· via an H$_2$O$_2$ intermediate with toxicity to microorganisms, and raise the possibility that such a mechanism may contribute to the microbicidal activity of phagocytes.

The role of hydroxyl radicals (OH·) in the microbicidal activity of phagocytes is controversial (1, 2). Phagocytes respond to stimulation with a respiratory burst, and much, if not all, of the extra oxygen consumed is converted to highly reactive oxygen species that contribute to the destruction of ingested organisms and extracellular targets (2). The superoxide anion (O$_2^-$) and H$_2$O$_2$ are formed by the respiratory burst, and their interaction in an iron-catalyzed reaction (Haber-Weiss reaction) to form a powerful oxidant has been proposed as follows:

\[
\begin{align*}
H_2O_2 + Fe^{2+} &\rightarrow Fe^{3+} + OH^- + OH^- \\
O_2^- + Fe^{3+} &\rightarrow Fe^{2+} + O_2 \\
O_2^- + H_2O_2 &\rightarrow O_2 + OH^- + OH^- \\
Fe &
\end{align*}
\]

Although OH· is widely assumed to be the highly reactive species formed (and will be used to designate the oxidant here), the formation of other oxidants, such as higher transition metal oxidation states (3), has been proposed. The formation of OH· by the Haber-Weiss reaction is limited by the very low solubility of ferric iron at neutral or alkaline pH due to the formation of precipitates of polynuclear hydrated iron complexes. Iron can be maintained in solution in a catalytically active form either by lowering the pH (4) or by certain chelating agents. Thus, iron bound to EDTA retains an aquo coordination site that can be oxidized and reduced (5, 6), and thus, EDTA considerably increases the formation of OH· by the iron-catalyzed Haber-Weiss reaction at neutral pH (7, 8). Other chelating agents, e.g., deferroxamine, are inhibitory, as they bind to all six coordination sites of iron displacing water, and these complexes are catalytically inactive.

A biological chelator of iron that, like EDTA, would facilitate its oxidation and reduction and thus act as a catalyst of OH· formation by the Haber-Weiss reaction in vivo has been sought. Iron-saturated lactoferrin has been reported to catalyze the Haber-Weiss reaction (9, 10); however, others have been unable to detect catalysis of OH· formation by iron-saturated lactoferrin in the absence of extraneous iron (11–13), and apolactoferrin or partially-saturated lactoferrin has been reported to inhibit the iron-catalyzed Haber-Weiss reaction, presumably by the chelation of free iron in an unreactive form (13, 14). Similarly, transferrin has been reported to catalyze the Haber-Weiss reaction in some studies (15, 16), but not others (13, 17), and the inhibition of OH· formation by unsaturated transferrin has been reported (13, 14). Other biologically relevant iron chelators reported to facilitate iron-dependent OH· formation, include ferritin (18), phosphate (19), α-picolinic acid (20), phosphonucleotides (21–23), and DNA (24), although several of the iron chelates are more

1 Abbreviations used in this paper: DMPO, 5,5-dimethyl-1-pyrroline N-oxide; EPR, electron paramagnetic resonance; O$_2^-$, superoxide anion; OH·, hydroxyl radical.
effective in the Fenton reaction (reaction a) than they are as catalysts of the Haber-Weiss reaction (25).

An additional mechanism by which iron-chelating agents can increase iron-dependent oxygen-centered free radical formation is by facilitating the autoxidation of iron. Chelating agents stimulate the autoxidation of Fe²⁺ best when the affinity of the chelator for Fe²⁺ greatly exceeds its affinity for Fe³⁺ (26). At pH 7.0, Fe³⁺-chelating agents enhance the autoxidation of Fe²⁺ in the order EDTA ~ dinitroliodiate > citrate > phosphate > oxalate (27). Deferoxamine binds Fe³⁺ with very high avidity, whereas it binds Fe²⁺ poorly, if at all, and thus would be expected to promote Fe²⁺ autoxidation. We have recently reported that the autoxidation of Fe²⁺ at pH 5.5 is greatly accelerated by defereroxamine with the formation of products (H₂O₂, OH⁻) that are toxic to bacteria (28), which is in sharp contrast to the inhibitory effect of defereroxamine on OH⁻ formation by the iron-catalyzed Haber-Weiss reaction. The prooxidant activity of defereroxamine also has been described by others, (29–31). In this paper, we report that apotransferrin and apolactoferrin also can accelerate the autoxidation of Fe³⁺ at acid pH with the formation of an oxidant or oxidants with cytotoxic properties.

Materials and Methods

Special Reagents. Human apotransferrin, human holotransferrin, and bovine apotransferrin were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Human milk lactoferrin was obtained from Calbiochem-Behring Corp., Freehold, NJ; superoxide dismutase (bovine erythrocyte, 3,150 U/mg), human albumin (essentially fatty acid free prepared from fraction V), and peroxide dismutase (bovine erythrocyte, 3,150 U/mg), human obtained from Worthington Biochemical Corp., Freehold, NJ; su-

Bactericidal Activity. *Escherichia coli* (ATCC 11775; American Type Culture Collection, Rockville, MD) was maintained on blood agar plates and, just before the experiment, transferred to Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) and grown on a Roto-Rack (Fisher Scientific Co., Pittsburgh, PA) rotating 15 times per minute for 2 h unless otherwise indicated. The organisms were collected by centrifugation, washed twice with 0.1 M Na₂SO₄, and suspended in 0.1 M Na₂SO₄ to the required absorbancy at 540 nm. The *E. coli* were incubated with the compo-

Statistical Analysis. The data are expressed as the mean ± SE. Statistical differences are determined using student's two-tailed t test for independent means (NS, p > 0.05). In the analysis of the bactericidal data, logarithmically transformed data are utilized for the determination of p values (34).

Results

Bactericidal Activity. Under the conditions used in Fig. 1, Fe³⁺ alone at relatively high concentration was toxic to *E. coli* in acetate buffer pH 5.0. When the concentration of Fe³⁺ was lowered to a level where little or no toxicity was observed, the further addition of human apotransferrin or apolactoferrin significantly increased toxicity. In Fig. 2, the Fe³⁺ concentration was maintained at 10⁻⁵ M, and the human apotransferrin and apolactoferrin concentrations were varied. The bactericidal activity increased with the apotransferrin or apolactoferrin concentrations to a maximum at 100 µg/ml (~1.3 × 10⁻⁶ M) and 25 µg/ml (~0.3 × 10⁻⁶ M), respectively, and then fell as the concentration was further increased.
Figure 1. Bactericidal effect of Fe²⁺ and apotransferrin or apolactoferrin. The reaction mixture contained 5 × 10⁻⁴ M sodium acetate buffer pH 5.0, 0.01 M Na₂SO₄, 1-3 × 10⁶ E. coli, FeSO₄ at the concentrations indicated, either alone (Δ), or with 100 μg/ml of human apotransferrin (●) or 25 μg/ml human apolactoferrin (○). The results are the mean of three to seven experiments. The asterisks indicate a significant difference between Fe²⁺ alone and Fe²⁺ + transferrin or Fe²⁺ + lactoferrin (all p < 0.001).

A bactericidal effect also was observed with Fe²⁺ and bovine apotransferrin with the optimum transferrin concentration being 25 μg/ml (data not shown). All subsequent bactericidal studies were performed with FeSO₄, human apotransferrin, and human apolactoferrin concentrations of 10⁻⁵ M, 100 μg/ml, and 25 μg/ml, respectively.

Table 1 demonstrates the effect of pH on the bactericidal effect of Fe²⁺, Fe²⁺ + apotransferrin, or Fe²⁺ + apolactoferrin. Fe²⁺ alone at 10⁻⁵ M, while ineffective at pH 4.5 and 5.0, was bactericidal when the pH was increased to 5.5-7.0 with either acetate or phosphate buffer. The further addition of apotransferrin significantly increased bactericidal activity in acetate buffer pH 4.5 and 5.0, and apolactoferrin increased bactericidal activity in acetate buffer pH 4.5-5.5. At the higher pH levels, there was a tendency for the chelators to inhibit the toxicity of iron. All subsequent studies were performed with acetate buffer pH 5.0.

The E. coli routinely used were in logarithmic growth phase, having been grown in broth for 2 h before isolation and use. As shown in Fig. 3, the bactericidal effect of Fe²⁺ and apotransferrin or apolactoferrin was high with organisms grown in broth for 1 or 2 h, and then declined as the growth period was extended to 24 h.

The time course of the toxicity of Fe²⁺ + apotransferrin and Fe²⁺ + apolactoferrin under our standard conditions is shown in Fig. 4. A significant bactericidal effect was observed at 5 min with both systems, with toxicity increasing with the incubation period as shown.

Table 1. Effect of pH

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Control</th>
<th>Fe²⁺</th>
<th>Fe²⁺ + apoTF</th>
<th>Fe²⁺ + apoLF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>organisms/ml × 10⁻⁶</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>4.5</td>
<td>2.30</td>
<td>1.92</td>
<td>0.15</td>
<td>0.20</td>
</tr>
<tr>
<td>Acetate</td>
<td>5.0</td>
<td>3.17</td>
<td>2.35</td>
<td>0.065</td>
<td>0.43</td>
</tr>
<tr>
<td>Acetate</td>
<td>5.5</td>
<td>2.78</td>
<td>1.44</td>
<td>0.75</td>
<td>0.45</td>
</tr>
<tr>
<td>Acetate</td>
<td>6.0</td>
<td>2.86</td>
<td>0.28</td>
<td>0.10</td>
<td>1.36</td>
</tr>
<tr>
<td>Phosphate</td>
<td>6.0</td>
<td>3.07</td>
<td>0.55</td>
<td>1.25</td>
<td>1.64</td>
</tr>
<tr>
<td>Phosphate</td>
<td>6.5</td>
<td>2.21</td>
<td>0.022</td>
<td>0.26</td>
<td>0.86</td>
</tr>
<tr>
<td>Phosphate</td>
<td>7.0</td>
<td>2.30</td>
<td>0.011</td>
<td>0.35</td>
<td>0.36</td>
</tr>
</tbody>
</table>

The reaction mixture contained 5 × 10⁻⁴ M sodium acetate or sodium phosphate buffers at the pH indicated, 0.01 M Na₂SO₄, 1-3 × 10⁶ E. coli, and, where indicated, 10⁻⁵ M FeSO₄, 100 μg/ml apotransferrin (apoTR), and 25 μg/ml apolactoferrin (apoLF). Results are the mean of three to seven experiments.

* Significantly different from control, p < 0.05.
† Significantly different from Fe²⁺ alone, p < 0.05.
In the Fe²⁺ + apotransferrin or apolactoferrin system, Fe²⁺ could not be replaced by Fe³⁺, and bactericidal activity was inhibited by catalase at 5.8 μg/ml, but not by heated catalase or by superoxide dismutase at 5 μg/ml (Table 2). Mannitol also was inhibitory at 0.1 M, as was NaCl. Comparable inhibition was observed in both systems when NaCl was replaced by equimolar concentrations of KCl, NaBr, or Na₂SO₄ (data not shown). Apotransferrin or apolactoferrin at the concentration used in Table 2 was ineffective in the absence of Fe²⁺, as was an equal concentration of holotransferrin or hololactoferrin (Table 2). Holotransferrin at higher concentration (1,000 μg/ml), however, was toxic to E. coli; this toxicity was unaffected by catalase at 5.8 or 58 μg/ml and by superoxide dismutase at 5 μg/ml, but was inhibited by mannitol and NaCl at 0.1 M (data not shown).

Earlier studies had indicated that the bactericidal activity of Fe²⁺ + H₂O₂ (Fenton's reagent) was considerably increased by the addition of iodide (36). Table 3 demonstrates the stimulatory effect of iodide at 10⁻⁵ M on the bactericidal effect of Fe²⁺ and apotransferrin, or Fe²⁺ and apolactoferrin.

### Table 2. Effect of Inhibitors

<table>
<thead>
<tr>
<th>Additions</th>
<th>Viable cell count</th>
<th>p^*</th>
<th>p†</th>
<th>Lactoferrin</th>
<th>p^*</th>
<th>p†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>organisms/ml x 10⁻⁶</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>3.05(6)</td>
<td>&lt;0.002</td>
<td></td>
<td>3.40(4)</td>
<td></td>
</tr>
<tr>
<td>Fe²⁺ + apoTF or apoLF</td>
<td></td>
<td>0.05(6)</td>
<td>&gt;0.05</td>
<td>0.19(4)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Fe²⁺, + Fe³⁺</td>
<td></td>
<td>3.83(3)</td>
<td>&lt;0.02</td>
<td>2.27(4)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>+ catalase</td>
<td></td>
<td>2.50(6)</td>
<td>&gt;0.01</td>
<td>2.64(4)</td>
<td>&lt;0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>+ heated catalase</td>
<td></td>
<td>0.03(6)</td>
<td>&gt;0.01</td>
<td>0.62(4)</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>+ SOD</td>
<td></td>
<td>0.08(6)</td>
<td>&gt;0.01</td>
<td>0.27(4)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>+ mannitol</td>
<td></td>
<td>0.92(4)</td>
<td>&gt;0.05</td>
<td>2.41(4)</td>
<td>&lt;0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>+ NaCl</td>
<td></td>
<td>2.09(5)</td>
<td>&gt;0.01</td>
<td>2.61(4)</td>
<td>NS</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ApoTF or apoLF</td>
<td></td>
<td>2.12(10)</td>
<td>&gt;0.01</td>
<td>2.74(6)</td>
<td>NS</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Holotransferrin or hololactoferrin</td>
<td></td>
<td>1.94(9)</td>
<td>&gt;0.01</td>
<td>2.47(3)</td>
<td>&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

The reaction mixture contained 5 x 10⁻⁴ M sodium acetate buffer, pH 5.0, 0.01 M Na₂SO₄, 10⁻⁵ M Fe₂(SO₄)₃, 100 µg/ml apotransferrin (apoTF), 100 µg/ml holotransferrin (holoTF), 25 µg/ml hololactoferrin (holoLF), 5.8 µg/ml catalase, 5 µg/ml superoxide dismutase (SOD), 0.1 M mannitol, and 0.1 M NaCl.

*p value for the difference from control (none).

†p value for the difference from Fe²⁺ + apoTF or apoLF.

[Table 2. Effect of Inhibitors](#)

Table 3. Stimulation of the Bactericidal Effect of Fe²⁺ and Apotransferrin or Apolactoferrin by Iodide

<table>
<thead>
<tr>
<th>Additions</th>
<th>Viable cell count</th>
<th>p^* organisms/ml x 10⁻⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.83</td>
<td></td>
</tr>
<tr>
<td>Fe²⁺ + apoTF</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Fe²⁺ + apoTF + I</td>
<td>0.000005</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Fe²⁺ + apoLF</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>Fe²⁺ + apoLF + I</td>
<td>0.000003</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The reaction mixture contained 5 x 10⁻⁴ M sodium acetate buffer, pH 5.0, 0.01 M Na₂SO₄, 3 x 10⁶ E. coli, and, where indicated, 10⁻⁵ M FeSO₄, 100 µg/ml apotransferrin (apoTF), 25 µg/ml apolactoferrin (apoLF), and 10⁻⁵ M NaI. The results are the mean of three to seven experiments. The asterisks indicate a significant difference from control (all p < 0.001).

*p value for the difference from Fe²⁺ + apoTF or Fe²⁺ + apoLF.

**Toxicity Induced by Fe²⁺ and Transferrin or Lactoferrin**
Iodination. Incubation of Fe²⁺ and apotransferrin or apolactoferrin with ¹²⁵I-iodide in the presence of albumin was associated with the conversion of iodide to a TCA precipitable form (iodination). When the Fe²⁺ concentration was maintained at 10⁻⁵ M, iodination increased with the apotransferrin or apolactoferrin concentrations to a maximum at 100 µg/ml (~1.3 × 10⁻⁸ M) and 50 µg/ml (~0.6 × 10⁻⁶ M), respectively, and then fell sharply as the chelator concentration was further increased (data not shown). Under the conditions used in Table 4, iodination by Fe²⁺ and apotransferrin or apolactoferrin was significantly decreased or abolished when either Fe²⁺ or the iron chelator was omitted, when Fe²⁺ was replaced by Fe³⁺, or when catalase was added. The inhibition by catalase was prevented by its heat-inactivation. Superoxide dismutase at 5 µg/ml did not significantly decrease iodination. Mannitol was inhibitory at 0.1 M; however, in contrast to its inhibition of bactericidal activity, NaCl at 0.1 M did not inhibit iodination by Fe²⁺ and apotransferrin, and only partially inhibited iodination by Fe²⁺ and apolactoferrin. No iodination was observed when Fe²⁺ + apotransferrin was replaced by holotransferrin at 100-1,000 µg/ml, or when Fe²⁺ + apolactoferrin was replaced by hololactoferrin at 10-500 µg/ml.

Autoxidation of Iron. The autoxidation of Fe²⁺ is greatly accelerated by apotransferrin or apolactoferrin at pH 5.0. Under the conditions used in Fig. 5, a rapid loss of Fe²⁺ (half maximal, ~10 s) was observed on incubation with apotransferrin or apolactoferrin at the concentrations routinely used for the measurement of bactericidal activity. Total disappearance of Fe²⁺ was not observed under these conditions, with ~32% of the Fe²⁺ remaining after a 15-min incubation with 100 µg/ml of apotransferrin, and ~54% remaining after a 15-min incubation with 25 µg/ml of apolactoferrin. However, when the apotransferrin and apolactoferrin concentrations were increased to 250 and 100 µg/ml, respectively, essentially complete disappearance of Fe²⁺ was observed at 15 min. Loss of Fe²⁺ was not observed in the absence of apotransferrin or apolactoferrin under the conditions used in Fig. 5.

That the loss of Fe²⁺ is due to its autoxidation is suggested by measurements of oxygen consumption. Little or no oxygen consumption was detected at the concentrations of Fe²⁺ (10⁻⁵ M) and apotransferrin (100 µg/ml) routinely used in bactericidal studies. Since the concentration of oxygen in air-saturated buffer at 37°C is 215 µg/ml, and the concentration of the Fe²⁺ used under our standard conditions is 10 µM, uptake of only a small percentage of the oxygen would be expected, thus making detection difficult. When the concentration of Fe²⁺ and apotransferrin were increased 2-, 5-, 10-, and 20-fold with the ratio kept constant, a rapid uptake of oxygen was observed that was essentially complete in 30 s. At the highest concentrations used (Fe²⁺, 2 × 10⁻⁴ M; apotransferrin, 2 mg/ml), no oxygen consumption was observed on the addition of either Fe²⁺ or apotransferrin alone. Assuming the oxidation of two-thirds of the Fe²⁺ (Fig. 5), approximately one molecule of oxygen is taken up for every three or four molecules of Fe²⁺ oxidized. Similar findings were observed with apolactoferrin, except that the oxygen/Fe²⁺ stoichiometry was 1:2-3.

Binding of Iron. The binding of iron on incubation of Fe²⁺ with apotransferrin at pH 5.0 was indicated by the association of ⁵⁹Fe²⁺ with the iron-binding protein (Fig. 6). In the absence of transferrin, ⁵⁹Fe²⁺ eluted from a Sephadex G-25 column in small amounts over an extended period. However, after a 15-min incubation of 10⁻⁵ M ⁵⁹FeSO₄ with 100 µg/ml of apotransferrin or apolactoferrin, essentially complete disappearance of Fe²⁺ was observed at 15 min. Loss of Fe²⁺ was not observed in the absence of apotransferrin or apolactoferrin under the conditions used in Fig. 5.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Fe²⁺ + apoTF</th>
<th>p*</th>
<th>Fe²⁺ + apoLF</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe²⁺</td>
<td>1.1 ± 1.1(3)</td>
<td>&lt;0.01</td>
<td>0.8 ± 0.7(4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ApoTF or apoLF</td>
<td>3.7 ± 0.1(3)</td>
<td>&lt;0.01</td>
<td>-1.8 ± 0.2(2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fe²⁺ + apoTF or ApoLF</td>
<td>45.7 ± 7.2(3)</td>
<td>85.2 ± 4.9(6)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Fe³⁺ + apoTF or apolF</td>
<td>4.1 ± 0.5(3)</td>
<td>&lt;0.01</td>
<td>1.4 ± 2.0(5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fe²⁺ + apoTF or apolLF + catalase</td>
<td>2.9 ± 0.5(3)</td>
<td>&lt;0.01</td>
<td>0.3 ± 0.4(3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fe²⁺ + apoTF or apolLF + heated catalase</td>
<td>32.4 ± 5.2(3)</td>
<td>NS</td>
<td>78.8 ± 9.6(3)</td>
<td>NS</td>
</tr>
<tr>
<td>Fe²⁺ + apoTF or apolLF + SOD</td>
<td>33.9 ± 6.3(3)</td>
<td>NS</td>
<td>70.1 ± 3.6(4)</td>
<td>NS</td>
</tr>
<tr>
<td>Fe²⁺ + apoTF or apolLF + mannitol</td>
<td>2.0 ± 0.1(3)</td>
<td>&lt;0.01</td>
<td>0.9 ± 0.8(5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fe²⁺ + apoTF or apolLF + NaCl</td>
<td>47.3 ± 7.1(5)</td>
<td>NS</td>
<td>27.3 ± 3.0(5)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The reaction mixture contained 5 × 10⁻⁵ M sodium acetate buffer, pH 5.0, 8 × 10⁻⁶ M NaI (4,000 pmol; 0.05 µCi⁵⁹I), 0.2 mg/ml albumin, and, where indicated, 10⁻⁵ M FeSO₄, 100 µg/ml apotransferrin, 50 µg/ml apolactoferrin, 10⁻⁶ M Fe(SO₄)₃, 5.8 µg/ml catalase, 5 µg/ml superoxide dismutase (SOD), 0.1 M mannitol, and 0.1 M NaCl in a final volume of 0.5 ml.

* p value for the difference from Fe²⁺ + apoTF or Fe²⁺ + apolF.
† Mean ± SE of no. of experiments.
Fe$^{2+}$ disappearance on incubation with apo-transferrin or apo-lactoferrin. The reaction mixture contained $5 \times 10^{-4}$ M sodium acetate buffer, pH 5.0, $10^{-5}$ M FeSO$_4$ alone (broken line) or $10^{-3}$ M FeSO$_4$, and either apo-transferrin (broken lines) ([O--O] 50 µg/ml; [Δ--Δ] 100 µg/ml; [■--■] 250 µg/ml) or apo-lactoferrin (solid lines) ([●--●] 25 µg/ml; [■--■] 50 µg/ml; [Δ--Δ] 100 µg/ml) in a final volume of 0.5 ml. After incubation for the times indicated, bathophenanthroline sulfonate was added for determination of Fe$^{2+}$ remaining in the reaction mixture. The results are the mean ± SE of three experiments.

μg/ml apo-transferrin at pH 5.0, 40.4 ± 0.7% (SE, n = 3) of the added iron eluted as a peak in fractions that corresponded to the transferrin protein peak, as indicated by 280-nm absorption. When apo-transferrin was replaced by an equivalent concentration of holotransferrin, 1.9 ± 1.9% (SE, n = 2) of the radioactivity was detected in the transferrin peak. Similarly, when $^{59}$Fe$^{2+}$ was incubated with 25 µg/ml apo-lactoferrin, 86.5 ± 0.5% (SE, n = 2) of the iron eluted with the protein peak, as compared with 17.5 ± 1.6% (SE, n = 3) when hololactoferrin was used.

**Formation of the (DMPO/OH)-Adduct.** The incubation of $10^{-5}$ M FeSO$_4$ and 100 µg/ml human apo-transferrin with the spin-trap DMPO in acetate buffer pH 5.0 produced an EPR signal with splitting constants of $\Delta H = 14.8$ G and a 1:2:1:2:1 intensity distribution (Fig. 7 C), which was not observed when either Fe$^{2+}$ or transferrin was added alone (Fig. 7 A and B). The signal was the same as that reported for the (DMPO/OH)-adduct (37-39) and that produced by FeSO$_4$ and H$_2$O$_2$ (Fenton's reagent), which generates OH· (data not shown). The production of the (DMPO/OH)-signal by Fe$^{2+}$ and transferrin was abolished by catalase (Fig. 7 D), but not by heated catalase (Fig. 7 E). The (DMPO/OH)-signal was consistently decreased but not abolished by superoxide dismutase at 25 µg/ml (Fig. 7 F), an effect that was partially reversed when heated superoxide dismutase was used (Fig. 7 G). When the superoxide dismutase concentration was lowered to 5 µg/ml, its inhibitory effect was lost, and when its concentration was raised to 50 µg/ml, a comparable inhibition was observed with the heated preparation, suggesting a nonspecific effect (data not shown). Methyl radicals (CH$_3$·), formed by the reaction of OH· with DMSO, react with DMPO to form the (DMPO/CH$_3$)·adduct, which has a characteristic EPR signal (1). When DMSO was added to the Fe$^{2+}$ + transferrin system, a complex EPR signal was observed (Fig. 7 H). Subtraction of the (DMPO/OH)-signal yielded a signal with splitting constants of $\Delta H = 16.2$ G and $\Delta H = 23.1$ G (Fig. 7 I), which is characteristic of the (DMPO/CH$_3$)·-adduct (40). No EPR signal was observed on the addition of holotransferrin at 100 µg/ml in the presence or absence of Fe$^{2+}$ (data not shown) or holotransferrin at 1,000 µg/ml (Fig. 7 J) under our experimental conditions. Similarly, under conditions identical to those used in Fig. 7, an EPR signal characteristic of the (DMPO/OH)-adduct was observed on the incubation of $10^{-5}$ M Fe$^{2+}$ with 25 µg/ml apo-lactoferrin, which was not seen with either Fe$^{2+}$ or apo-lactoferrin alone, or when apo-lactoferrin was replaced by holotransferrin in the presence or absence of Fe$^{2+}$ (data not shown). (DMPO/OH)-adduct formation by Fe$^{2+}$ and apo-lactoferrin was largely inhibited by catalase (1.2 µg/ml), but not by heated catalase. A small inhibition by superoxide dismutase (25 µg/ml) was observed that also was present when the heated preparation was used.

**Discussion**

We report here that incubation of Fe$^{2+}$ with apo-transferrin or apo-lactoferrin results in the autoxidation of Fe$^{2+}$
with the formation of oxidants that convert iodide to an iodinating species and are toxic to *E. coli*. Evidence for the formation of two oxygen reduction products, H₂O₂ and OH⁻, was obtained. Rather precise conditions were required: an acid pH (4.5–5.5); Fe⁺⁺ at a concentration (10⁻⁵ M) just below that at which it was toxic alone; apotransferrin or apolactoferrin over a narrow concentration range above which activity was lost; and organisms in early logarithmic growth phase. Our findings will be discussed in relation to the following sequence of reactions:

\[
2\text{Fe}^{2+} + \text{apoTF (or apoLF)} + 2\text{O}_2 \rightarrow 2\text{Fe}^{3+} + 2\text{O}_3
\]

TF (or LF)

\[
2\text{O}_3 + 2\text{H}^+ \rightarrow \text{O}_2 + 2\text{H}_2\text{O}_2
\]

(a)

\[
\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+}\text{OH}^{-} + \text{OH}^{-}
\]

(b)

where TF is transferrin, LF is lactoferrin, apotransferrin, and apolactoferrin. This sequence of reactions would predict an O₂/Fe²⁺ stoichiometry of 1:3, which is approximately what was found.

The autoxidation of Fe²⁺ has a second order dependence on the OH⁻ concentration (41) and thus is favored by an increase in pH. At the pH used here (5.0), autoxidation is very slow, but is greatly accelerated by apotransferrin or apolactoferrin, as indicated by the disappearance of Fe²⁺, the uptake of oxygen, and the binding of iron to the protein. It has been suggested that Fe²⁺ can bind to apotransferrin at the specific binding site, and that the Fe²⁺ at this site is highly susceptible to oxidation (42). Other investigators, however, have been unable to detect appreciable binding of Fe²⁺ to transferrin (43, 44), and it is the generally held view that iron binds to transferrin and lactoferrin largely if not entirely in the Fe³⁺ form, with each molecule of transferrin or lactoferrin capable of binding two atoms of Fe³⁺ to specific iron-binding sites. Thus, transferrin or lactoferrin, in common with other chelators such as deferoxamine (28), which have a much greater affinity for Fe³⁺ than for Fe²⁺, may accelerate the autoxidation of Fe²⁺ in part by the chelation of the Fe²⁺ formed. However, this does not appear to be the sole mechanism since, under our optimum conditions, Fe²⁺ disappearance exceeded the iron-binding capacity of transferrin and lactoferrin. Iron is released from holotransferrin as the pH is lowered below neutrality (45), with one of the two iron-binding sites dissociating at a higher pH than the other (46, 47). Lactoferrin binds iron more avidly than does transferrin (48) but, like transferrin, has one iron-binding site that is more acid labile than the other (33). At pH 5.0, some dissociation of the iron-binding sites would be expected, and possibly transient binding occurs with release of iron into the medium. Some nonspecific binding of iron to the protein cannot be excluded.

Although the formation of O₃ would be anticipated as a consequence of the one electron reduction of oxygen by Fe²⁺ (reaction d), we were unable to appreciably modify the toxicity by the addition of superoxide dismutase, although there was some suggestion of a heat-reversible inhibition of (DMPO/OH⁻) formation. Possible explanations compatible with an O₃ intermediate are the formation of an O₃ complex inaccessible to superoxide dismutase, or the rapid spontaneous dismutation of O₃ at the pH used, making catalysis unnecessary. The formation of H₂O₂ by the Fe²⁺-transferrin or Fe²⁺-lactoferrin system and its involvement, either directly or indirectly, in the toxicity is indicated by catalase inhibition of (DMPO/OH⁻) formation, iodination, and bactericidal activity. In each instance, the inhibition by catalase was partially or totally prevented by its heat inactivation. H₂O₂ may be formed by the dismutation of O₃ (reaction e) or by the divalent reduction by oxygen without an O₃ intermediate.

The formation of OH⁻ was suggested by the detection by EPR of the (DMPO/OH⁻) adduct on the addition of the spin-trap DMPO to the Fe²⁺-transferrin or Fe²⁺-lactoferrin

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Figure 7. Radical formation measured by EPR. The reaction mixture contained 5 × 10⁻⁴ M sodium acetate buffer, pH 5.0, 0.1 M DMPO, and, where indicated, 10⁻⁵ M FeSO₄, 100 µg/ml human apotransferrin (apoTF), 1.000 µg/ml human holotransferrin (holoTF), 1.2 µg/ml catalase, 25 µg/ml superoxide dismutase (SOD), and 0.7 M DMSO in a final volume of 0.5 ml. The catalase and SOD were heated where indicated. The tracings are the sum of three or four replicates, with the tracing for DMPO in buffer alone subtracted from each. Tracing I is a difference spectrum of tracing H minus tracing C. The arrows in tracing I indicate the points where peak lines crossed zero, from which splitting constants AN = 16.2 G and AH = 23.1 G were calculated.

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system. The (DMPO/OH· adduct can be formed either by
the reaction of OH· with DMPO or by the reduction of
the (DMPO/OOH· adduct formed by reaction of O2· with
DMPO as follows:

\[
\text{OH}^- + \text{DMPO} \rightarrow (\text{DMPO}/\text{OH}·) \\
\uparrow \\
\text{O}_2^- + \text{DMPO} \rightarrow (\text{DMPO}/\text{OOH}·)
\]

The strong inhibition of (DMPO/OH· adduct formation
by catalase and the little or no inhibition by superoxide dis-
mutase would argue against an intermediate requirement for
(DMPO/OOH·). DMSO reducts with OH· to form the methyl radical (CH₃·), which can be detected as the (DMPO/CH₃)· adduct. The detection of the (DMPO/CH₃)· adduct on the addition of DMSO to the Fe²⁺-transferrin system is further evidence for the formation of OH·.

The H₂O₂, generated by the autoxidation of Fe²⁺, would be expected to react with excess Fe²⁺ (Fenton's reagent) to generate OH· (reaction a). It is of interest in this regard that the Fe²⁺ was not totally utilized at the optimal transferrin or lactoferrin concentration used, indicating that Fe²⁺ would be available for interaction with the H₂O₂ formed. When the transferrin or lactoferrin concentration was increased to a level where the Fe²⁺ totally disappeared, toxicity was lost. The potentiation of bactericidal activity by iodide (36) and the inhibition of bactericidal activity by the OH· scavenger mannitol at 0.1 M is compatible with OH· involvement in the toxicity. High salt concentration (0.1 M NaCl, NaBr, KCl, Na₂SO₄) also inhibited bactericidal activity, raising the possibility of a nonspecific solute effect. However, 0.1 M mannitol also abolished iodination, whereas equimolar NaCl was ineffective (transferrin) or only partially inhibitory (lactoferrin), raising the possibility that hypotonicity is an additional requirement for bactericidal activity.

The importance of the composition of the bacterial cell wall in the toxicity is indicated by the requirement for early growth phase E. coli. The chemical composition of bacterial cell walls varies with the phase and rate of growth of the organisms (49–52), raising the possibility that structural modification of the cell wall, as well as toxicity, may influence the accessibility of the oxidant to essential chemical targets on the cell surface. Phenotypic tolerance, that is, the resistance of nongrowing bacteria to a variety of antibiotics, is a well recognized phenomenon. In earlier studies, it was proposed that the antimicrobial effect of partially or fully unsaturated transferrin or lactoferrin (53, 54) was due to the chelation of iron required for the growth of the organisms. This mechanism is unlikely to be operative here, since the addition of Fe²⁺ was required.

It is not known whether transferrin- or lactoferrin-
dependent autoxidation of Fe²⁺ with the generation of toxic oxidants can occur in vivo. Conditions in the circulation would be unfavorable for such a reaction by virtue of the absence of appreciable free iron, the relatively high pH, and the presence of abundant protein and other scavengers of oxygen radicals. Transferrin binds to cell surface receptors on macrophages (55–58), as well as a number of other cell types, and the transferrin-receptor complex is endocytosed. The fall in pH in the endocytic vacuole results in the dissociation of iron from the transferrin, and the apotransferrin bound to its receptor is returned to the cell surface, where the complex dissociates, releasing transferrin into the circulation. Reduction of the iron to the ferrous form in the endocytic vacuole or its membrane has been proposed (59). Although this process is designed primarily to supply iron for cellular needs, the conditions in the endocytic vacuole may favor radical formation, namely, the presence of Fe²⁺ and apotransferrin in an acidic environment, with transferrin serving both as a source of iron and as a stimulus of Fe²⁺ autoxidation. Hydroxyl radicals would have to be formed adjacent to an ingested organism for an antimicrobial effect to occur. After endocytosis, transferrin is detected in a juxtanuclear compartment in CHO (60) and K562 (61) cells, and it is not known whether passage into the phagosome occurs in macrophages. It should be noted in this regard that iron-saturated transferrin prevents the inhibition of Legionella pneumophila multiplication by activated cultured human monocytes, presumably by providing the iron required for the growth of the organisms (62).

Lactoferrin is present in high concentration in the specific (secondary) granules of neutrophils and is released into the phagosome after microbial ingestion (63). Iron-unsaturated lactoferrin has antimicrobial properties (64–73) that were, in some studies, favored by a low pH (5.0–6.0) and the use of organisms in early exponential growth phase (69, 73). In general, Fe²⁺ was not added in these studies, and in one instance in which it was (69), no effect on the bactericidal effect of apolactoferrin was observed; however, the experimental design would allow the detection of an inhibition, but not a potentiation, of bactericidal activity. Most studies indicate a fall in pH in the phagosome to a level comparable with that used here (74), although an early rise may occur (75, 76). It is not known whether adequate amounts of free Fe²⁺ are available in the phagosome; a potential source of iron is its release by oxidative attack on the ingested organism (77, 78) or its reductive release from ferritin through the action of O₂ (79, 80). Although lactoferrin has been reported to limit the formation of OH· by the chelation of iron required for the Haber-Weiss reaction (1), the studies reported here raise the possibility that under some conditions potentiation of OH· formation by lactoferrin may occur. Similarly, a number of microorganisms contain iron chelators (siderophores) with a high affinity for Fe³⁺ and a low affinity for Fe²⁺ (81), which would be expected to facilitate the autoxidation of Fe²⁺ with autoinhibition through the formation of toxic oxidants.

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References


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Address correspondence to Seymour J. Klebanoff, Department of Medicine, SJ-10, University of Washington, Seattle, WA 98195

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