Prooxidant Activity of Transferrin and Lactoferrin

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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-pyroline *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_2O_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·)[1] 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_2O_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:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH\cdot \quad (a)$$

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2 \quad (b)$$

$$O_2^- + H_2O_2 \xrightarrow{Fe} O_2 + OH^- + OH\cdot \quad (c)$$

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., deferoxamine, 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.

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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 Fe^{2+} best when the affinity of the chelator for Fe^{3+} greatly exceeds its affinity for Fe^{2+} (26). At pH 7.0, Fe^{3+}-chelating agents enhance the autoxidation of Fe^{2+} in the order EDTA ~ dinitrilodiacetate > citrate > phosphate > oxalate (27). Deferoxamine binds Fe^{3+} with very high avidity, whereas it binds Fe^{2+} poorly, if at all, and thus would be expected to promote Fe^{2+} autoxidation. We have recently reported that the autoxidation of Fe^{2+} at pH 5.5 is greatly accelerated by deferoxamine with the formation of products (H_2O_2, OH·) that are toxic to bacteria (28), which is in sharp contrast to the inhibitory effect of deferoxamine on OH· formation by the iron-catalyzed Haber-Weiss reaction. The prooxidant activity of deferoxamine also has been described by others, (29–31). In this paper, we report that apotransferrin and apolactoferrin also can accelerate the autoxidation of Fe^{2+} 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., San Diego, CA, and its iron was removed to form apolactoferrin as described (32). Briefly, 10 mg of lactoferrin were dissolved in 2 ml of 0.2 M sodium acetate buffer containing 40 mM EDTA and 0.2 M sodium phosphate, pH 4.0. The solution was kept overnight at 4°C and dialyzed for 3 d against 4 liters of water with three water changes, and lyophilized. Iron-saturated hololactoferrin was prepared from apolactoferrin as described (33). Catalase (CTR; bovine liver, 84,150 U/mg) was obtained from Worthington Biochemical Corp., Freehold, NJ; superoxide dismutase (bovine erythrocyte, 3,150 U/mg), human albumin (essentially fatty acid free prepared from fraction V), and DMSO from Sigma Chemical Co., St. Louis, MO; 5,5-dimethyl-1-pyrroline N-oxide (DMPO) from Aldrich Chemical Co., Milwaukee, WI; and $Na^{125}I$ (NEZ033) and $^{59}FeSO_4$ (NEZO49) from DuPont-New England Nuclear, Boston, MA. The catalase was dialyzed overnight against water before use. The catalase was heated at 100°C for 20 min, and the superoxide dismutase was heated at 121°C for 30 min in an autoclave where indicated.

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 Na_2SO_4 and suspended in 0.1 M Na_2SO_4 to the required absorbency at 540 nm. The *E. coli* were incubated with the components indicated in the legends to the figures and tables in a final volume of 0.5 ml for 60 min at 37°C unless otherwise indicated, and the viable cell count was determined by the pour-plate method using Trypticase soy agar (34).

Iodination. The reagents indicated in the legends to the tables were incubated in polystyrene test tubes (12 × 75 mm) for 60 min at 37°C without shaking, and iodination was determined as previously described (28). Each experimental value was determined in duplicate and was averaged to give a single value for statistical analysis.

Fe^{2+} Measurement. The components of the reaction mixture (see legends to figures) in a final volume of 0.5 ml were incubated at 37°C in a shaking water bath for the periods indicated. 50 μl of 0.01 M bathophenanthroline sulfonate was added, and the OD at 535 nm was compared with a ferrous sulfate standard curve (35).

Oxygen Consumption. Oxygen consumption was determined with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) at 37°C and expressed as nmol/ml based on a concentration of 215 μM O_2 in air-saturated buffer at 37°C.

Binding of $^{59}Fe^{2+}$ to Transferrin. The components of the reaction mixture (see legends to figures) containing $^{59}Fe^{2+}$ were incubated for 15 min, and the total reaction mixture (0.5 ml) was separated at room temperature on a prepacked disposable 16 × 50-mm Sephadex G25 column (9.1-ml bed volume) (PD-10, Sephadex G-25M; Pharmacia Fine Chemicals, Uppsala, Sweden) that had been prewashed with 30 ml of 5×10^{-4} M sodium acetate buffer, pH 5.0. The fractions (0.5 ml) were eluted with the same buffer at a flow rate of ~1 ml/min and counted with a gamma scintillation counter. A standard containing the total amount of ^{59}Fe added to the reaction mixture was counted for calculation of percent recovery. The protein content of the fractions was estimated by the absorption at 280 nm.

Electron Paramagnetic Resonance (EPR) Spectroscopy. The components of the reaction mixture (see legends to figures) were mixed and immediately transferred to a flat cell placed in the cavity of an EPR spectrometer (E3; Varian Associates, Inc., Palo Alto, CA), and the EPR spectrum was determined at room temperature. Measurements were with a scan speed of 12.5 Gauss/min, modulation amplitude of 5 G, time constant of 3 s, X band frequency of 9.43 GHz, field centered at 3,345 G, receiver gain at 10^6, and power at 20 mW. Where indicated, scans from the XY recorder of the EPR instrument were digitized at 0.1-Gauss intervals using a digitizing tablet and accompanying software (Sigma-Plot; Jandel Scientific, San Francisco, CA). Digitized scans were transferred to a spreadsheet program (Lotus 123; Lotus Development Corporation, Cambridge, MA), where redundant values were averaged, missing values linearly interpolated, multiple scans summed, and subtraction of scans performed.

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, Fe^{2+} alone at relatively high concentration was toxic to *E. coli* in acetate buffer pH 5.0. When the concentration of Fe^{2+} 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 Fe^{2+} 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

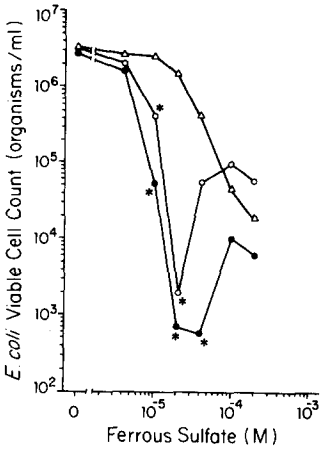

Figure 1. Bactericidal effect of Fe^{2+} and apotransferrin or apolactoferrin. The reaction mixture contained 5 × 10^{-4} M sodium acetate buffer pH 5.0, 0.01 M Na_2SO_4, 1–3 × 10^6 *E. coli*, $FeSO_4$ at the concentrations indicated, either alone (Δ), or with 100 μg/ml of human apotransferrin (●) or 25 μg/ml human apolactoferrin (O). The results are the mean of three to seven experiments. The asterisks indicate a significant difference between Fe^{2+} alone and Fe^{2+} + transferrin or Fe^{2+} + lactoferrin (all $p < 0.001$).

increased. A bactericidal effect also was observed with Fe^{2+} and bovine apotransferrin with the optimum transferrin concentration being 25 μg/ml (data not shown). All subsequent bactericidal studies were performed with $FeSO_4$, 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 Fe^{2+}, Fe^{2+} + apotransferrin, or Fe^{2+} + apolactoferrin. Fe^{2+} 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.

Table 1. *Effect of pH*

Buffer	pH	Viable cell count			
		Control	Fe^{2+}	Fe^{2+} + apoTF	Fe^{2+} + apoLF
		organisms/ml × 10^{-6}			
Acetate	4.5	2.30	1.92	0.15*‡	0.20*‡
Acetate	5.0	3.17	2.35	0.065*‡	0.43*‡
Acetate	5.5	2.78	1.44*	0.75*	0.45*‡
Acetate	6.0	2.86	0.28*	0.10*	1.36*
Phosphate	6.0	3.07	0.55*	1.25*	1.64*
Phosphate	6.5	2.21	0.022*	0.26*	0.86*
Phosphate	7.0	2.30	0.011*	0.35*	0.36*

The reaction mixture contained 5 × 10^{-4} M sodium acetate or sodium phosphate buffers at the pH indicated, 0.01 M Na_2SO_4, 1–3 × 10^6 *E. coli*, and, where indicated, 10^{-5} M $FeSO_4$, 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 Fe^{2+} alone, $p < 0.05$.

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 Fe^{2+} 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 Fe^{2+} + apotransferrin and Fe^{2+} + 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.

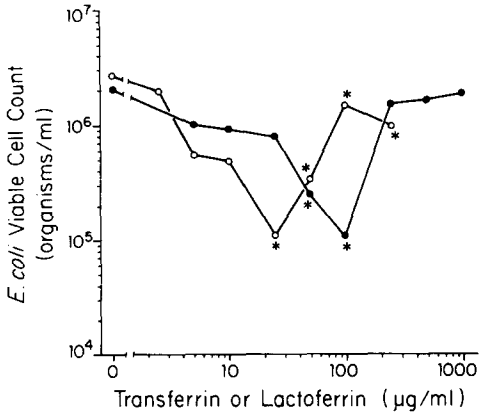

Figure 2. Effect of transferrin or lactoferrin concentration. The reaction mixture contained 5 × 10^{-4} M sodium acetate buffer, pH 5.0, 0.01 M Na_2SO_4, 1–2 × 10^6 *E. coli*, 10^{-5} M $FeSO_4$, and either apotransferrin (●) or apolactoferrin (O) at the concentrations indicated. The results are the mean of three to five experiments. The asterisks indicate a significant difference from control without transferrin or lactoferrin ($p < 0.05$).

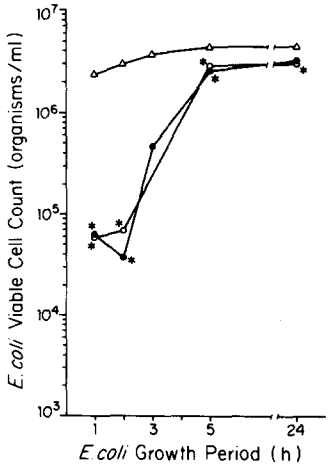

Figure 3. Effect of *E. coli* growth period. The reaction mixture contained 5 × 10^{-4} M sodium acetate buffer, pH 5.0, 0.01 M Na_2SO_4, 2–5 × 10^6 *E. coli* grown in broth for the periods indicated, and further additions as follows: none (Δ); 10^{-5} M $FeSO_4$ + 100 μg/ml apotransferrin (●); 10^{-5} M $FeSO_4$ + 25 μg/ml apolactoferrin (O). The results are the mean of three to seven experiments. The asterisks indicate a significant difference from the control without additions ($p < 0.05$).

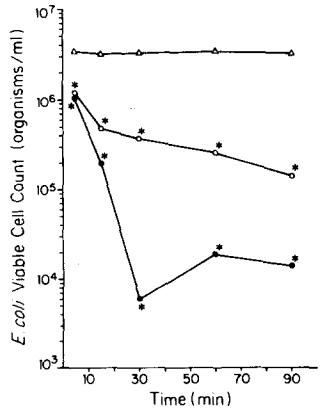

Figure 4. Effect of incubation period. The reaction mixture contained 5×10^{-4} M sodium acetate buffer, pH 5.0, 0.01 M Na_2SO_4, 1–3 × 10^6 *E. coli*, either alone (control) (△), or with 10^{-5} M $FeSO_4$ + 100 μg/ml apotransferrin (●) or 10^{-5} M $FeSO_4$ + 25 μg/ml apolactoferrin (○). The incubation period was varied as indicated. Results are the mean of three to seven experiments. The asterisks indicate a significant difference from control (all $p < 0.001$).

In the Fe^{2+} + apotransferrin or apolactoferrin system, Fe^{2+} could not be replaced by Fe^{3+}, 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 Na_2SO_4 (data not shown). Apotransferrin or apolactoferrin at the concentration used in Table 2 was ineffective in the absence of Fe^{2+}, 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).

Table 3. *Stimulation of the Bactericidal Effect of Fe^{2+} and Apotransferrin or Apolactoferrin by Iodide*

Additions	Viable cell count	p^*
	organisms/ml × 10^{-6}	
None	2.83	
Fe^{2+} + apoTF	0.35	
Fe^{2+} + apoTF + I	0.00005	<0.002
Fe^{2+} + apoLF	0.11	
Fe^{2+} + apoLF + I	0.00003	<0.001

The reaction mixture contained 5×10^{-4} M sodium acetate buffer, pH 5.0, 0.01 M Na_2SO_4, 3 × 10^6 *E. coli*, and, where indicated, 10^{-5} M $FeSO_4$, 100 μg/ml apotransferrin (apoTF), 25 μg/ml apolactoferrin (apoLF), and 10^{-5} M NaI. The results are the mean of three to four experiments.
* p value for the difference from Fe^{2+} + apoTF or Fe^{2+} + apoLF.

Earlier studies had indicated that the bactericidal activity of Fe^{2+} + H_2O_2 (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 Fe^{2+} and apotransferrin, or Fe^{2+} and apolactoferrin.

Table 2. *Effect of Inhibitors*

Additions	Viable cell count					
	Transferrin	p^*	$p^‡$	Lactoferrin	p^*	$p^‡$
			organisms/ml × 10^{-6}			
None	3.05(6)§			3.40(4)		
Fe^{2+} + apoTF or apoLF	0.05(6)	<0.002		0.19(4)	<0.001	
− Fe^{2+}, + Fe^{3+}	3.83(3)	NS	<0.02	2.27(4)	<0.001	<0.001
+ catalase	2.50(6)	NS	<0.002	2.64(4)	<0.02	<0.001
+ heated catalase	0.03(6)	<0.01	NS	0.62(4)	<0.001	<0.05
+ SOD	0.08(6)	<0.01	NS	0.27(4)	<0.001	NS
+ mannitol	0.92(4)	<0.05	<0.05	2.41(4)	<0.02	<0.001
+ NaCl	2.09(5)	NS	<0.01	2.61(4)	NS	<0.001
ApoTF or apoLF	2.12(10)	NS	<0.001	2.74(6)	NS	<0.001
HoloTF or holoLF	1.94(9)	NS	<0.001	2.47(3)	NS	<0.01

The reacton mixture contained 5×10^{-4} M sodium acetate buffer, pH 5.0, 0.01 M Na_2SO_4, 3 × 10^6 *E. coli*, and, where indicated, 10^{-5} M $FeSO_4$, 10^{-5} M $Fe_2(SO_4)_3$, 100 μg/ml apotransferrin (apoTF), 100 μg/ml holotransferrin (holoTF), 25 μg/ml apolactoferrin (apoLF), 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 Fe^{2+} + apoTF or apoLF.
§ Mean of no. of experiments.

Iodination. Incubation of Fe^{2+} and apotransferrin or apolactoferrin with ^{125}I-iodide in the presence of albumin was associated with the conversion of iodide to a TCA precipitable form (iodination). When the Fe^{2+} 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^{-6} 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 Fe^{2+} and apotransferrin or apolactoferrin was significantly decreased or abolished when either Fe^{2+} or the iron chelator was omitted, when Fe^{2+} was replaced by Fe^{3+}, 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 Fe^{2+} and apotransferrin, and only partially inhibited iodination by Fe^{2+} and apolactoferrin. No iodination was observed when Fe^{2+} + apotransferrin was replaced by holotransferrin at 100–1,000 μg/ml, or when Fe^{2+} + apolactoferrin was replaced by hololactoferrin at 10–500 μg/ml.

Autoxidation of Iron. The autoxidation of Fe^{2+} is greatly accelerated by apotransferrin or apolactoferrin at pH 5.0. Under the conditions used in Fig. 5, a rapid loss of Fe^{2+} (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 Fe^{2+} was not observed under these conditions, with ~32% of the Fe^{2+} 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 Fe^{2+} was observed at 15 min. Loss of Fe^{2+} was not observed in the absence of apotransferrin or apolactoferrin under the conditions used in Fig. 5.

That the loss of Fe^{2+} is due to its autoxidation is suggested by measurements of oxygen consumption. Little or no oxygen consumption was detected at the concentrations of Fe^{2+} (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 μM, and the concentration of the Fe^{2+} 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 Fe^{2+} 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 (Fe^{2+}, 2×10^{-4} M; apotransferrin, 2 mg/ml), no oxygen consumption was observed on the addition of either Fe^{2+} or apotransferrin alone. Assuming the oxidation of two-thirds of the Fe^{2+} added (Fig. 5), approximately one molecule of oxygen is taken up for every three or four molecules of Fe^{2+} oxidized. Similar findings were observed with apolactoferrin, except that the oxygen/Fe^{2+} stoichiometry was 1:2–3.

Binding of Iron. The binding of iron on incubation of Fe^{2+} with apotransferrin at pH 5.0 was indicated by the association of $^{59}Fe^{2+}$ with the iron-binding protein (Fig. 6). In the absence of transferrin, $^{59}Fe^{2+}$ eluted from a Sephadex G-25 column in small amounts over an extended period. However, after a 15-min incubation of 10^{-5} M $^{59}FeSO_4$ with 100

Table 4. *Iodination by Fe^{2+} and Apotransferrin or Apolactoferrin*

Additions	Iodination			
	Fe^{2+} + apoTF	p^*	Fe^{2+} + apoLF	p^*
	pmol			
Fe^{2+}	1.1 ± 1.1(3)‡	<0.01	0.8 ± 0.7(4)	<0.001
ApoTF or apoLF	3.7 ± 0.1(3)	<0.01	−1.8 ± 0.2(2)	<0.001
Fe^{2+} + apoTF or ApoLF	45.7 ± 7.2(3)		85.2 ± 4.9(6)	
Fe^{3+} + apoTF or apoLF	4.1 ± 0.5(3)	<0.01	1.4 ± 2.0(5)	<0.001
Fe^{2+} + apoTF or apoLF + catalase	2.9 ± 0.5(3)	<0.01	0.3 ± 0.4(3)	<0.001
Fe^{2+} + apoTF or apoLF + heated catalase	32.4 ± 5.2(3)	NS	78.8 ± 9.6(3)	NS
Fe^{2+} + apoTF or apoLF + SOD	33.9 ± 6.3(3)	NS	70.1 ± 3.6(4)	NS
Fe^{2+} + apoTF or apoLF + mannitol	2.0 ± 0.1(3)	<0.01	0.9 ± 0.8(5)	<0.001
Fe^{2+} + apoTF or apoLF + NaCl	47.3 ± 7.1(5)	NS	27.3 ± 3.0(5)	<0.001

The reaction mixture contained 5×10^{-4} M sodium acetate buffer, pH 5.0, 8×10^{-6} M NaI (4,000 pmol; 0.05 μCi ^{125}I), 0.2 mg/ml albumin, and, where indicated, 10^{-5} M $FeSO_4$, 100 μg/ml apotransferrin, 50 μg/ml apolactoferrin, 10^{-5} M $Fe_3(SO_4)_2$, 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 Fe^{2+} + apoTF or Fe^{2+} + apoLF.

‡ Mean ± SE of no. of experiments.

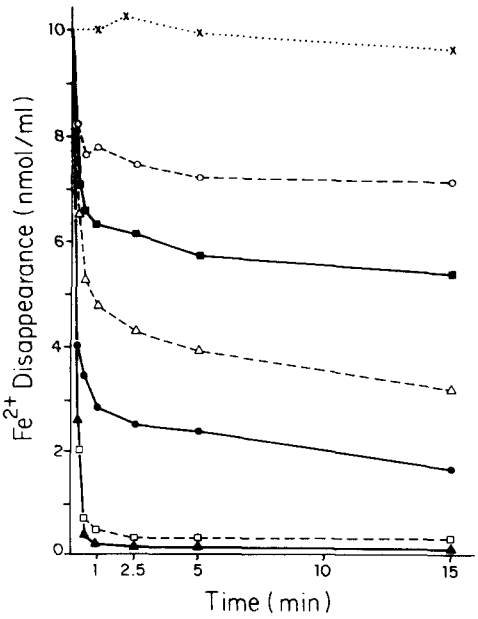

Figure 5. Fe^{2+} disappearance on incubation with apotransferrin or apolactoferrin. The reaction mixture contained 5 × 10^{-4} M sodium acetate buffer, pH 5.0, 10^{-5} M $FeSO_4$ alone (x · · · x) or 10^{-5} M $FeSO_4$, and either apotransferrin (*broken lines*) ([O---O] 50 μg/ml; [Δ---Δ] 100 μg/ml; [□---□] 250 μg/ml) or apolactoferrin (*solid lines*) ([■——■] 25 μg/ml; [●——●] 50 μg/ml; [▲——▲] 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.

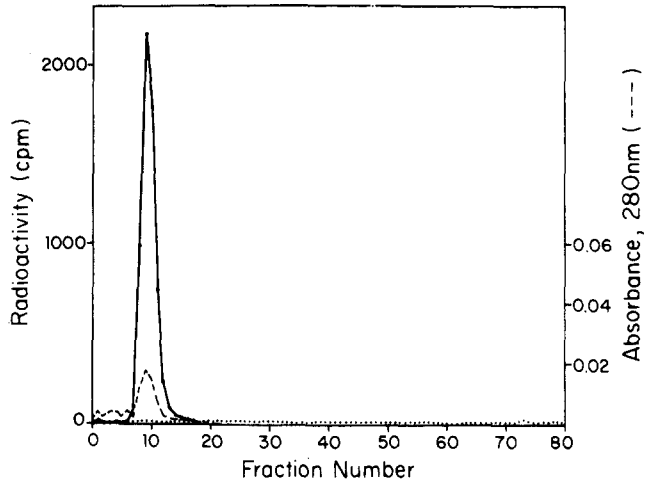

Figure 6. Binding of $^{59}Fe^{2+}$ to apotransferrin. The reaction mixture contained 5 × 10^{-4} M sodium acetate buffer, pH 5.0, 10^{-5} M $FeSO_4$ (~14,000 cpm) (*dotted line*) or 10^{-5} M $^{59}FeSO_4$ + 100 μg/ml human apotransferrin (apoTF) (*solid line*) in a final volume of 0.5 ml. After separation on a Sephadex G-25 column, the ^{59}Fe present in the fractions ($FeSO_4$ alone, *dotted line*; $FeSO_4$ + apoTF, *solid line*) and the absorbency at 280 nm ($FeSO_4$ + apoTF, *broken line*) was determined. The results are the mean of three experiments.

μg/ml apotransferrin 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 apotransferrin 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 apolactoferrin, 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 apotransferrin with the spin-trap DMPO in acetate buffer pH 5.0 produced an EPR signal with splitting constants of $a_N = a_H = 14.8$ G and a 1:2:2:1 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_2O_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 $A_N = 16.2$ G and $A_H = 23.1$ 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 apolactoferrin, which was not seen with either Fe^{2+} or apolactoferrin alone, or when apolactoferrin was replaced by holotransferrin in the presence or absence of Fe^{2+} (data not shown). (DMPO/OH)· adduct formation by Fe^{2+} and apolactoferrin 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 apotransferrin or apolactoferrin 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, H_2O_2 and OH·, was obtained. Rather precise conditions were required: an acid pH (4.5–5.5); Fe^{2+} 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:

$$2Fe^{2+} + \underset{\text{TF (or LF)}}{\text{apoTF (or apoLF)}} + 2O_2 \rightarrow 2Fe^{3+} + 2O_2^- \quad \text{(d)}$$

$$2\,O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \quad \text{(e)}$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+}OH^- + OH\cdot \quad \text{(a)}$$

where TF is transferrin, LF is lactoferrin, apoTF is apotransferrin, and apoLF is apolactoferrin. This sequence of reactions would predict an O_2/Fe^{2+} stoichiometry of 1:3, which is approximately what was found.

The autoxidation of Fe^{2+} 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 Fe^{2+}, the uptake of oxygen, and the binding of iron to the protein. It has been suggested that Fe^{2+} can bind to apotransferrin at the specific binding site, and that the Fe^{2+} at this site is highly susceptible to oxidation (42). Other investigators, however, have been unable to detect appreciable binding of Fe^{2+} to transferrin (43, 44), and it is the generally held view that iron binds to transferrin and lactoferrin largely if not entirely in the Fe^{3+} form, with each molecule of transferrin or lactoferrin capable of binding two atoms of Fe^{3+} 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 Fe^{3+} than for Fe^{2+}, may accelerate the autoxidation of Fe^{2+} in part by the chelation of the Fe^{3+} formed. However, this does not appear to be the sole mechanism since, under our optimum conditions, Fe^{2+} 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 O_2^- would be anticipated as a consequence of the one electron reduction of oxygen by Fe^{2+} (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 O_2^- intermediate are the formation of an O_2^- complex inaccessible to superoxide dismutase, or the rapid spontaneous dismutation of O_2^- at the pH used, making catalysis unnecessary. The formation of H_2O_2 by the Fe^{2+}-transferrin or Fe^{2+}-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. H_2O_2 may be formed by the dismutation of O_2^- (reaction e) or by the divalent reduction by oxygen without an O_2^- intermediate.

The formation of OH· was suggested by the detection by EPR of the (DMPH/OH)· adduct on the addition of the spin-trap DMPO to the Fe^{2+}-transferrin or Fe^{2+}-lactoferrin

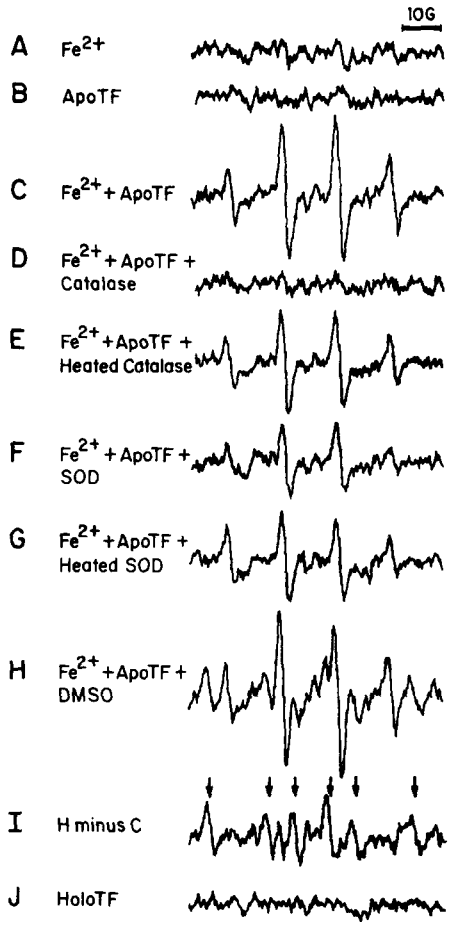

Figure 7. Radical formation measured by EPR. The reaction mixture contained 5×10^{-4} M sodium acetate buffer, pH 5.0, 0.1 M DMPO, and, where indicated, 10^{-5} M $FeSO_4$, 100 μg/ml human apotransferrin (apoTF), 1,000 μg/ml human holotransferrin (holoTF), 1.2 μg/ml catalase, 25 μg/ml superoxide dismutase (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 $A_N = 16.2$ G and $A_H = 23.1$ G were calculated.

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 O_2^- with DMPO as follows:

$$OH\cdot + DMPO \rightarrow (DMPO/OH)\cdot$$
$$\uparrow$$
$$O_2^- + DMPO \rightarrow (DMPO/OOH)\cdot$$

The strong inhibition of (DMPO/OH)· adduct formation by catalase and the little or no inhibition by superoxide dismutase would argue against an intermediate requirement for (DMPO/OOH)·. DMSO reacts with OH· to form the methyl radical (CH_3), which can be detected as the (DMPO/CH_3)· adduct. The detection of the (DMPO/CH_3)· adduct on the addition of DMSO to the Fe^{2+}-transferrin system is further evidence for the formation of OH·.

The H_2O_2, generated by the autoxidation of Fe^{2+}, would be expected to react with excess Fe^{2+} (Fenton's reagent) to generate OH· (reaction a). It is of interest in this regard that the Fe^{2+} was not totally utilized at the optimal transferrin or lactoferrin concentration used, indicating that Fe^{2+} would be available for interaction with the H_2O_2 formed. When the transferrin or lactoferrin concentration was increased to a level where the Fe^{2+} 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, Na_2SO_4) 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 tonicity, 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 Fe^{2+} was required.

It is not known whether transferrin- or lactoferrin-dependent autoxidation of Fe^{2+} 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 Fe^{2+} and apotransferrin in an acidic environment, with transferrin serving both as a source of iron and as a stimulus of Fe^{2+} 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, Fe^{2+} 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 Fe^{2+} 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 O_2^- (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 Fe^{3+} and a low affinity for Fe^{2+} (81), which would be expected to facilitate the autoxidation of Fe^{2+} with autoinhibition through the formation of toxic oxidants.

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