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The Effect of Human Serum Transferrin and Milk Lactoferrin on Hydroxyl Radical Formation from Superoxide and Hydrogen Peroxide*

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The effect of transferrins on hydroxyl radical formation from the superoxide anion and hydrogen peroxide generated by the xanthine-xanthine oxidase system has been studied by EPR using 5,5-dimethyl-1-pyrroline *N*-oxide as a spin trap. Neither diferriclactoferrin nor diferrictransferrin were found capable of promoting hydroxyl radical formation via the Haber-Weiss reaction even in the presence of EDTA in concentrations up to 1 mM. Activity observed by other authors may have been due to the presence of extraneous iron or an active protein impurity. Partially saturated transferrin and lactoferrin present in normal subjects may protect cells from damage by binding iron that might catalyze hydroxyl radical formation from superoxide and hydrogen peroxide. In any event, the hydroxyl radical formation observed in active neutrophils during phagocytosis cannot be associated with lactoferrin activity.

The reaction of superoxide, O_2^-, with either ascorbate or hydrogen peroxide can generate the highly reactive hydroxyl radical, $OH^\cdot$ (1, 2), which has been proposed as one of the major species responsible for oxygen toxicity (3). The direct reaction of O_2^- with H_2O_2, the Haber-Weiss reaction, however, proceeds at a significant rate only in the presence of a transition metal ion, with iron as the most likely candidate to be active *in vivo* (4):

$$O_2^- + H_2O_2 \xrightarrow{Fe} OH^\cdot + OH^- + H_2O.$$

The iron-catalyzed Haber-Weiss reaction may promote lipid peroxidation with consequent cell damage (5–8).

During phagocytosis, neutrophils have been shown to produce $OH^\cdot$ which may participate in bacterial killing (3). Two important mechanisms have been proposed for $OH^\cdot$ production, one involving the H_2O_2/Cl^-/myeloperoxidase system (3, 8) and the other an iron-catalyzed Haber-Weiss reaction (9). The importance of myeloperoxidase in bacterial killing has been widely confirmed (3) but the role of an iron-catalyzed Haber-Weiss reaction is still uncertain. The observation that the iron-binding protein lactoferrin occurs in neutrophils (10) has led to a number of studies examining the catalytic potential of lactoferrin in the Haber-Weiss reaction but with conflicting results (9, 11–14). Ambruso and Johnston (11) observed considerable $OH^\cdot$ formation with both milk and neutrophil lactoferrin. Accordingly, they suggested that lactoferrin functions as a regulator of $OH^\cdot$ production and, therefore, an important contributor to the microbicidal activity of neutrophils. Recent work by Bannister *et al.* (9) appears to offer support for this proposal. However, Winterbourn (12) found no evidence for enhancement of $OH^\cdot$ production by lactoferrin except, possibly, in the presence of EDTA (13). This view is consistent with the observation that partially saturated lactoferrin can inhibit, rather than promote, lipid peroxidation (14). There have been similarly conflicting reports that the serum iron-binding protein transferrin can (15) and cannot (13, 16) enhance $OH^\cdot$ production.

The methods used to detect $OH^\cdot$ production in these studies with transferrins include the formation of ethylene from methional (12, 13) or α-keto-γ-methiolbutyric acid (9, 11), a thiobarbituric acid-reactive product from deoxyribose (14, 16), a diphenol from salicylic acid (15) and a long-lived hydroxyl radical adduct with DMPO[1] (9, 15). Because of the potential importance of the involvement of lactoferrin and transferrin in $OH^\cdot$ formation we have investigated their effect on $OH^\cdot$ production using the xanthine-xanthine oxidase system to generate O_2^- and H_2O_2 (17). The $OH^\cdot$ generated was trapped using DMPO, and the resulting DMPO-$OH^\cdot$ radical was studied by EPR spectroscopy. This method of detection of $OH^\cdot$ was preferred since the DMPO-$OH^\cdot$ spectrum is readily distinguished from the adducts formed by other radicals and thus may be more specific for detecting $OH^\cdot$ than many of the other methods which have been used. The use of DMPO as a spin-trap for $OH^\cdot$ has been discussed by other authors (18, 19).

MATERIALS AND METHODS

Lactoferrin was isolated from human breast milk by the method of Blackberg and Hernell (20) using heparin-Sepharose CL-6B (Pharmacia Fine Chemicals). All sample preparations gave a single band with an apparent molecular weight near 80,000 on SDS-gel electrophoresis. Both lactoferrin and human serum transferrin (Behringwerke) were saturated with iron by adding an excess of Fe^{3+}-nitrilotriacetate (1:3, pH 4.0) to a solution containing 0.1 M $NaHCO_3$. After incubation for at least 1 h at room temperature the solutions were dialyzed at 4 °C against 0.1 M ClO_4^-, 10 mM phosphate buffer, pH 7.4 (3 changes), then dialyzed against the required phosphate or Hepes buffer. Some samples were also passed through a column of Chelex-

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[1] The abbreviations used are: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTPA, diethylenetriamine pentaacetic; DFO, desferrioxamine.

100 resin (Bio-Rad) to insure complete removal of any excess Fe^{3+}. Samples of diferriclactoferrin had A_{470}/A_{410} ratios between 1.25 and 1.30, while diferrictransferrin had ratios between 1.35 and 1.38.

Buffers were depleted of metal ion contaminants by extraction with dithizone (diphenylthiocarbazone, J. T. Baker Chemical Co.) in CCl_4 or by passage through a column of Chelex-100. All glassware was acid-washed with 30% nitric acid and rinsed thoroughly with distilled, deionized water.

The spin-trap DMPO (Aldrich) was dissolved in distilled-deionized water (1 g/10 ml) and impurities were removed using neutral decolorizing charcoal as described by Beuttner and Oberley (19). Portions of this solution were stored in liquid nitrogen until required. All operations using DMPO were performed in subdued light to minimize formation of light-induced degradation products (21).

Xanthine oxidase (0.6 unit/mg), bovine serum albumin, and diethylenetriammine pentaacetic acid, DTPA, (2 times crystallized) were purchased from Sigma, xanthine from Calbiochem-Behring, and desferrioxamine mesylate (Desferal) from the Ciba-Geigy Corp. All other chemicals used were the best grade commercially available.

EPR spectra were obtained at room temperature using an aqueous solution cell and a Varian E9 EPR spectrometer. In a typical experiment all other reagents were mixed prior to addition of DMPO and the reaction was then initiated by adding the required amount of xanthine oxidase solution. In most experiments the DMPO and xanthine oxidase were added immediately but in some cases the mixture was allowed to equilibrate for about 18 h (see "Results"). Spectra could be obtained in as little as 1½ min after initiation.

RESULTS

Figs. 1 and 2 illustrate the results of a typical series of experiments. The spectrum of the DMPO-$OH^{\cdot}$ adduct obtained in the absence of any iron catalyst or EDTA is shown in Fig. 1*a*; the observed intensity increased with increasing xanthine (40 nM to 50 μM), DMPO (5 to 100 mg/ml), and xanthine oxidase (5 to 100 mg/ml) concentration. Addition of EDTA (Fig. 1*b*) gave little if any increase in intensity indicating that the system is essentially free of any readily available extraneous iron. Deliberate addition of iron as ferrous ammonium sulfate (1 to 20 μM) in the presence of EDTA gave the expected large increase in DMPO-$OH^{\cdot}$ concentration (see Fig. 1*c*) because of the well-documented catalytic behavior of Fe-EDTA (1, 2, 4). Addition of diferriclactoferrin, in contrast (see Fig. 2*a*), at concentrations from 1 to 30 μM, in the absence or presence of EDTA (20 μM to 1 mM) and in 10 mM phosphate or Hepes buffers, pH 7.4, resulted in no experimentally significant increase in DMPO-$OH^{\cdot}$ production. Similar results (see Fig. 2*b*) were obtained with diferrictransferrin (1 to 30 μM). Deliberate addition of Fe^{2+} to diferriclactoferrin either before or after addition of EDTA resulted in the DMPO-$OH^{\cdot}$ spectrum expected for the same concentration of Fe^{2+}-EDTA. Thus, both diferriclactoferrin and diferrictransferrin show no catalysis of $OH^{\cdot}$ formation from the xanthine-xanthine oxidase system as long as sufficient care is taken to remove any available extraneous iron. Our results indicate that dialysis against 0.1 M $NaClO_4$ or NaCl and/or slow passage of the iron-loaded protein down a column of Chelex-100 is sufficient to remove extraneous iron.

Both partially saturated transferrin (12) and lactoferrin (14) prevent catalysis of $OH^{\cdot}$ formation presumably by binding any catalytically active iron. However, during the course of our experiments we observed that apotransferrin can lower the activity of Fe-EDTA even in the presence of 5 mM EDTA, an amount sufficient to remove iron completely from transferrin (22). Equilibration of 10 μM ferrous ammonium sulfate, 5 mM EDTA, and 5 μM apotransferrin (10 μM in Fe binding sites) for 18 h at room temperature gave no increase in absorbance at 470 nm, indicating that no specific iron-protein complex is formed. The results of the EPR experiments are given in Table I, comparing apotransferrin with DTPA, DFO, apolactoferrin, and bovine serum albumin for ability to reduce

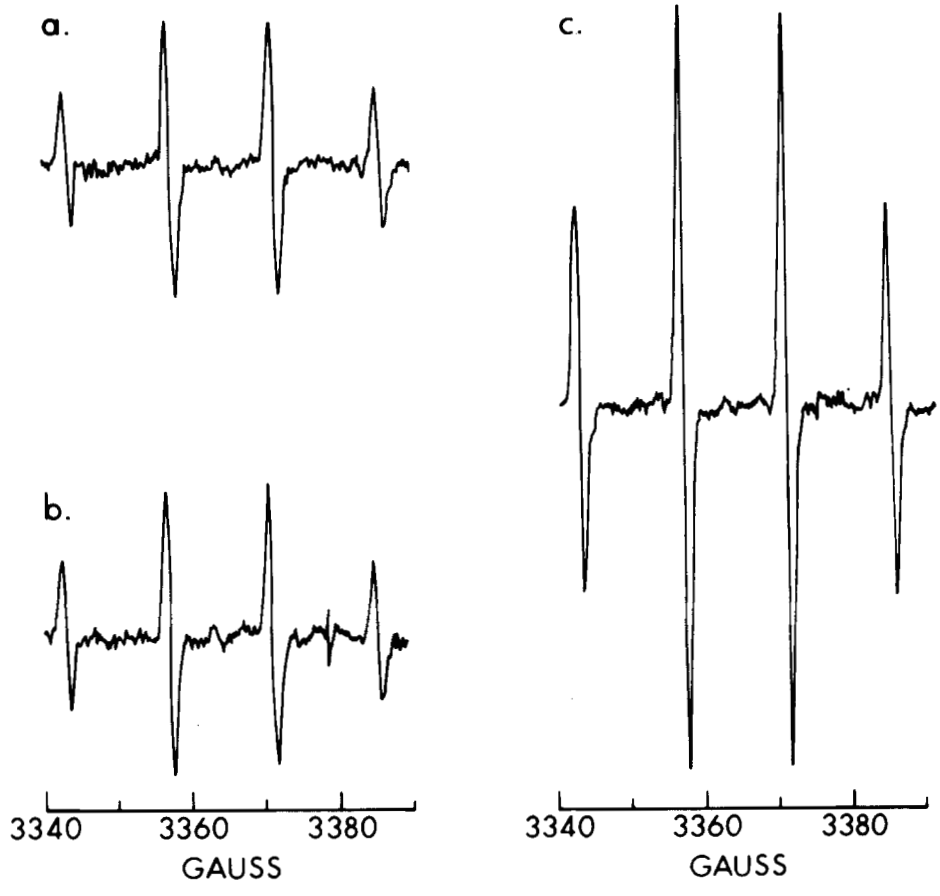

FIG. 1. **EPR spectra of DMPO-$OH^{\cdot}$ generated from the xanthine-xanthine oxidase reaction.** *a*, control: 50 μM xanthine, 40 mM DMPO, 0.6 mg/ml of xanthine oxidase in 10 mM phosphate buffer, pH 7.4; *b*, control plus 1 mM EDTA; *c*, control plus 1 mM EDTA and 10 μM ferrous ammonium sulfate. Instrumental details: field set, 3370 G; scan range, 200 G; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; receiver gain, 1×10^4; time constant, 1.0 s; microwave frequency, 9.470 GHz; microwave power, 20 milliwatts; scan time, 16 min. The scan was started 5 min after initiating the reaction.

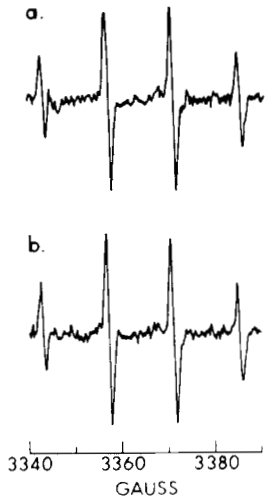

FIG. 2. **EPR spectra of DMPO·OH generated in the presence of diferriclactoferrin and diferric transferrin.** *a*, control plus 1 mM EDTA and 10 μM diferriclactoferrin; *b*, control plus 1 mM EDTA and 10 μM diferrictransferrin. Composition of control and instrumental details as given in Fig. 1.

TABLE I

Effect of some proteins and chelators on the catalytic activity of 10 μM Fe^{2+} in the presence of 5 mM EDTA

Protein or chelator	Increase over control on addition of 10 μM Fe^{2+} [a]	Decrease in Fe-EDTA activity
	%	
None	215	0
Apotransferrin, 5 μM	85	60
Apolactoferrin, 5 μM	165	23
Bovine serum albumin, 5 μM	185	14
Desferrioxamine, 5 μM	115	47
Desferrioxamine, 5 mM	−70	133
DTPA, 5 mM	135	37

[a] Control: xanthine, 50 μM; EDTA, 5 mM; DMPO, 40 mM; xanthine oxidase, 0.6 mg/ml; Hepes, 10 mM; plus the protein or chelator listed in Column 1. Final pH of reaction mixture, 7.4.

the activity of 10 μM Fe^{2+} in the presence of 5 mM EDTA. Both DTPA and DFO (23) reduce iron-catalyzed $OH^{\cdot}$ formation. In the presence of either, therefore, a lowering of DMPO-$OH^{\cdot}$ would be expected due to competition between the chelating agent and EDTA for available iron. The extent of reduction in activity should depend on the relative binding constants of EDTA and DFO or DTPA, provided sufficient time is allowed for equilibration. The results for 5 mM DTPA and 10 μM DFO given in the table tend to confirm this prediction. In the presence of 5 mM DFO, however, the amount of DMPO-$OH^{\cdot}$ formed was even less than that observed in the control experiment carried out under the same conditions except for the absence of added iron. The formation of the Fe·DFO complex was confirmed by the expected increase in absorbance at 428 nm (24). The reasons for this apparent "inhibition" of $OH^{\cdot}$ formation by the Fe-DFO complex are not clear. The 60% reduction in DMPO-$OH^{\cdot}$ observed with 5 μM apotransferrin, and the much smaller decreases with apolactoferrin (23%) and bovine serum albumin (14%), could be due to either (i) binding of the anionic $(Fe\cdot EDTA\cdot H_2O)^-$ complex by the proteins, all of which bind anions (25, 26) thus lowering the catalytic concentration of the complex, or (ii) reaction of the proteins with $OH^{\cdot}$ thus reducing availability of the radical for reaction with DMPO. It should be noted, however, that neither diferrictransferrin nor diferriclactoferrin reduced the yield of DMPO-$OH^{\cdot}$ in any of our experiments, perhaps because the reactivities of the iron-loaded proteins with $OH^{\cdot}$ differ from those of the apoproteins.

DISCUSSION

The results reported here show that neither diferriclactoferrin nor diferrictransferrin can catalyze $OH^{\cdot}$ formation via the Haber-Weiss reaction and confirm similar findings of earlier studies (12, 13, 16). Even in the presence of EDTA (up to 1 mM), however, we saw no enhancement of $OH^{\cdot}$ formation, contrary to one report by others (13). We have also shown that apotransferrin and, to a lesser extent, apolactoferrin can reduce the apparent activity of Fe^{3+} even in the presence of sufficient EDTA (5 mM) to prevent iron binding by the protein. The mechanism for this apparent inhibition may be (i) the formation of protein bound iron-EDTA complexes which are catalytically inactive or (ii) reaction of the apoproteins with $OH^{\cdot}$, thus reducing the observed amount of DMPO-$OH^{\cdot}$ adduct. The latter is a property that may be shared by many other proteins (16).

In vivo, in normal subjects, both transferrin and lactoferrin are only partially saturated with iron (27, 28). These proteins can be expected to sequester any free iron and thus prevent cell damage such as lipid peroxidation from any $OH^{\cdot}$ generated by an iron-catalyzed Haber-Weiss reaction. Cell damage by this mechanism would only be expected in disorders characterized by iron overload (6). Our results also indicate that the formation of $OH^{\cdot}$ observed in activated neutrophils during phagocytosis (3) cannot be associated with lactoferrin activity.

Most previous reports (9, 11, 15) of enhancement of $OH^{\cdot}$ formation by both lactoferrin and transferrin may be attributable, at least in part, to a failure to exclude extraneous iron. The study by Bannister *et al.* (9), however, warrants additional comment. Since their experiments were performed using 1 mM DTPA which would completely inhibit the activity of extraneous iron (23), their observation that lactoferrin enhances $OH^{\cdot}$ formation must have some other explanation. The first possibility is that there are intrinsic differences in the reactivity of iron bound to pig neutrophil lactoferrin and the human milk lactoferrin used in this study. We consider this possibility unlikely because the properties of lactoferrins isolated from different sources are extremely similar (29). Perhaps a better explanation is that neutrophil lactoferrin preparations may be contaminated with small amounts of a highly active protein impurity, possibly myeloperoxidase (9, 11). Ambruso and Johnston (11) reported their neutrophil lactoferrin preparation to be 5000 times more active than Fe^{2+}-EDTA. In contrast, the results reported by Bannister *et al.* (9) indicate that lactoferrin has an activity approximately half that of Fe^{2+}-EDTA. The reasons for this discordance are not clear, although it seems likely that methodological inconsistencies contributed to the variation in activity.

Because of its extremely high reactivity the hydroxyl radical is usually detected by observing the formation of a reaction product of hydroxyl radical with a second chemical species. Methods that have been used include monitoring (i) the loss of the tryptophan absorbance at 278 nm (1), (ii) the formation of ethylene from thiols such as methional (13) or α-keto-γ-methiolbutyric acid (9, 11), (iii) the formation of diphenols from phenols such as salicylic acid (15, 23), (iv) the formation of thiobarbituric acid reactive products on reaction with deoxyribose (16), and (v) the formation of a spin-trapped adduct by EPR spectroscopy using a nitrone such as DMPO (18, 19). Each of these methods has its own disadvantages. For example the reaction of $OH^{\cdot}$ with tryptophan produces only small absorbance changes (23), while the formation of ethylene from

methional can occur by reaction with radicals other than $OH^{\cdot}$ (30). Even the reaction with DMPO, which has the considerable advantage that the ESR spectrum of the DMPO-$OH^{\cdot}$ adduct is readily distinguishable from other radical adducts, must be used with proper controls in order to prevent erroneous conclusions (18, 19). However, because of the high reactivity of $OH^{\cdot}$, *all* the above methods detect only a fraction of the $OH^{\cdot}$ formed; the rest reacts both with itself and most organic molecules in a number of parallel and poorly understood pathways. Not all of these pathways are first-order, so that none of the techniques give a quantitative measure of the rate of $OH^{\cdot}$ production. In biological systems, the absence of a reaction product between $OH^{\cdot}$ and the detecting molecule must be interpreted with care since most biological fluids contain many species which may react with $OH^{\cdot}$ (16).

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