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Oxygen radical-induced erythrocyte hemolysis by neutrophils. Critical role of iron and lactoferrin.

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Research Article

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Oxygen Radical-induced Erythrocyte Hemolysis by Neutrophils

Critical Role of Iron and Lactoferrin

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Abstract

Human neutrophils (PMN), when stimulated with such chemotaxins as phorbol myristate acetate (PMA), destroy erythrocytes and other targets. Cytotoxicity depends on PMN-generated reactive oxygen metabolites, yet the exact toxic specie and its mode of production is a matter of some dispute. Using ^{51}Cr-labeled erythrocytes as targets, we compared various reactive-O_2 generating systems for their abilities to lyse erythrocytes as well as to oxidize hemoglobin to methemoglobin.

PMA-activated PMNs or xanthine oxidase plus acetaldehyde were added to target erythrocytes in amounts that provided similar levels of superoxide. PMNs lysed 68.3±2.9% (SEM) of targets, whereas the xanthine oxidase system was virtually impotent (2.3±0.8%). In contrast, methemoglobin formation by xanthine oxidase plus acetaldehyde was significantly greater than that caused by stimulated PMNs ($P < 0.001$). A similar dichotomy was noted with added reagent H_2O_2 or the H_2O_2-generating system, glucose plus glucose oxidase; neither of these caused ^{51}Cr release, but induced 10–70% methemoglobin formation. Thus, although O_2^- and H_2O_2 can cross the erythrocyte membrane and rapidly oxidize hemoglobin, they do so evidently without damaging the cell membrane.

That a granule constituent of PMNs is required to promote target cell lysis was suggested by the fact that agranular PMN cytoplasts (neutroplasts), although added to generate equal amounts of O_2^- as intact PMNs, were significantly less lytic to target erythrocytes ($P < 0.01$). Iron was shown to be directly involved in lytic efficiency by supplementation studies with 2 μM iron citrate; such supplementation increased PMN cytotoxicity by ~30%, but had much less effect on erythrocyte lysis by neutroplasts (~3% increase), and no effect on lysis in the enzymatic oxygen radical–generating systems. These results suggest a critical role for an iron-liganding moiety that is abundantly present in PMN, marginally so in neutroplasts, and not at all in purified enzymatic systems—a moiety that we presume catalyzes very toxic O_2 specie generation in the vicinity of juxtaposed erythrocyte targets. The obvious candidate is lactoferrin (LF), and indeed, antilactoferrin IgG, but not nonspecific IgG, reduced PMN cytotoxicity by >85%. Re-adding 10^{-8} M pure LF to neutroplasts increased their ability to promote hemolysis by 48.4±0.9%—to a level near that of intact PMNs. We conclude that O_2^- and H_2O_2 are not sufficient to mediate target cell lysis, but require iron bound to LF, which, in turn, probably generates and focuses toxic O_2 radicals, such as $^{\cdot}OH$, to target membrane sites.

Introduction

The mechanism by which polymorphonuclear leukocytes (PMNs)[1] and other phagocytes destroy target cells has been the subject of intense research in recent years. A welter of confusing and frequently conflicting data has accumulated, although it is generally agreed that the ability of PMNs to sequentially reduce oxygen to superoxide (O_2^-) (1) and hydrogen peroxide (2) initiates target cell lysis. Moreover, a role seems secure for transition metal catalysts, such as iron; they are thought to foster, through the Haber-Weiss reaction, production of even more deleterious oxygen species, such as the hydroxyl radical ($^{\cdot}OH$) (3–5).

In several recent reports (6–13), the intact, radiolabeled erythrocyte has been used as a convenient target cell for PMN-mediated damage and various toxic oxygen species-generating systems have been analyzed. Several mechanisms of erythrocyte destruction have been proposed, and we find it difficult to find a unifying thread. To illustrate (but not to rigorously review conflicting data): xanthine oxidase plus acetaldehyde—a superoxide generating system—has been reported to lyse erythrocytes, which is inhibited by scavengers of $^{\cdot}OH$ (6); moreover, in another study using the same enzymatic system (but erythrocyte ghosts as targets), damage could be abrogated by superoxide dismutase (SOD) (7). Others have used similar O_2^-- and H_2O_2-generating systems and found that hemolysis only occurs in the presence of the PMN lysosomal constituent, myeloperoxidase (MPO) (8). This suggests that the H_2O_2/halide/MPO microbicidal system that generates hypohalous acid (9) and chloramines (10) might be critical to PMN-mediated erythrocyte lysis. This conclusion is also supported by studies with granulocytes: when stimulated with phorbol myristate acetate (PMA), they lyse murine erythrocytes evidently through generation of H_2O_2, but not O_2^- or $^{\cdot}OH$ (11). Recently, Weiss (12) proposed that PMN-derived O_2^- is toxic for erythrocytes by its interaction with hemoglobin, and showed that only SOD was consistently protective. In a later study (13), he showed that O_2^- and H_2O_2 from PMA-stimulated PMNs cross the erythrocyte membrane and rapidly produce methemoglobin, yet cause little or no damage to the cell during their transit.

We assume that much of the conflicting data in these reports reflects differences in the experimental conditions, such as dif-

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1. *Abbreviations used in this paper:* GHBSS, HBSS containing 1% gelatin; LF, lactoferrin; MPO, myeloperoxidase; PMA, phorbol myristate acetate; SOD, superoxide dismutase.

ferent incubation times, different ratios and proximity of target and effector cells, and variations in O_2^- and H_2O_2 concentrations. Moreover, since in our present studies we demonstrated a critical role for lysosomal lactoferrin in PMN-mediated hemolysis, we think that varying degranulation of PMNs in previous studies may underlie some of the differences in reported results.

In the present study, we investigated the capacity of PMNs, when stimulated with PMA, to lyse erythrocytes and produce methemoglobin. Results were compared with effects on erythrocytes of enzymically generated O_2^- and H_2O_2. In our hands, significant hemolysis is only induced by stimulated PMNs and not by the enzymatic systems, which do, however, cause hemoglobin oxidation to methemoglobin. This suggests that a fixed constituent of PMNs might be critical to their hemolytic function. Using a novel technique to prepare lysosome-depleted and enucleate cytoplasts from parent PMNs (14), we demonstrate that an iron-liganding moiety of PMN specific granules, namely lactoferrin (LF) (15), is that critical constituent. We believe LF is doubly reactive: it provides catalytic iron for production of very toxic oxygen species, such as $^{\cdot}OH$, and, because of its highly cationic and membrane-adsorbing properties, it also focuses toxic O_2 species directly onto target surfaces. These studies have been presented in preliminary form elsewhere (16).

Methods

Reagents. Phorbol myristate acetate, xanthine oxidase (grade I, buttermilk), glucose oxidase (type V, aspergillus niger), superoxide dismutase (type I, bovine blood, 2,750 U/mg protein), catalase (bovine liver, 20,000 U/mg protein, thymol free), LF (human milk, ~98% pure), thiourea, H_2O_2 (30%), ferric citrate, and *N*-formyl-methionine-leucine-phenylalanine were purchased from Sigma Chemical Co. (St. Louis, MO), mannitol (25%) was from Abbott Laboratories (Irving, TX), deferoxamine mesylate from Ciba Pharmaceutical Co. (Summit, NJ), urea from Mallinckrodt Inc. (St. Louis, MO), and acetaldehyde (99%) from Aldrich Chemical Co. (Milwaukee, WI). Anti-human LF (IgG fraction rabbit) and rabbit IgG were obtained from Cappel Laboratories (Cochranville, PA). In some experiments, deferoxamine was saturated with $FeCl_3$, as described elsewhere (17), and SOD and catalase were heat-inactivated by autoclaving stock solutions at 120°C for 30 min, followed by sonification to separate aggregates.

Isolation and radiolabeling of erythrocytes. Human venous blood was obtained from normal volunteers after informed consent. Heparinized (5 U preservative-free heparin/ml blood) erythrocytes were centrifuged (4 min, 500 *g*) three times with isotonic NaCl with removal of plasma and buffy coat, then resuspended in phosphate-buffered Hanks' balanced salt solution (HBSS) (Gibco Laboratories, Grand Island, NY) which contained 0.1% gelatin (GHBSS) at a concentration of 10^8 cells/ml and incubated for 30 min at 37°C with 20 μCi of $Na_2\ ^{51}CrO_4$/ml cells (Amersham Corp., Arlington Heights, IL) for radioactive labeling. After four washes (1', 1,000 *g*), the erythrocytes were suspended in GHBSS at 2×10^7/ml.

Preparation of PMNs. Human blood (40 ml) was drawn into a plastic syringe containing 20 ml hydroxy-ethyl-starch (Hespan, American Hospital Supply Corp., Irvine, CA) and 200 U preservative-free heparin. The mixture was allowed to sediment at room temperature, the supernatant collected and centrifuged at 400 *g* for 5 min at 4°C, and the pellet resuspended in 0.2 ml ice-cold HBSS. Residual erythrocytes were lysed in 15 ml of ice-cold distilled water and after 25 s, isotonicity was reconstituted by addition of 5 ml of 3.6% NaCl. This suspension was centrifuged in the cold at 400 *g* for 5 min, the pellet resuspended in 5 ml of HBSS, carefully layered on top of Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) made up to a density of 1.075, and then centrifuged at 27,750 *g* for 30 min at 4°C. The resulting PMNs (>95%) were washed once and suspended at 10^7/ml in ice-cold GHBSS. Viability was assessed by trypan blue exclusion and exceeded 95%.

Preparation of enucleated cytoplasts of PMNs. Enucleated cytoplasts of PMNs (neutroplasts) were prepared according to a method of Roos et al. (14). In brief, PMNs prepared as above were suspended in a 12.5% weight per volume Ficoll 70 solution (Pharmacia Fine Chemicals) containing cytochalasin B (Sigma Chemical Co.) at 5 μg/ml. This suspension was layered on a discontinuous gradient of 16% Ficoll and 25% Ficoll and centrifuged at 100,000 *g* for 30 min at 37°C. The band of neutroplasts was harvested from the interface of the 12.5%/16% Ficoll layers and washed three times in HBSS. The neutroplasts were counted by hemocytometry, as well as with a Coulter counter, and suspended at a concentration of 2×10^7/ml in ice-cold GHBSS. The neutroplasts were ascertained to be virtually granule-free by electron microscopic examination (not shown), as well as by measurement of released β-glucuronidase, elastase, MPO, and LF (Table I) by methods described previously (18–21).

Superoxide production. Production of superoxide by PMA-activated PMN and neutroplasts and by xanthine oxidase plus acetaldehyde was measured as the reduction of ferricytochrome *c* (horseheart type I [Sigma Chemical Co.]) using a modification of the method described by Johnston et al. (22). The standard reaction mixture contained 75 μM ferricytochrome *c* with or without 100 μg SOD and enough GHBSS to obtain a final volume of 1 ml. PMNs or neutroplasts were added at various concentrations and were stimulated with PMA (10 μg/ml) in a 37°C shaking incubator. After 15 min, the mixtures were promptly centrifuged at 4°C and 175 *g* for 10 min and the absorbance of the supernatants was determined at 550 nm. The rate of superoxide dismutase inhibitable reduction of ferricytochrome *c* by xanthine oxidase (330 mU/ml) plus acetaldehyde (5 mM) was measured at 550 nm by continuous recording using a Beckman spectrophotometer (model 25) and recorder. At a temperature of 37°C maximum reduction was observed after ~9 min. Nanomoles of reduced cytochrome *c* were calculated from the increase in the absorbance using the extinction coefficient $E_{550\ nm} = 2.11 \times 10^4\ M^{-1}\ cm^{-1}$.

^{51}Cr release assay. Hemolysis was measured using a ^{51}Cr release assay as previously described by Weiss (12) with slight modification. Briefly, 3.3×10^5 or 1.7×10^6 PMN/ml or 6.7×10^6 neutroplasts/ml were incubated with radioactively-labeled erythrocytes (3.3×10^6/ml) in GHBSS (pH 7.4). At the latter two concentrations PMNs and neutroplasts generated virtually equal amounts of superoxide on stimulation with PMA as measured in the ferricytochrome *c* reduction assay. Experiments were performed in duplicate in Microtest plates (Corning Glass Works, Corning, NY), and the final volume of each reaction mixture was 0.3 ml. PMA at a concentration of 10 ng/ml was added to activate PMN or neutroplasts, and cell contact was initiated by centrifugation at 60 *g* for 3 min at room temperature; increasing the *g* force fourfold so as to ensure even closer effector/target cell contact had no amplifying effects on target cell lysis. The samples were then placed in a humidified atmosphere of 95% air/5% CO_2 at 37°C for 30 or 60 min. After the incubation period, cells were again pelleted (175 *g* for 3 min) and 150-μl

Table I. Neutroplasts Are Devoid of Releasable Lysosomal Constituents

Lysosomal constituents per milligram of protein	PMNs	Neutroplasts
β-Glucuronidase (μg)	0.4±0.07	0.004
Elastase (*nmol/min*)	81±5	1.2±0.1
MPO (*mU*)	0.26±0.06	0.002
LF (μg)	12±2	0.1

PMNs (2.5×10^6) and neutroplasts (1×10^7) were stimulated with *N*-formyl-methionine-leucine-phenylaline (10^{-7} M) and cytochalasin B (50 μg/ml) for 15 min at 37°C. The cells were centrifuged and the released lysosomal contents were measured in the supernatants as described in Methods. Results are expressed as means±SE.

samples of supernatant were removed for determination (Beckman Gamma 5500 [Beckman Instruments, Irvine, CA]) of the percentage of ^{51}Cr release using the formula $(A-B/C-B) \times 100$. *A* represents counts per minute in the supernatant of samples containing erythrocyte alone (*B* = spontaneous release; always <1.5%) and *C* represents the total counts per minute of erythrocytes added to each sample. In other experiments erythrocytes were incubated with xanthine oxidase (330 mU/ml) plus acetaldehyde (5 mM)—concentrations generating virtually identical levels of superoxide as that of PMA-activated PMN or neutroplasts used in these studies. As a source of H_2O_2, glucose oxidase (1,100 mU/ml) plus glucose (5 mM, present in GHBSS), or reagent H_2O_2 (0.9 mM) was used.

Other techniques. Methemoglobin and total hemoglobin were determined by a slightly modified Evelyn and Malloy method (23). The immunofluorescence technique to detect PMN and neutroplast-associated LF was that of Masson et al. (24) and used a fluorescein-labeled rabbit antilactoferrin obtained from Cappel Laboratories.

Statistical analysis. The standard error was taken as an estimate of variance. Statistical differences were determined by the *t* test.

Results

Dichotomous production of methemoglobin and erythrocyte lysis by enzymically generated oxygen species and by PMNs. As shown previously by others (12, 25), PMA-stimulated PMNs lyse ^{51}Cr-labeled erythrocytes and do so more efficiently with increasing effector/target cell ratios (Fig. 1, left; solid lines). If the incubation period was increased to 3 h, further hemolysis to a maximum of ~90% was noted (data not shown). In contrast, a superoxide-generating enzymatic system, xanthine oxidase plus acetaldehyde—although added so as to produce approximately the same amount of superoxide as PMNs in these studies (42.2±3.1 nmol cytochrome *c* reduced/1.7 × 10^6 PMN/ml/15 min vs. 39.8±3.4 nmol/9 min by xanthine oxidase/acetaldehyde; $n = 5$)—causes virtually no hemolysis.[2] Likewise, another enzymatic system that generates H_2O_2, glucose oxidase plus glucose, provokes no hemolysis either—in this case, exhibited levels of H_2O_2 were two- to tenfold of those produced by PMNs in these same experiments (Fig. 1, left). In ancillary studies, reagent H_2O_2 was added to erythrocytes in even higher concentrations—up to 0.9 mM—and only 1.7±0.9% hemolysis was fostered. A reverse phenomenon is noted regarding oxidation of hemoglobin in the target erythrocytes: to wit, stimulated PMNs provoke little methemoglobin formation, whereas enzymically generated superoxide of H_2O_2 oxidizes large quantities of target cell hemoglobin (Fig. 1, right). This dichotomy suggests either that toxic O_2 species other than O_2^- or H_2O_2 are required for hemolysis or that their focusing to membrane, rather than cytosolic, sites are critical for lysis—or both.

We examined the possibility that a granule constituent of PMNs might be involved in producing, or focusing, lytic O_2

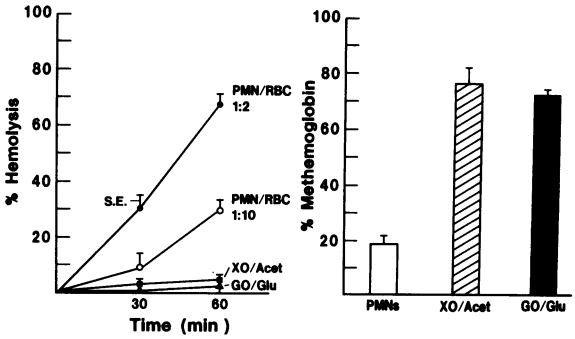

Figure 1. Dichotomous methemoglobin (*right*) production and erythrocyte (RBC) lysis (*left*) fostered by PMN and by enzymically generated oxygen species. *Left:* percent ^{51}Cr released by 3.3 × 10^6 erythrocytes/ml after 30- or 60-min incubation at 37°C; PMA (10 ng/ml)-activated PMN were added at effector/target cell ratios of 1:2 (●) and 1:10 (○); alternatively, xanthine oxidase (XO) (330 mU/ml) + 5 mM acetaldehyde (Acet) (■) or glucose oxidase (GO) (1,100 mU/ml) + 5 mM glucose (Glu) (▲) were exhibited. *Right:* percent methemoglobin formation in 3.3 × 10^7 erythrocytes/ml by PMA-activated PMN (1.7 × 10^6/ml), XO + acetaldehyde (hatched bar) or GO + glucose (solid bar), at the concentrations and incubation conditions used on the *left*. Values are the mean±SE of assays in at least four separate experiments performed in duplicate.

species by using granule-poor neutroplasts. Despite the fact that we used in these studies neutroplasts in numbers that generate the same amount of assayable superoxide after stimulation with PMA as do native PMNs (Fig. 2, left), these neutroplasts are significantly ($P < 0.001$) less efficient hemolysins (Fig. 2, right).

Evidence that toxic oxygen species are involved in PMN or neutroplast-engendered hemolysis: inhibitor studies. Although our neutroplast studies demonstrate that PMN-granule constituents are evidently required for maximum hemolytic efficiency, the inhibitor studies shown in Table II also implicate toxic O_2 species as necessary effectors of lysis. Thus, both superoxide dismutase and catalase (but not their heat-inactivated congeners) markedly inhibit hemolysis induced either by PMA-stimulated PMNs or their neutroplasts. Since O_2^- or H_2O_2 seem unlikely hemolysins themselves (Fig. 1), two scavengers of hydroxyl radical, mannitol and thiourea, were studied; both significantly inhibit PMN and neutroplast-mediated hemolysis—the latter particularly so (97.1±0.9% inhibition of PMN-mediated hemolysis); urea, a closely related congener of thiourea, yet unable to scavenge $^{\cdot}OH$, is without inhibitory effect. Moreover, iron, a potent

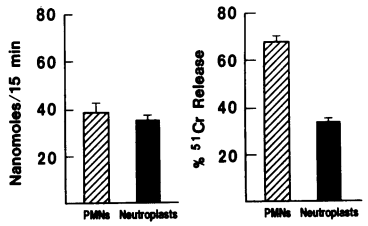

Figure 2. Effect of PMNs and neutroplasts, generating equal amounts of superoxide (*left*), on erythrocyte (RBC) lysis (*right*). *Left:* nanomoles of superoxide generated after 15 min at 37°C by PMA (10 ng/ml)-activated PMN (1.7 × 10^6/ml) or neutroplasts (6.7 × 10^6/ml). *Right:* percent ^{51}Cr release by 3.3 × 10^6 erythrocytes/ml after 60-min incubation at 37°C with PMA-activated PMN or neutroplasts at the concentrations used on the *left;* no significant difference in superoxide production by PMNs and neutroplasts was evident over this longer incubation period. Values are the mean±SE of assays in at least five separate experiments performed in duplicate.

2. Extended incubation with the xanthine oxidase system for as long as 4 h also did not promote significant hemolysis, and the exclusion of gelatin, a potential oxidant scavenger, in the incubation media only minimally increased hemolysis at 4 h to 5.4±1.0%; others (6) using different xanthine oxidase/erythrocyte ratios, report more significant hemolysis with xanthine oxidase, but only after 4 h. The quantity of superoxide estimated to be released in the enzymic and neutrophil (1.7 × 10^6 cells/ml) systems were deduced from measurements made at 15 min. In fact, the total amount of cytochrome *c* reducing-equivalents were approximately fivefold higher for the enzymic system compared to the PMN system when assayed at 1 h. To fully appreciate this difference, addition of catalase to the assay system was required in part to prevent the reoxidation of cytochrome *c* by the H_2O_2 produced during this more prolonged period.

*Table II. Effect of Inhibitors of Toxic Oxygen Species on Hemolysis of Erythrocytes by Stimulated PMN and Neutroplasts**

Inhibitor	% Inhibition of erythrocyte lysis	
	PMN	Neutroplast
SOD (10 μg/ml)	93.4±1.0 (8)	98.4±0.4 (3)
SOD heated	12.7±3.2 (3)	ND
Catalase (500 μg/ml)	66.0±4.7 (9)	89.4±1.9 (3)
Catalase heated	6.6±1.0 (3)	ND
Thiourea (5 mM)‡	97.1±0.9 (7)	87.8±1.1 (4)
Urea (5 mM)	0±11.1 (3)	ND
Mannitol (50 mM)§	44.5±5.0 (9)	66.9±1.8 (4)
Deferoxamine (1 mM)	96.3±0.7 (14)	97.6±0.3 (6)
Deferoxamine iron-saturated (1 mM)	18.3±4.4 (4)	ND
Na phytate (1 mM)	95.4±3.6 (4)	ND

* ^{51}Cr-labeled erythrocytes (3.3×10^6/ml) were incubated for 60 min at 37°C with PMA (10 ng/ml)-activated PMN (1.7×10^6/ml) or neutroplasts (6.7×10^6/ml). The absolute ^{51}Cr release without depicted inhibitors is shown in Fig. 2. Values are mean±SE with number of experiments in parentheses. ND, not done.
‡ Thiourea in the doses used had no inhibitory effect on superoxide generation by PMA-stimulated PMNs.
§ Mannitol inhibition of erythrocyte lysis occurred equally in isotonic medium or in hypertonic (330 mosmol) medium.

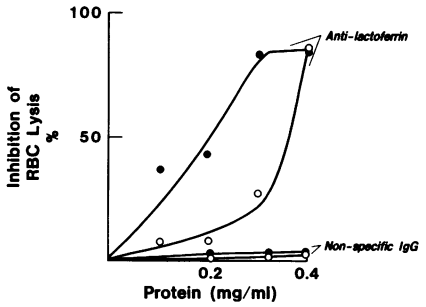

Figure 4. Inhibition of PMN and neutroplast-induced erythrocyte (RBC) lysis by antibody to LF. Percent inhibition of hemolysis of ^{51}Cr-labeled erythrocyte (3.3×10^6/ml) incubated with PMA (10 ng/ml)–activated PMN (1.7×10^6/ml) (○) or neutroplasts (6.7×10^6/ml) (●) in the presence of antibody to LF or nonspecific IgG. Values are the mean±SE of assays in at least four separate experiments performed in duplicate.

catalyst of ·OH production via the Haber-Weiss reaction (3, 4), is an important component in PMN and neutroplast hemolysis; its chelation by deferoxamine prevents hemolysis, while nonchelating, "spent" (iron-saturated) deferoxamine manifests only a slight inhibitory effect on PMN hemolysis. Deferoxamine blocks generation of ·OH (26), but has no effect on its substrates, O_2^- or H_2O_2; thus, it is not surprising that deferoxamine does not inhibit PMN-mediated methemoglobin formation (19.4±1.2 vs. 18.4±1.7%).

Evidence that LF is a critical constituent in PMN-mediated hemolysis. That iron is an important catalyst in PMN-mediated hemolysis was further validated with iron citrate supplementation studies. Addition of as little as 0.2 μM Fe^{+++} as citrate to PMA-stimulated intact PMNs significantly ($P < 0.05$) increases their hemolytic capacity (data not shown). Supplementation with 2 μM Fe citrate (but not with sodium citrate) increases hemolysis by 35.7±8.7% ($P < 0.02$). Addition of the same amount of Fe^{+++} (2 μM) to neutroplasts detectably, but only minimally (2.5±1.3%; $P < 0.10$), enhances their hemolytic capacity. In contrast, iron citrate does not discernibly awaken a capability of the enzymatic systems, xanthine oxidase/acetaldehyde or glucose oxidase/glucose, to provoke hemolysis (data not shown).

These results suggest a critical role in hemolysis for an iron-liganding moiety that is abundantly present in intact PMNs, marginally so in granule-poor neutroplasts, and not at all in purified enzymatic systems. The specific-granule constituent, LF, is an obvious candidate. Indeed, although we were barely able to detect LF in neutroplasts by a relatively crude ELISA assay (21) (Table I), immunofluorescent studies using a fluorescent rabbit anti-LF antibody demonstrated it in small amounts. That is, native PMNs brightly stain with the antibody (Fig. 3, left), while neutroplasts manifest a "dusting" of LF on their surfaces (Fig. 3, right). This tiny amount is evidently strategically placed to foster hemolysis, since addition of anti-LF immunoglobulin to PMA-stimulated neutroplasts inhibits their ability to lyse target erythrocytes in a dose-dependent fashion (Fig. 4, solid circles). As might be expected, the antibody, but in greater amounts, is also able to prevent intact PMN-mediated cytotoxicity (Fig. 4, open circles); nonspecific rabbit IgG is ineffectual.

To further validate LF's importance in lytic efficiency, we

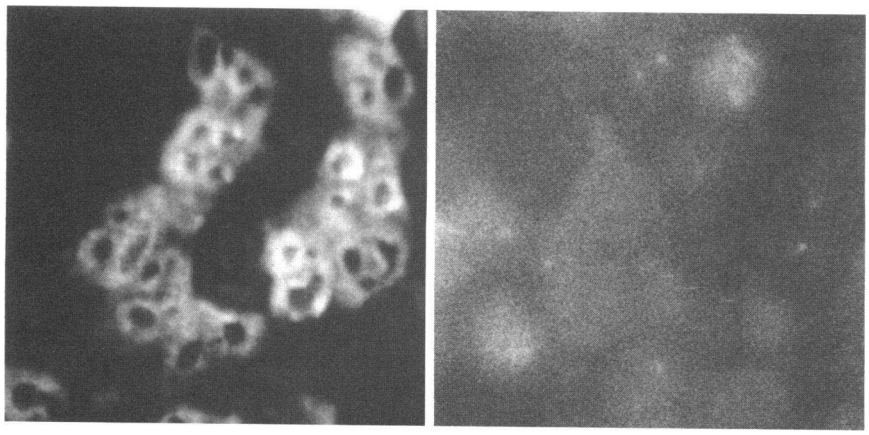

Figure 3. In *a*, neutrophils brightly stain with a fluorescein-tagged rabbit anti-LF (× 400). In *b*, neutroplasts (× 1,000) only faintly stain for LF.

added back purified LF to our various hemolytic assay systems. As little as 10^{-10} M purified LF significantly increases ($P < 0.05$) neutroplast's cytotoxic potential (data not shown). At higher concentration (10^{-8} M), LF significantly "re-arms" neutroplasts ($P < 0.01$) (Fig. 5, right) rendering them more hemolytic (48.4±0.9% increase; $n = 6$); co-addition of anti-LF antibody with LF prevents this reassembled hemolytic activity (data not shown). Perhaps not surprisingly, exogenous LF has no significant effect on hemolytic potential of the already-replete intact PMN (Fig. 5, left bars). Finally, exogenously added LF awakens cytolytic potential of the usually indolent xanthine oxidase/acetaldehyde mixture; hemolysis significantly increases when LF is added to this enzymic, superoxide-generating system (Table III).

*Table III. Lactoferrin Increases Erythrocyte Hemolysis by Acetaldehyde/Xanthine Oxidase**

Experiment	% ^{51}Cr Release	
	Acet/XO	Acet/XO + LF
1	1.0±0.3	10.3±4.4
2	1.1±0.5	12.7±0.6
3	4.5±0.8	20.1±1.9
4	0.6±0.2	5.3±2.0

* Erythrocytes (3.3×10^6/ml) in HBSS were incubated with xanthine oxidase (XO) (330 mU/ml) plus acetaldehyde (Acet) (5 mM) with or without LF (LF) (100 nM) for 4 h at 37°C. Values represent mean±SE of quadruplicate incubations.

Discussion

Our studies demonstrate that stimulated PMNs lyse erythrocytes (and by extrapolation, perhaps other cells as well) not solely by producing the toxic oxygen species, O_2^- and H_2O_2; the iron-liganding lysosomal granule constituent, LF, is also critically important to lytic function. Thus, enzymically-generated O_2^- or H_2O_2 can be added in amounts equal to, or many-fold higher than, those produced by PMNs, yet cause virtually no target cell lysis. Nonetheless, their oxidant potential cannot be doubted from the abundant quantities of methemoglobin they form (Fig. 1). Evidently both O_2^- and H_2O_2 can cross erythrocyte membranes without significantly damaging them.

A role for one, or another, PMN-granule constituent in redirecting oxidant potential to the erythrocyte membrane was uncovered in the present studies by the use of granule-depleted neutroplasts. As shown by Roos et al. (14), who originally described their preparation, and by Korchak et al. (27), neutroplasts are efficient toxic O_2 species generators; yet their hemolytic potency is significantly less than PMNs (Fig. 2). An iron-containing moiety would seem to provide neutroplast's sparse, but real, lytic function, since deferoxamine completely abolishes their hemolytic potential (Table II)—as it does with intact PMNs as well. The simplest explanation: LF iron fosters from O_2^- and H_2O_2 the generation of the highly toxic O_2 specie, $^{\cdot}OH$, which, in turn, might be particularly membrane reactive. That LF can promote $^{\cdot}OH$ generation is controversial (28, 29), and even if it does, this simple construct may not be fully explicative of our data. To be sure, others have shown that addition of iron-saturated LF to stimulated PMNs enhances ethylene generation—a presumed assay of $^{\cdot}OH$ (30)—and our present studies demonstrate that PMN or neutroplast-engendered hemolysis is largely inhibited by the $^{\cdot}OH$ scavengers, thiourea and mannitol. However, if iron-catalyzed $^{\cdot}OH$ generation is the only critical factor in hemolysis, addition of iron itself to enzymically generated O_2^- or H_2O_2, or to both, should promote hemolysis; it does not (data not shown). Yet, if added to PMNs and less so to neutroplasts, iron does promote increased hemolysis. However, LF added to xanthine oxidase/acetaldehyde (Table III) enhances lysis, suggesting focusing of oxidants at the erythrocyte membrane. This suggests that $^{\cdot}OH$, or a similarly potent iron-catalyzed toxic O_2 specie, may be necessary, but is not sufficient, to cause target cell membrane damage.

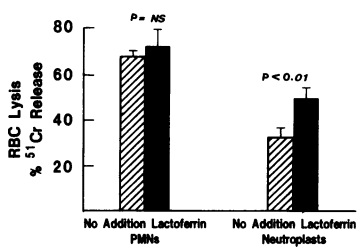

Figure 5. Effect of "re-arming" neutroplasts with LF on their erythrocyte (RBC) lytic activity. Percent ^{51}Cr release by 3.3×10^6 erythrocytes/ml after 60-min incubation at 37°C with PMA (10 ng/ml)–activated PMN (1.7×10^6/ml) (*left* bars) or neutroplasts (6.7×10^6/ml) (*right* bars) without (hatched bars) or in the presence (solid bars) of added LF (10^{-7} M). Values are the mean±SE of seven (PMN) and six (neutroplast) experiments in duplicate.

Our results indicate that LF is the special constituent that allows iron-catalyzed toxic O_2 species to efficiently work their damage. When isolated from PMNs, LF is only modestly iron-replete and has excess iron-binding capacity. That hemolytic efficiency of PMNs is increased by iron citrate supplementation suggests this excess capacity can be used to promote increased toxic O_2 specie formation. But, in addition to acting as an iron donor for O_2-specie catalysis, we speculate that LF does more: it seems particularly well-suited to focus its catalyzed products directly onto membranes of target cells. That is, LF is highly cationic (pI = 9.0), which suggests that it might be readily absorbable to negatively charged cellular membranes. Indeed, self-adsorbtion of LF onto the surfaces of stimulated PMNs has been shown by others (31), and with immunofluorescent techniques we detect dusting of neutroplast membranes with this granule constituent (Fig. 3). In ancillary studies (not shown), we found immunofluorescent LF adsorbed to erythrocyte membranes when LF was added to the target erythrocytes. This ease of adsorbability may underlie the ability of miniscule amounts (10^{-10}M) of added exogenous LF to rearm neutroplasts into efficient hemolysins (Fig. 5). Another way in which the cationic nature of LF might be involved in its enhancement of hemolysis is by promoting closer contact between target and effector membranes. Ancillary studies in which polylysine was added to PMA-stimulated granulocytes partially support this suggestion in that hemolysis was increased by ~30%. However, the significance of this is diluted: to wit, negatively charged polyglutamate does not inhibit hemolysis, whereas anti-LF antibody completely inhibits it, even though effector and target cells are pelleted together by centrifugation (Fig. 4). In addition, the role of tight effector-target adhesion mediated by LF is buttressed by the data in Table III, demonstrating only modest enhancement of hemolytic activity in the enzymatic system despite abundant oxidants produced.

Our tactic of using the opposing systems: (*a*) deferoxamine vs. iron supplementation; and (*b*) anti-LF antibody vs. LF rearming of neutroplasts, provides strong support for our con-

clusion that LF and its constituent iron is crucial to PMN-engendered cytotoxicity. Some caveats, however, need be acknowledged. First, we used very large concentrations of deferoxamine (1 mM) in these studies as well as in our previous studies of iron-driven autotoxicity of stored PMNs (17); this concentration of deferoxamine was greater than needed to sequester iron from LF, particularly in that its association constant for iron is several orders of magnitude greater than that of LF. Could this super-abundance allow deferoxamine to act in ways other than simply as an iron-chelator, perhaps, in fact, as a direct O_2-species scavenger? Such a construct is suggested in that iron-saturated deferoxamine, which presumably can no longer chelate iron, does modestly inhibit (18.3±4.4%) PMN-induced hemolysis as compared to the 96.3±0.7% inhibition wrought by iron-deficient deferoxamine (Table II). However, in ancillary studies performed to assess superoxide scavenging by deferoxamine, we could not find any such; whether measured in PMA-stimulated granulocytes or with the xanthine oxidase/acetaldehyde system, assayable superoxide production remained constant with or without added deferoxamine (1 mM), respectively (43.1±4.3 vs. 39.8±3.4 nmol $O_2^-/1.7 \times 10^6$ PMNs/15 min). In contrast, others have recently reported (32) some decrease in cytochrome *c* reduction during very brief exposure (3 min) to deferoxamine, but not its iron-saturated congener; we are unable to confirm these results in our longer incubations, which also differ in using acetaldehyde, instead of xanthine, as substrate for superoxide generation. To further buttress our conclusion that iron is critical to maximum PMN-provoked hemolysis, we also used another iron-chelator shown by Graf et al. (26) to prevent the Haber-Weiss Reaction—sodium phytate; as with deferoxamine, phytate (1 mM) markedly inhibits (by 95%) PMN-engendered hemolysis (Table II).

Second, we do not wish to denigrate the role of lysosomal enzymes other than LF in facilitating PMN-mediated cytotoxicity. Others have marshalled impressive evidence that MPO-dependent production of hypohalous acids (9) and of chloramines (10, 33, 34) is an important cellular-damaging strategem of PMNs (35, 36). Despite the fact that our neutroplasts contain no detectable MPO, and when rearmed with only LF become efficient hemolysins, we cannot exclude that a similar fine dusting of MPO might have been discerned on neutroplasts—particularly if we had used analogous immunofluorescent techniques to seek it, as we had done for LF. Moreoever, our rearming of neutroplasts with iron-saturated LF[3] significantly increased hemolysis, but not quite to levels wrought by intact PMNs; this allows the supposition that both LF and MPO systems might act additively in facilitating target cell dissolution. Indeed, in preliminary data reported elsewhere (37), we have demonstrated that rearming neutroplasts with MPO (and not LF) causes significant increases in erythrocyte, as well as endothelial cell, lysis; in the latter situation, increased lift-off of cells from substratum was also noted.

Finally, to buttress our conclusion that LF is critical, perhaps indispensable, for PMN cytotoxicity, we would like to perform cell-lysis studies with LF-deficient PMNs. Patients harboring such cells exist and, in fact, are infection-prone (31, 38), but are not available to us; moreoever, since only a fine "dusting" of PMN or target cell surfaces with LF is evidently sufficient for efficient target cell lysis to occur, such studies, when performed, might not be compelling.

We conclude that O_2^- and H_2O_2 are not sufficient to mediate target cell lysis but may require iron bound by LF. This specific granule constituent probably focuses particularly toxic oxygen species, such as $^{\cdot}OH$, onto target membrane sites.

3. The LF used in re-arming studies was of uncertain saturation after purification. However, it was used at levels of 10^{-10}–10^{-7} M in media that contained 0.5 μM Fe^{+++}, guaranteeing its full saturation with the iron ligand.

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