Studies on the Molecular Mechanisms of Human Fc Receptor-mediated Phagocytosis

Amplification of Ingestion Is Dependent on the Generation of Reactive Oxygen Metabolites and Is Deficient in Polymorphonuclear Leukocytes from Patients with Chronic Granulomatous Disease

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Abstract

Human PMN and monocytes both possess a mechanism for amplifying Fc receptor-mediated phagocytic function, which is dependent on activation of the respiratory burst. The pathway for augmentation of phagocytosis requires superoxide anion, hydrogen peroxide, and lactoferrin and is independent of the hydrogen peroxide-MPO-halide system. In neither cell type is this mechanism induced upon exposure to the opsonized target. PMN require an additional signal for stimulation of the respiratory burst; this is not true of monocytes. On the other hand, monocytes require an exogenous source of lactoferrin in order to activate this pathway for enhanced ingestion. The dependence of this pathway for both PMN and monocytes on superoxide anion, hydrogen peroxide, and cell-bound lactoferrin is consistent with a role for locally generated reactive oxygen metabolites, possibly hydroxyl radicals, in phagocytosis amplification. Patients with chronic granulomatous disease, who are genetically deficient in the ability to activate the respiratory burst, are unable to amplify Fc receptor-mediated phagocytosis. Thus, these patients may have a previously unrecognized defect in the recruitment of phagocytic function at inflammatory sites.

Introduction

Studies undertaken to elucidate the molecular mechanisms by which Fc receptor-mediated phagocytic signals are transduced have shown that human monocytes and neutrophils (PMN) display functionally distinct pathways for ingestion. PMN are less able than monocytes to bind, ingest, and/or lyse immune targets opsonized with equivalent concentrations of IgG (1–3). In addition, phagocytosis mediated by these two cell types may be regulated by different molecular mechanisms. First, PMN but not monocyte Fc-mediated ingestion is markedly augmented by stimulation with tumor-promoting phorbol esters, the polyene antibiotic amphotericin B (AmB),[1] and a low molecular weight, heat-labile cytokine designated YM-10E (4, 5). Second, monocyte Fc-mediated ingestion is inhibited by pertussis toxin treatment (6) while PMN baseline ingestion is unaffected by this toxin (4). These differences in Fc receptor phagocytic function, together with the different structures and avidities for IgG of the Fc receptors expressed on these two cell types (7, 8) suggest that these Fc receptors subserve different functions.

To elucidate the pathway through which phorbol esters, amphotericin B, and YM-10E stimulate PMN Fc-mediated ingestion, we investigated whether there might be a common mechanism through which these diverse reagents act and whether there was any significance of this mechanism to regulation of monocyte Fc-mediated ingestion. Because AmB stimulates Fc-mediated ingestion and because it is known to exert some of its biological and toxic effects by the generation of reactive oxygen metabolites (9), we have investigated activation of the respiratory burst as a possible mechanism for regulation of phagocytic function. Superoxide and hydrogen peroxide production are known to accompany phagocytosis (10). In fact, products of the respiratory burst have been proposed to limit PMN ingestion of opsonized target particles via oxidative modification of phagocytic receptors by the H_2O_2-myeloperoxidase (MPO)-halide system (11, 12). We report here that both PMN and monocytes possess a mechanism for augmentation of phagocytosis that requires activation of the respiratory burst NADPH oxidase. This pathway for amplification of phagocytosis does not utilize the H_2O_2-MPO-halide system but does require superoxide anion, hydrogen peroxide, and the iron-binding protein lactoferrin. Both PMN and monocytes are able to increase their ingestion of opsonized targets significantly when these three molecules are present. However, in neither cell type is this pathway for enhanced phagocytosis induced simply upon exposure to an opsonized particle. PMN require an additional stimulatory signal for sufficient activation of the respiratory burst, while monocytes require an exogenous source of lactoferrin. These additional requirements potentially allow this pathway to be carefully regulated in vivo by other cells and secreted molecules at inflammatory sites. The

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1. *Abbreviations used in this paper:* AmB, amphotericin B; CGD, chronic granulomatous disease; EIgG, sheep erythrocyte opsonized with rabbit IgG anti-E; EC3b, sheep erythrocyte opsonized with C3b; MPO, myeloperoxidase; PDBu, phorbol dibutyrate; PI, phagocytic index, No. of EIgG ingested/100 PMN; RBA, RPMI 1640 containing 2 mM glutamine, 1% BSA, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin; YM-10E, the culture supernatant effluent from a YM-10 Centricon unit.

molecular mechanism of ingestion induced by activation of this pathway is distinct from the mechanism for baseline (unstimulated) phagocytosis, which is inhibited by the reactive oxygen metabolites of the H_2O_2-MPO-halide system. Moreover, because this augmented phagocytosis requires activation of the NADPH oxidase, PMN from patients with chronic granulomatous disease (CGD) have a previously unrecognized defect in this mechanism for enhancing ingestion that may contribute to their abnormal host defense against bacterial infections.

Methods

Special reagents. The following reagents were purchased from Sigma Chemical Co., St. Louis, MO: sodium benzoate, hypoxanthine, xanthine oxidase (grade III, 10 U/ml, pH 7.8), catalase (bovine liver, 52,000 U/mg), superoxide dismutase (bovine erythrocytes, 3,000 U/mg), cytochrome *c* (type VI, horse heart), lactoferrin (0.8 bound Fe/mol protein), iron-saturated lactoferrin, phenylalanine, FMLP, phorbol dibutyrate (PDBu), and AmB. PDBu (1 mg/ml), FMLP (22.9 mM), and AmB (2 mM) were prepared as stock solutions in DMSO and were diluted into aqueous medium immediately before use. Equivalent concentrations of vehicle were used to control for solvent effects. The hydroxyl radical scavengers, ethanol, DMSO, sodium benzoate, phenylalanine (13), and glucose as a control, were dissolved in Hanks' balanced salt solution containing 4.2 mM $NaHCO_3$, 10 mM Hepes, 1.2 mM Ca, and 1.2 mM Mg (HBSS) at a stock concentration of 1 M and the pH adjusted to 7.4. Rabbit $F(ab')^2$ anti-human lactoferrin and goat $F(ab')^2$ anti-murine IgM were purchased from Cooper Biomedical, Malvern, PA.

Isolation of human PMN and monocytes. Heparinized peripheral blood was obtained from eight normal volunteers and from two male patients with CGD. PMN were isolated by the method of Boyum (14) with modifications (5). CGD PMN were confirmed to be incapable of generating detectable quantities of superoxide anion. Human peripheral blood monocytes were isolated by Percoll density gradient centrifugation and cultured as described (15, 16). Monocytes were used both freshly isolated (day 0) and after culture for up to 7 d.

Preparation of phagocytic target particles. Sheep erythrocytes (E) were purchased from Whittaker M.A. Bioproducts, Walkersville, MD. Rabbit IgG anti-E was purchased from Diamedix, Miami, FL. EIgG were prepared as described (5) using either a 1/500 or a 1/2,500 dilution of antibody for assay of PMN or monocyte phagocytosis, respectively. E opsonized with C3b (EC3b) were prepared as described (17). Zymosan was purchased from Sigma Chemical Co., St. Louis, MO and was prepared by boiling in PBS for 10 min. After washing three times, it was suspended in veronal-buffered saline at 1.0×10^8 particles/ml.

Preparation of cytokine (YM-10E)-containing culture supernatant. Phagocytosis-stimulating culture supernatant was prepared as described (5) by a modification of the protocol of Griffin and Griffin (18). On the day of the experiment, both control and cytokine-containing supernatants were fractionated by ultrafiltration through YM-10 Centricon microconcentrators (Amicon Corp., Danvers, MA) as described (5). All phagocytosis experiments were performed using YM-10 effluents; therefore, cytokine-containing preparations are referred to as YM-10E.

Preparation of PMN supernatant for stimulation of monocyte phagocytosis. PMN (2.0×10^6/ml) in RPMI 1640 containing 2 mM glutamine, 1% BSA, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin (RBA) and 150 μl of EIgG (5.0×10^8/ml) were centrifuged at 50 *g* for 5 min before incubation at 37°C for 45 min. This mixture was centrifuged at 200 *g* for 10 min and the supernatant sterile filtered through 0.45-μm filters and kept on ice for immediate use. The lactoferrin concentration of the supernatants was 493±84 ng/ml, $n = 7$, as determined by the method of Metcalf et al. (19).

Phagocytosis assay. PMN phagocytosis was assessed by a fluid-phase assay as described (5). PMN were suspended in either RBA or HBSS containing 1% BSA at 2.0×10^6 cells/ml. Phagocytic indices in RBA or HBSS were identical. Equal volumes of PMN (50 μl) and either control buffer (RBA or HBSS) or various stimuli diluted in control buffer were incubated with 15 μl of either EC3b, EIgG (both at 5.0×10^8 E/ml), or zymosan (1.0×10^8) at 37°C in 5% CO_2. The final concentrations of the reagents that had been previously determined as optimal for EIgG phagocytosis stimulation were 50% YM-10E, 15 ng/ml PDBu, and 4 μM AmB; for stimulation of EC3b phagocytosis, the final optimal concentrations were 1 μM FMLP and 15 ng/ml of PDBu. For inhibition experiments, the following reagents were included in these reaction mixtures at the indicated concentrations: 25 μg SOD, 3,100 U catalase, 40 mM glucose, 40 mM benzoate, 6.2 μg $F(ab')^2$ antilactoferrin, or 6.2 μg control $F(ab')^2$ antibody, antimurine IgM. After incubation for 30 min, the noningested E were lysed with 0.83% ammonium chloride. Phagocytosis was assessed by light microscopy and was quantitated as a phagocytic index (PI = No. of E ingested by 100 phagocytes). Ingestion of zymosan was quantitated by phase microscopy. Monocyte phagocytosis was assessed by an adherent assay (5). Monocytes at 1.0×10^5/ml in RBA (0.25 ml) were allowed to adhere to each well of 8 well Lab-Tek chambers (Miles Laboratories, Naperville, IL) for 45 min at 37°C in 5% CO_2. Adherent cells were > 90% esterase positive. Nonadherent cells were removed by washing and 0.25 ml RBA, PMN supernatant, or various concentrations of lactoferrin were added as well as 30 μl of EIgG (5.0×10^8/ml). For inhibition experiments, the following reagents were included in these reaction mixtures at the concentrations indicated above: SOD, catalase, glucose, benzoate, anti-lactoferrin, or control antibody. After 30 min at 37°C in 5% CO_2, ingestion was determined as above.

Xanthine oxidase-hypoxanthine generation of superoxide anion. To control for nonspecific effects on superoxide anion generation, some reagents used to inhibit phagocytosis were tested in a cell-free system for generation of superoxide anion. 1-ml reaction mixtures were prepared at 0°C (ice H_2O bath) containing 2 mg hypoxanthine, 0.1 U xanthine oxidase, and 60 μM cytochrome *c*, and either HBSS without BSA, 40 mM glucose, 40 mM benzoate, 40 mM ethanol, 40 mM DMSO, 200 ng/ml lactoferrin, 2 μg/ml lactoferrin, 62 μg/ml antilactoferrin diluted in HBSS, heat-denatured SOD, or catalase. Duplicate tubes contained 50 μg/ml SOD. The reactions were incubated at 37°C for 5 min and then held at 0°C for spectrophotometric assessment. Superoxide anion was quantitated as the superoxide dismutase-inhibitable reduction of cytochrome *c*, based on an extinction coefficient of 21,000 $m^{-1}cm^{-1}$ at 550 nm (20). Neither glucose, benzoate, lactoferrin, nor antilactoferrin had any significant effect on cell-free generation of superoxide anion production. The inclusion of either ethanol or DMSO significantly reduced superoxide anion production (61 and 54% inhibition, respectively). Neither heat-denatured SOD nor catalase significantly reduced superoxide anion production.

Quantitation of superoxide anion generated by PMN stimulation and by xanthine oxidase-hypoxanthine. To assess activation of the respiratory burst under the conditions of phagocytosis enhancement, superoxide anion was quantitated by cytochrome *c* reduction. Reaction mixtures (0.75 ml) were prepared at 0°C (ice-water bath) containing 1.0×10^6 PMN, 75 μl of either E or EIgG, 80 μM cytochrome *c*, and either HBSS with 1% BSA, 50% YM-10E, or 11.25 ng of PDBu diluted in HBSS with 1% BSA. Duplicate tubes contained 50 μg of SOD. To allow the target particles to interact optimally with the PMN, the reaction mixtures were centrifuged at 50 *g* for 5 min before incubation at 37°C for 15 min. After centrifugation at 12,500 *g* for 5 min at 4°C, the supernatants were held at 0°C for spectrophotometric assessment as above. Superoxide levels are reported as the SOD inhibitable nmol of cytochrome *c* reduced/1.0×10^6 PMN/15 min.

To assess the effect of extracellular generation of superoxide anion or reactive oxygen metabolites dependent on superoxide anion on EIgG ingestion by both normal and CGD PMN, 1.0×10^6 PMN were incubated with 2.0 mg hypoxanthine, various concentrations of xanthine oxidase, 75 μl of EIgG, 80 μM cytochrome *c*, and either HBSS with 1% BSA or 11.25 ng of PDBu in a final volume of 0.75 ml. Duplicate tubes contained 50 μg of SOD. The reaction mixtures were

treated as above for quantitation of superoxide anion levels. To quantitate phagocytosis, identical reaction mixtures (excluding cytochrome *c*) were incubated for 30 min at 37°C with 5% CO_2 and phagocytosis assessed as described above.

Results

YM-10E, PDBu-, and AmB-stimulated ingestion of EIgG are dependent on the activation of the respiratory burst NADPH oxidase. Previous work has shown that PMN IgG-mediated ingestion is markedly augmented by stimulation with YM-10E, PDBu, or AmB (4, 5). Both nonstimulated and stimulated ingestion occur via the Fc receptor as MAb 3G8-treatment of PMN inhibits both attachment and ingestion of EIgG by stimulated and nonstimulated PMN. In addition, unopsonized E are never bound or ingested by either nonstimulated or stimulated PMN (4, 5). The mechanism for amplification of phagocytosis does not involve an increase in Fc receptor number or in EIgG binding (4, 5). In fact, when PMN are allowed to bind EIgG at 4°C and the unbound EIgG washed away before incubation with either buffer or YM-10E, the marked increase in ingestion observed with stimulation is still evident (Gresham, H. D., unpublished observation). Therefore, increased ingestion is not simply a result of increased binding of the target particle as the result of a direct effect on the Fc receptor. We next examined the effect of these phagocytosis enhancing agents on the rate of ingestion. To assess the kinetics of ingestion, we examined nonstimulated and PDBu- and YM-10E-stimulated ingestion as a function of time (Fig. 1). Fc-mediated ingestion, whether stimulated or nonstimulated reached a plateau within 25–30 min and the level of ingestion was stable for 45–50 min. Interestingly, there was not a significant difference in the amount of ingestion by either nonstimulated or stimulated PMN until after 20 min of incubation. This was true whether the stimulus was added with the opsonized E or if the PMN were stimulated for 15 min before incubation with the target (data not shown). Previous work had shown that the optimal time of stimulation before incubation with the target was 15 min (5). These data appeared to indicate that stimulated ingestion was dependent on some process occurring relatively late (after 20 min) in the phagocytic process. Because AmB also stimulated EIgG ingestion in a similar fashion and is known to cause the generation of reactive oxygen metabolites, we investigated activation of the respiratory burst as a possible common mechanism through

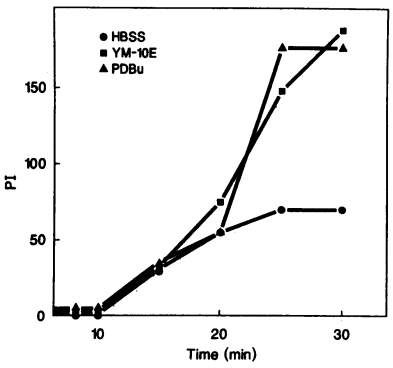

Figure 1. Effect of time on nonstimulated and YM-10E- and PDBu-stimulated ingestion of EIgG. Equal volumes (50 μl) of PMN (2.0 × 10^6/ml) and either HBSS, YM-10E (50% f.c.), or PDBu (15 ng/ml f.c.) were incubated in a fluid-phase assay with 15 μl of EIgG at 37°C. At the indicated times, phagocytosis was stopped by placing the samples in an ice-H_2O bath. Phagocytosis was assessed as a phagocytic index. PI, No. of EIgG ingested/100 PMN. Data are representative of three to four determinations.

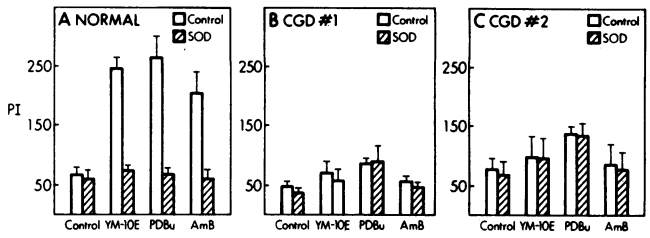

Figure 2. Effect of YM-10E, PDBu, and AmB on ingestion of EIgG by normal and CGD PMN. (*A*) Normal PMN (2.0 × 10^6/ml) and PMN from two patients with CGD (*B*) and (*C*) were incubated with equal volumes (50 μl) of control buffer, YM-10E (50% f.c.), PDBu (15 ng/ml f.c.), or AmB (4 μM f.c.) in the presence of 15 μl EIgG for 30 min at 37°C. Duplicate samples contained 50 μg SOD. In (*A*) and (*B*), the EIgG were opsonized with a 1/500 dilution of antibody whereas in (*C*), the EIgG were opsonized with a 1/1,000 dilution of antibody to result in equivalent levels of baseline ingestion. Phagocytosis was assessed as a phagocytic index. PI, No. EIgG ingested/100 PMN.

which these reagents might act to stimulate phagocytic function. As shown in Fig. 2 *A*, the inclusion of SOD in the reaction mixture completely abrogated YM-10E, PDBu-, and AmB-stimulated ingestion. There was no effect of SOD on baseline or nonstimulated ingestion. Furthermore, when ingestion was performed under anaerobic conditions (in a sealed nitrogen chamber), baseline ingestion was not inhibited while PDBu-stimulated ingestion was completely abrogated (data not shown). These data indicate that Fc-mediated ingestion stimulated with YM-10E, PDBu, or AmB is completely dependent on the activation of the respiratory burst and the generation of superoxide anion.

PMN from patients with CGD fail to manifest cytokine (YM-10E)-, phorbol dibutyrate-, or AmB-stimulated ingestion of EIgG. PMN from patients with CGD are genetically deficient in the ability to activate the respiratory burst NADPH oxidase (21) and have been used to elucidate respiratory burst-dependent (22) and independent (23, 24) functions of phagocytic cells. Functions not manifested by CGD PMN may depend on activation of the respiratory burst NADPH oxidase. Because Fc-mediated ingestion by normal PMN stimulated with YM-10E, PDBu, or AmB was completely inhibited by the inclusion of SOD in the reaction mixture while baseline or nonstimulated ingestion was unaffected by the inclusion of SOD, we examined whether PMN from patients with CGD could enhance phagocytosis in response to YM-10E, PDBu, or AmB. In contrast to normal cells, Fc-mediated ingestion by CGD PMN was not amplified by stimulation with either YM-10E, PDBu, or AmB (Fig. 2 *B*). Furthermore, the inclusion of SOD in the reaction mixture had no effect on the level of ingestion observed. An identical pattern of response was observed with PMN from a second patient with CGD. This patient's PMN exhibited a baseline level of ingestion twice that of the normal control, so the data illustrated in Fig. 2 *C* were obtained with EIgG opsonized with half of the IgG concentration used in Fig. 2 *A* and *B*. Even though the PMN of the two patients we examined expressed variable levels of baseline ingestion, neither patient's PMN responded like normal PMN to stimulation by either YM-10E, PDBu, or AmB no matter what IgG concentration was used to opsonize the E. A possible explanation for the inability of these cells to enhance phagocytosis could be that the PMN of these patients did not express a

normal amount of Fc receptor. However, PMN from both patients expressed normal levels of MAb 3G8 binding (anti-PMN Fc receptor), as detected by fluorescence flow cytometry, and normal levels of Fc-mediated EIgG binding (data not shown). Thus, these data are further evidence that an intact mechanism for activation of the respiratory burst is required for phagocytosis amplification by YM-10E, PDBu, and AmB. Moreover, these data also demonstrate that patients with CGD have a previously unrecognized defect in a potentially important pathway for enhancing phagocytic function.

PDBu-stimulated ingestion of EC3b or zymosan is also dependent on activation of the respiratory burst NADPH oxidase. To examine the specificity of this reactive oxygen metabolite-dependent mechanism for Fc-mediated ingestion, we examined EC3b ingestion stimulated by FMLP, PDBu, or FMLP combined with PDBu. As shown in Fig. 3 *A*, the inclusion of SOD in the reaction mixture had no effect on the small amount of spontaneous EC3b ingestion or on phagocytosis stimulated with FMLP. However, the much greater EC3b ingestion stimulated with PDBu or with a combination of PDBu and FMLP was inhibited 65 and 53%, respectively. These data indicate that both Fc- and C3b-mediated ingestion can occur through both respiratory burst-dependent and independent mechanisms for phagocytosis. Interestingly, even though this concentration of FMLP is capable of activating the respiratory burst, FMLP-stimulated ingestion of EC3b was not affected by SOD. Therefore, for EC3b, the amount of ingestion by either mechanism may be modulated by the individual reagents or combination of reagents used to stimulate ingestion and the cell transduction mechanisms that these reagents activate. Due to the added complexity involved in the study of EC3b-mediated ingestion, we chose to examine the respiratory burst-dependent mechanism in greater detail by examining stimulated Fc-mediated phagocytosis.

To further assess the specificity of this mechanism for IgG- and C3b-mediated ingestion of sheep E, we examined the ingestion of unopsonized zymosan in response to PDBu. As shown in Fig. 3 *B*, ingestion of zymosan by buffer-treated

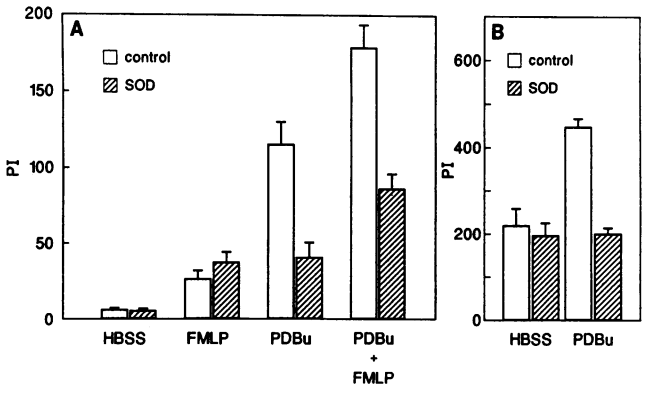

Figure 3. Effect of SOD on nonstimulated, FMLP-, PDBu-, or FMLP plus PDBu-stimulated ingestion of EC3b and nonstimulated or PDBu-stimulated ingestion of zymosan. Equal volumes of PMN (50 μl) and either HBSS, FMLP (1 μM f.c.), PDBu (15 ng/ml f.c.), or FMLP (1 μM) plus PDBu (15 ng/ml) were incubated with 15 μl of either EC3b (*A*) or unopsonized zymosan (*B*) in a fluid-phase assay for 30 min at 37°C. Duplicate samples contained 50 μg of SOD. PI, No. of EC3b or zymosan particles ingested/100 PMN. Data are represented as the mean±SEM, $n = 3$.

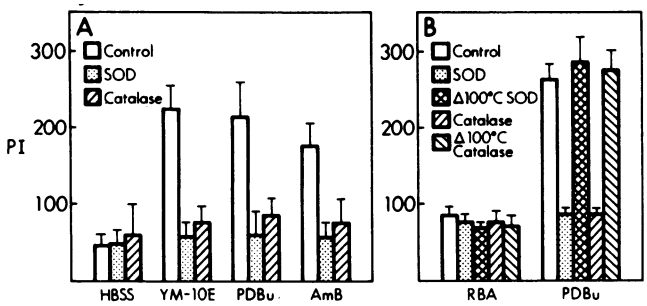

Figure 4. Effect of superoxide dismutase and catalase, native and heat-denatured, on nonstimulated and YM-10E-, PDBu-, or AmB-stimulated ingestion of EIgG. (*A*) Equal volumes of PMN (2.0 × 10^6/ml) and HBSS, YM-10E (50% f.c.), PDBu (15 ng/ml f.c.), or AmB (4 μM f.c.) were incubated with EIgG in the absence (control) or the presence of either superoxide dismutase (50 μg) or catalase (3,100 U) for 30 min at 37°C. PI, No. of EIgG ingested/100 PMN. Data are represented as the mean±SEM, $n = 4$. (*B*) Catalase and superoxide dismutase were heated to 100°C for 10 min and compared with native superoxide dismutase and catalase for effects on nonstimulated and PDBu-stimulated ingestion of EIgG, as described in A. PI, No. of EIgG ingested/100 PMN. Data are represented as the mean±SEM, $n = 3$.

PMN was unaffected by the inclusion of SOD. However, ingestion of zymosan by PDBu-stimulated PMN was inhibited by the inclusion of SOD. These data indicate that the respiratory burst-dependent mechanism for enhancing ingestion is not restricted to specific opsonin-dependent ingestion (IgG, C3b) nor to a single class of target particles (i.e., sheep E).

Catalase inhibits YM-10E-, PDBu-, and AmB-stimulated ingestion of EIgG by normal PMN. Because the inclusion of SOD in the phagocytosis assay abrogated YM-10E-, PDBu-, and AmB-stimulated ingestion, we concluded that a reactive oxygen metabolite might be responsible for stimulating Fc-mediated ingestion. SOD catalyzes the destruction of superoxide anion into superoxide anion and H_2O_2. Therefore any reaction dependent on superoxide anion or on one of its metabolites would be inhibited in the presence of SOD. We also studied the effect of catalase, which mediates the destruction of H_2O_2, on stimulated phagocytosis. As shown in Fig. 4 *A*, the inclusion of catalase in the reaction mixture also completely inhibited YM-10E, PDBu-, and AmB-stimulated ingestion. This preparation of catalase did not contain SOD because its inclusion in the xanthine oxidase-hypoxanthine system did not inhibit superoxide anion reduction of cytochrome *c*. Neither SOD nor catalase which had been denatured by incubation at 100°C for 10 min were capable of inhibiting stimulated phagocytosis (Fig. 4 *B*). Heat-denatured SOD was also incapable of inhibiting cytochrome *c* reduction in the xanthine oxidase-hypoxanthine system, indicating that heat denaturation did inhibit the SOD function of this preparation. Inhibition by catalase showed that stimulation of phagocytosis was dependent on H_2O_2, which suggested that the biologically important H_2O_2-myeloperoxidase (MPO)-halide system (22) might be involved in mediating phagocytosis enhancement. However, the inclusion of NaN_3 (an inhibitor of MPO) at concentrations from 0.1 to 10 mM had no effect on either PDBu-stimulated ingestion or on baseline levels of ingestion (data not shown). Moreover, the biological effects of H_2O_2-MPO-halide activity are not inhibited by SOD alone, because inclusion of SOD in a superoxide anion generating system actually increases the level

of H_2O_2. However, stimulated ingestion is inhibited by SOD alone (Figs. 2–4). Therefore, the H_2O_2-MPO-halide system is unlikely to be responsible for mediating stimulation of Fc-mediated ingestion.

IgG-Fc receptor interaction is known to activate the respiratory burst and generate superoxide anion and H_2O_2 (25); therefore, SOD and catalase could be simply inhibiting the ability of PMN to phagocytose EIgG and not specifically inhibiting a distinct mechanism for phagocytosis enhancement. To increase phagocytosis without stimulation, E were opsonized with higher concentrations of antibody up to an amount (1/200 dilution) twice that required for maximal rosetting (4, 5). The concentration of antibody (1/500 dilution) used to demonstrate the respiratory burst-dependent mechanism of ingestion is sufficient to obtain 76–87% rosetting PMN (4, 5). The resulting ingestion of the more heavily opsonized E was as high as that observed for YM-10E-, PDBu-, and AmB-stimulated ingestion of E bearing fewer IgG molecules (Fig. 5). In contrast to stimulated ingestion (Fig. 4 *A*), this ligand-dependent unstimulated ingestion was not inhibited by the inclusion of either SOD or catalase in the phagocytosis assay (Fig. 5). Therefore, SOD and catalase inhibit YM-10E-, PDBu-, and AmB-stimulated ingestion specifically, rather than simply nonspecifically inhibiting the extent of Fc-mediated ingestion.

Sodium benzoate and anti-lactoferrin inhibit YM-10E-, PDBu-, and AmB-stimulated ingestion of EIgG. Inhibition of amplified phagocytosis by SOD alone suggested an important role for superoxide anion in the pathway for stimulation. Because superoxide anion and H_2O_2 were both required for YM-10E-, PDBu-, and AmB-stimulated ingestion, we concluded that the reactive oxygen metabolite involved in this mechanism for phagocytosis enhancement might be a free radical generated during the respiratory burst. One such free radical is hydroxyl radical, which can be generated in vitro by the reaction of superoxide anion and H_2O_2 in the iron-catalyzed Haber-Weiss reaction (26). To pursue this possibility, we examined several hydroxyl radical scavengers for their ability to limit stimulated Fc-mediated ingestion. These included DMSO, ethanol, sodium benzoate, and phenylalanine (13). Neither DMSO nor ethanol were useful because at concentrations at which they scavenge hydroxyl radical (40 mM) both inhibited superoxide production and at concentrations of

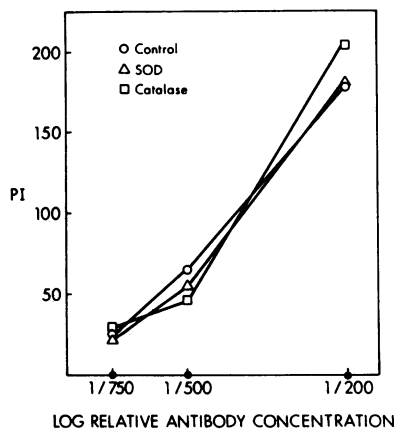

Figure 5. Effect of superoxide dismutase and catalase on ingestion of EIgG opsonized with increasing concentrations of anti-E. Equal volumes of PMN and RBA were incubated with EIgG opsonized with either a 1/750, 1/500, or 1/200 dilution of anti-E in the absence (control) and presence of either SOD (50 μg) or catalase (3,100 U). Phagocytosis was assessed after 30 min at 37°C. PI, No. of EIgG ingested/100 PMN.

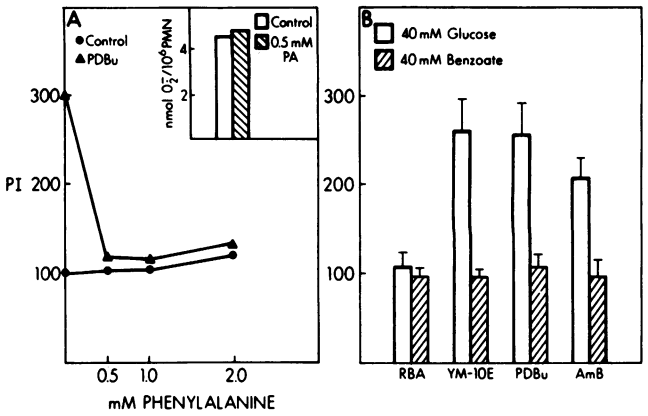

Figure 6. Effect of phenylalanine and sodium benzoate on nonstimulated and YM-10E-, PDBu-, or AmB-stimulated ingestion of EIgG. (*A*) Equal volumes (50 μl) of PMN and buffer control or PDBu (15 ng/ml) were incubated with EIgG in the presence of increasing concentrations of phenylalanine for 30 min at 37°C. PI, No. of EIgG/100 PMN. (*Inset*) Effect of 0.5 mM phenylalanine on PDBu-stimulated generation of superoxide anion. (*B*) Equal volumes of PMN, buffer control, YM-10E (50% f.c.), PDBu (15 ng/ml f.c.), or AmB (4 μM f.c.) were incubated with EIgG in the presence of either 40 mM glucose or 40 mM sodium benzoate. Phagocytosis was assessed after 30 min at 37°C. PI, No. of EIgG ingested/100 PMN. Data are represented as the mean±SEM, $n = 4$.

80–140 mM both significantly inhibited baseline levels of ingestion. However, neither 40 mM sodium benzoate nor 0.5 mM phenylalanine inhibited superoxide production or baseline nonstimulated phagocytosis. The inclusion of 40 mM sodium benzoate in the xanthine oxidase-hypoxanthine reaction (described in Methods) resulted in the production of 33 nmol of superoxide anion as compared to 31.3 nmol in the buffer control. The inclusion of 0.5 mM phenylalanine in the PMN-EIgG-PDBu reaction mixture resulted in 4.7 nmol of superoxide anion as compared to 4.4 nmol in the buffer control (Fig. 6 *A*). In contrast, this same concentration of phenylalanine was capable of completely inhibiting PDBu-stimulated ingestion without any effect on baseline ingestion (Fig. 6 *A*). Similarly, the inclusion of 40 mM benzoate, as compared to 40 mM glucose as a control (27), completely abrogated YM-10E-, PDBu-, and AmB-stimulated ingestion without affecting baseline levels of phagocytosis (Fig. 6 *B*). If the reactive oxygen metabolite responsible for stimulating phagocytosis were truly hydroxyl radical, then a source of iron would be required to catalyze the reaction. Lactoferrin, a 77,000-D iron-binding protein present in PMN secondary granules (28) has been reported variously as both a catalyst (29) and an inhibitor of hydroxyl radical formation (30). As shown in Fig. 7 *A*, $F(ab')^2$ antilactoferrin inhibited PDBu-stimulated ingestion in a dose-dependent manner though it did not completely reduce stimulated ingestion to the level of baseline phagocytosis. Antilactoferrin at all concentrations had no effect on baseline ingestion. Equivalent concentrations of antilysozyme (another constituent of PMN secondary granules) had no effect on either PDBu-stimulated or nonstimulated Fc-mediated ingestion (data not shown). $F(ab')^2$ antilactoferrin, as compared to an equivalent concentration of a control antibody, $F(ab')^2$ anti-murine IgM, also inhibited YM-10E- and AmB-stimulated ingestion of EIgG (Fig. 7 *B*). In concert, these data indicate that the mechanism responsible for mediating stimulated phagocy-

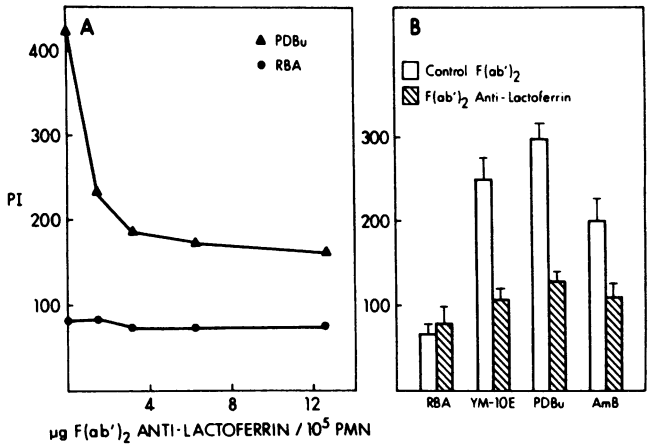

Figure 7. Effect of anti-lactoferrin on non-stimulated and YM-10E-, PDBu-, or AmB-stimulated ingestion of EIgG. (*A*) The dose-response of anti-lactoferrin was examined by incubating equal volumes of PMN and either buffer control or PDBu (15 ng/ml f.c.) with EIgG in the absence and the presence of increasing concentrations of $F(ab')^2$ anti-human lactoferrin. Phagocytosis was assessed after 30 min at 37°C. PI, No. of EIgG ingested/100 PMN. Data are representative of three experiments. (*B*) The effect of antilactoferrin on stimulated ingestion was assessed by incubating equal volumes of PMN and buffer control, YM-10E (50% f.c.), PDBu (15 ng/ml f.c.), or AmB (4 μM f.c.) with EIgG in the presence of either 6.2 μg $F(ab')^2$ anti-murine IgM (control) or 6.2 μg $F(ab')^2$ anti-human lactoferrin. Phagocytosis was assessed after 30 min at 37°C. PI, No. of EIgG ingested/100 PMN. Data are represented as the mean±SEM, $n = 3$.

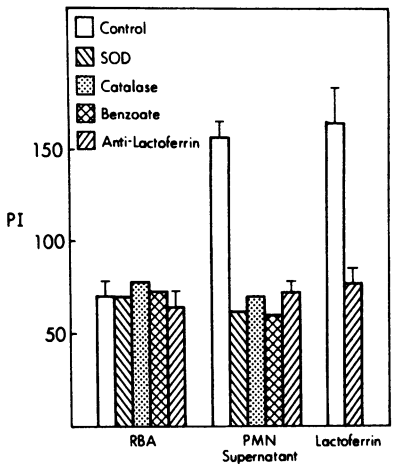

Figure 8. Effect of PMN supernatant and human lactoferrin on monocyte ingestion of EIgG. Adherent monocytes (days 1–3) in either buffer control, a supernatant of IgG-stimulated PMN containing 493±84 ng/ml of lactoferrin, or 200 ng/ml of purified human milk lactoferrin were incubated with EIgG in the absence (control) or presence of SOD, catalase, benzoate, or antilactoferrin. Control samples also included 40 mM glucose and 6.2 μg/ml of control antibody. Phagocytosis was assessed after 30 min at 37°C. PI, No. of EIgG ingested/100 monocytes. Data are represented as the mean±SEM of two to eight determinations.

tosis is dependent on superoxide anion, hydrogen peroxide, and lactoferrin and its effects are inhibited by phenylalanine and sodium benzoate. One possible reactive oxygen metabolite that could be created under these conditions is hydroxyl radical.

Monocyte Fc-mediated ingestion stimulated by PMN supernatant and lactoferrin is dependent on reactive oxygen metabolites. In our previous studies of Fc-mediated phagocytosis (4–6), we have noted that monocyte and PMN Fc-mediated ingestion appear to be regulated very differently. Monocyte Fc-mediated ingestion is not enhanced by YM-10E, PDBu, or AmB stimulation. One possibility for the failure of monocytes to respond to these reagents and therefore fail to demonstrate respiratory burst-dependent phagocytosis is that monocytes do not possess lactoferrin. Engagement of monocyte Fc receptors during the attachment and ingestion of EIgG is known to activate the respiratory burst with the generation of superoxide anion and H_2O_2 (31), products necessary for phagocytosis enhancement. Therefore, we examined whether monocyte Fc-mediated ingestion would be stimulated by a respiratory burst-dependent mechanism in the presence of a source of lactoferrin. Incubation of monocytes with either an IgG-stimulated PMN supernatant (493±84 ng of lactoferrin/ml) or with purified human milk lactoferrin markedly increased monocyte Fc-mediated ingestion (Fig. 8). This same effect was observed with iron-saturated lactoferrin but not with lactoferrin depleted of iron by dialysis against 0.1 M citric acid (32) (data not shown). Moreover, this enhancement was inhibited specifically by the inclusion of SOD, catalase, benzoate, and $F(ab')^2$ antilactoferrin (Figs. 8 and 9). As with PMN, baseline ingestion was unaffected by any of these reagents (Figs. 8 and 9). The effect of lactoferrin was dose dependent, reaching an optimal concentration at 200 ng/ml (Fig. 9 *A*), while greater than optimal doses (1–2 μg/ml) reduced ingestion down to the level of baseline phagocytosis (Fig. 9, *A* and *B*). The addition of 200 ng/ml of lactoferrin to unstimulated PMN had no effect on their ingestion of EIgG. Monocytes and PMN bind lactoferrin specifically (28, 33) and PMN stimulated by various means

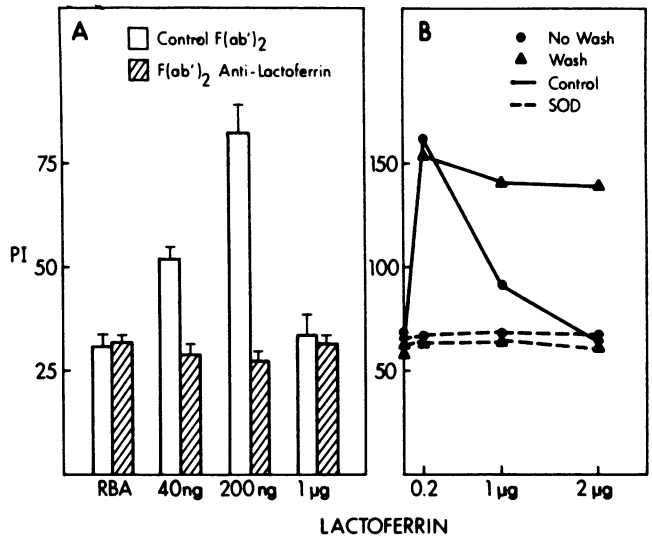

Figure 9. Dose-dependent effect of lactoferrin and its alteration by washing on monocyte ingestion of EIgG. (*A*) Adherent monocytes (day 0) in buffer control or increasing concentrations of purified human milk lactoferrin were incubated with EIgG in the presence of either 6.2 μg/ml $F(ab')^2$ anti-murine IgM (control) or 6.2 μg/ml $F(ab')^2$ antilactoferrin. Phagocytosis was assessed after 30 min at 37°C. PI, No. of EIgG ingested/100 PMN. Data are represented as the mean±SEM, $n = 3$. (*B*) Adherent monocytes (day 1) were incubated in buffer control or increasing concentrations of lactoferrin for 2 h at 4°C. After incubation, half of the samples were washed (*triangles*) and the other half left with the lactoferrin (*circles*). After the addition of the EIgG, incubation continued in the absence (control) or presence of 50 μg superoxide dismutase (*dashed lines*). Phagocytosis was assessed after 30 min at 37°C. PI, No. of EIgG ingested/100 monocytes. Data are represented as the mean of two determinations.

translocate lactoferrin to the cell surface where some of it remains cell-bound while the excess enters the fluid-phase (34). Because higher levels of fluid-phase lactoferrin appeared to prevent stimulated ingestion, we assessed the role of cell-bound lactoferrin on Fc-mediated ingestion. Monocytes were incubated with increasing concentrations of lactoferrin at 4°C for 2 h (28) and then ingestion of EIgG assessed with and without washing of the adherent cells. As shown in Fig. 9 *B*, incubation of monocytes with 1 and 2 μg/ml of lactoferrin reduced the level of ingestion observed compared with 200 ng/ml lactoferrin. However, after removal of unbound lactoferrin by washing, ingestion remained at the stimulated level for all concentrations of lactoferrin tested. All of the stimulated ingestion either by washed or nonwashed cells was prevented by the inclusion of SOD (Fig. 9 *B*). These data indicate that cell-bound lactoferrin may be responsible for mediating stimulation of ingestion and that increases in unbound lactoferrin may actually act to limit oxidative-dependent ingestion. These observations are consistent with the hypothesis that lactoferrin can function as both a catalyst and an inhibitor of hydroxyl radical production.

Extracellular generation of reactive oxygen metabolites is not sufficient to stimulate either normal or CGD PMN phagocytosis. To examine the effect of extracellular generation of superoxide anion and H_2O_2 on the stimulation of Fc-mediated ingestion, we first quantitated the levels of superoxide anion generated under the conditions optimal for stimulation of ingestion. We chose to examine superoxide anion production as it is the first product generated by activation of the respiratory burst, is required for the generation of H_2O_2 and the reactive oxygen metabolites such as hydroxyl radical, and is absolutely required for stimulated ingestion. As shown in Table I, PMN incubated in buffer alone did not generate significantly different amounts of superoxide anion whether E or EIgG were also included in the reaction mixture. Therefore, under the conditions used to assess phagocytosis (fluid-phase assay, lower concentrations of IgG used to opsonize E), engagement of the Fc receptor did not enhance superoxide anion generation over buffer alone. In contrast, YM-10E stimulation of PMN did result in a significant increase in superoxide anion levels when EIgG but not E were present in the reaction mixture. Stimulation with PDBu resulted in significant increases in superoxide anion levels whether E or EIgG were included in the mixture. These data indicate that under the conditions that are optimal for examination of Fc-mediated ingestion, the respiratory burst is activated and superoxide anion generated. Moreover, YM-10E stimulation of PMN does not activate the respiratory burst; it requires the contribution of the ligand, IgG, for significant levels of superoxide anion to be generated. In contrast, PDBu stimulation alone is sufficient to activate the respiratory burst.

To examine extracellular generation of reactive oxygen metabolites, we employed the xanthine oxidase-hypoxanthine system, which is known to generate superoxide anion, H_2O_2, and hydroxyl radical when incubated in the presence of chelated iron (30). Our reaction mixtures contained various concentrations of xanthine oxidase to vary the level of superoxide anion generated. All other concentrations and reaction conditions were identical to those in Table I. As shown in Table II, incubation of normal PMN with concentrations of xanthine oxidase that generated levels of superoxide anion identical to those in Table I did not stimulate Fc-mediated ingestion. Supernatants of the reaction mixtures contained sufficient levels of lactoferrin, as measured by ELISA, to support respiratory burst-dependent ingestion. In addition, there was no significant effect on EIgG-mediated rosettes (data not shown). In contrast, generation of H_2O_2 by the glucose oxidase-glucose system, performed as described by Gaither et al. (12), in the presence of EIgG and PMN inhibited Fc-mediated rosettes as well as Fc-mediated ingestion (data not shown) (11, 12). In addition, xanthine oxidase at concentrations of 0.1 U/ml also inhibited Fc-mediated ingestion. Thus, the level of xanthine oxidase used to generate superoxide anion at levels equivalent to those generated during stimulation of phagocytosis was not sufficient to generate enough H_2O_2 to inhibit Fc-mediated functions in our fluid-phase assay. We concluded from these data that simple extracellular generation of superoxide anion and H_2O_2, even at equivalent concentrations generated by YM-10E and PDBu stimulation in the presence of EIgG, was not sufficient to stimulate Fc-mediated ingestion. In addition, these data indicate that the reactive oxygen metabolite that enhances ingestion is unlikely to have it effect on the target particle, because in the above system the target was exposed to the extracellularly generated oxygen products and ingestion

Table I. Effect of Stimulation on PMN Superoxide Anion Production

Stimulus	Target	Superoxide anion production*
Control	E	1.32±0.34
	EIgG	1.01±0.34
YM-10E	E	1.42±0.13
	EIgG	4.21±0.42
PDBu	E	5.31±0.89
	EIgG	5.40±0.42

* Superoxide anion levels were measured as SOD-inhibitable nanomoles of cytochrome *c* reduced/1.0 × 10^6 PMN/15 min. See Methods for details of the assay. The data are represented as the mean±SEM of three to four determinations.

Table II. Effect of Extracellular Generation of Superoxide Anion on Normal and CGD PMN Ingestion of EIgG

PMN	Stimulus	XO*	Superoxide levels‡	PI§
Normal	Control	0	0.9±0.1	55.7±5.5
		0.9	5.5±0.5	45.3±6.6
		1.8	12.9±0.8	48.0±8.7
		3.6	20.4±1.2	40.7±2.9
CGD	Control	0	0	54.5±14.5
		0.9	5.6±0.9	34.5±10.5
		1.8	13.1±1.4	41.0±11.0
	PDBu	0	0	114.0±6.0
		0.9	5.5±0.4	67.5±5.5
		1.8	12.5±2.8	91.0±22.9

* Xanthine oxidase (XO) is added in units $\times 10^{-3}$.
‡ Superoxide is measured as SOD-inhibitable nanomoles of cytochrome *c* reduced/1.0 × 10^6 PMN/15 min.
§ PI, no. of EIgG ingested/100 PMN.

was not increased. We also studied CGD PMN to assess whether stimulation with PDBu (which might activate all cellular components necessary for phagocytosis enhancement except the respiratory burst) in addition to the extracellular generation of superoxide anion would provide a sufficient signal to stimulate Fc-mediated ingestion. As shown in Table II, incubation of CGD PMN, either non- or PDBu-stimulated, with varying concentrations of xanthine oxidase was not sufficient to engage the respiratory burst-dependent mechanism of ingestion. PDBu alone did enhance slightly CGD PMN ingestion, but as shown in Fig. 2, this enhancement was not inhibited by SOD and was not equivalent to the level of enhancement observed with PDBu stimulation of normal PMN. Therefore, these data indicate that while the respiratory burst-dependent mechanism is dependent on superoxide anion and on its metabolites, there may be restrictions on where these metabolites are generated as to their ability to stimulate ingestion. Furthermore, the defect observed in the CGD PMN can not be overcome simply by providing a stimulant and a source of reactive oxygen metabolites.

Discussion

Unstimulated PMN are not highly phagocytic as compared to the phagocytes of the monocyte/macrophage lineage. However, when stimulated by a variety of inflammatory products, they will enhance their ingestion of IgG- and C3b-opsonized targets. Presumably, this regulatory control of phagocytosis acts to limit the potentially damaging products of ingestion, such as toxic oxygen metabolites and lysosomal enzymes, to areas of infection or preexisting inflammation. Data presented in this paper indicate that transduction of signals for phagocytic function can be divided into distinct mechanisms; one independent of and one dependent on the generation of reactive oxygen metabolites. Evidence that these pathways are distinct comes from the fact that activation of the respiratory burst is absolutely required for YM-10E, PDBu, and AmB stimulation of Fc-mediated ingestion (Fig. 2 *A*) and for PDBu-stimulated EC3b and zymosan ingestion (Fig. 3, *A* and *B*). In contrast, the mechanism in PMN for baseline or unstimulated phagocytosis is actually limited by products of oxygen metabolism, via oxidative modification of Fc and C3b receptors by the H_2O_2-MPO-halide system (11, 12). Further evidence that the two pathways of ingestion are distinct comes from studies on the effects of cholera toxin, pertussis toxin, and elevated intracellular cAMP levels on Fc-mediated phagocytosis. In PMN, elevated intracellular cAMP concentrations inhibit baseline levels of Fc-mediated ingestion, whereas ingestion stimulated by YM-10E, PDBu, or AmB is unaffected (4). Moreover, cholera toxin and pertussis toxin affect phagocytosis in a manner reciprocal to cAMP; while they have no effect on baseline levels of ingestion, they entirely block ingestion stimulated by YM-10E and AmB (4).

In the present work, we have examined in detail the respiratory burst dependent pathway for amplification of Fc-mediated phagocytosis. This mechanism is dependent on superoxide anion, hydrogen peroxide, and lactoferrin (Figs. 2, 4, 7–9) and is independent of the H_2O_2-MPO-halide system, since SOD alone inhibits augmented phagocytosis (Figs. 2, 4, 8, and 9), and NaN_3, an inhibitor of MPO, does not. The results obtained with CGD PMN indicate the absolute necessity for activation of the respiratory burst (Fig. 2, *B* and *C*); these PMN contain normal levels of lactoferrin (24), yet they do not demonstrate YM-10E-, PDBu-, or AmB-stimulated ingestion. Previous studies of phagocytic function in this disease have indicated that CGD PMN manifest normal to hyperphagocytic capabilities (12, 22, and 23). The hyperphagocytic response observed with adherent CGD PMN as compared with normal adherent PMN is thought to be due to absence of H_2O_2-MPO-halide inhibition of baseline ingestion (12). However, in our fluid-phase assay the quantities of reactive oxygen metabolites generated were not sufficient to inhibit nonstimulated Fc-mediated function via activation of the H_2O_2-MPO-halide system. Thus, the variability in CGD baseline or nonstimulated levels of ingestion may be dependent in part on the method of assay (e.g., adherent vs. fluid-phase) and on the heterogeneity of the manifestations of the CGD defect (12). Most importantly, even though the PMN of the two patients we examined expressed variable levels of baseline ingestion in our fluid-phase assay, they did not respond like normal PMN to YM-10E, PDBu, or AmB stimulation.

Both PMN and monocytes possess a respiratory burst-dependent mechanism for Fc-mediated ingestion; however, the requirement for its demonstration is different in the two cell types. For both cells, stimulation of ingestion absolutely requires both activation of the respiratory burst, with generation of superoxide anion and H_2O_2, and lactoferrin. Normal monocytes possess an intact mechanism for activation of the respiratory burst, but unlike PMN they are unable to manifest respiratory burst-dependent phagocytosis since they do not contain lactoferrin. In the presence of purified milk lactoferrin or PMN lactoferrin provided as a supernatant from IgG-stimulated PMN, monocyte Fc-mediated ingestion is stimulated in a respiratory burst-dependent manner (Figs. 8 and 9). There is a further difference between monocytes and PMN. Ligation of the monocyte Fc receptor by IgG is apparently sufficient to engage this mechanism in the presence of lactoferrin. In contrast, demonstration of this reactive oxygen metabolite-dependent pathway in PMN absolutely requires external stimulation by agents such as phorbol esters, AmB, and YM-10E (Figs. 1, 2, and 4). Some of these agents (e.g., PDBu) are known to stimulate PMN to produce O_2^- and H_2O_2 as well as to secrete lactoferrin, whereas others, such as YM-10E and AmB, require an additional contribution of the opsonized target (Table I). It is intriguing to speculate that the stimulation of PMN phagocytosis observed with chemotactic peptides (35), lymphokines, and cytokines such as colony-stimulating factor (GM-CSF) (36) and tumor necrosis factor (TNF) (36, 37), and peptides such as tuftsin (38) is due to activation of the respiratory burst-dependent pathway either by the stimulus alone or in conjunction with the opsonized target.

Because the respiratory burst-dependent mechanism of ingestion requires superoxide anion, H_2O_2, and lactoferrin, the reactive oxygen metabolite involved may be hydroxyl radical. In previous investigations of PMN function, these requirements have been interpreted as evidence of hydroxyl radical production. Studies on the production of hydroxyl radical by PMN and the role of lactoferrin in its generation are controversial. Recent work using spin-trapping techniques has indicated that PMN do not produce detectable quantities of hydroxyl radical in the absence of additional exogenous sources of chelated iron (Fe^3+/DETAPAC) (39) and that lactoferrin secreted into the fluid-phase acts to limit hydroxyl radical production (30). We investigated the role of hydroxyl radical in

respiratory burst-dependent phagocytosis by the addition of hydroxyl radical scavengers and of antilactoferrin to the phagocytosis assay. The only radical scavengers that could be used in our experiments because they did not inhibit either superoxide anion production or nonstimulated baseline phagocytosis were phenylalanine and sodium benzoate. Phenylalanine and benzoate completely abrogated YM-10E-, PDBu-, and AmB-stimulated ingestion without affecting baseline phagocytosis (Fig. 6) and benzoate also inhibited monocyte lactoferrin-induced ingestion (Fig. 8). The addition of antilactoferrin as compared to a control antibody also abrogated YM-10E-, PDBu-, and AmB-stimulated ingestion (Fig. 7) as well as monocyte lactoferrin-induced ingestion (Figs. 8 and 9) without affecting baseline phagocytosis. In addition, partially and fully Fe-saturated lactoferrin were capable of inducing monocyte respiratory burst-dependent ingestion but Fe-depleted lactoferrin was not. This indicates that the Fe-binding function of lactoferrin is absolutely required to engage this mechanism of ingestion. While these data do not demonstrate that the role of lactoferrin in stimulated phagocytosis is to produce hydroxyl radical, they are consistent with this interpretation.

Although we do not know the precise mechanism by which the reactive oxygen metabolite acts to amplify ingestion, our data indicate that its affect is on the phagocytic process and not on a specific receptor, ligand, or a particular target particle. For instance, both IgG- and C3b-mediated ingestion of sheep E are enhanced as well as the ingestion of unopsonized zymosan (Figs. 2 and 3). Also since activation of this mechanism does not result in any change in receptor number or ligand-binding capability (4, 5), the reactive oxygen metabolite is unlikely to have any effect on the phagocytic receptor. Furthermore, our data provide evidence that there are restrictions on the location or production within the cell of the oxidative metabolites involved in augmentation of phagocytosis. For example, the generation of superoxide anion and H_2O_2 by the xanthine oxidase-hypoxanthine system in a mixture containing lactoferrin, unstimulated PMN, and EIgG was not sufficient to stimulate Fc-mediated ingestion (Table II). In addition, stimulation of CGD PMN with PDBu as well as extracellular generation of reactive oxygen metabolites was not sufficient to engage this mechanism for augmenting ingestion. Our data on monocyte Fc-mediated ingestion indicate that the role lactoferrin plays in stimulating ingestion occurs at the cell membrane or within the phagolysosome. The following data support this hypothesis: (*a*) the effect of lactoferrin on monocyte Fc-mediated ingestion was not reversed by washing (Fig. 9); (*b*) antilactoferrin completely inhibited all stimulated Fc-mediated ingestion by monocytes, indicating that the lactoferrin responsible was accessible to the antibody (Figs. 8 and 9); (*c*) antilactoferrin was not able to completely inhibit PDBu-stimulated ingestion by PMN (Fig. 7 *A*), consistent with intragranular and/or intralysosomal lactoferrin stimulating ingestion while remaining inaccessible to inhibition by antibody; and (*d*) inclusion of the chelated iron complex, Fe^3 + DETAPAC, in the monocyte phagocytosis assay did not stimulate Fc-mediated ingestion, consistent with the observation that the iron-containing molecule needs to be cell-bound (unpublished observations). Thus, the inability of extracellular generation of superoxide anion to enhance ingestion and the need for lactoferrin to be cell-bound provide evidence that the reactive oxygen metabolite might be generated and have its effect near or within the phagolysosome. Because we believe that the affect of the reactive oxygen metabolite is on the phagocytic process, its action may be directly on phagolysosome formation possibly by affecting such processes as membrane fusion or cytoskeletal function.

In summary, we believe these data suggest a model for the recruitment of a respiratory burst-dependent mechanism for augmentation of phagocytosis in vivo. At inflammatory sites, where PMN collect, many substances are present that could initiate this mechanism for stimulation of ingestion. These substances include chemotactic peptides, cytokines and lymphokines (YM-10E, TNF, etc.), peptides such as tuftsin, and components of the extracellular matrix. Upon stimulation of PMN by these substances in the presence of opsonized pathogens, the respiratory burst is activated, lactoferrin translocated to the PMN surface, and phagocytosis stimulated by the generation of a reactive oxygen metabolite, possibly hydroxyl radical. Lactoferrin secreted into the fluid phase would help to limit hydroxyl radical damage to host tissue, as proposed by Britigan et al. (30). Monocytes that subsequently infiltrate into the site would be exposed to secreted PMN lactoferrin and could utilize the reactive oxygen metabolite-dependent mechanism to enhance phagocytic destruction of the invading opsonized pathogens. This model implies that recruitment of increased phagocytic function at inflammatory sites is a normal process of host defense. In this regard, patients with CGD, who have been previously thought as having normal or even hyperphagocytic capacity, may actually lack an important pathway for amplification of phagocytic function.

Acknowledgments

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