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Interferon-γ Corrects the Respiratory Burst Defect *In Vitro* in Monocyte-Derived Macrophages from Glycogen Storage Disease Type 1b Patients

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ABSTRACT. Glycogen storage disease (GSD) type 1b is accompanied by decreased respiratory burst activity in peripheral blood phagocytic cells (*i.e.* monocytes and neutrophils). To elucidate whether this depressed respiratory burst was due to an intrinsic defect of phagocytic cells or due in part to *in vivo* host factors, we examined superoxide anion (O_2^-) production in monocytes from five GSD 1b patients cultured 9 d *in vitro* to allow for differentiation into macrophages (MDM). O_2^- production in MDM was measured in response to concanavalin A, fMet-Leu-Phe, and phorbol myristate acetate (PMA) stimulation. GSD 1b MDM had significantly depressed O_2^- generation with fMet-Leu-Phe and concanavalin A stimulation; however, unlike peripheral blood monocytes, GSD 1b MDM responded to PMA stimulation with O_2^- production comparable to healthy control donors. The cytokine interferon-γ (IFN-γ) has been shown to enhance O_2^- production in MDM. When GSD 1b MDM were cultured in the presence of IFN-γ (1×10^5 U/L), O_2^- production in response to fMet-Leu-Phe, concanavalin A, and PMA was enhanced to rates similar to those of control MDM cultured in the presence of IFN-γ. Thus, the respiratory burst defect observed in circulating phagocytic cells is also present *in vitro* in cultured GSD 1b MDM. However, in contrast to circulating phagocytic cells, depressed O_2^- production in GSD 1b MDM is selective to receptor-mediated activation, but not to PMA stimulation. This defect is correctable after short-term treatment with IFN-γ, suggesting a role for IFN-γ in treating the phagocytic defect in this disease. (*Pediatr Res* 34: 265–269, 1993)

Abbreviations

GSD, glycogen storage disease
1a and 1b, types 1a and 1b of GSD
O_2^-, superoxide anion
MDM, monocyte-derived macrophage
IFN-γ, interferon-γ
PMA, phorbol myristate acetate
Con A, concanavalin A
Ca^{2+}, calcium

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In both GSD 1a and 1b, hepatic defects prevent the conversion of glucose-6-phosphate to glucose resulting in hypoglycemia during fasting (1, 2). In GSD 1a, the enzyme glucose-6-phosphatase is defective (1, 2), whereas in GSD 1b, the glucose-6-phosphate translocase is affected (3–5). The clinical features of GSD 1b are similar to those of GSD 1a with the exception that patients with GSD 1b are often neutropenic and suffer from recurrent bacterial infections suggesting an impairment in their immune system (6–11).

There is evidence of functional defects in the phagocytic cells from GSD 1b patients that limit their ability to destroy invading microorganisms. Multiple functional abnormalities have been identified including chemotaxis, bactericidal activity, respiratory burst activity, and glucose transport (12–18). We previously demonstrated that the defect in respiratory burst activity was associated with impaired calcium mobilization and decreased intracellular calcium stores (18, 19). It is not apparent whether these phagocytic cell dysfunctions are a result of multiple defects or of a more fundamental membrane defect.

Previous studies on the respiratory burst of monocytes from GSD 1b patients have focused exclusively on circulating cells (15, 18); however, *in vivo*, the circulating monocytes migrate into tissue, where they adhere and over time differentiate into mature macrophages (20). Both cell adherence and differentiation modulate the kinetics of the O_2^- generation (21–24). It is not known whether GSD 1b macrophages also have defective respiratory burst activity.

Macrophages can be functionally modified *in vitro* through exposure to cytokines, such as IFN-γ. IFN-γ is a potent macrophage activator that is secreted by T cells. Among its varied effects, *in vitro* incubation with IFN-γ has been shown to stimulate cell differentiation and enhance respiratory burst activity in MDM (25–27). In addition, IFN-γ has been used *in vivo* to improve respiratory burst activity in patients with chronic granulomatous disease, leprosy, and HIV infection (28–31).

The purposes of this study were *1*) to determine whether the respiratory burst activity in macrophages was also defective in GSD 1b and *2*) to establish the efficacy of IFN-γ treatment on the depressed respiratory burst in GSD 1b. Similar to circulating phagocytic cells, MDM from GSD 1b patients had a selective defect in O_2^- generation in response to the ligands fMet-Leu-Phe and Con A. Accordingly, GSD 1b phagocytic cells have an intrinsic defect in respiratory burst activity that is not dependent on cellular environment. However, unlike the peripheral blood phagocytic cells, MDM from GSD 1b patients do not have a depressed respiratory burst in response to PMA. Therefore, the defect in GSD 1b respiratory burst activity in response to PMA stimulation is corrected after *in vitro* culture and cell differentiation. *In vitro* culture of MDM from GSD 1b patients with IFN-γ enhanced O_2^- production to levels similar to controls in

response to either fMet-Leu-Phe or Con A. Moreover, a comparison of the cell cultures revealed that GSD 1b MDM showed morphologic differences in comparison to control cells that were ameliorated by IFN-γ treatment. Thus, MDM from GSD 1b patients have a selective defect in respiratory burst activity to ligand stimulation that can be corrected *in vitro* with IFN-γ.

MATERIALS AND METHODS

Study population. The study population consisted of five unrelated GSD 1b patients: two men aged 28 and 24 y and three girls aged 4, 9, and 13 y. Written permission was obtained from patients or their parents for all blood samples in accordance with policies of the Institutional Review Board at the Children's Hospital of Philadelphia. Control blood samples were obtained from healthy adults routinely used as donors in our laboratory.

Monocyte isolation and culture of MDM. Monocytes were isolated from heparinized venous blood (1×10^4 U/L) and cultured as previously described (32). Mononuclear cells, separated from whole blood by Ficoll-Hypaque centrifugation, were suspended in Dulbecco's Modified Eagle Media (DMEM; GIBCO Laboratories, Grand Island, NY). The cell suspension was plated onto gelatin-coated tissue culture flasks and incubated at 37°C for 45 min in 5% CO_2. Nonadherent cells were removed by several washings. Adherent monocytes were detached by incubation with 5 mM EDTA in DMEM with 10% horse serum (HS; GIBCO Laboratories), washed, and resuspended in DMEM supplemented with 10% FCS (Hyclone Laboratories, Logan, UT), 10% HS, L-glutamine (4 mM), penicillin (1×10^5 U/L), and streptomycin (100 mg/L). Cells were plated at $2.0–3.5 \times 10^5$ cells/polylysine-coated glass coverslip/well in 24-well tissue culture plates. After 24 h of incubation at 37°C in 5% CO_2, nonadherent cells were removed by aspiration and fresh medium was added in the presence or absence of 1×10^5 U/L of recombinant human IFN-γ (1×10^5 U/L = 100U/mL) (Hoffman-La Roche, Nutley, NJ). Cell cultures were maintained at 37°C in 5% CO_2 for 9 d.

O_2^- generation. The generation of O_2^- by MDM was measured on the 9th d of culture in the well plate as superoxide dismutase-inhibitable cytochrome *c* reduction by endpoint analysis (33).

DNA assay. DNA from MDM cultures was assayed according to a procedure adapted from West *et al.* (34). Ten mM EDTA, pH 12.3, were added to each well and incubated for 90 min at 37°C. After the plates were cooled on ice, the pH was lowered to pH 7.0 with 1 M KH_2PO_4. The supernatants were incubated for 20 min with 9.0×10^{-7} M Hoescht no. 33258 (bisBenzimide; Sigma Chemical Co., St. Louis, MO) and diluted in 100 mM NaCl-10 mM Tris, pH 7.0. Fluorescence was measured using an excitation wavelength of 350 nm and an emission wavelength of 455 nm. A standard curve from 0.31 to 10 μg DNA was prepared from calf thymus DNA and sample concentrations were calculated allowing for a linear conversion of log(fluorescence) to log(μg DNA).

Materials. PMA was purchased from Sigma, stored as a concentrated stock solution in DMSO, and diluted before use. fMet-Leu-Phe (Sigma) was stored in stock solution of ethanol and diluted in buffer before use. Con A (Sigma) was prepared fresh before use.

RESULTS

Stimulation of MDM by a variety of stimuli elicits the assembly and activation of the NADPH oxidase, which catalyzes the reduction of oxygen to O_2^-, the respiratory burst (35). We compared the respiratory burst activity of GSD 1b and control MDM activated by fMet-Leu-Phe and by Con A, both surface receptor-mediated agonists, and also by the phorbol ester PMA, which acts at a site distal to plasma membrane receptors, thereby bypassing part of the receptor-activated signaling pathway. As shown in Figure 1*A*, stimulation of control MDM with either

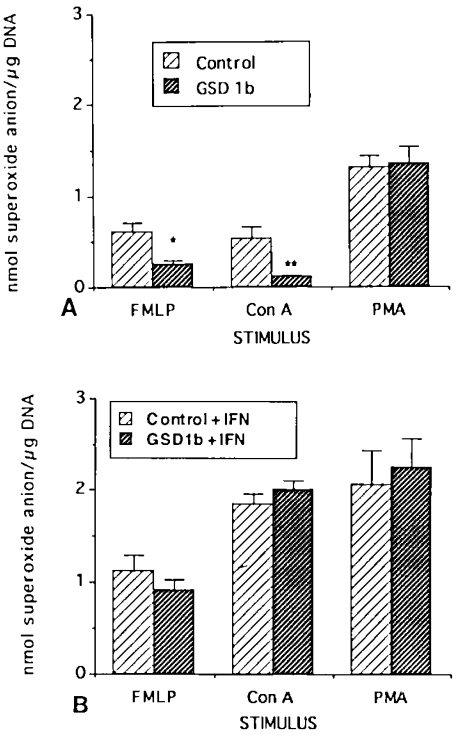

Fig. 1. *A*, O_2^- generation in control and GSD 1b MDM. MDM were cultured for 9 d. O_2^- was determined on d 9 as superoxide dismutase-inhibitable cytochrome *c* reduction by endpoint analysis after 60 min of stimulation in response to PMA (0.1 mg/L), fMet-Leu-Phe (10^{-6} M), or Con A (100 mg/L). Con A was added after a 5-min incubation of MDM with 5 mg/L cytochalasin B. Values are mean ± SEM of triplicate determinations for three to four individual patients with matched controls and are expressed as nmol O_2^-/μg DNA. *, $p < 0.02$ in fMet-Leu-Phe–stimulated control MDM *vs* GSD 1b MDM; **, $p < 0.03$ in Con A–stimulated control MDM *vs* GSD 1b MDM. *B*, O_2^- generation in control and GSD 1b MDM cultured with IFN-γ. MDM were cultured for 9 d in the presence of IFN-γ (1×10^5 U/L). O_2^- was determined on d 9 of culture in response to PMA, fMet-Leu-Phe, and Con A as described in *A*. Values are mean ± SEM of triplicate determinations for three to four patients with matched controls and are expressed as nmol O_2^-/μg DNA.

PMA, fMet-Leu-Phe, or Con A results in O_2^- production. Maximal O_2^- production was obtained with PMA (1.35 ± 0.18 nmol O_2^-/μg DNA control MDM, mean ± SEM, $n = 4$, which is equivalent to 12.7 nmol $O_2^-/10^6$ cells). This rate of PMA-triggered O_2^- production in MDM is less than that of O_2^- production in freshly isolated monocytes and neutrophils as previously reported (18, 19, 22–24).

fMet-Leu-Phe (10^{-6} M)-stimulation of GSD 1b MDM had significantly depressed O_2^- generation as compared with controls (0.25 ± 0.05 nmol O_2^-/μg DNA GSD 1b MDM *versus* 0.61 ± 0.1 nmol O_2^-/μg DNA control MDM, $n = 4$, $p < 0.02$). This reduction in O_2^- production in GSD 1b MDM (42% of controls) was similar to the reduction in respiratory burst activity we reported in GSD 1b monocyte suspensions (32% of controls, $n = 3$ patients) and neutrophils (17% of controls, $n = 3$ patients) (18, 19). Defective generation of O_2^- was also observed in GSD 1b MDM stimulated with Con A (0.11 ± 0.03 nmol O_2^-/μg DNA GSD 1b MDM *versus* 0.53 ± 0.12 nmol O_2^-/μg DNA control MDM, $p < 0.03$, $n = 3$). In contrast, there was no difference between the rates of O_2^- generation elicited by PMA in MDM from GSD 1b patients and control donors (1.35 ± 0.18 nmol O_2^-/μg DNA and 1.30 ± 0.14 nmol O_2^-/μg DNA, respectively, $n = 4$). Thus, MDM from GSD 1b patients have a selective impairment of O_2^- production in response to fMet-Leu-Phe and

Con A but not to PMA stimulation compared with healthy controls.

Previous studies have demonstrated that the addition of IFN-γ during cell culture enhances the respiratory burst activity of MDM upon subsequent stimulation (26, 27). Control MDM cultured in the presence of IFN-γ had increased respiratory burst activity independent of the stimulus used (Fig. 1*B*). The O_2^- generation in control cells cultured with IFN-γ was increased in response to PMA (59%, p = NS), fMet-Leu-Phe (79%, $p < 0.04$), and Con A (245%, $p < 0.01$) stimulation compared with control MDM cultured in the absence of IFN-γ. IFN-γ also increased O_2^- production in GSD 1b MDM stimulated with fMet-Leu-Phe (259%, $p < 0.01$), Con A (1700%, $p < 0.01$), or PMA (67%, $p < 0.01$) compared with GSD 1b MDM cultured with medium alone (Fig. 1*B*). There was no significant difference between the O_2^- generated by IFN-γ–treated GSD 1b MDM by PMA, fMet-Leu-Phe, and Con A and their respective controls. Thus, *in vitro* treatment of MDM with IFN-γ corrected the defective response to fMet-Leu-Phe and Con A stimulation in MDM from GSD 1b patients.

Figure 2 shows representative phase-contrast photomicrographs of MDM from control and GSD 1b patients cultured in the presence or absence of 1×10^5 U/L IFN-γ. The control cultured MDM demonstrated the morphologic changes that accompany maturation of monocytes into MDM (Fig. 2*B*): evidence of cytoplasmic spreading and increased cell diameter (36). In contrast, the GSD 1b MDM had smaller cell diameter and less cytoplasmic spreading (Fig. 2*A*). GSD 1b MDM cultured in the presence of IFN-γ responded with morphologic changes similar to those of control cells cultured with IFN-γ addition (Fig. 2*C* and *D*). Thus, the changes in respiratory burst activity of the GSD 1b MDM upon IFN-γ treatment are paralleled by morphologic changes.

DISCUSSION

We and others have documented the defective respiratory burst activity in circulating phagocytic cells from GSD 1b patients (10, 11, 14, 15, 18, 19). The results of the present study demonstrate that MDM cells from GSD 1b patients are also defective in their ability to generate O_2^- in response to ligand (*i.e.* fMet-Leu-Phe and Con A) stimulation. The reproducibility of the observed depressed respiratory burst in GSD 1b phagocytic cells cultured short-term *in vitro* indicates that the defect is intrinsic and is not a result solely of *in vivo* host factors.

This study also demonstrates that the PMA-stimulated respiratory burst activity in GSD 1b MDM is equivalent to that of controls. Seger *et al.* (10) observed normal NADPH-dependent O_2^- production in neutrophil lysates from a single GSD 1b patient, which is consistent with our observation. These results suggest that the components that make up the NADPH oxidase are present in the cell and are fully functional. Thus, the GSD 1b patient's phagocytic cell defect differs from chronic granulomatous disease, which is distinguished by absent protein components of the NADPH oxidase enzyme complex and in which long-term *in vivo* therapy with IFN-γ failed to improve the blunted respiratory burst (37–39).

The basis for the observation that PMA-stimulated O_2^- production is defective in freshly isolated monocytes from GSD 1b patients but not *in vitro* in MDM is not apparent but may be a result of alterations in the cellular environment. The presence of an inhibitory factor or the absence of a growth factor in the plasma of these patients may be responsible for the depressed PMA-triggered O_2^- production. Alternatively, monocyte adherence and differentiation induces alterations in protein synthesis and cellular metabolism (22, 36). This results in decreased respiratory burst activity compared with peripheral blood mono-

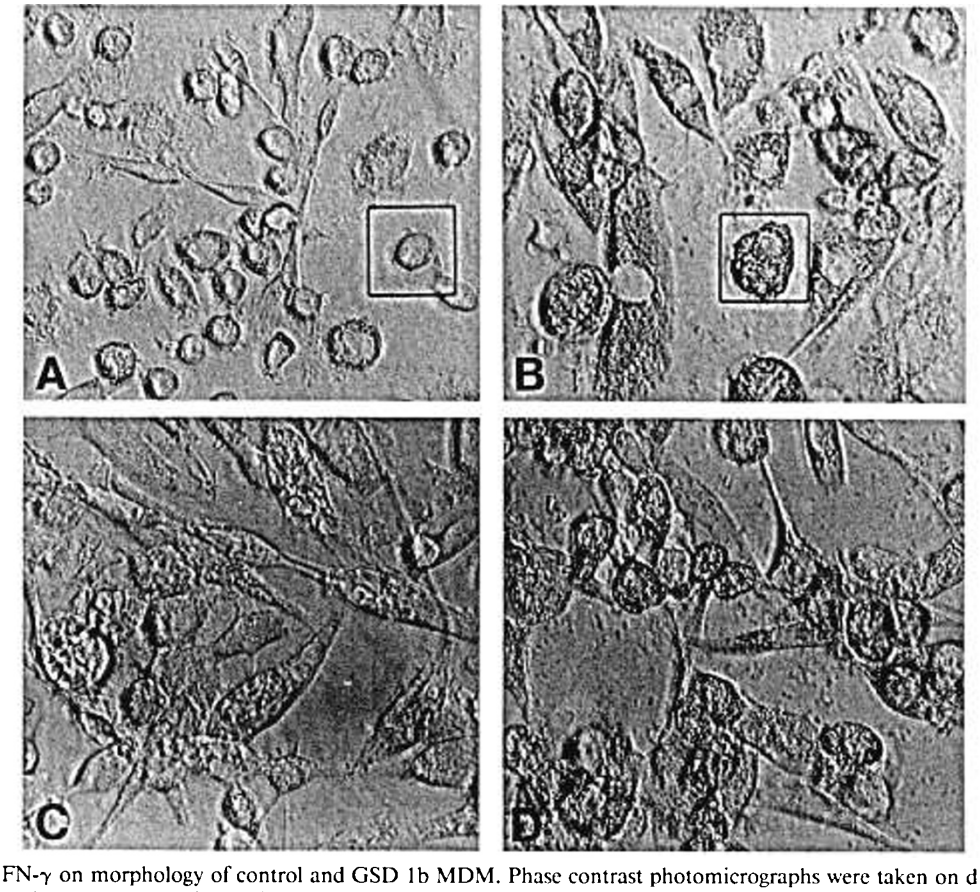

Fig. 2. Effect of IFN-γ on morphology of control and GSD 1b MDM. Phase contrast photomicrographs were taken on d 9 of culture. *A*, GSD 1b MDM and *B*, control MDM cultured in medium alone (×400). *C*, GSD 1b MDM and *D*, control MDM cultured in the presence of IFN-γ (1×10^5 U/L) (×400).

cytes and enhanced membrane depolarization and Ca^{2+} mobilization upon stimulation (21–24). Thus, cellular alterations induced by cell adherence or differentiation may restore the PMA-stimulated respiratory burst upon GSD 1b monocyte maturation.

IFN-γ addition during cell culture enhanced the respiratory burst activity in both control and GSD 1b MDM, thereby correcting the defective fMet-Leu-Phe– and Con A–elicited respiratory burst activity in GSD 1b MDM. There are several different sites where IFN-γ could alter respiratory activity in these patients' MDM; these include a direct effect on components of the NADPH oxidase enzyme complex, its assembly in the plasma membrane, or the signaling pathways that regulate the respiratory burst.

During monocyte maturation to macrophage, there is a down-modulation in the cells' ability to generate O_2^- (23, 24). This has been attributed to down-modulation of the mRNA for constituents of the NADPH oxidase, specifically the membrane-associated gp91 and the cytosolic p47 components (40, 41). The culture of monocytes with IFN-γ during maturation increases the expression of gp91 and p47, paralleling the increase in respiratory burst activity in these cells (40, 41). In addition, IFN-γ alters the signaling pathways involved in regulating respiratory burst activity, specifically modulation of intracellular Ca^{2+} levels. Incubation with IFN-γ increased the free intracellular Ca^{2+} levels in human monocytes (42) and in the monocytic cell line U937 (43). Furthermore, Ca^{2+} ionophores and phorbol esters, together, mimic the IFN-γ–enhanced cytocidal activity in macrophages (44). As stated previously, our results suggest that the respiratory burst defect is not due to decreased expression of the components of the NADPH oxidase itself. Thus, although up-regulation of gp91 and p47 after IFN-γ treatment may be responsible for the overall increase in the potential rate of the respiratory burst, it is more likely that IFN-γ corrects the respiratory burst defect in GSD 1b MDM by modulation of receptor expression and/or the signal transduction pathway through changes in intracellular Ca^{2+} levels.

The morphologic changes associated with IFN-γ treatment of GSD 1b MDM suggests an IFN-γ–mediated effect on signal transduction. GSD 1b MDM did not show cytoplasmic spreading or morphologic alterations concomitant with monocyte differentiation unless cultured in the presence of IFN-γ, which then resulted in morphologic changes similar to control cells. After adherence, a rise in intracellular Ca^{2+}, similar to that in respiratory burst activity, is a necessary precedent to spreading in neutrophils (45). We have previously demonstrated depressed Ca^{2+} mobilization in GSD 1b monocytes that is associated with decreased intracellular Ca^{2+} stores. This is suggestive of a common signaling defect affecting cytoplasmic spreading and respiratory burst activity in GSD 1b patients, possibly at the level of or before Ca^{2+} mobilization.

The mechanism(s) by which IFN-γ corrects the deficient ligand-elicited respiratory burst in GSD 1b MDM remains to be elucidated. Defining the specific alteration(s) by which IFN-γ normalizes the deficient respiratory burst activity in GSD 1b MDM will provide further insight into the defect in GSD 1b respiratory burst activity. In summary, the O_2^- production in MDM from GSD 1b patients was depressed in response to fMet-Leu-Phe and Con A but not PMA stimulation. This selective defect in ligand-triggered O_2^- generation can be corrected *in vitro* with IFN-γ, which suggests that IFN-γ should be considered for the treatment of this defect in patients with GSD 1b.

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