*short report*
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# NADPH OXIDASE ACTIVITY AND CHEMOTAXIS BY NEUTROPHILS IN TWO PATIENTS WITH GLYCOGEN STORAGE DISEASE TYPE Ib TREATED WITH RECOMBINANT HUMAN GRANULOCYTE-MONOCYTE COLONY-STIMULATING FACTOR

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**ABSTRACT**
**Polymorphonuclear neutrophils play an important role against pathogens through the production of toxic oxygen metabolites by the NADPH oxidase enzyme, which reduces oxygen to superoxide anion in the respiratory burst. Neutropenia, infectious complications and impaired neutrophil function are often reported in glycogen storage disease type Ib (GSDIb), a metabolic disorder characterized by increased glycogen and decreased glucose-6-phosphatase (G-6-P) activity in the liver. Two children with GSDIb and associated neutropenia with recurrent bacterial infections were treated daily with different doses of rHu-GM-CSF. NADPH oxidase activity and chemotaxis in patients were assessed before and during therapy in stimulated and unstimulated neutrophils. During rHu-GM-CSF treatment, any increase found in the NADPH oxidase activity of patients was not significant with respect to that in controls. In one patient chemotaxis was greater than of controls. This finding suggests that in patients with GSDIb both neutropenia and PMN abnormalities may be responsible for infections, and PMN dysfunction probably depends on the degree of inherited functional G-6-P deficit.**

*Key words: NADPH oxidase, GSDIb, rHu-GM-CSF, neutropenia, infections*



Polymorphonuclear leukocytes (PMN) release oxygen free radicals in infection sites. Superoxide anion ($O_2^-$) is produced primarily through the activation of the plasma membrane-bound NADPH oxidase complex (respiratory burst oxidase [EC 1.6.99.6]) that requires the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) for the conversion of molecular oxygen to superoxide anion.[1] Glycogenosis Ib (GSD Ib) is a metabolic disease due to G-6-P translocase deficiency; about two thirds of these patients present associated chronic severe neutropenia with neutrophil dysfunction and are susceptible to recurrent oral and perianal mucosal infection and inflammatory bowel disease, such as Crohn's disease.[2] Recent reports have shown the effectiveness of GM-CSF or G-CSF treatment against GSDIb infections. Elsewhere we described two children with GSDIb and the effect of treatment with rHu-GM-CSF on NADPH oxidase activity and chemotaxis.[3]

### *Case report*

Two patients (AA, a 10-year-old boy and CP, a 7-year-old boy) with hepatomegaly, fasting hypoglycemia and hyperlactacidemia were diagnosed as having GSDIb on the basis of findings from enzymatic investigation of fresh liver biopsy. Neutropenia (PMN $<0.8\times10^9$/L) was associated with severe recurrent infections: oral

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mucosal ulceration and perianal abscesses. Since antibiotic therapy was ineffective, the children were treated subcutaneously for 12 and 15 months, respectively, with rHu-GM-CSF, (in AA at a dosage of 3 µg/kg/day for 13 days, which was subsequently increased to 5 µg/kg per day; in CP at 5 µg/kg/day for 7 days and then 3 µg/kg/day for 8 days). After a pause of 11 days the treatment was repeated at a dose of 3 µg/kg/day, then every other day for 16 days and, finally, at a definitive dosage of 3 µg/kg/day.

### *Materials and Methods*

PMN obtained from whole blood samples collected by venipuncture and anticoagulated with EDTA (Vacutainer Systems, Becton-Dickinson, Meylan, France) were separated on a Ficoll-Hypaque density gradient (Histopaque-1077, Histopaque-1119, Sigma Diagnostics, St. Louis, Mo., USA). Fifty µL of the PMN suspension at $5\times10^9$ PMN/L were incubated at room temperature in the individual flat-bottom wells of a polystyrene microtiter plate (Kima, Piove di Sacco, Padua, Italy) with 50 µL of PBS and 50 µL of phorbol myristate acetate (PMA) (1.625 µmol/L, Sigma Diagnostics, Mo, USA) to initiate the respiratory burst. After 10 min. of mixing, 50 µL of NBT (2.4 mmol/L, Sigma Diagnostics, Mo., USA) were added to each individual well and the rate of NBT reduction was monitored at 490 nm for 30 min. by a photometer for microplates (Autoreader II, Ortho Diagnostic Systems, Milan, Italy). Each test was carried out five times and was also performed with resting PMN, without stimulation of the respiratory burst.[4] NADPH oxidase activity was expressed as the mean of absorbance (A) values in the 30-min period and measured as $A\times10^{-3}$/min. The coefficient of variation (CV%) of five replications was calculated for twenty samples (CV 5.09%, range 1.41 to 10.55%).

Cell migration was measured with the modified double chamber filter method of Boyden, using blind-well chambers (Costar, Nucleopore Italia, Concorezzo, Milan) with a volume of 200 µL Zymosan-activated serum (ZAS) or control medium (PBS) in the bottom of the well and a 200 µL PMN suspension containing 500 cells/µL in the top compartment, separated by 5 µm pore polycarbonate PVP free filters (Costar, Nucleopore Italia, Concorezzo, Milan). Following an incubation of 60 min. at 37°C, the filters were fixed in methanol, stained with May-Grünwald Giemsa solution, dried and mounted on a Bürker hemocytometric chamber for counting. The results were expressed as neutrophil number/$\times10^9$/L(mean±SD) in the bottom side filter.

The tests were performed within 5 h. of blood collection.

### *Results*

Before rHu-GM-CSF treatment no abnormalities in PMN function were observed in the two patients. Administration of rHu-GM-CSF increased the mean average PMN number in both of them: from below 0.60±0.15 to above 2.10±0.68 in AA and from 0.92±0.27 to 2.67±1.55 PMN$\times10^9$/L in CP; however, this treatment did not significantly enhance NADPH oxidase activity values, and neither patient showed a significant increase in enzyme activity with respect to daily controls (unpaired t-test, Table 1) .

Unfortunately, chemotaxis could not be performed before rHu-GM-CSF therapy because of the patients' neutropenic state; it was significantly increased in both of them without stimulation (AA 81±58, controls 8.3±15, $p=0.05$; CP 43.6±46.9, controls 6±9.2, $p=0.04$) and in only one (AA) with ZAS stimulation (AA 211±18, controls 34.6±18.5, $p= 0.002$; CP 138±63.5, controls 76± 12.2, p=n.s., paired t-test).

### *Discussion*

Neutropenia, a constant feature of GSDIb, is not related to metabolic control of the disease or to therapy. Studies on functional tests of neutrophils from patients with this condition have reported contradictory results. Most of the reports have documented various quantitative and qualitative anomalies in PMN, such as diminished chemotaxis, decreased respiratory burst, diminished random and directed migra-

| | *NADPH oxidase activity in PMNs before rHu-GM-CSF treatment* | |
|---|---|---|
| | resting PMNs | PMA-stimulated PMNs |
| Controls (n=2) | 1.22 | 3.44 |
| A.A. (n=2) | 1.15 | 1.39 |
| Control (n=1) | 1.47 | 4.15 |
| C.P. (n=1) | 1.40 | 2.80 |
| | *NADPH oxidase activity in PMNs during rHu-GM-CSF treatment* | |
| | resting PMN | PMA-stimulated PMNs |
| Controls (n=23) | 1.02 ± 0.43 | 2.63 ± 1.04 |
| A.A. (n=23) | 0.77 ± 0.61 | 2.45 ± 1.94 |
| Controls (n=23) | 1.19 ± 0.76 | 3.01 ± 1.18 |
| C.P. (n=23) | 1.36 ± 0.89 | 2.69 ± 1.27 |

Table 1. NADPH oxidase activity in isolated neutrophils of GSDIb patients before and during rHu-GM-CSF treatment. Superoxide anion production was measured as NBT reduction at 490 nm in a microplate reader. The absorbance values were expressed as A x $10^{-3}$/min (mean±SD). NADPH oxidase activity by neutrophils from the patients was compared to daily normal controls, and neither patient showed a significant increase in enzyme activity with respect to controls, *(unpaired t-test).*



tion, decreased nitrozolium blue test reduction and a defect in bactericidal activity.[5-7] Bone marrow examination in GSDIb patients sometimes shows maturation arrest and moderate myeloid hyperplasia without ultrastructural cytologic abnormalities.[2] Patients presenting neutropenia are susceptible to infections and some, including ours, have normal PMN function.[8]

GSDIb is an autosomally transmitted recessive condition with genetic heterogeneity. The relationship between neutrophil abnormalities and metabolic aberrations in glycogenosis is not fully understood. PMN dysfunction in GSDIb is probably related to the degree of G-6-P defect inherited, and in patients who have a greater enzyme deficiency PMN abnormalities may be consequent to impaired glucose metabolism due to a defect in glucose uptake, as recently described by Bashan *et al.*[9] In one patient, who was treated with higher doses of rHu-GM-CSF, chemotaxis was increased with respect to control. In fact, GM-CSF primes PMN for chemoattractants to increase fMLP receptor availability.[10]

The nature of the neutropenia remains unclear, although it undoubtedly caused the infections observed in our subjects. In order to prevent infections, patients with GSDIb have been treated with lithium or corticosteroids and antibiotics. Our patients were given rHu-GM-CSF because antibiotic therapy proved ineffective. RHu-G-CSF has been widely and successfully used in the treatment of neutropenia in Shwachman's syndrome[11] and its clinical applicability is steadility expanding.[12] RHu-GM-CSF has been less commonly used but it has been observed that GM-CSF has a greater effect on neutrophil function than G-CSF, since the latter is less potent and more selective in its stimulation of neutrophils.[10]

In conclusion, rHu-GM-CSF treatment resulted in the prompt correction of neutropenia, produced a dramatic decrease in the frequency and severity of infections and eliminated mouth ulcers, resulting in markedly improved quality of life in these GSDIb patients.

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