Title: Partial correction of neutrophil dysfunction by oral galactose therapy in glycogen storage disease type Ib

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Abstract

Glycogen storage disease type Ib (GSD-Ib) is characterized by impaired glucose homeostasis, neutropenia and neutrophil dysfunction.

Mass spectrometric glycomic profiling of GSD-Ib neutrophils showed severely truncated N-glycans, lacking galactose. Experiments indicated the hypo-glycosylation of the electron transporting subunit of NADPH oxidase, which is crucial for the defense against bacterial infections. In phosphoglucomutase 1 (PGM1) deficiency, an inherited disorder with an enzymatic defect just one metabolic step ahead, hypogalactosylation can be successfully treated by dietary galactose. We hypothesise the same pathomechanism in GSD-Ib and started a therapeutic trial with oral galactose and uridine. The aim was to improve neutrophil dysfunction through the correction of hypoglycosylation in neutrophils. The GSD-Ib patient was treated for 29 weeks. Monitoring included glycomics analysis of the patient’s neutrophils and neutrophil function tests including respiratory burst activity, phagocytosis and migration. Although no substantial restoration of neutrophil glycosylation was found, there was partial improvement of respiratory burst activity.
Key words

glycogen storage disease; galactose; CDG; neutrophil dysfunction; glycosylation

Abbreviations

CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CDG, congenital disorder of glycosylation; DHR, dihydrorhodamine; E. coli, Escherichia coli; EDTA, ethylenediaminetetraacetic acid; FICT, fluorescein isothiocyanate; fMLP, formyl-methionyl-leucyl-phenylalanine; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; G6PC, glucose 6-phosphate catabolic; G6PT, glucose 6-phosphate transporter; GCSF, granulocyte-colony stimulating factor; GSD-1b, glycogen storage disease type 1b; HMPS, hexose-monophosphate shunt; HPLC, high performance liquid chromatography; LacNAc, N-acetyllactosamine; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight mass spectrometer; MS, mass spectometric; NADPH, nicotinamide adenine dinucleotide phosphate; PBMC, peripheral blood monocyte; PBS, phosphate-buffered saline; PGM1, phosphoglucomutase 1; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; UDP-Gal, uridine diphosphate galactose; UDP-Glc, uridine diphosphate glucose

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1. Introduction

Glucose 6-phosphate (G6P) is a central intermediate in the hexose monophosphate shunt (HMPS) and is closely linked to glycogen and galactose metabolism through isomerisation to glucose 1-phosphate (G1P) which subsequently can undergo epimerisation to galactose-1-phosphate (Fig. 1).

It also is part of the final pathway of gluconeogenesis and glycogenolysis. In the final steps of these pathways the glucose 6-phosphate transporter (G6PT) translocates G6P into the lumen of the endoplasmic reticulum (ER) where it is hydrolysed by glucose 6-phosphatase (G6Pase) to glucose and inorganic phosphate (1).

There are three G6Pase activities, G6PC1, G6PC2 and G6PC3, in humans. Localized in the lumen of the ER, G6PC1 is predominantly expressed in liver and kidney but also at lower levels in intestine, G6PC2 is expressed in the pancreas, while G6PC3 is expressed ubiquitously (1-4).

Mutations in the gene G6PC1 cause the metabolic disorder glycogen storage disease type Ia (GSD-Ia) (5). Patients suffer from severe hypoglycemia, hepatomegaly, lactic acidosis, hyperuricemia, hyperlipidemia and hypoglycemic seizures starting early in life (6). G6PC3 deficiency is associated with neutropenia and neutrophil dysfunction, but not with the metabolic phenotype of GSD-Ia (7).

Both enzymes are dependent on the transport of the substrate G6P into the lumen of the ER accomplished by the G6PT (8, 9). This transporter is deficient in glycogen storage disease type Ib (GSD-Ib) (Fig. 1) and is encoded by the gene SLC37A4 (10). GSD-Ib patients show a mixed phenotype of both diseases mentioned previously: dysregulated glucose homeostasis and neutropenia with neutrophil dysfunction (11, 12). Since the discovery of G6PC3 and its functional complex with G6PT (3), studies of neutrophil dysfunction and neutropenia have focused on these two enzymes. Investigations on human and murine neutrophils with inactivating mutation in either G6PT or G6PC3 showed that both deficiencies lead to
increased ER stress and apoptosis (7, 13, 14). Further studies on both diseases revealed that cycling G6P/glucose is significant for energy homeostasis and their impairment is associated with neutrophil dysfunction (15-17). The therapy available to face these problems is based on administration of granulocyte-colony stimulating factor (GCSF), that increases numbers but does not affect function (18). In addition, vitamin E supplementation seems to be another suitable option to improve neutropenia (19). There is evidence, that vitamin E, acting as an antioxidant agent, can improve neutropenia and reduce the frequency and severity of infections in GSD-Ib patients.

Hayee et al. (20) introduced a new explanation for neutrophil dysfunction in patients with deficiency in G6PT and those lacking G6PC3 and built a bridge between both syndromes and congenital disorders of glycosylation (CDG). Their research revealed that the electron-transferring subunit of the neutrophil enzyme complex nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, gp91phox, is hypoglycosylated in these patients. High-sensitivity mass spectrometric (MS) methodologies revealed a deficient galactosylation in complex-type antennae of N- and O-glycans expressed by neutrophils.

CDG is a rapidly growing group of multisystem disorders caused by inherited enzyme defects in the biosynthesis of glycans and their attachment to proteins or lipids. In addition, CDG can also be caused by defects in pathways involving the provision of substrates and precursors of oligosaccharide synthesis or disorders of translocation of required sugars into the right compartment (21). Recently, phosphoglucomutase 1 (PGM1) deficiency, also named glycogen storage disease XIV, could be identified as a CDG (22). MS analysis of serum transferrin of PGM1-deficient patients demonstrated truncated glycans lacking galactose. It is most likely that the lack of UDP-galactose (UDP-Gal) is the underlying pathomechanism. There is evidence that the defective conversion of G6P to G1P and vice versa, accomplished by PGM1 leads to a decreased ratio of UDP-Gal to UDP-glucose (UDP-Glc), suggesting that UDP-Gal is the limiting factor for N-glycosylation. This assumption is supported by the fact...
that oral galactose supplementation leads to the almost complete disappearance of truncated glycans lacking galactose as well as normalization of the UDP-Gal to UDP-Glc ratio in PGM1-deficient cells when treated with galactose and uridine.

These results gave rise to the question whether galactose supplementation could improve hypoglycosylation in GSD-Ib. Deficient G6P transport through the ER membrane could lead to an accumulation of cytoplasmatic G6P and G1P (Fig. 1). This in turn would lead to an increased ratio of UDP-Glc over UDP-Gal which could cause protein hypogalactosylation analogous to the previously mentioned pathomechanism.

In order to verify this hypothesis we treated a GSD-Ib patient with oral galactose for 6 weeks, followed by a galactose-free interval of 9 weeks, and then a second oral galactose regime lasting 23 weeks. To exclude a lack of uridine as a limiting factor, uridine was added for the last 9 weeks of treatment. For monitoring, we analysed neutrophil glycosylation by mass spectrometry and investigated neutrophil function at fixed time points. No lasting effect on the hypoglycosylation of the complex-type N-glycans was observed. Nevertheless, neutrophil function partially improved, as revealed by the production of reactive oxidant species (ROS) in response to phorbolmyristyl acetate (PMA).
2. Materials and Methods

2.1. Case report

The clinical phenotype of the 23 year old patient is a short stature, a doll-like-face with prominent cheeks, inflamed gums, hepatoo- and splenomegaly as well as neutropenia. Diagnosis of a GSD type Ib was delayed until she was 6 months of age, suffering from multiple hypoglycemic seizures that were incorrectly interpreted as myoclonia. Hypoglycemia is avoided by frequent feedings (10 meals) in the daytime and continuous noctural nasogastric infusion.

Her statomotor and psychomotor development was impaired. She began to walk at the age of two years. At the age of 4 years, she spoke two-word phrases.

Treatment with GCSF began in the first year of life after an episode of Crohn-like disease requiring partial colectomy. With GCSF therapy neutrophils fluctuated around an average of 1.58 tsd/µL (n=16). In isolated cases the degree of moderate (0.5-1 tsd/µL) or even a severe neutropenia (<0.5 tsd/µL) was reached. Due to the treatment, splenomegaly and hypersplenism occurred. A partial splenic embolisation was performed at the age of 8 6/12 years.

At the age of 2 years, two episodes of seizures occurred, the latter a status epilepticus. A treatment with anticonvulsants was initiated. Initially triggered by high temperatures or hypoglycemic states, seizures were later independent from these factors. Several adaptations of anticonvulsant therapy were required.

Despite GCSF treatment and neutrophil counts close to the reference range, the patient suffered from a number of different serious infections. Most of them required antibiotic treatment and many of them a hospitalization.

Already in the first year of life, due to peritonitis and pararectal infection caused by Crohn-like disease, the patient received a colostomy. In the following years, numerous ear infections,
chronic gum inflammation, various abscesses requiring surgical intervention, bronchiolitis,
several infections of the bladder, pneumonia, and other minor infections occurred.
Due to frequent infections, a permanent antibiotic prophylaxis was started in September 1996
and was continued for nearly 10 years, till July 2006. Nevertheless it could not prevent the
frequent infections mentioned above.
Informed consent was obtained from parents in their authorized capacity. The local Ethics
Committee approved the study.

2.2. Mutation analysis
Mutations were characterized in SLC37A4 performed as described previously (23). DNA was
analyzed by ABI Prism 3700 capillary electrophoresis system (Applied Biosystems®, Life
Technologies GmbH, Germany).

2.3. Patient blood
Blood was drawn from a peripheral vein for standard clinical laboratory measurements. For
MS-analysis, neutrophil pellets were isolated from EDTA blood. For mutation analysis EDTA
blood was collected from the patient and both parents.

2.4. Isolation of neutrophils
Neutrophils were isolated as described previously (20). Briefly, ETDA blood was diluted with
phosphate-buffered saline (PBS) and layered over Ficoll-Paque™ (GE Healthcare Bio-
Sciences AB, Uppsala). After centrifugation the peripheral blood monocyte (PBMC) fraction,
which lay above the Ficoll-Paque gradient, was removed. Neutrophils, which sedimented with
erthrocytes below the Ficoll-Paque gradient were isolated by sedimentation with dextran
solution. Neutrophils containing supernatant was separated and treated by hypotonic lysis of
red blood cells. The neutrophil pellet was snap-frozen and freezed at -80°C.
2.5. Dietary supplementation

Galactose and uridine was supplied by Vitaflo Pharma GmbH, Bad Homburg, Germany.

Galactose and uridine supplementation was administered as aqueous solution in 5 daily doses.

After increase of the dose at week 20 up to 1.5 g per kilogram body weight daily, the additional 0.5 g per kilogram per body weight daily was administered through nasogastric drip continuously over night. In the last 9 weeks of treatment, 150 mg of uridine per kilogram of body weight per day were added. \( \frac{2}{3} \) of uridine was administered in 5 daily doses and \( \frac{1}{3} \) continuously over night.

2.6. Determination of total and differential leukocyte counts

Total and differential leukocyte counts were determined by standard laboratory procedure.

2.7. Galactose 1-phosphate and glycogen quantification in erythrocytes

Galactose 1-phosphate concentration in red cells was determined using (C-14) UDP-Gal and a mini column chromatography as described elsewhere (24). Glycogen level was determined by a spectrophotometric measurement of glucose using amylglucosidase as described previously (25).

2.8. Sonography of the liver

In the course of the treatment, the liver investigated by standarizied b-mode sonography of the abdomen.

2.9. Glycomic profile analysis

For glycomics experiments, isolated neutrophils were subjected to sonication in the presence of detergent (CHAPS) and treated as described previously (26). Permethylated N-glycans
prepared were analyzed on a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Life Technologies), as described previously (20).

2.10. Leukocyte function

Oxidative burst reaction in leukocytes was tested with the Phagoburst™ (Glycotope Biotechnology, Heidelberg) assay in whole blood according to the manufacturer’s instructions. In summary, unlabeled opsonized E. coli bacteria and PMA serve as stimulants to determine the percentage of phagocytic cells which produce ROS, and thereby converting dihydorhodamine (DHR) to rhodamine, analysed by flow cytometry with a BD Facscanto 337175 cell analyser (BD Biosciences, Franklin Lakes, NJ). Further characterization of leukocyte function included phagocytosis (Phagotest™ Glycotope Biotechnology, Heidelberg) and migration of phagocytes (Migratest™ Glycotope Biotechnology, Heidelberg). Both assays were performed according to the manufacturer’s instructions. In short, for Phagotest™ FITC-labelled opsonized E. coli bacteria were incubated for 10 minutes with whole blood. Quantitative determination of phagocytosis was measured via flow cytometry (BD Facscanto). For Migratest™ patient leukocytes were placed in cell culture inserts with a pore size of 3,0 µm and were allowed to migrate towards a concentration gradient of the chemoattractant fMLP, which allows determination of successfully migrated neutrophils. Migrated neutrophils were identified and quantified by flow cytometry (BD Facscanto) and additionally L-selectin shedding of activated cells was measured.
3. Results

3.1. Patients and parents genotype

DNA sequencing of all eight exons of the G6PT gene, SLC37A4, confirmed the diagnosis of GSD-Ib. The patient is homozygous for the mutation c.411G>A (W137X) in exon 3. Both parents have the same mutation in the G6PT gene in the heterozygote state.

3.2. Clinical phenotype

Infections during the last 3 years are summarized in Figure 3. As shown in the graphic, in the last 3 years the patient suffered from various purulent skin infections. When galactose therapy was started, the patient had an abscess in her right cheek and left axilla. Both were treated with antibiotics and both necessitated surgical intervention. One month later, again an abscess of the left axilla required incision and antibiotic treatment. Apart from the ongoing inflammation of the gum, the patient showed no new infections or abscesses during the whole period of therapy for 32 weeks. Purulent skin inflammation did not reoccur for more than half a year after stopping treatment.

3.3. Determination of the total and differential leukocyte counts

Absolute neutrophil counts over time are summarized in Figure 2. Despite daily treatment with GCSF (300 µg, 2 times a day) the patient suffered most of the time from neutropenia. Twice her neutrophil count reached the normal range in week 29 and 34, but decreased again before stopping treatment.

3.4. Sugar metabolite and glycogen in erythrocytes

Galactose 1-phosphate and glycogen concentrations in erythrocytes were normal for the whole period of therapy.
3.5. Sonography of the liver

Liver dimensions were measured at 4 fixed time points (week 0, 5, 24, 38). The anterior axillary line varied from initial 29 cm to 20 cm at week 5 and again 29 cm in week 24. In week 38 the liver measured 21 cm in the midclavicular line, 2 cm off the previous measurement.

3.6. Neutrophil N-glycans

Glycome analysis of the healthy and GSD-Ib neutrophils were in agreement with the studies of Hayee et al. (20). Control neutrophils showed N-glycans including complex up to tetraantennary structures mainly terminated with sialic acis and Lewis^X epitopes, many of them extended with N-acetyllactosamine (LacNAc) units (Fig. 5A). Only a minority of core-fucosylated bi- and tri-antennary structures were found to be agalactosylated. In contrast, neutrophils of the GSD-Ib patient showed truncated antennae, deficient in galactose, which were not detected in the healthy control. In order to compare the glycomes of the samples over time, we calculated ratios of the relative abundances of the ions for agalactosylated tetra-antennary N-glycans (m/z 2326 and 2530) to their mature tetra-antennary N-glycan counterparts, capped with galactose (m/z 3503, 4039, 4213 and 4400). According to the hypothesis, these ratios should decrease with time. In the course of therapy they fluctuated but showed no clear decrease (see Fig. 4).

3.7. Leukocyte function

In accordance with the observation of Hayee et al. the oxidative burst reaction in leukocytes of the GSD-Ib patient (48% ROS positive cells after PMA stimulation) was diminished compared with the result of control (99% ROS positive cells) before treatment. The difference of the E.coli stimulated neutrophils, 81% positive cells in patient, compared to 97% in control,
was smaller. After supplementation of galactose, ROS production improved to a peak value of 87% positive cells after PMA stimulation and 95% after the stimulation with E.coli. This value was reached after 14 weeks of continuous treatment with galactose (4 weeks with 1g/kg BW/day and 10 weeks with 1,5g/kg BW/day). The additional of 150mg/kg BW/day uridine for a further 9 weeks resulted in no further improvement. Stopping the treatment led to a decrease of ROS production in patients leukocytes again to 65% positive cells after PMA stimulation and 76% after stimulation with E.coli. While the ROS positive leukocytes presented a peak closely to the negative control (unstimulated cells) (dashed line in Fig. 5) before treatment, a second peak appeared after supplementation of galactose with a more robust respiratory burst than initially. This second peak remained in the course of therapy and disappeared after the end of treatment. Taken together, this data suggest that galactose supplementation induced a new population with a more robust respiratory burst than the initial population. The latter did not vanish during treatment. Phagocytic activity was normal and did not change during therapy (Fig. 7).

3.8. Migration and L-selectin shedding

Before therapy with galactose was started, 31.7% of patient neutrophils were able to migrate towards a concentration gradient of fMLP in comparison to 71.8% of healthy control neutrophils. Migratory activity of patient neutrophils was impaired and could not be restored by galactose therapy (18.1% migrated neutrophils after 1 month of galactose treatment). In addition, L-selectin shedding of migrated neutrophils was measured by flow cytometry, indicating activation of migrated cells. Only 6.15% of migrated cells were L-selectin negative after migration, indicating a hampered activation of these cells, which could not be restored with galactose therapy (4.42% negative cells after 1 month of galactose treatment).
4. Discussion

In this study, the amount of oral galactose and uridine sufficient to correct PGM1-deficiency, could not improve the hypoglycosylation of neutrophils in a GSD-Ib patient. The ratio of agalactosylated tetra-antennary N-glycans to mature tetra-antennary N-glycans fluctuated without a clear decrease during the course of therapy. Furthermore, it could be shown that after supplementation of galactose a subpopulation of neutrophils arose, whose respiratory burst after PMA stimulation was comparable to that in control. This subpopulation diminished 4 weeks after interrupting oral supplementation.

The underlying biochemical cause of hypoglycosylation in GSD-Ib remains unclear. Having in mind the results of Hayee et al. that the stage of truncated glycans observed in GSD-Ib patients could be attributed to low levels of UDP-Gal, the main question is how the lack of UDP-Gal could be explained. Similar to the pathway affected in PGM1-deficiency (22), the cause of hypoglycosylation remains comprehensible. The deficient G6P transport through the ER membrane should lead to an accumulation of G6P and, after isomerisation, G1P. This in turn may lead to an increased ratio of UDP-Glc over UDP-Gal, which could cause protein hypogalactosylation. But this hypothesis is not supported by previous studies that intracellular G6P levels in GSD-Ib neutrophils are decreased (17). Moreover, the concentration of UDP-Gal in leukocytes from subjects with low-lactose diets, similar in patient with standard dietary therapy for GSD-Ib (27, 28), did not differ from that determined in normal individuals (29). Further investigations on GSD-Ib cells and their metabolite concentrations are necessary.

Nevertheless, studies of the effect of galactose loading on UDP-Gal concentration in leukocytes in normal subjects showed an exaggerated response on oral administered galactose (30). After a dose of 20 g galactose, UDP-Gal concentration in leucocytes increased more than threefold over the normal range, similar to those who achieved a 50g galactose dose. The ratio of UDPGlc/UDPGal decreased from 1,87 ± 0,13 to 0,17 ± 0,09 after both doses, the
lower with a briefer duration. At 30 minutes after oral ingestion of 20 g galactose the plasma concentration achieved a peak of 0.54 mM. At a comparatively lower dose of 12g we were unable to reach comparable results in the study of galactose kinetic of the GSD-Ib patient (data not shown). The lack of increase may be attributed to the fact that the patient was not tested under optimal conditions, e.g. previous fasting or fasting while measurement of galactose kinetic, which we made for two reasons. First, we want to simulate the galactose blood kinetic in the normal daily routine. Secondly, it is not possible to keep a GSD-Ib patient without eating, since patients are always at risk of developing hypoglycemia, considering the fact that galactose does not raise blood glucose due to the defect of these patients. The question is whether the small amount of ingested galactose was sufficient to increase UDP-Gal level in leukocytes. On the other hand, the amount of administered galactose in patient with GSD-Ib is limited by the risk of lactate acidosis (28). This is, by the way, the only side effect of galactose in GSD-Ib that poses a problem and can be handled by regular blood tests. The risk of increased storage of glycogen can be managed by reduced calories in diet. To handle the problem of increased glycogen storage due to the galactose supplementation, we monitored the glycogen concentration in erythrocytes and the liver dimensions by sonography before and in the course of therapy. Erythrocyte glycogen concentrations were in normal range during the whole course of therapy. Dimensions of the liver were most likely dependent on different physicians. The hazard of the deterioration of the metabolic situation was taken into account by monitoring the blood sugar by obligate measurements and sampling measurements of blood lactate 2 or 3 hours after ingestion of galactose. It was at no time hazardous. However, in PGM1-deficiency the dietary supplementation of 0.5 to 1.0 g galactose per kilogram per day, divided into three to six daily doses could improve hypoglycosylation on transferrin. Probably the origin of transferrin plays a crucial role. Transferrin is liver derived, like most serum glycoproteins. It is important to bear in mind that galactose is subject to the
first-pass effect. Therefore, there is an amount of galactose for disposal of the hepatocytes and the glycosylation of liver derived proteins. Due to the fact that transferrin is formed by the liver, G6PC3 deficient patient will not be diagnosed by classic diagnostic CDG blood test, because the liver uses G6PC1 instead of G6PC3. However, G6PT is expressed ubiquitously, suggesting that glycosylation defects also concern transferrin. However, transferrin glycosylation analyzed by high performance liquid chromatography (HPLC) was normal in the GSD-Ib patient (data not shown).

We further investigated the respiratory burst of neutrophils in response to PMA and E.coli stimulation by flow-cytometry. This assay is commonly used as a neutrophil function test in order to detect ROS production (31). ROS are used to harm pathogens directly by oxidizing damage as well as indirectly by nonoxidative pathways like inducing phagocyte death (31).

Despite the fact that some viruses, bacteria and protozoans are capable to thrive under oxidative stress or even enhance pathogen burden most bacterial infections are partially vulnerable to ROS production (32, 33). Therefore it can be assumed that the ability of ROS production, especially the provision of large amounts of ROS required for effective killing, is associated with more potent microbial killing capacity (33, 34). However, the neutrophils have to be recruited to the inflammation site to enable the deadly effect. This recruitment includes rolling, adhesion and transmigration of the neutrophils and is impaider in neutrophils deficient in G6PT, as we could show through the ability to migrate in response to fMLP (35).

Taking into account the clinical phenotype of our patient diminished microbicidal killing capacity is obvious in chronical gum inflammation and recurring various purulent skin infection. Recording these events during therapy and beyond, the missing purulent skin inflammation for up to twelve months supports increased microbial killing capacity caused by increased ROS production.

On the other hand impaired cell migration could not be restored with galactose supplementation. It even seems that cell migration deteriorated during therapy as the
decreased responses to fMLP revealed. First, impaired cell migration in G6PT-deficient neutrophils can not be attributed to altered fMLP receptor expression (36). Further, fMLP signaling seems not to be affected by galactose supplementation demonstrated by ROS production. Compared to PMA, fMLP is a much weaker activator of respiratory burst and supplied ROS is most likely part of signal cascade promoting migration (31). Interestingly results of ROS production in response to fMLP differ only slightly compared to a healthy control (data not shown). Moreover, galactose therapy did not influenced ROS production stimulated by fMLP. Consequently it can be assumed that decreased migratory response of patient’s neutrophils to fMLP during therapy is not due to lower level expression of the fMLP receptors or dampened receptor signaling. The reason for further reduction migratory capacity in response to fMLP during oral galactose suplementation remains unclear.

It is known that respiratory burst is affected by antibiotic therapy (37, 38). At the beginning of the galactose supplementation the patient was treated with amoxicillin in combination with clavulanic acid. Reynaret et al. showed that amoxicillin acts as an activator of oxygen production without any other stimuants, but does not seem to influence oxygen production induced by PMA (38). Thus, the initial value of the respiratory burst activity of the GSD-Ib patients neutrophils was not due to antibiotic therapy. Another course of antibiotic therapy was necessary from week 8 until week 11 due to an abscess in the left axilla. Initially treatment consisted of vancomycin in combination with flucloxacillin, followed by cefuroxim. How far these antibiotics affected the respiratory burst cannot be estimated. For the beta-lactam antibiotic penicillin G no effect on respiratory burst was found (37).

Because of the evidence of hypoglycosylation of the GSD-Ib neutrophils the central question is, whether the described mechanism is responsible for neutrophil dysfunction. Despite the observation that neither assembly of the oxidase complex nor electron transport are subject to mature glycosylation of gp-91^{phox} in cell-free assay (20), hypoglycosylation...
could lead to a decreased respiratory burst. It can be assumed that a sub-population with improved hypoglycosylation was too low, to generate a adequate signal in MS analysis.

Even if the assembly of the oxidase complex does not require glycosylation, as DeLeo et al. could show in tunicamycin-treated cells, an inhibitor of N-linked glycosylation, the treatment also results in a reduced amount of the respective proteins (39). It is therefore conceivable that the reduced amount of proteins leads to reduce oxygen production. To pursue the question, whether restoration of hypoglycosylation is responsible for improved respiratory burst activity, it would be necessary to isolate the subpopulation with robust ROS production, to analyze its glycomic profile separately.

The observed neutropenia in GSD-Ib could be caused by either decreased production of neutrophils in bone marrow, enhanced levels of apoptosis in circulating neutrophils with shortened lifespan, or increased egress to inflamed tissues (40-42). As Visser et al. could show hematopoietic progenitors of GSD-Ib patients cultured in medium containing high concentration of glucose up to 450 mg dL\(^{-1}\) (25 mmol L\(^{-1}\)) led to normal percentage and absolute numbers of differentiated neutrophils (42). These observations are supported by Jun et al. (17), who could show decreased glucose uptake and reduced levels of intracellular G6P in GSD-Ib neutrophils. Having this in mind, the isolated increased neutrophil count in the course of therapy could be credit to the additional galactose offered, despite the low concentration.

Due to the lack of evidence of improved hypoglycosylation, the hypothesis that the N-glycosylation on gp-91\(^{phox}\) may protect from the degenerative contents of the phagocytic vacuole, which may account for reduced respiratory burst can not be verified (43).

In summary, these findings suggest that the hypoglycosylation of GSD-Ib neutrophils may not be attributed to the hypothesized pathway. As a result, it remains unclear whether the hypoglycosylation in gp-91\(^{phox}\) can account for neutrophil dysfunction and neutropenia in GSD-Ib patients. Nevertheless, the observation that dietary galactose supplementation led to a
rising subpopulation with robust ROS production gave rise to the question which role
galactose plays in sugar metabolism of neutrophils in GSD-Ib. CDG patients with
hypogalactosylation have been treated with up to 3.75 g galactose per kg body weight and day
(44). Treatment with higher galactose amounts in GSD-Ib may be worthwhile.
Authorship

R.L. and T.M. designed and performed the research and wrote the manuscript. A.A., S.H. and A.D. performed glycomic profile analysis of neutrophils. H.W. and D.F. performed respiratory burst assay. T.P. performed Galactose 1-phosphate and glycogen quantification in erythrocytes.
Conflict of Interest Disclosure

The authors declare no conflict of interest.
References


