Abnormal Distribution of Complex Carbohydrates in Neutrophils of a Patient With Lactoferrin Deficiency

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Previous studies have identified patients with susceptibility to bacterial infection associated with lactoferrin deficiency in dysmorphic neutrophils containing abnormal or no secondary granules and abnormal nuclear segmentation. We have investigated the subcellular distribution of vicinal glycol-containing complex carbohydrates in marrow and blood myeloid cells of such a patient using the periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP) staining method and have examined the response of these neutrophils to the degranulating agents N-formylmethionyl-leucyl-phenylalanine (FMLP) and phorbol myristate acetate (PMA). As in normal specimens, immature primary granules were strongly PA-TCH-SP reactive; however, unlike normal specimens, masking of PA-TCH-SP reactivity did not occur in mature primary granules. Endoplasmic reticulum demonstrated moderately strong PA-TCH-SP staining, in contrast to absent staining of this organelle in normal promyelocytes and consistent with abnormal primary granule genesis. Small abnormal elongated granules (0.1–0.2 μm in diameter) were identified at the myelocyte state of development and were the predominant granule type in late neutrophils. These granules were identified as secondary granules on the basis of their PA-TCH-SP positivity and were differentiated from primary and tertiary granules on the basis of a lack of peroxidase, acid phosphatase, and sulfate staining. When the neutrophils were exposed to PMA, cell aggregation occurred, and the abnormal granules degranulated in a manner similar to the degranulation observed with normal secondary granules. Although PA-TCH-SP staining of the plasma membrane appeared normal, a decrease in FMLP receptors was demonstrated. Thus, a defect(s) is present in complex carbohydrate distribution and staining that involves primary and secondary granules and possibly the plasmalemma of neutrophils from this patient. This results in abnormal packaging of primary granules and synthesis of normal numbers of secondary granules that are qualitatively and morphologically abnormal, but can be recruited to degranulate with PMA.

Several investigators have now reported patients with susceptibility to bacterial infections and neutrophil dysfunction associated with absent secondary or specific granules and abnormal nuclear lobulation.1–4 Primary or azurophilic granules morphologically appeared to be normal or increased in peroxidase-stained specimens. The neutrophils of these patients contained abundant small vesicle-like granules. On the basis of their morphological appearance at the myelocyte stage of development, Strauss et al.1 hypothesized that they were abnormal secondary granules. Light microscopic studies demonstrated an absence or decrease in alkaline phosphatase1–3 and lactoferrin staining.5 Alkaline phosphatase may not be a definitive marker of secondary granules, since, in humans, alkaline phosphatase has been localized in a vesicle fraction distinct from secondary granules.5,6 Similarly, ultrastructural immunocytochemical and iron binding studies have localized lactoferrin in mature primary granules in our laboratory,7 in contrast to the interpretation of biochemical and light microscopic data, indicating that lactoferrin is a component of secondary granules.6 It was also reported that the chemotaxis of neutrophils from these patients seemed to be impaired.1,2 In addition, the bacterial killing ability appeared decreased in some studies.1,2 Recently, Boxer et al.8 have demonstrated that not only chemotaxis, but also adherence, aggregation, and the ability to decrease cell surface charge after N-formylmethionyl-leucyl-phenylalanine (FMLP) stimulation were diminished to some extent.

In this study, we examined neutrophils from such a patient to determine whether abnormalities in complex carbohydrate distribution might exist. Our ultrastructural studies demonstrate undescribed abnormalities in the packaging of vicinal glycol-containing complex carbohydrates in primary and secondary granules. Furthermore, the studies indicate that secondary granules are present in normal numbers, but are unrecognizable morphologically because of their abnormal appearance. Although these granules can be induced to degranulate with phorbol myristate acetate (PMA), the recruitment of FMLP binding sites is altered.

MATERIALS AND METHODS

The clinical data regarding the patient studied have been reported previously.1,8 A decrease in cytoplasmic lactoferrin was documented
using an immunoperoxidase method at the light microscope level. Rare neutrophils from the patient demonstrated normal lactoferrin content.

**Ultrastructural Cytochemistry**

Blood and marrow specimens were obtained in a heparinized syringe by venipuncture and needle aspiration of the posterior iliac crest, after obtaining informed consent from the patient. Normal marrow samples from healthy volunteers were used as controls. The specimens were centrifuged at 1,500 g for 3 min. After removing the plasma and overlaying the buffy coat with fixative, the buffy coats were removed, minced, and fixed in 3% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.35, for 1 hr at 4°C-10°C. After rinsing in cacodylate sucrose buffer, the specimens were processed for routine morphology or cytochemistry. Peroxidase staining was accomplished by incubation in a 3,3′-diaminobenzidine solution, according to the method of Graham and Karnovsky. Acid phosphatase staining was performed by incubation in substrate medium containing β-glycerophosphate, as described by Barka and Anderson. Staining of sulfated glycoconjugates was performed by incubation of specimens in a high-ion diamine (HID) solution, according to the method of Spicer. Specimens processed for routine morphology, acid phosphatase, and peroxidase were postfixed in 1% OsO4 in cacodylate buffer, whereas specimens stained with HID or processed for postembbedment staining of vicinal glycols were not osmicated. Some specimens to be stained for vicinal glycols were exposed for 3 hr at 37°C with 20 ng/ml phorbol myristate acetate (PMA) for 5 and 15 min. The tubes were then centrifuged at 4°C, and the supernatant fluids were assayed for the presence of granule protein, lactoferrin, and the cytoplasmic enzyme, lactacid dehydrogenase.

Thin sections of unosmicated specimens, with or without α-amylase digestion, were collected on stainless steel grids and stained for vicinal glycols according to the periodate-thiocarbohydrazide-silver proteinate (PA-TCH-SP) method of Théry, as described previously. All specimens were routinely dehydrated in graded alcohols and propylene oxide and embedded in Spurr low-viscosity medium.

Effect of Phorbol Myristate Acetate on Granules

To examine degranulation, aliquots consisting of 5 × 106 cells/ml were incubated at 37°C with 20 ng/ml phorbol myristate acetate (PMA) for 5 and 15 min. The tubes were then centrifuged at 1,500 g for 10 min at 4°C, and the supernatant fluids were assayed for the presence of the granule protein, lactoferrin, and the cytoplasmic enzyme, lactacid dehydrogenase.

Cells obtained immediately before exposure to PMA, and 5 and 15 min after exposure were centrifuged into pellets and fixed in 3% glutaraldehyde, 0.1 M cacodylate sucrose buffer, and processed for morphological examination as described above.

**Measurement of FMLP Receptors and Reversibility of 3H-FMLP Binding**

Purified polymorphonuclear cells (PMNs) obtained from healthy donors or a lactoferrin-deficient patient were washed twice with Hanks’ buffered salt solution (HBSS) and resuspended in HBSS. The cells were then incubated at 37°C for 30 min in HBSS with ionophore A23187, a degranulating stimulus (kindly provided by Dr. Robert Hamill, Eli Lilly and Co., Indianapolis, IN). Control cells were incubated with HBSS only. After the incubation, both stimulated and control cells were centrifuged at 350 g for 10 min at 4°C. The precipitates were washed twice and resuspended in Ca- and Mg-free HBSS (mHBSS) for binding assays. Receptor and binding studies were carried out according to the procedure of Fletcher and Gallin, with minor modifications.

To measure PMN receptors for the FMLP, tosyl-L-phenylalanyl chloromethane (TPCK, Calbiochem, San Diego, CA) at 10-4 M and 3H-FMLP (formylmethionyl-leucyl-3H-phenylalanine; New England Nuclear, Boston, MA) at varying final concentrations (10-180 nM) were added to 1 ml (5 × 106 cells) of cell suspension (stimulated or control). Duplicate samples containing the above 3H-FMLP (55.6 Ci/mM) and 10-4 M nonradioactive FMLP (Peninsula Labs, San Carlos, CA) were assayed simultaneously to determine nonspecific binding. The cells were then incubated in an ice bath for 1 hr with constant shaking. The assay was terminated by adding 4.5 ml of ice-cold HBSS, and the cell suspensions were washed twice with HBSS (5 ml each). The precipitates were finally dissolved in 0.5 ml of H2O, and radioactivity was counted in 10 ml of Aquasol-2.

For the reversibility of 3H-FMLP binding, 3H-FMLP (total binding) and 3H-FMLP plus nonradioactive FMLP (nonspecific binding) were added to 2 separate 50-ml polypropylene tubes. In an ice bath, the cell suspensions (5 × 106/ml mHBSS) were added to start the assay. The final concentrations were 40 nM for 3H-FMLP and 40 µM for cold FMLP. The reaction mixtures were kept in a melting ice bath with constant shaking. At various time points, triplicate aliquots (250 µl) were collected. To displace the surface-bound 3H-FMLP from the cell, a paralleled set of aliquots from the total binding tube were added to a separate set of tubes containing an excess of unlabeled FMLP. At certain time intervals, triplicate aliquots of these cells were collected to determine the residual cell-associated radioactivity. The assay was terminated, washed, resuspended, and counted as described above. The different components of the binding were obtained as follows: (1) total specific binding – total binding – nonspecific binding; (2) displaceable binding – total specific binding – residual radioactivity after the addition of excess nonradioactive FMLP; and (3) nondisplaceable binding – residual radioactivity after the addition of nonradioactive FMLP – nonspecific binding.

**RESULTS**

Electron Microscopy

The ultrastructural morphology of the myeloid cells in this disorder has been described previously. Elongated abnormal small granules, 0.1–0.2 µm in diameter, were observed with increasing frequency from the myelocyte to the band neutrophil stage of development. In band and segmented neutrophils (Fig. 1), these granules outnumbered dense primary granules by a ratio of approximately 4:1. In morphological preparations, these abnormal granules appeared much smaller and less dense when compared to secondary granules in normal neutrophils. The small granules contained moderately dense content, unlike tubulovesicular structures, which were also present in patient and normal neutrophils.

Peroxidase, acid phosphatase, and sulfate staining was performed to determine the relationship of the abnormal granules to primary and tertiary granules. Primary granules demonstrated normal peroxidase (Fig. 2), acid phosphatase, and sulfate staining. In most primary granules of late neutrophils or PMNs,
staining of acid phosphatase and sulfate appeared masked (Fig. 3). From 1 to 4 tertiary granules measuring 0.1–0.3 μm in diameter were stained in profiles of late neutrophils and appeared normal (Fig. 3). Positive identification of tertiary granules was possible on the basis of their acid phosphatase and sulfate staining (Fig. 3) and of Golgi reactivity for these components in segmented neutrophils as described for normal human neutrophils. In contrast, positively stained profiles similar to the abnormal granules were not observed with the peroxidase, acid phosphatase, and sulfate methods.

In unosmicated specimens not treated with α-amylase, strong particulate staining of glycogen was observed in myeloid cells. This staining was most abundant in segmented neutrophils, and it obscured the staining of cytoplasmic organelles (Fig. 4). Consequently, staining of organelles was evaluated in α-amylase-treated specimens.

PA-TCH-SP moderately stained endoplasmic reticulum and Golgi lamellae in promyelocytes (Fig. 5), in contrast to normal promyelocyte endoplasmic reticulum, which lacked staining with this method as reported previously. Staining, however, was not observed in the nuclear envelope with this method, despite the observed peroxidase reactivity in both the nuclear envelope and the endoplasmic reticulum of promyelocytes. The matrix and membrane of immature primary granules stained moderately.

The abnormal small granules were first noted at the myelocyte stage of development and stained intensely with the PA-TCH-SP method (Fig. 6). Often, the granule membrane staining was more intense than the matrix staining and was similar to the intensity of the matrix and membrane staining seen in normal secondary granules (Fig. 7). Several canaliculi and tubulovesicular structures demonstrated PA-TCH-SP reactivity in patient and normal myeloid cells. These structures were generally more elongated, pleomorphic, and were less intensely stained (Fig. 7) than the abnormal granules. These vesicles frequently contacted and appeared to contact or fuse with immature primary granules of promyelocytes and myelocytes.

Primary granules of the patient at the myelocyte stage of development continued to demonstrate moderate PA-TCH-SP staining. In contrast, masking was observed in more mature primary granules of normal myelocytes. At least three stages of maturation were identified in normal myelocytes: P-1 granules demonstrated PA-TCH-SP staining of flocculent matrix material and were often contacted by PA-TCH-SP-reactive vesicles that presumably transport glycoprotein to the forming granule; P-2 granules demonstrated moderate PA-TCH-SP staining throughout the matrix; and P-3 granules lacked matrix staining. In the patient myelocytes, granules with the P-3 pattern of staining were markedly decreased or absent.

The abnormal small granules were the predominant granule type seen in segmented neutrophils and generally outnumbered primary granules by a ratio of 4:1 (Fig. 8). The staining of the abnormal granules was similar to that seen in the patient's myelocytes. In normal segmented neutrophils, PA-TCH-SP strongly stained normal secondary granules (Fig. 9), which appeared much larger than the abnormal small granules of the patient. Primary granules of the patient continued to demonstrate moderate PA-TCH-SP staining (Fig. 8), whereas this staining was masked in primary granules of normal segmented neutrophils (Fig. 9). Some pleomorphic tubulovesicular structures in patient and normal neutrophils demonstrated weak to moderate PA-TCH-SP staining.

**PMA-Treated Specimens**

A progressive decrease in the abnormal small granules was observed with increasing time of exposure to PMA. After 1 min exposure, the abnormal small granules were observed fusing with the plasmalemma and cytoplasmic vacuoles or indentations (Fig. 10). Several cells were observed with an accumulation of vesicular membranes at one pole that were adherent to the plasmalemma and appeared to be a collection of extruded granules (Fig. 11). In specimens exposed for 15 min, the small granules were markedly depleted and were outnumbered by the larger, more dense primary granules (Fig. 12). In addition, the neutrophils

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**Fig. 1.** This segmented neutrophil contains several dense primary granules (P) and numerous abnormal small granules (arrows) that appear less dense than primary granules (enlarged in inset). In longitudinal section, these granules appear to be disc- or cup-shaped, whereas in transverse section, they appear more round. The morphology does overlap with short tubulovesicular structures; however, the latter structures are more pleomorphic and lack matrix density (×22,000; inset, ×55,000).

**Fig. 2.** Diaminobenzidine stains peroxidase in primary granules in this segmented neutrophil from the patient. Few or no normal-appearing secondary granules are present. Staining is not seen in small granule profiles (arrows, enlarged in inset). M, mitochondria. (×22,000; inset, ×55,000.)

*The thin sections for Figs. 1 and 10–12 were stained with uranyl acetate and lead citrate, whereas the other figures are from cytochemical preparations and were not counterstained. The specimens for Figs. 1, 2, and 10–12 were postfixed in OsO₄, whereas those for the other figures were not. The specimens for Figs. 1–9 are from marrow, and those for Figs. 10–12 are from blood.*
Fig. 3. HID-TCH-SP stains sulfate in presumed tertiary granules (arrows) from the patient. Staining is masked in larger primary granules. Profiles of the stained granules do not resemble the frequency or morphology of the abnormal granules (×28,500).

Fig. 4. Without α-amylase digestion, PA-TCH-SP intensely stains cytoplasmic glycogen of this segmented neutrophil from the patient. The reactive glycogen particles obscure the staining of cytoplasmic granules (×11,250).

Fig. 5. PA-TCH-SP stains vicinal glycol-containing glycoconjugates in this α-amylase-digested specimen. This early promyelocyte from the patient contains several dilated segments of endoplasmic reticulum (ER), with moderate PA-TCH-SP reactivity (enlarged in far right inset) not seen in normal specimens. Only a few primary granules (P) are present. The Golgi lamellae (G) are intensely PA-TCH-SP reactive, with increasing staining on the mature face (enlarged in inset). (×13,120; inset, ×40,000; far right inset, ×50,000.)

Fig. 6. The patient's myelocyte from an α-amylase-digested PA-TCH-SP-stained specimen contains several reactive primary granules, a Golgi zone (G), and intensely reactive abnormal small granules (enlarged in inset, arrows). Primary granules (P) stain intensely, and some weak staining persists in endoplasmic reticulum (ER). These abnormal primary granules are not as readily appreciated in morphological specimens (cf., Fig. 1). (×22,000; inset, ×55,000.)

Fig. 7. This normal marrow myelocyte (after α-amylase digestion) contains several intensely reactive secondary granules (S). Less intense staining is present in immature primary granules (P-2), and staining is completely masked in mature primary granules (P-3). Very immature primary granules (P-1) or condensing vacuoles are contacted by PA-TCH-SP-reactive tubulovesicular structures (arrows), which presumably transport glycoprotein matrix material to the forming primary granule (enlarged in B, arrows). (A) ×22,000; (B) ×55,000.
Fig. 8. This segmented neutrophil from the patient (after α-amylase digestion) demonstrates numerous intensely PA-TCH-SP-reactive abnormal small granules (enlarged in upper left inset). Primary granules (P) are also PA-TCH-SP reactive (enlarged in upper right inset) and do not demonstrate the masking of staining observed in normal primary granules (as is shown in Figs. 7 and 9). Specimen digested with α-amylase (×22,000; insets, ×62,000).

Fig. 9. This normal segmented neutrophil (after α-amylase digestion) contains abundant PA-TCH-SP-reactive secondary granules (S). Staining is present in the membrane and matrix of the normal secondary granule. Vicinal glycols in mature primary granules (P) are masked to staining (enlarged in B). (A) ×22,000; (B) ×65,000.
appeared in large aggregates in the 5- and 15-min exposed specimens.

**Binding of Chemoattractant FMLP**

Figure 13 indicates the representative Scatchard plot for the effects of the degranulating stimulus A23187 on \(^3\text{H}\)-FMLP binding of PMN from a normal donor and the lactoferrin-deficient patient. This plot was derived from the method of linear regression analysis to obtain the best fitted lines. Comparison of the calculated available FMLP binding sites per PMN (Table 1, without A23187 exposure) shows that the lactoferrin-deficient patient had only 36% of that found in normal cells (10,245 \(^3\text{H}\)-FMLP binding sites/PMN). Under the present conditions, the binding of \(^3\text{H}\)-FMLP to normal PMN was enhanced by 46% by exposure to 10 nM of A23187. On the contrary, such an exposure caused a decrease of \(^3\text{H}\)-FMLP binding sites to 72% of the unstimulated cells in lactoferrin-deficient PMN. Therefore, it is apparent that the binding of \(^3\text{H}\)-FMLP to PMN in response to the degranulating agent A23187 was different between the normal and lactoferrin-deficient cells. Furthermore, the total specific saturable binding of the \(^3\text{H}\)-FMLP to the patient’s PMN was only 16% of that observed from the normal cells (Table 2). This drastic decrease could be attributed to a decrease in both displaceable and nondisplaceable binding sites.

**DISCUSSION**

This study demonstrates a variety of defects in neutrophils related to complex carbohydrate distribution and staining in a patient with lactoferrin deficiency. Specifically, complex carbohydrates appear to be abnormally packaged in both the primary and secondary granules of this patient. In contrast to previous speculation, the number of secondary granules is not truly decreased. Using the \(\alpha\)-amylase PA-TCH-SP method, the abnormal small granules appear intensely reactive, as is seen for larger normal secondary granules.\(^{4-9}\) and is consistent with the high content of glycoprotein enzymes, which may include acid hydrolases.\(^{25}\) The endoplasmic reticulum staining indicates increased amounts of glycoprotein, which could result from delayed transit of glycoprotein from the endoplasmic reticulum or increased synthesis of glycoprotein by this organelle. Other studies have demonstrated increased amounts or reactivity of the primary granule substances, peroxidase,\(^{1,8}\) and lysozyme,\(^{8}\) consistent with increased synthesis of primary granule components. The masking of vicinal glycol groups in normal primary granules may reflect interaction with other added granule substances.\(^{14,19,24}\) Thus, the lack of masking in this patient could represent either an abnormal glycoprotein or lack of a granule substance responsible for masking of vicinal glycols.

The deficiency of lactoferrin observed in these patients may represent a primary rather than a secondary granule defect. Recent ultrastructural studies\(^7\) have localized iron-binding protein(s), including lactoferrin, in mature primary granules and not in secondary granules. The lactoferrin-positive primary granules are secreted with PMA and function differently than immature primary granules.\(^7\) Thus, the decrease in lactoferrin observed in this patient appears to be consistent with the primary granule maturation defect in complex carbohydrates demonstrated with the PACT-SH method. The production of some lactoferrin in the neutrophils of this patient, as demonstrated by glycol-reactive groups to become masked with granule maturation, as is seen in normal primary granules\(^{14,19}\) and eosinophil-specific granules.\(^{24}\) Similarly, PACT-SH moderately stained the normally unreactive endoplasmic reticulum of promyelocytes. The PACT-SH-reactive material in endoplasmic reticulum and immature primary granules presumably represents glycoprotein enzymes, which may include acid hydrolases.\(^{25}\) The endoplasmic reticulum staining indicates increased amounts of glycoprotein, which could result from delayed transit of glycoprotein from the endoplasmic reticulum or increased synthesis of glycoprotein by this organelle. Other studies have demonstrated increased amounts or reactivity of the primary granule substances, peroxidase,\(^{1,8}\) and lysozyme,\(^{8}\) consistent with increased synthesis of primary granule components. The masking of vicinal glycol groups in normal primary granules may reflect interaction with other added granule substances.\(^{14,19,24}\) Thus, the lack of masking in this patient could represent either an abnormal glycoprotein or lack of a granule substance responsible for masking of vicinal glycols.

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<tr>
<th>Stimulus</th>
<th>Number of FMLP Receptors/PMN</th>
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<tbody>
<tr>
<td>Control</td>
<td>10,245 (100)</td>
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<tr>
<td>A23187 (10 nM)</td>
<td>21,830 (146)</td>
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*Entries in parentheses represent the percentages of the values observed in the control or unstimulated cells.

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<tr>
<th>Cell Origin</th>
<th>(\text{(^3\text{H})-FMLP (fmole Bound} / 5 \times 10^8 \text{PMN})^*)</th>
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<tr>
<td>Normal</td>
<td>131.7 (100)†</td>
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<tr>
<td>Lactoferrin-deficient</td>
<td>21.7 (16)</td>
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*Values for total specific and displaceable bindings were obtained as described in Materials and Methods.

†Entries in parentheses represent the percentages of the values observed in normal cells.
immunoassay methods in previous studies,\textsuperscript{8} indicates that a complete deletion of a gene for lactoferrin synthesis has not occurred. Similarly, we have observed some iron-binding activity in mature primary granules of these cells (unpublished) using previously described histochemical methods.\textsuperscript{7}

A deficiency of alkaline phosphatase has been described in these patients. In humans, alkaline phosphatase may be localized in vesicles distinct from secondary granules.\textsuperscript{5,6} Thus, membrane-limited organelles, other than lysosomes, appear to be involved in the pathophysiology of this disorder.

The observed decrease in plasmalemma FMLP receptors and in their recruitment has been previously characterized in another patient with lactoferrin deficiency.\textsuperscript{4} Our data would suggest that the observed plasma membrane defect in this disorder may then be a consequence of abnormal secondary granule membrane structure, rather than absence of granule membranes, since these membranes appear to be present and can be induced to fuse with the plasmalemma, as evidenced in our morphological studies of PMA-treated cells.

The abnormal nuclear segmentation, nuclear pocket formation, and nuclear membrane duplication described previously\textsuperscript{13} could be related to a defect in the nuclear envelope, which shares protein synthetic functions with the endoplasmic reticulum. Both of these organelles appeared to be functioning abnormally in this patient on the basis of PA-TCH-SP staining and

\textbf{Fig. 13.} This graph depicts the decrease in recruitment of FMLP receptors in the patient's neutrophils compared to controls.
nuclear membrane duplication. Defects in nuclear segmentation and nuclear membranes have been observed in other disorders with abnormal granule genesis. Thus, the observation of several morphological and functional abnormalities of membrane-limited organelles in the neutrophils of this patient suggests a more generalized defect affecting many aspects of neutrophil membrane genesis and/or glycoprotein synthesis.

REFERENCES

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