Intracellular Localization of Glycosyl-Phosphatidylinositol–Anchored CD67 and FcRIII (CD16) in Affected Neutrophil Granulocytes of Patients With Paroxysmal Nocturnal Hemoglobinuria

By Carolina R. Jost, Marleen L. Gaillard, Jack A.M. Fransen, Mohamed R. Daha, and Leo A. Ginsel

Immunoelectron microscopical studies performed in healthy human neutrophils showed the presence of glycosyl-phosphatidylinositol (GPI)-linked CD67 in granules. The use of immunogold double-labeling of CD67 and lactoferrin (LF; as marker for specific granules) or CD67 and myeloperoxidase (MPO; as marker for azurophilic granules) showed that CD67 occurred only in the specific granules. Furthermore, flow cytometry showed that CD67 has a low level of expression on the plasma membrane of these cells. In paroxysmal nocturnal hemoglobinuria (PNH)-affected neutrophils, CD67 was not detected in any intracellular compartment by immunoelectron microscopy, and flow cytometry showed no CD67 on the plasma membrane. In earlier studies, FcRIII was found on the plasma membrane, in electron-lucent vesicles, and in the Golgi complex of healthy neutrophils, and in the Golgi complex of some of the PNH-affected neutrophils. Here we have studied FcRIII in PNH-affected cells of three other patients and found, by immunoelectron microscopy, that the receptor can not be detected in these cells. However, flow cytometry showed that FcRIII was not completely absent on the plasma membrane of the affected cells, but that the level of expression on these cells was low. Thus, PNH patients can differ from one another with respect to the occurrence of affected neutrophils that have a detectable level of FcRIII in the Golgi complex. In summary, these findings show not only that the expression of the two GPI-linked proteins, CD67 and FcRIII, is markedly lower on the plasma membrane, but also that neither occurred in any of the intracellular compartments of affected neutrophils of the PNH patients examined in this study.

© 1991 by The American Society of Hematology.

P A R O X Y S M A L nocturnal hemoglobinuria (PNH) is an acquired clonal disorder of the hematopoietic stem cells. PNH patients have abnormal erythrocytes that are unusually susceptible to complement lysis. 1 This increased sensitivity to complement lysis is due to the absence on the plasma membrane of a number of factors that regulate the complement cascade, ie, decay-accelerating factor (DAF), 2 homologous restriction factor (HRF), 3 and C8-binding protein (C8bp). 4 DAF, HRF, C8bp, and other proteins showing deficiency in PNH belong to the class of glycosyl-phosphatidylinositol (GPI)-linked proteins. 5,6 The multiple membrane protein deficiencies in PNH cells suggest that the primary defect is not in the production of any of these individual proteins, but is probably due to a defect in the assembly of, or in the linkage to, the GPI anchor. This is consistent with the finding that normal DAF genes and mRNA transcripts are found in affected PNH cells. 7

In PNH patients, not only the erythrocytes, but the neutrophils, eosinophils, monocytes, and occasionally lymphocytes are also affected and can be deficient in, or have a reduced expression of, these GPI-linked proteins on the plasma membrane. The percentage of the cells that are affected can vary among patients. 8

FcRIII is a GPI-linked protein, 9,10 and we recently studied its intracellular localization in healthy neutrophils 9,10 and PNH-affected cells 1 by immunoelectron microscopy. FcRIII was found to occur on the plasma membrane, in electron-lucent vesicles mainly present in the juxtanuclear area, and in the Golgi complex of healthy neutrophils. 9 In PNH neutrophils, the expression of FcRIII on the plasma membrane amounts on average to 10% of the normal levels, as shown by flow cytometry. 1 In addition, we reported that FcRIII was present in the Golgi complex of affected neutrophils. 9

The present immunoelectron-microscopical study was undertaken to visualize the intracellular localization of a number of GPI-linked proteins in other PNH patients. However, since only CD67 and FcRIII could be localized intracellularly by immunoelectron microscopy, we limited our study to these two proteins.

MATERIALS AND METHODS

Antibodies. Monoclonal antibody B13.9, which is directed against CD67, has been described elsewhere as an antibody directed against gp90. 11 In this report, it will be referred to as anti-CD67. Monoclonal antibody CLB-FcR-gran1, directed against FcRIII (CD16), has been described elsewhere 12 and has been used previously in immunocytochemistry. 10 In this report, it will be referred to as anti-FcRIII. In this study, we also used a polyclonal antibody directed against DAF 13 and the monoclonal antibodies CLB-gran/Bly1 and CLB-gran/5, directed against CD24 and gp70, 14 respectively. All monoclonal antibodies except CLB-gran/5 were clustered in the International Workshops on Leukocyte Differentiation Antigens and were kindly donated by C.E. van der Schoot and T.W.J. Huizinga of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, Amsterdam, The Netherlands. The polyclonal antibody against lactoferrin (LF) has been used in immunocytochemistry. 15 This antibody and the rabbit anti-mouse IgG were both purchased from Cappel Laboratories, West Chester, PA. The polyclonal antibody against myeloperoxidase (MPO), a kind gift from D. Roos from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, has been described 16 and has also been used in immunocytochemistry. 10

From the Laboratory for Electron Microscopy, University of Leiden; and the Department of Nephrology, Leiden University Hospital, Leiden, The Netherlands.

Submitted February 11, 1991; accepted August 9, 1991.

Supported by Grant No. 900 512 074 from the Netherlands Organization for Scientific Research (NWO). Address reprint requests to Leo A. Ginsel, PhD, Department of Cell Biology and Histology, University of Nijmegen, PO Box 9101, NL 6500 HB, Nijmegen, The Netherlands.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1991 by The American Society of Hematology.

0006-4971/91/7811-0015$3.00/0

3030

Cell preparation. Leukocytes were obtained from blood of healthy individuals heparinized with 143 U tromboliquine/10 mL blood (Organon Technika B.V., Boxtel, Holland). After removal of erythrocytes by dextran sedimentation for 1 hour at room temperature, the remaining cells were washed three times in phosphate-buffered Ringer’s solution. The cell preparation was composed primarily of neutrophils, the cells of other types being predominantly erythrocytes, eosinophils, and monocytes. These cells were used in the immunogold-labeling studies.

For the immunofluorescence studies, cells from this cell preparation were diluted in phosphate-buffered Ringer’s solution and further separated over isotonic Percoll (1.076 g/cm³, 1,000 × g for 20 minutes at 20°C) to remove the monocytes. The remaining erythrocytes in the granulocyte-rich pellet were lysed with isotonic 0.89% NH₄Cl at 4°C. The resulting cell preparation was composed primarily of neutrophils, the remaining cells being predominantly cosinophils.

The HL-60 promyelocyte cell line was kindly provided by Dr A.J. Verhoeven of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service.

Immunocytochemistry. Immunocytochemical procedures have been described elsewhere. Cells were fixed in a graded (1% to 8%) formaldehyde series in NaHCO₃ buffer at pH 7.4 (freshly prepared from paraformaldehyde) for 2 hours at room temperature. Fixed cells were pelleted in 10% gelatin in phosphate-buffered saline (PBS), after which the pellet was postfixed overnight in 1% formaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). On the following day, small gelatin blocks containing cells were infused with 2.5 mol/L sucrose in PBS for 25 minutes, mounted on a specimen holder, and frozen in liquid nitrogen. The pellets were not kept in the postfixation solution longer than one night, because the antigenicity of FcRIII is reduced by postfixation. Cryosectioning was performed according to Tokuyasu. The sections were preincubated on drops of incubation buffer containing 0.1% bovine serum albumin (BSA), 0.1% “60 Bloom” gelatin, and 0.15% glycine in PBS (BGG-PBS), and labeled with the monoclonal antibody against FeRIII, CD67, or polyclonal antibodies directed against MPO or LF. All antibodies were diluted in incubation buffer, and incubated with anti-CD67 or anti-FcRIII either at 4°C for 16 hours or at room temperature for 60 minutes. The antibodies were visualized with protein A conjugated to 5- or 10-nm colloidal gold particles. Because of the low affinity of protein A for some classes of murine IgG, a second antibody (rabbit anti-mouse IgG) and protein A conjugated to gold were used to visualize anti-FcRIII and anti-CD67. Incubations with all polyclonal antibodies and with protein A-gold were performed at room temperature for 60 minutes.

Controls included omission of the primary antibody incubation step and, for anti-CD67, incubation with a nonspecific murine IgG of the same class as the anti-CD67 immunoglobulin (IgG1). Immunogold double-labeling of CD67 and MPO or LF was performed essentially according to Geuze et al, incubation with the monoclonal antibody always being the first step in the labeling procedure.

Finally, the cryosections were embedded in a mixture of methylcellulose (methocel 25 mPa·s) and uranylacetate. All sections were examined with a Philips EM 200 (Eindhoven, Holland) at 80 kV.

Quantitative analysis of the immunogold labeling. To study the localization of CD67 in neutrophils, we determined the density of the gold particles over the specific and azurophilic granules, as well as over the nucleus (as control). For this analysis, we used micrographs taken from an immunogold double-labeling experiment in which CD67 and MPO or LF had been labeled. We measured the total surface areas of the nucleus and of the monoprotein-containing azurophilic and LF-containing specific granules in 20 representative cell sections and counted the gold particles representing CD67 in these areas. The density of these gold particles was calculated for each of the cells included in the counts and the average labeling density for 20 cells is given as the number of gold particles per square micron ± SD. The surface area of the nucleus and of the specific and azurophilic granules was determined using a sheet of transparent paper carrying a lattice and placed over a micrograph, according to Weibel et al. The same quantitative analysis was used when examining the presence of CD67 in the azurophilic granules of HL-60 cells.

To compare the labeling of CD67 and FeRIII in healthy and PNH-affected cells, we determined the density of the labeling over an area containing the cytoplasmic matrix and all of the cytoplasmic organelles, but not the nucleus. This intracellular labeling density was calculated from measurements and counts performed in electron micrographs of cells sectioned through both nucleus and juxtanuclear area. The size of the area used for this analysis was obtained by subtracting the nuclear area from the total cell area, both areas having been measured with an X-Y tablet (Kontron, München, Germany; MOP-AMO2). The intracellular gold particles were counted (not including those present on the plasma membrane) and the labeling density over this intracellular area was expressed as the number of gold particles per square micron. The labeling densities of CD67 and FeRIII in each patient and in the control donors are given as the mean (±SD) of the labeling densities of 20 individual cells. These determinations were performed for three PNH patients and three healthy control donors. PNH and control cells were always isolated and prepared for cryosectioning simultaneously, and the labeling experiments were performed at the same time.

Less than 10% of the patients’ granulocytes were not affected by PNH, as shown by flow cytometry, and in the electron microscope the heavily labeled nonaffected cells could be easily distinguished from the affected cells that were not labeled. For this quantitative analysis, only affected cells of the PNH patients were used.

Immunofluorescence. The cells were fixed in paraformaldehyde and washed in BGG-PBS before use. Incubation with anti-FcRIII or anti-CD67 was performed at the appropriate antibody concentration in BGG-PBS for 1 hour at 4°C, and cells were then washed and incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit-anti-mouse immunoglobulin (Nordic Immunological Laboratories, Tilburg, The Netherlands). The FITC conjugate was diluted in PBS containing 10% heat-inactivated human serum and 0.5% BSA and was allowed to stand for 1 hour at 4°C before use to avoid nonspecific cross-reaction with human immunoglobulin present on the cell surface of neutrophils. For the controls, either the primary antibody incubation step was omitted or the cells were incubated with a nonspecific murine IgG.

The fluorescence intensities were measured by flow cytometry (FACStar, Becton Dickinson, Mountain View, CA).

RESULTS

Ultrastructure. In ultrathin cryosections of formaldehyde-fixed neutrophils, the cellular organelles were well preserved (Fig 1). The wide range in size and electron density of the granules in these cells made it difficult to discriminate between the various types of granule solely on the basis of morphological criteria, although the granules containing LF generally had a denser matrix (Fig 2a) than that of MPO-containing granules (Fig 2b). In the juxtanuclear area between the lobes of the nucleus, a small Golgi complex and an occasional centriole were found. A few mitochon-
Fig 1. Localization of CD67 in the human neutrophil. The cryosections were incubated first with a monoclonal antibody against CD67, then with rabbit anti-mouse IgG, and finally with protein A conjugated to 10-nm colloidal gold. CD67 (arrows) is present in granules. Note the absence of CD67 in the plasma membrane (pm). Bar = 0.5 μm.

dria were present, but elements of the rough endoplasmic reticulum were not observed.

Localization of CD67 in healthy neutrophils. We initially tried to determine the intracellular localization of five different GPI-linked molecules (ie, FcRIII, DAF, CD24, gp70, and CD67) in healthy neutrophil granulocytes by immunoelectron microscopy. FcRIII had already been studied with this technique, and of the others, only CD67 could be detected within cells.

Immunogold labeling of cryosections showed that CD67 was present in granules (Fig 1). To distinguish between specific and azurophilic granules in this respect, we performed immunogold double-labeling experiments. For CD67, we used 10-nm gold particles, and for LF (as marker for the specific granules) and MPO (as marker for the azurophilic granules), we used 5-nm gold particles. CD67 was found in the specific granules containing LF (Fig 2a), but there were also a few 10-nm gold particles over the azurophilic granules containing MPO (Fig 2b). We assume that CD67 occurs inside the specific granules, because the gold particles were found primarily along the inner surface of the limiting membrane of these granules. With this technique, CD67 could not be detected on the plasma membrane (Fig 1) or in the Golgi complex (not illustrated).

Assessment of the density of the gold particles over the nucleus and over the specific and azurophilic granules showed the highest labeling density over the specific granules (57.8 ± 17.1) and lower densities over the azurophilic granules (13.1 ± 4.5) and the nucleus (1.1 ± 0.8). However, when incubating with a nonspecific IgG1 antibody (Fig 2c), we observed an equally high labeling over the azurophilic granules (20.1 ± 5.0), while the labeling over the nucleus and the specific granules were both low (0.4 ± 0.3 and 1.1 ± 1.2, respectively). These data suggest that the gold particles present over the azurophilic granules represent background labeling.

To further examine the possible presence of CD67 in the azurophilic granules, we studied the intracellular localization of CD67 in the promyelocyte cell line HL-60. We found that the labeling density over the MPO-containing azurophilic granules in these cells was low (1.9 ± 1.0) and comparable to the labeling over the azurophilic granules when using a nonspecific IgG1 antibody (1.1 ± 0.7). On these grounds, we consider CD67 to be present in the specific granules, but absent from the azurophilic granules.

PNH patients. Before the granulocytes of the patients were studied at the ultrastructural level, immunofluorescence flow cytometry was applied to cells of the healthy donors and PNH patients.

To determine the percentage of affected neutrophils in
each patient, we labeled FcRIII. FcRIII is abundantly present on the cell surface of healthy granulocytes and shows reduced expression on affected cells. Greater than 90% of the cells of each of the three patients showed reduced expression of FcRIII on the plasma membrane, indicating that greater than 90% of the granulocytes were affected. The flow cytometric findings for FcRIII in two PNH patients (no. 1 and 3) in two healthy controls are shown in Fig 3.

This technique was also used to determine the level of expression of CD67 and FcRIII on the plasma membrane of the cells under study. CD67 had a low level of expression on the plasma membrane of healthy controls and did not occur on the cell surface of the PNH-affected neutrophils (Fig 3). FcRIII showed a strong expression on control cells and a low level on the affected cells (Fig 3). The number of Fc receptors on the affected neutrophils was considerably lower for patient 1 than for patient 3 (Fig 3). Immunofluorescence analysis of CD67 and FcRIII was not performed for cells of patient 2.

Electron microscopically, two types of cell could be distinguished among the CD67-labeled granulocytes of the PNH patients: a few cells were normally labeled, but the majority were labeled with only a few gold particles scattered randomly over the cell. To assess the decreased labeling of these cells, we determined intracellular labeling densities of CD67 in healthy and affected neutrophils. The results are shown in Tables 1 and 2. The labeling densities of CD67 in the healthy donors ranged from 5.9 ± 1.9 to 14.9 ± 4.4 gold particles/µm² (Table 1), and those in the affected cells of the PNH patients, from 0.6 ± 0.2 to 2.6 ± 1.2 gold/µm² (Table 2).

In neutrophils from all three healthy donors, FcRIII was localized on the plasma membrane, in the Golgi complex, and in electron-lucent vesicles, as we had found previously (not illustrated). The labeling densities over the cytoplasm of these healthy donors varied considerably, ranging from 6.9 ± 3.3 to 25.0 ± 12.6 gold particles/µm² (Table 1). After immunogold labeling of FcRIII in the PNH-affected cells, only a few gold particles were seen randomly distributed over the cell. Quantification showed that these densities were all low, ranging between 2.2 ± 0.6 and 2.7 ± 0.8 gold particles/µm² (Table 2).
These are the affected cells. Patient fluorescence than seen in healthy controls. Note that the level of the weak, whereas FcRIII is abundantly present on these cells. In PNH fluorescence on affected granulocytes of patient of membrane-bound proteins initially present in these occurring in human neutrophil granulocyte. The level of the basis for the increased surface expression of a number is associated with the transport of storage compartments to 11,30,32-34

CD67 is an 85- to 95-Kd GPI-linked membrane protein occurring in human neutrophil granulocytes. The level of expression is low on nonstimulated cells and enhanced on neutrophils stimulated by any of a number of soluble stimuli, presumably due to exocytosis of the above-mentioned compartments.

The present immuno electron microscopic study was undertaken to visualize CD67 at the ultrastructural level. Immunogold double-labeling showed that CD67 occurred only in the specific granules. The finding of CD67 primarily on the luminal surface of the specific granules is consistent with the hypothesis that GPI anchor attachment only occurs after the polypeptide has been translocated across the membrane of the endoplasmic reticulum. We failed to detect CD67 on the plasma membrane after immunogold labeling, probably because the level of expression on these cells is too low to be detected by immuno electron microscopy.

The present study has shown that the localization of CD67 and FcRIII, two GPI-linked molecules, does not coincide in neutrophil granulocytes. Both molecules are present on the cell surface, but CD67 was also found in the specific granules and FcRIII in small electron-lucent vesicles and the Golgi complex as well. These findings do not point to a role of the GPI anchor in the targeting of such molecules, like that shown for the GPI anchor in the MDCK cell line. In cells of this line, all GPI-linked proteins occur on the apical surface, and in these polarized epithelial cells, the GPI anchor is thought to contain apical targeting information.37,38

PNH patients. Using immunoelectron microscopy, we failed to detect intracellular FcRIII and CD67 in neutrophils of three PNH patients. Furthermore, flow cytometry demonstrated that CD67 is absent from the plasma membrane of PNH-affected cells and showed that the level of expression of FcRIII on the cell surface of these cells is low.

The finding that CD67 and FcRIII could not be detected intracellularly in PNH-affected neutrophils and showed a reduced level of expression on the plasma membrane can be explained by assuming that the rate at which these molecules are synthesized is low, or for CD67, that biosynthesis of this molecule does not occur in these cells. This assumption is consistent with results reported by Huizinga et al, who found that the amount of soluble FcRIII identified in the plasma of PNH patients is much lower than that in the plasma of healthy donors, suggesting a low level of shedding or secretion of FcRIII by these cells.

However, the occurrence of multiple GPI-linked membrane-protein deficiencies and the presence of normal mRNA for DAF (another GPI-linked molecule) in PNH-affected cells, suggested that the primary defect in these cells lies not in the biosynthesis of any of these proteins, but rather in some step in the assembly of, or linkage to, their

Table 1. Intracellular Labeling Density of CD67 and FcRIII in Three Control Donors

<table>
<thead>
<tr>
<th>Control No.</th>
<th>CD67 (Gold Particles per μm² of Cytoplasm)</th>
<th>FcRIII (Gold Particles per μm² of Cytoplasm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.9 ± 4.4</td>
<td>17.7 ± 4.4</td>
</tr>
<tr>
<td>2</td>
<td>6.9 ± 1.9</td>
<td>6.9 ± 3.3</td>
</tr>
<tr>
<td>3</td>
<td>10.1 ± 0.9</td>
<td>25.0 ± 12.8</td>
</tr>
</tbody>
</table>

Table 2. Intracellular Labeling Density of CD67 and FcRIII in Three PNH Patients

<table>
<thead>
<tr>
<th>PNH Patient No.</th>
<th>CD67 (Gold Particles per μm² of Cytoplasm)</th>
<th>FcRIII (Gold Particles per μm² of Cytoplasm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.6 ± 1.2</td>
<td>2.6 ± 0.9</td>
</tr>
<tr>
<td>2</td>
<td>0.6 ± 0.2</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>3</td>
<td>2.1 ± 2.3</td>
<td>2.7 ± 0.8</td>
</tr>
</tbody>
</table>

Fig. 3. Results of immunofluorescence flow-cytometric analysis of granulocytes of PNH patients and normal healthy controls. I — — — , control; I — — — , CD67; II — — — , FcRIII. Incubation with anti-FcRIII, anti-CD67, or a nonspecific IgG was performed at the appropriate antibody concentration for 1 hour at 4°C before the cells were washed and incubated with FITC-conjugated rabbit anti-mouse IgG. The expression of CD67 on the plasma membrane of healthy controls is relatively weak, whereas FcRIII is abundantly present on these cells. In PNH patients 1 and 2, the plasma membrane shows virtually no CD67. Patient 1 has a small population of cells with a high level of expression of FcRIII. The fluorescence of these cells is comparable to that of the control cells, which suggests that the former are normal cells not affected by the disease. This patient also shows a large category of neutrophils with a low level of expression of FcRIII, suggesting that these are the affected cells. Patient 3 has virtually no normal cells with a high level of fluorescence, and more of the cells with a lower level of fluorescence than seen in healthy controls. Note that the level of the fluorescence on affected granulocytes of patient 3 is considerably higher than that on the corresponding cells of patient 1.

DISCUSSION

Human peripheral blood neutrophil granulocytes possess a number of intracellular storage compartments: specific and azurophilic granules,8,20-27 nucleated granules,28 and one containing alkaline phosphatase.29,30 Neutrophil activation is associated with the transport of storage compartments to the plasma membrane,29-32 and this process is believed to be the basis for the increased surface expression of a number of membrane-bound proteins initially present in these compartments.11,30,32-34

The present study has shown that the localization of CD67 and FcRIII, two GPI-linked molecules, does not coincide in neutrophil granulocytes. Both molecules are present on the cell surface, but CD67 was also found in the specific granules and FcRIII in small electron-lucent vesicles and the Golgi complex as well. These findings do not point to a role of the GPI anchor in the targeting of such molecules, like that shown for the GPI anchor in the MDCK cell line. In cells of this line, all GPI-linked proteins occur on the apical surface, and in these polarized epithelial cells, the GPI anchor is thought to contain apical targeting information.37,38

PNH patients. Using immunoelectron microscopy, we failed to detect intracellular FcRIII and CD67 in neutrophils of three PNH patients. Furthermore, flow cytometry demonstrated that CD67 is absent from the plasma membrane of PNH-affected cells and showed that the level of expression of FcRIII on the cell surface of these cells is low.

The finding that CD67 and FcRIII could not be detected intracellularly in PNH-affected neutrophils and showed a reduced level of expression on the plasma membrane can be explained by assuming that the rate at which these molecules are synthesized is low, or for CD67, that biosynthesis of this molecule does not occur in these cells. This assumption is consistent with results reported by Huizinga et al, who found that the amount of soluble FcRIII identified in the plasma of PNH patients is much lower than that in the plasma of healthy donors, suggesting a low level of shedding or secretion of FcRIII by these cells.

However, the occurrence of multiple GPI-linked membrane-protein deficiencies and the presence of normal mRNA for DAF (another GPI-linked molecule) in PNH-affected cells, suggested that the primary defect in these cells lies not in the biosynthesis of any of these proteins, but rather in some step in the assembly of, or linkage to, their

Table 2. Intracellular Labeling Density of CD67 and FcRIII in Three PNH Patients

<table>
<thead>
<tr>
<th>PNH Patient No.</th>
<th>CD67 (Gold Particles per μm² of Cytoplasm)</th>
<th>FcRIII (Gold Particles per μm² of Cytoplasm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.6 ± 1.2</td>
<td>2.6 ± 0.9</td>
</tr>
<tr>
<td>2</td>
<td>0.6 ± 0.2</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>3</td>
<td>2.1 ± 2.3</td>
<td>2.7 ± 0.8</td>
</tr>
</tbody>
</table>
common GPI anchor. Thus, if GPI molecules are synthesized but not secreted and cannot be detected intracellularly, posttranslational regulation must be considered to reconcile these findings.

An example of posttranslational regulation is provided by the retention of misfolded and unassembled proteins in the endoplasmic reticulum, as reviewed by Rose et al.4 This retention has been reported to lead to the degradation of newly synthesized proteins even before they reach the Golgi complex.1,4,12

Furthermore, we cannot categorically rule out the possibility that newly synthesized molecules that have not become attached to the membrane via the GPI anchor are processed differently and in this way have lost antigenic sites that are usually present on the membrane-bound GPI-linked molecules. If so, FcRIII might not be recognized by the antibody used in this study.

All PNH patients show reduced expression of GPI-linked molecules, but the origin of this defect remains elusive and might not be the same in all patients. In an earlier study on the intracellular localization of FcRIII in granulocytes of another patient,6 we found (1) unaffected cells showing a normal labeling pattern; (2) affected cells without label on the plasma membrane, but with a labeled Golgi complex; and (3) affected cells that were not labeled at all. The occurrence of the second of these three categories indicates that this patient's affected neutrophils were capable of biosynthesis of FcRIII. This is in agreement with the persistent occurrence of FcRIII on the plasma membrane even though the receptor density is low on the cells of these patients.

After the finding of three categories of neutrophils in this patient, we found that three other PNH patients showed only two of these categories (1 and 3) of neutrophils. This indicates that there are differences among patients, and is in accordance with the finding that the number of Fc receptors on affected neutrophils varies considerably among patients and ranges from 3% to 19% of the normal number.8 Furthermore, three categories of erythrocyte, varying in their sensitivity to complement lysis, are found in PNH patients, and differences among patients have also been illustrated by the finding that the percentages in which these three categories occur vary among patients.

In sum, the results concerning the intracellular localization of GPI-linked molecules in three PNH patients show that these molecules cannot be localized intracellularly by immuno-electron microscopy. This could be because GPI-linked molecules are not or rarely synthesized in neutrophils of these patients, but could also be due to posttranslational regulation and modulation, which could lead to the early degradation of newly synthesized molecules or to loss of antigenic determinants. Furthermore, the occurrence of patients with only two categories of neutrophil, in contrast to the earlier patient with three such categories, suggests that not all PNH patients are the same in this respect.

ACKNOWLEDGMENT
The authors are grateful to Dr W. Fibbe for providing us with blood of PNH patients and to C.E. van der Schoot and T.W.J. Huizinga for helpful discussions. Moreover, we thank J.J.M. Onderwater for technical assistance, L.D.C. Verschragen for preparing the photographic material, and I. Seeger for reading the English text.

REFERENCES
Intracellular localization of glycosyl-phosphatidylinositol-anchored CD67 and FcRIII (CD16) in affected neutrophil granulocytes of patients with paroxysmal nocturnal hemoglobinuria

CR Jost, ML Gaillard, JA Fransen, MR Daha and LA Ginsel