Evidence for glycosylphosphatidylinositol (GPI)-anchored eosinophil-derived neurotoxin (EDN) on human granulocytes

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Abstract Eosinophil-derived neurotoxin (EDN) is one of the four basic proteins stored in specific eosinophil granules. Here we demonstrate that EDN can also be detected at the surface of granulocytes. Reduction of EDN membrane expression after phosphatidylinositol-specific phospholipase C treatment suggests that a glycosylphosphatidylinositol (GPI) anchor is involved in the membrane association of EDN. The presence of a GPI anchor was confirmed by a lower expression of membrane EDN on granulocytes from patients with paroxysmal nocturnal hemoglobinuria which present cells lacking GPI anchor proteins. Furthermore, metabolic labeling with GPI anchor components supports biochemical evidence of GPI anchoring of EDN.

Key words: Neutrophil; Eosinophil; Cell surface molecule; Eosinophil-derived neurotoxin; Paroxysmal nocturnal hemoglobinuria

1. Introduction

Eosinophil-derived neurotoxin (EDN) is one of the four cationic proteins present in eosinophil granules. It is found in the granule matrix as shown by immuno-electron microscopy [1]. EDN is also present in basophils and in granules of neutrophils [2,3]. In various eosinophilic diseases, this protein is detected in blood or in body fluids. Elevated EDN levels have been measured in urine collected from patients with Churg–Strauss syndrome, atopic asthma, or helminth diseases [4–6], in tears collected from conjunctival allergic disorders [7], in sera of patients with asthma [8], and in cerebrospinal fluid of children with *Baylisascaris procyonis* encephalitis [9]. Moreover, EDN levels may provide information on eosinophil activation in diseases. For example, urinary EDN is a useful marker for atopic dermatitis activity [10] and the measurement of EDN in feces may be used for evaluating disease activity and predicting relapse in patients with inflammatory bowel disease [11].

EDN possesses neurotoxic activities exhibited in the Gordon phenomenon [12] and exerts an inhibitory effect on lymphocyte proliferation [13]. EDN has a weak helminthotoxic activity as revealed by high concentrations needed to kill microfilariae of *Bruga malayi* or *B. pahangi* [14]. EDN has been implicated in host defense against respiratory virus pathogens via its ribonucleolytic activity [15,16]. Indeed, EDN presents a strong structural homology with proteins of the ribonuclease superfamily [17]. Recombinant EDN promotes a decrease in respiratory syncytial virus infectivity that correlates with the loss of viral genomic RNA, suggesting that EDN is responsible for the direct ribonucleolytic destruction of extracellular virus [15]. The mechanisms and the conditions of eosinophil activation in which the ribonuclease enzymatic activity of EDN is exhibited remain to be investigated.

Until now, EDN has been described as a protein stored in the large cytoplasmic granules of granulocytes and secreted after activation. In this study, we demonstrate that EDN is also present on the eosinophil and neutrophil membranes as a glycosylphosphatidylinositol (GPI)-anchored protein.

2. Materials and methods

2.1. Purification of peripheral blood cells

Human venous blood samples were obtained from seven healthy donors, four hypereosinophilic patients and three patients with paroxysmal nocturnal hemoglobinuria (PNH) after written consent. Mononuclear cells (MNC) and granulocytes were collected after centrifugation on Ficoll-Paque (Tech Gen International, Les Ulis, France). MNC were obtained from the interface and granulocytes were depleted of erythrocytes by lysis, successively with 0.2% NaCl and 1.6% NaCl. Granulocytes, collected from seven healthy donors and three PNH patients, contained more than 95% neutrophils as evaluated with a variant of the May-Grünwald-Giemsa method (RAL kit, Vasse Industries, Paris, France). Eosinophils were purified from three healthy subjects and four hypereosinophilic patients by using a negative selection technique with CD16 antibody (Ab). Granulocytes were subjected to a magnetic cell separation system using CD16 Ab conjugated with magnetic beads (Variomacs; Miltenyi Biotec, Bergisch Gladbach, Germany), as described by Hansel et al. [18]. The degree of eosinophil purity evaluated with RAL kit was greater than 95% (MNC were the predominant contaminating cells).

2.2. Purification of EDN and preparation of polyclonal anti-EDN antibody

EDN was purified from urine of patients with blood hypereosino-

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Abbreviations: EDN, eosinophil-derived neurotoxin; GPI, glycosylphosphatidylinositol; PNH, paroxysmal nocturnal hemoglobinuria; MNC, mononuclear cells

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philia according to a protocol described previously [19]. Polyclonal anti-EDN Ab was obtained by immunization of rabbits, which were inoculated with 200 μg of purified EDN as 30 intradermal injections in complete Freund's adjuvant, according to Vaïkutakis [20]. Total IgG from pooled sera of immunized rabbits was purified after incubation with acrylic beads coupled with protein A (Sigma, Saint Louis, MO, USA). Anti-EDN polyclonal IgG Ab fraction (anti-EDN Ab) was diluted at a 1:1 ratio with glycerol (Sigma) and stored at −20°C until use. IgG fraction, prepared with the same procedure from non-immunized rabbit sera (control Ab), was used as negative control.

2.3. Flow cytometry analysis

Cells (3×10^5) in 50 μl of phosphate-buffered saline (PBS) containing 1 mg/ml human γ-globulins (Sigma) were first incubated with rabbit anti-EDN Ab or control Ab used at 1:20 dilution for 30 min at 4°C. After two washes with PBS, cells were incubated with FITC-labeled goat $F(ab')_2$ anti-rabbit IgG antibody (Pharmingen BD Biosciences, San Diego, CA, USA) for 30 min at 4°C and then washed twice with PBS. Cells were analyzed using a flow cytometer (Elite ESP™, Beckman Coulter, Brea, CA, USA). To gate in lymphocyte or monocyte population, 3×10^5 MNC were incubated with FITC-conjugated CD3 or CD14 Ab (Beckman Coulter), respectively. CD16, CD15 or CD11b expression was investigated using FITC-conjugated CD16 or CD15 Ab (Beckman Coulter) and PE-labeled CD11b Ab (Dako, Glostrup, Denmark). Results were expressed either as percentage of positive cells, determined by subtraction of binding of unrelated rabbit or mouse antibodies, or as mean fluorescence intensity.

To investigate the presence of GPI-anchored protein, granulocytes were pre-incubated with phosphatidylinositol-specific phospholipase C (PI-PLC) derived from *Bacillus cereus* (0.01–1 U/ml) (Calbiochem, San Diego, CA, USA) in RPMI 1640 for 1 h at 37°C. Cells were then washed twice with PBS, and stained for flow cytometric analysis as described above.

2.4. Immunostaining

Granulocytes and MNC (0.8×10^5), collected from healthy subjects, were resuspended in 100 μl of PBS, loaded on slides by cytocentrifugation (Thermo Shandon, UK) and fixed for 20 min with 4% paraformaldehyde at room temperature. After three washes, the preparations were incubated for 1 h, at room temperature, with rabbit anti-EDN Ab or control Ab, diluted 1:1000 with Tris–HCl (0.05 M, pH 7) containing human γ-globulins (1 mg/ml). After three washes, binding of antibodies was detected by incubating cells with alkaline phosphatase-goat anti-rabbit IgG (dilution 1:1000; Sigma) for 1 h. Slides were then developed with BCIP/NBT (Sigma).

2.5. Sandwich-ELISA EDN

Rabbit anti-EDN Ab was used to evaluate plasma EDN levels (ng/ml) by using a sandwich ELISA as described previously [7]. Plasma samples were tested in duplicate, at a dilution of 1:100 or 1:500.

2.6. Western blotting analysis

Cells were centrifuged 5 min at $300\times g$ and extraction buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin and 100 mM vanadate, Sigma) was added to the pellet (10 μl per 1×10^6 cells). After six freeze–thaw cycles, tubes were centrifuged at $10\,000\times g$, 15 min at 4°C. Supernatants, containing nuclear and cytosolic proteins, were removed carefully and kept at −80°C until use. Protein extracts (20 μg) or purified EDN (1 μg) were applied to a 12% SDS–polyacrylamide gel and electro-transferred to nitrocellulose membrane. EDN was detected by successive incubations with rabbit anti-EDN Ab (1:2500) and then with peroxidase-coupled donkey anti-rabbit IgG (1:10 000) (Amersham Biosciences, Buckinghamshire, UK). Immunoreactivity was determined after incubation with the enhanced chemiluminescence detection reagents (ECL, Amersham Biosciences).

2.7. Metabolic labeling of neutrophils and immunoprecipitation

Peripheral blood neutrophils were incubated for 4 h at 37°C with 0.5 mCi [9,10(*n*)-^{3}H]palmitic acid or with 0.5 mCi [9,10(*n*)-^{3}H]myristic acid (Amersham Biosciences) coupled with serum albumin, in 10 ml DMEM medium, or with 0.5 mCi [1-^{3}H]ethan-1-ol-2-amine hydrochloride (Amersham Biosciences) in 10 ml DMEM medium, or with 0.5 mCi D-[6-^{3}H]glucosamine hydrochloride (Moravek Biochemicals, Brea, CA, USA) or with 0.5 mCi D-[2-^{3}H]mannose (Amersham Biosciences) in glucose-free DMEM medium (Invitrogen) supplemented with 2.5 mM sodium pyruvate. After incubation, cells were washed two times with PBS and the proteins were extracted with 1 ml of lysis buffer (20 mM Tris, 140 mM NaCl, 1 mM EDTA, protease inhibitor cocktail (Sigma), 1 mM iodoacetic acid (Sigma), 0.5% Triton X-100). After 30 min on ice, tubes were centrifuged at $10\,000\times g$, 15 min at 4°C. Supernatants were removed and specifically immunoprecipitated overnight with 5 μg rabbit anti-EDN Ab and 1 h with protein G-Sepharose. Beads were eluted with Laemmli buffer [21] and samples were subjected to a 12% SDS–polyacrylamide gel electrophoresis and autoradiography analysis.

3. Results

3.1. Expression of EDN at the surface of human granulocytes

Detection of EDN was investigated using flow cytometric analysis at the surface of blood leukocytes. Monocytes, lymphocytes and neutrophils were collected from seven healthy subjects whereas eosinophils were obtained from three healthy subjects and four hypereosinophilic patients. The percentage of EDN-positive cells was 79.5 ± 15.5% for neutrophils (Fig. 1A), 48.2 ± 24.4% for eosinophils (Fig. 1B), 1.1 ± 1.7% for CD14-positive MNC (Fig. 1C), and 2.1 ± 3.3% for CD3-positive MNC (Fig. 1D). Similar variations in the intensity of surface expression of EDN with regard to cell specificity were noted (mean fluorescence intensity: neutrophils: 16.9 ± 22.3, eosinophils: 7.5 ± 8, CD14-positive MNC: 0.5 ± 0.1, and CD3-positive MNC: 0.2 ± 0.1). In agreement with results observed by flow cytometry analysis, high expression of EDN at the surface of granulocytes was also revealed after immunostaining performed on cytopreparations of polymorphonuclear cells (Fig. 1G). In contrast, no staining was noted on MNC, as shown in Fig. 1H.

3.2. EDN is present on the surface of cells that synthesize the protein

To understand the origin of the membrane EDN, we examined if there was a correlation between eosinophil surface EDN and plasma EDN levels that have been found to be elevated in hypereosinophilic diseases. As shown in Table 1, high or low EDN surface expression was observed on eosinophils collected either in patients or in healthy donors. No

Table 1
Blood eosinophil counts, plasma EDN levels, and membrane EDN expression on eosinophils collected from healthy donors and from hypereosinophilic patients

	Healthy donors			Hypereosinophilic patients			
	1	2	3	1	2	3	4
Blood eosinophil count ($\times10^9$/l)	0.2	0.4	0.4	6.2	2.6	2.4	2.7
Plasma EDN levels (ng/ml)	20	60	78	631	736	200	390
EDN-positive eosinophils (%)	60.5	32.2	90.7	59.4	17.5	22.2	46.5

EDN surface expression is independent of the plasma EDN level.

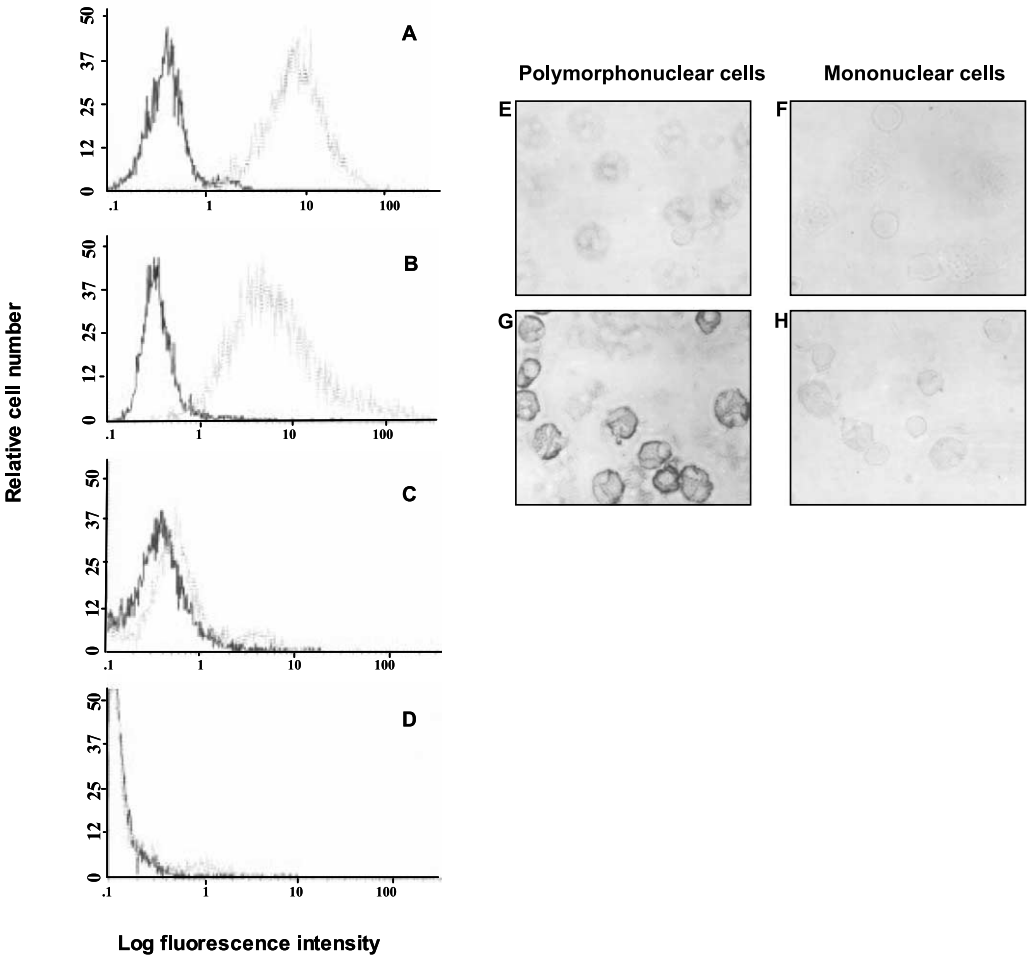

Fig. 1. Membrane EDN is expressed on neutrophils and eosinophils but not on monocytes and lymphocytes. Cells were first incubated with rabbit anti-EDN Ab (dotted line) or control Ab (solid line) in presence of human γ-globulins, then stained with FITC-labeled $F(ab')_2$ fragment of goat anti-rabbit IgG Ab. Surface expression of EDN on neutrophils (A), eosinophils (B), monocytes (C) gated with FITC-conjugated CD14 Ab, and on lymphocytes (D) gated with FITC-CD3 Ab was analyzed by flow cytometry. Histograms are representative of each cell population tested in one healthy subject. Cytocentrifuged polymorphonuclear (E,G) and mononuclear (F,H) cells were fixed with paraformaldehyde and incubated with control Ab (E,F) or rabbit anti-EDN Ab (G,H) in the presence of human γ-globulins. The binding is revealed by alkaline phosphatase-goat anti-rabbit IgG Ab and BCIP/NBT.

statistical difference was noted between the percentage of EDN-positive eosinophils from healthy and hypereosinophilic patients ($P = 0.1$, non-parametric Mann–Whitney test). EDN surface expression appears to be independent of the plasma EDN level ($R = -0.5$, $P = 0.13$, Spearman's correlation test). One possibility was that membrane EDN had an intracellular origin. We tested this hypothesis by evaluating EDN synthesis with Western blot analysis. As shown in Fig. 2, expression of EDN was observed in neutrophils and eosinophils but not in MNC. These data indicate that membrane EDN is detected on cell populations that synthesize EDN protein.

3.3. Low EDN expression on the surface of cells isolated from PNH patients

Next, we investigated how EDN could be linked to the plasma membrane. Analysis of the EDN amino acid sequence [22] does not suggest that EDN is a transmembrane protein, since a sequence of about 26 hydrophobic amino acids required for transmembrane anchor is not found either in the C-terminal or in the N-terminal EDN sequence. However, the hydrophobicity profile of the COOH-terminus published EDN sequence [22] reveals that the end of the polypeptide sequence is a short hydrophobic domain (residues 125–134) preceded by 13 hydrophilic residues. Similar structure and hydrophobicity profile are shared with GPI-anchored proteins [23].

To determine the involvement of a GPI anchor in the membrane association of EDN, we evaluated the presence of EDN

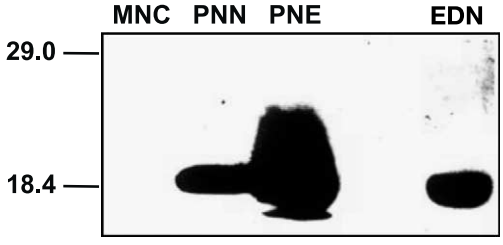

Fig. 2. EDN is expressed in polymorphonuclear eosinophils (PNE) and in polymorphonuclear neutrophils (PNN) but not in MNC. Western blotting of 20 μg of protein extracts probed with rabbit anti-EDN Ab. The positive control contains 1 μg of purified EDN (lane EDN).

on granulocytes isolated from patients with PNH. PNH is an acquired defect of hematopoietic precursor cells in the biosynthesis of the GPI anchor and may affect clone progeny in the different hematopoietic lineages. Membrane EDN expression together with CD11b expression (a non-GPI-linked protein) were investigated on neutrophils isolated from PNH patients with 10%, 40% and 95% abnormal cells and from control

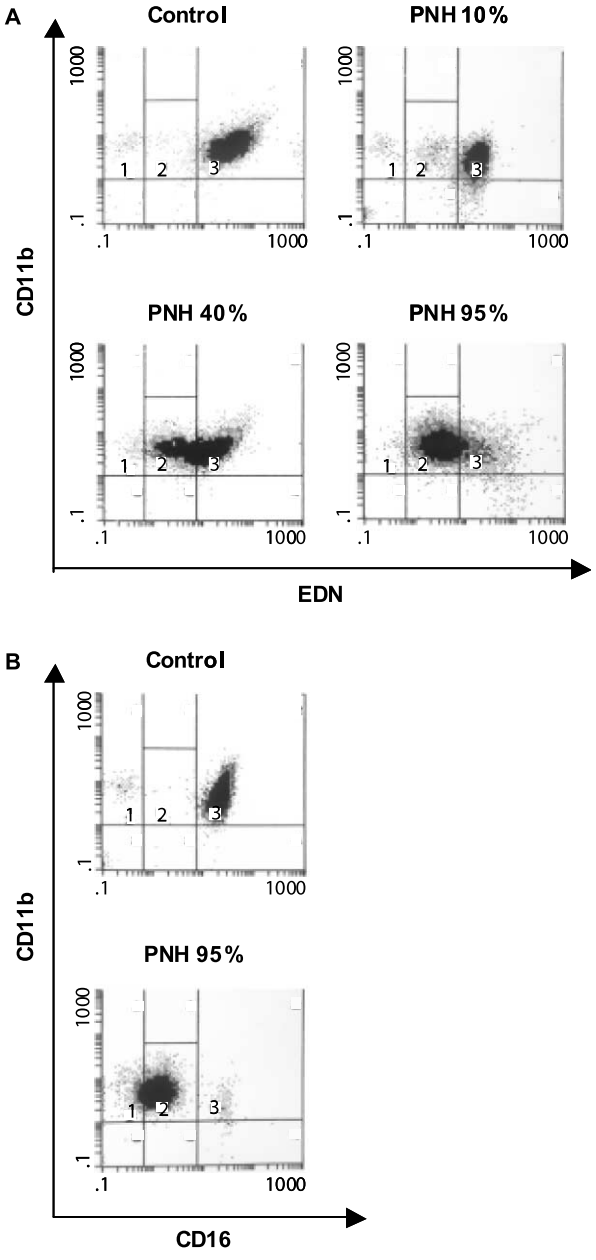

Fig. 3. Membrane EDN expression parallels the percentage of abnormal cells isolated from PNH patients. Neutrophils isolated from one representative healthy control and from three PNH patients having 10%, 40% and 95% abnormal cells. Double staining with PE-CD11b Ab and rabbit anti-EDN Ab with FITC-goat anti-rabbit IgG (A). Double staining with PE-CD11b Ab and FITC-CD16 Ab (B). Two quad-stat regions were placed in each graph. Quad-stat region 1 separates negative cells for CD11b and EDN expression (determined with corresponding unrelated Ab) and the vertical line of quad-stat 3 is placed at the lowest value of the fluorescence intensity of EDN-positive cells from the healthy control sample. The rectilinear region 2 corresponds to CD11b-positive cells with low expression of EDN.

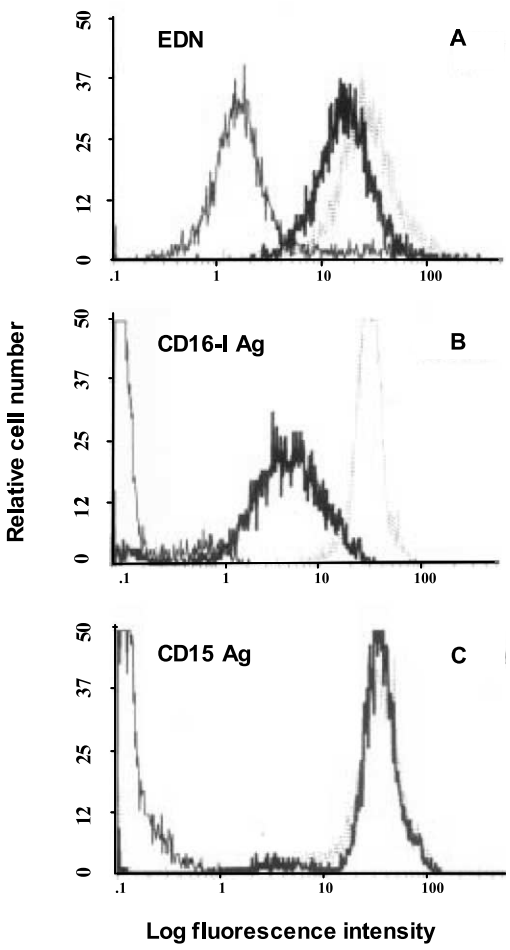

Fig. 4. Modulation of EDN membrane expression after treatment with PI-PLC. Respective numbers of CD15- CD16-, and EDN-positive cells were evaluated with (thick line) and without (dotted line) treatment with PI-PLC (0.1 U/ml) (thin line: control Ab). Histograms illustrate one representative experiment.

subject in comparison with CD16-1 (a GPI-anchored protein)/CD11b expression. As shown in Fig. 3A, neutrophils from the control subject presented high density EDN expression. In PNH patients, we observed that high density EDN expression shifted into low density EDN expression, according to the extent of deficiency in the abnormal clone of these PNH patients. The percentages of cells in the rectilinear region 2 are 6.5%, 40% and 75% for patients having 10%, 40% and 95% abnormal cells, respectively. Similar shifts of CD16-1 expression were observed on granulocytes from the PNH patient having 95% abnormal cells (Fig. 3B). No variation in CD11b expression was observed on cells from either healthy or PNH subjects.

3.4. *Membrane EDN expression is sensitive to PI-PLC treatment*

To back up the results, we evaluated by flow cytometric analysis the EDN expression on neutrophils treated with PI-PLC. The effect of hydrolysis induced by PI-PLC on EDN membrane expression was compared to the effect on the carbohydrate CD15 antigen, which is not GPI-anchored, and to the expression of CD16-1 antigen, a GPI-anchored protein [24]. PI-PLC treatment at 0.1 U/ml (Fig. 4) induced a drastic

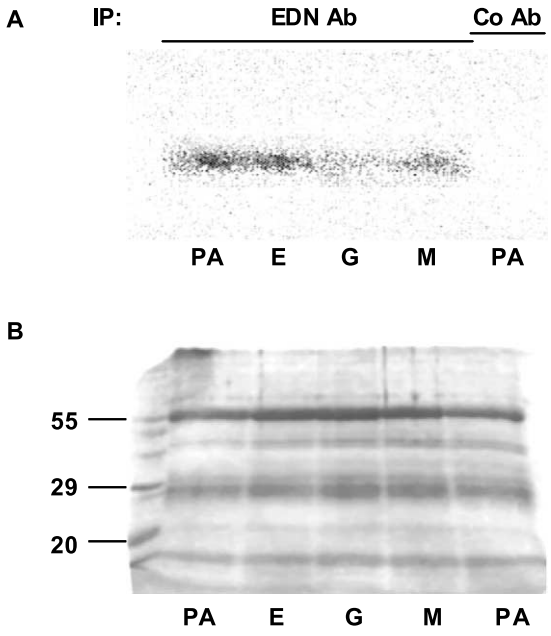

Fig. 5. A GPI anchor is involved in the membrane association of EDN. Autoradiography after metabolic labeling of neutrophils with tritiated GPI anchor components (PA: [^{3}H]palmitic acid, GlcN: [^{3}H]glucosamine, Man: [^{3}H]mannose, EtN: [^{3}H]ethanolamine), and immunoprecipitation with anti-EDN Ab or control Ab (A). Coomassie blue staining of the SDS–PAGE (B).

decrease in the intensity of fluorescence on EDN-positive cells (reduction of 56.5 ± 15.8%, $n = 4$) and on CD16-positive cells (reduction of 59.5 ± 18.5%, $n = 4$), while CD15 expression was unchanged. A higher concentration of PI-PLC (1 U/ml) was not more effective in EDN release from the membrane (not shown).

3.5. *EDN is linked to the membrane through a GPI anchor molecule*

To demonstrate that EDN is directly linked to a GPI anchor we performed metabolic labeling of neutrophils with tritiated GPI anchor components such as ethanolamine, glucosamine, mannose, myristic acid, and palmitic acid. After immunoprecipitation with rabbit anti-EDN Ab, proteins extracts were subjected to SDS–PAGE and autoradiography. We obtained a band corresponding to the size of EDN but with various intensities, depending on the labeling. In Fig. 5A the amount of incorporated radioactivity was 12 000 cpm with [^{3}H]palmitic acid (lane 1), 5300 cpm with [^{3}H]glucosamine (lane 2), 1700 cpm with [^{3}H]mannose (lane 3), and 2800 cpm with [^{3}H]ethanolamine (lane 4). Labeling of neutrophils with [^{3}H]myristic acid did not result in incorporation of radioactivity into EDN (not shown). Coomassie blue staining of the gel showed that the same amounts of proteins were loaded on each lane (Fig. 5B). No signal was obtained after immunoprecipitation with the control rabbit serum of cell extract labeled with [^{3}H]palmitic acid (Fig. 5A, lane 5). Taken together with the PI-PLC assay, these results show that the membrane association of EDN on granulocytes involves a GPI anchor.

4. Discussion

EDN is a cationic protein known to be present in granules of eosinophils and neutrophils. Our results indicate that EDN can be detected at the surface of neutrophils and eosinophils as revealed by flow cytometric analysis and immunostaining using rabbit polyclonal anti-EDN Ab. This antibody is specific for EDN and recognizes a 18 kDa protein in urine of hypereosinophilic patients as well as in eosinophils and neutrophils, revealed by Western blot analysis. Moreover, in the sandwich ELISA EDN, this antibody does not cross-react with ECP, the most closely related protein. Numerous immunochemical studies have already been performed on eosinophils to detect the presence of intracellular EDN. Staining was usually preceded by a cell permeabilization step that altered the plasma membrane, leading to a release of endogenous PI-PLC [25]. This procedure may explain why EDN has not yet been detected as a membrane protein. Here we used paraformaldehyde-fixed cells that keep the integrity of their plasma membrane.

Absence of transmembrane anchor sequence and hydrophobicity profile of the COOH-terminus published EDN sequence led us to investigate the presence of EDN at the surface of granulocytes collected from patients with PNH, an acquired defect of hematopoietic precursor cells in the biosynthesis of GPI. A cell population with very low density expression of EDN was detected and its extent paralleled those of abnormal clones detected with other GPI-anchored molecules: CD16 on neutrophils and CD14, CD66 on monocytes (not shown). The cleavage of GPI anchors by bacterial PI-PLC is the most common criterion used in GPI anchor identification. The release of EDN from the membrane is induced by PI-PLC up to about 60%. Cleavage to a similar extent is described for other GPI-anchored proteins [26,27]. The sensitivity of GPI-anchored proteins towards PI-PLC is dependent on the specificity of the cells tested [28], the lipid fluidity of the membrane [27], and the origin of the phospholipase [29]. The sensitivity of neutrophil membrane EDN might be related to the properties of PI-PLC obtained from *B. cereus* used in our experiments. In spite of the low metabolism of the differentiated cells, metabolic labeling of neutrophils with components common to all GPI molecules was successful. Together with the cleavage by PI-PLC, the incorporation of radioactivity shows that a GPI anchor is involved in the membrane association of EDN. Alternative splicing is a mechanism known to generate transmembrane, GPI-anchored, or secreted forms of several proteins [30,31]. An alternative splicing could explain the presence of a membrane EDN isoform containing a GPI anchor, and of a granule EDN isoform susceptible to be secreted. An alternatively spliced mRNA of VCAM leads to a GPI-anchored protein [32] detected by incorporation of [^{3}H]-ethanolamine and by cleavage of VCAM after PI-PLC treatment. In the case of EDN, we have found incorporation of radioactivity after labeling with [^{3}H]ethanolamine, [^{3}H]palmitic acid, [^{3}H]glucosamine, and [^{3}H]mannose.

These new findings raise questions about the physiopathologic functions of EDN on the surface of polymorphonuclear cells. EDN is mainly described as a protein with ribonuclease activity. Domachowske and colleagues have demonstrated that eosinophils are capable of inhibiting retroviral transduction of human target cells [33]. Purified EDN has been shown to exert this antiviral activity [15,16] that is specifically blocked by a ribonucleolytically inactivated mutant of EDN [34]. In this context, it would be of interest to evaluate the role of surface EDN. In the absence of eosinophil degranulation

that releases the cationic proteins, EDN present at the surface or cleaved by the action of specific phospholipases on the GPI anchor could directly exert this antiviral activity. Domachowske et al. have obtained results suggesting that regions within the first 50 amino acids of human EDN are required for an antiviral activity [34]. With regard to the EDN sequence, one putative GPI binding site for EDN protein might be found between the Cys 111 and Asp 112 amino acids since the two amino acids found usually immediately after the GPI binding site are combinations of A, N, D, G, and S residues [35]. In this case, the N-terminal end is conserved leading to the maintenance of enzyme activity. It would give one more clue to support the idea of the implication of eosinophils in the immune response in viral infections.

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