Neutrophil-Specific Granule Deficiency Includes Eosinophils

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Neutrophil-specific granule deficiency is a disorder of leukocyte maturation associated with decreased levels of mRNA for a distinct subset of granule proteins. Our work indicates that this disorder, previously thought to be limited to the neutrophil lineage, can also include eosinophils. Immunofluorescence staining led to the discovery of a small but distinct population of peripheral white blood cells containing eosinophil peroxidase (EPO). Unlike normal eosinophils, these EPO⁺ cells do not have large, eosin-staining cytoplasmic granules, and are indistinguishable from granule-deficient neutrophils by light microscopy. The EPO⁺ cell lineage did resemble the normal eosinophil lineage in its ability to respond dramatically to granulocytomacrophage colony-stimulating factor (GM-CSF); the size of the EPO⁺ peripheral cell population increased ~70-fold over baseline in response to GM-CSF administration. The EPO⁺ cells contained eosinophil Charcot-Leyden crystal protein, but were deficient in three eosinophil-specific granule proteins; neither eosinophil cationic protein, eosinophil-derived neurotoxin, nor major basic protein could be detected in these EPO⁺ cells, despite the presence of mRNA transcripts for each of the three absent proteins. This is a US government work. There are no restrictions on its use.

NEUTROPHIL-SPECIFIC granule deficiency is a rare disorder in which an increased susceptibility to bacterial infection is coupled to specific defects in neutrophil structure and function. Defects in neutrophil chemotaxis, disaggregation, receptor upregulation, and bactericidal activity¹,² are found in conjunction with specific structural abnormalities. These abnormalities include large, cleft nuclei and the absence of secondary or specific cytoplasmic granules. Only trace amounts of lactoferrin, vitamin B-12 binding protein, and collagenase, all specific granule proteins, are detected in peripheral blood neutrophils from affected individuals.¹ Reduced levels of the primary granule proteins, defensins, have also been reported.³ Lomax et al⁴ showed that only trace quantities of lactoferrin mRNA could be detected in bone marrow from an individual with specific granule deficiency. Although lactoferrin could not be detected in granule-deficient peripheral neutrophils, nasal secretions from the same individual contained normal amounts of this protein. These findings suggested that the granule protein deficiencies were the result of a neutrophil-specific transcription defect. The experiments described in this report indicate that specific granule deficiency is not limited to neutrophils and suggest that granule protein biosynthesis in the neutrophil and eosinophil lineages may be regulated via a common mechanism.

MATERIALS AND METHODS

Preparation of peripheral white blood cells (WBCs). Ten milliliters of heparinized blood from a patient with specific granule deficiency and a normal volunteer were diluted 1:1 with 3% dextran in 0.85% sodium chloride to sediment red blood cells (RBCs); cells remaining in supernatant were washed twice with cold Hanks’ Balanced Saline Solution (HBSS), and RBCs remaining were lysed with cold ammonium chloride buffer (150 mmol/L ammonium chloride/10 mmol/L potassium carbonate/0.1 mmol/L EDTA). The WBCs were washed twice and either frozen at −80°C or resuspended in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and fixed on glass slides via a Shandon Cytofun 2 centrifuge (Shandon, Inc, Pittsburgh, PA) (1,000 rpm for 6 minutes), followed by immersion in methanol for 5 minutes at room temperature.

Immunofluorescence staining. Peripheral WBCs fixed on slides were hydrated for 10 minutes at room temperature in PBS and incubated with a 1:4 dilution (all dilutions in PBS) of normal goat serum (Jackson Immunoresearch Laboratories, Inc, West Grove, PA) in a humidified chamber at 37°C for 1 hour.Slides were washed three times with PBS and then incubated as above with primary antibody (either 1:200 mouse monoclonal antibody [MoAb] 263ASC anti-eosinophil peroxidase [anti-EPO] alone or together with 1:300 rabbit anti-major basic protein [anti-MBP], anti-eosinophil-derived neurotoxin/eosinophil cationic protein [anti-EDN/ECP], or anti–Charcot-Leyden crystal [anti-CLC]). Fixed cells were washed three times with PBS and then incubated as described above with fluorescent-tagged secondary antibody (either 1:1,000 dichlorotriazinyl amino fluorescein [DTAF]-conjugated goat antimouse IgG alone or together with 1:750 tetramethyl rhodamine isothiocyanate [TRITC]-conjugated goat antirabbit IgG; Jackson Immunoresearch Laboratories, Inc, West Grove, PA) with 1% BSA/PBS). Slides were washed three times in PBS, air-dried, and mounted in 10% polyvinyl alcohol/10 mmol/L propyl gallate and viewed with a Zeiss fluorescence microscope (Carl Zeiss, Inc, Germany). One percent Chromotrope 2R (Aldrich Chemical Co, Milwaukee, WI) was used to block nonspecific fluorescence binding to eosinophil granules.

Detection of genomic DNA sequences. Normal and specific granule-deficient peripheral leukocytes prepared and frozen as described above were resuspended at 5,000/μL in 10 mmol/L Tris-HCl/50 mmol/L KCl/2.5 mmol/L MgCl₂/0.5% Tween 20 (pH 8.3) with 100 pg/μL proteinase K (Sigma Chemical Co, St Louis, MO). Cells were incubated for 1 hour at 56°C and then for 10 minutes at 95°C. Polymerase chain reaction (PCR) was performed using 10 μL of this digest as a template and 1 μmol/L primers (see below) in a final volume of 100 μL; three-temperature PCR (95°C for 20 seconds, 55°C for 20 seconds, and 72°C for 30 seconds for 40 cycles, with 3 minutes at 72°C for extension) was performed using standard reagents (GeneAmp; Perkin-Elmer Cetus, Emeryville, CA) in a Perkin-Elmer Cetus 9600 thermocycler. Ten microliters of reaction products was evaluated by agarose gel electrophoresis; ethidium-stained bands were transferred to a nitrocellulose membrane and hybridized sequentially to each of three 5'-P⁻³²-labeled gene-spe-
Fig 1. Immunofluorescence staining of specific granule-deficient (A) and normal (C) leukocytes. In (A) and (C), fixed cells were incubated with a 1:200 dilution of mouse MoAb 263ASC (anti-EPO) followed by a 1:1,000 dilution of DTAF-conjugated goat anti-mouse IgG. (B and D) Phase contrast micrographs of the fields shown in (A) and (C), respectively; the arrow in (B) identifies the cell staining with MoAb 263ASC shown in (A).

Fig 3. Immunofluorescence staining of normal and specific granule-deficient leukocytes. The specific granule-deficient cells were taken from the patient at day 6 of GM-CSF administration. Specific granule-deficient (A and B) and normal control cells (C and D) were incubated simultaneously with MoAb 263ASC, anti-EPO (1:200), and rabbit anti-EDN/ECP (1:300), followed by DTAF-conjugated goat anti-mouse IgG (1:1,000) together with TRITC-conjugated goat antirabbit IgG (1:750). Identical fields were photographed through appropriate filters to detect fluorescein and rhodamine fluorescence.

Fig 4. Immunofluorescence staining of normal and specific granule-deficient leukocytes. The conditions are same as in Fig 3, except that the first antibody solution contained MoAb 263ASC (1:200) with rabbit anti-MBP (1:300).

Fig 5. Immunofluorescence staining of normal and specific granule-deficient leukocytes. The conditions are same as in Fig 3, except that the first antibody solution contained MoAb 263ASC (1:200) with rabbit anti-CLC (1:300).
cific oligonucleotide probes (see below). The nitrocellulose membrane was immersed in 0.2X SSPE at 65°C for 1 hour to remove radiolabeled probe between hybridizations.

Amplification primers and hybridization primers are listed below: base pairs (bp) refer to the site at which the oligonucleotide sequence is located in genomic maps in Hamann et al22 and Barker and et al4 and shown as the response of granule-deficient leukocyte populations in Hamann et al22 and Barker and et al4 and shown as the response of granule-deficient leukocyte populations to intravenous administration of GM-CSF (112 μg/d). Fixed cells were prepared on days indicated and treated as described in Fig 1. EPO+ cells were counted in four independent high power (40 x) fields (error bars). Total leukocyte counts were obtained from Wright’s-stained peripheral blood smears.

![Figure 2](image_url)

**Fig 2.** EPO+ cell (m) and total peripheral leukocyte counts (square) are shown as the response of granule-deficient leukocyte populations to intravenous administration of GM-CSF (112 μg/d). Fixed cells were prepared on days indicated and treated as described in Fig 1. EPO+ cells were counted in four independent high power (40 x) fields (error bars). Total leukocyte counts were obtained from Wright’s-stained peripheral blood smears.

Primers are numbered according to the cDNA sequences reported in the literature41: MBP (5'-3'), ATGAAACTCCCTATTCTCTGCT (bp 69-92); (3'-5'), TCAGTAGAACGTAAGAGGAAAG (bp 714-737); ECP (5'-3'), AGATGATTCTATCCAGGTGAA (bp 406-426); and EDN, 5′-TTAACATAGCCCTCCCAACC (bp 406-426).

**RESULTS**

Shown in Fig 1 is a comparison of peripheral WBCs from a normal donor with those from an individual with specific granule deficiency. The normal eosinophils (Fig 1C and D) have bilobed nuclei and prominent cytoplasmic granules. The mouse MoAb 263ASC, specific for EPO, detects this protein in normal eosinophils (Fig 1C). The MoAb 263ASC recognizes an epitope unique to EPO; despite 68% sequence homology between EPO and neutrophil myeloperoxidase (MPO),12,13 the neutrophils in this field remain unstained.

The eosinophils from an individual with specific granule deficiency were identified by their reactivity with MoAb 263ASC (Fig 1A). These EPO+ cells do not have prominent cytoplasmic granules (Fig 1B, cell at arrow); both EPO+ and EPO+ granulocytes have atypically large, cleft nuclei and are otherwise indistinguishable from one another at the light microscopic level. The EPO+ cells represent less than 1% of this patient’s baseline total WBC count (Fig 2).

Administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) resulted in an ~70-fold expansion of the peripheral EPO+ cell population (Fig 2). At day 0, the total WBC count was 5,700/μL, including 0.2% EPO+ cells. At day 6, the WBC count increased slightly (7,800/μL), whereas the pool of peripheral EPO+ cells had expanded to 10% of the total, or from 11 to 780 EPO+ cells/μL. Peripheral eosinophilia is a common reaction to GM-CSF therapy in individuals with disorders unrelated to specific granule deficiency14 and can occur without a concomitant neutrophilia.15 Proliferation in response to GM-CSF is a characteristic common to the granule-deficient EPO+ cells and normal eosinophils. No morphologic changes were observed at the light microscopic level in either the EPO+ or EPO+ granulocyte lineages in response to GM-CSF administration.

In addition to EPO, the eosinophil-specific granule contains three other distinct proteins: MBP, ECP, and EDN.16 Shown in Figs 3 and 4 are the results from experiments in which fixed peripheral WBCs are double-stained with MoAb 263ASC anti-EPO and polyclonal rabbit antiserum against EDN/ECP or MBP, respectively. One hundred independent EPO+ cells were scored per experiment. No MBP, EDN, or ECP was detected in the specific granule-deficient EPO+ cells either before (not shown) or after 6 days of GM-CSF administration (Figs 3B and 4B); all proteins stained appropriately in normal eosinophil controls (Figs 3C and D and 4C and D). The antisera prepared against EDN is equally reactive against ECP (H.F. Rosenberg, unpublished results), which is likely due to their 67% amino acid sequence identity.10,13 The brown tinge seen on the fluorescein-stained cells pictured in Figs 4C and 5C (below) results from the detection of EPO by the MoAb 263ASC.
Fig 6. Detection of genes encoding EDN, MBP, and ECP in genomic DNA from normal and granule-deficient leukocytes using the PCR. Preparation of genomic templates, gene-specific amplification primers, and hybridization probes was described in Materials and Methods. (A) Ethidium bromide-stained agarose gel containing PCR products from each set of gene-specific (EDN, MBP, and ECP) primers using both normal (NL) and granule-deficient (PT) genomic DNA templates. PCR products were transferred to nitrocellulose and hybridized to independent ECP-specific (B), MBP-specific (C), and EDN-specific oligonucleotides (D). (E) The genomic structures determined for EDN, ECP, and MBP, along with the relative positions of the amplification primers and hybridization probes.

from interference from the intense red fluorescence of the rhodamine counterstain.

Shown in Fig 5 are the results from experiments in which the WBCs are double-stained with MoAb 263ASC anti-EPO and polyclonal rabbit antisera specific for the eosinophil CLC protein, a constituent of crystalloid-free eosinophil granules that can be distinguished from the crystalloid-containing eosinophil-specific granules. In contrast to the specific granule protein deficiencies shown for the EPO cells, both normal eosinophils and specific granule-deficient EPO cells contain detectable levels of CLC protein (Fig 5B and D).

Although MBP, EDN, and ECP were not found in peripheral EPO cells, gene sequences for all three absent proteins were detected in genomic DNA isolated from granule-deficient WBCs. Gene-specific primers were used to amplify segments of genomic DNA encoding these proteins (see Materials and Methods and Fig 6E). Amplification products obtained using genomic DNA from granule-deficient WBCs as a template were of appropriate size and identical to that produced from DNA from normal WBCs for each of the three genes (Fig 6A). In addition, each amplification product hybridized uniquely to gene-specific oligonucleotides derived from nucleotide sequence spanned by the amplification reaction (Fig 6B through D).

Using reverse-transcriptase PCR, mRNA transcripts for MBP, EDN, ECP, and CLC were detected in total bone marrow RNA from the individual with specific granule deficiency (Fig 7). Amplification products from each pair of gene-specific primers were of appropriate size: MBP at 668 bp, EDN at 431 bp, CLC at 429 bp, and ECP at 216 bp (see Materials and Methods). In addition, each product contained characteristic restriction sites and hybridized to genespecific oligonucleotides (data not shown).

DISCUSSION

Although neutrophil-specific granule deficiency is an extremely rare disorder of leukocyte maturation, evaluation of this disease will add much to our understanding of granule protein regulation and biosynthesis. Previous work suggested that specific granule deficiency was the result of a neutrophil-specific transcription defect; Lomax et al.
showed that, despite the absence of lactoferrin in granule-deficient neutrophils and the detection of only trace amounts of mRNA for lactoferrin in specific granule deficiency bone marrow, there were normal levels of lactoferrin in nasal secretions. We have shown here that granule protein abnormalities similar to those reported in neutrophils are found in eosinophils. Although there were no peripheral leukocytes with characteristic eosin or fast green-staining granules, there was a small subset of peripheral WBCs that contained eosinophil peroxidase and that increased in number in response to GM-CSF administration. It is interesting to note that another individual with specific granule deficiency initially reported by Strauss et al\textsuperscript{19} was reported to have a normal eosinophil count and normal eosinophil granules; furthermore, this other individual’s granule-deficient neutrophils had remnants of specific granule membranes\textsuperscript{19,24} whereas none were observed in the individual described in this report.\textsuperscript{25} This diversity of presentation suggests that the specific granule-deficient phenotype may result from two or more independent genetic lesions.

Although the specific granule-deficient eosinophils contained EPO and CLC, no EDN, MBP, or ECP could be detected. There are no published reports of this eosinophil phenotype occurring elsewhere, either as an isolated condition or in conjunction with another developmental abnormality. It is interesting to compare this phenotype with that of congenital EPO deficiency, an otherwise benign clinical condition in which EPO is missing from the eosinophil-specific granule.\textsuperscript{26} It is not clear as to whether EPO deficiency represents a defect in primary peroxidase gene structure or an aberrancy at the transcriptional or translational level.

The specific granule-deficient phenotype also includes abnormalities of neutrophil primary granules. Gallin et al\textsuperscript{2} showed that neutrophil primary granules from a patient with specific granule deficiency were significantly less dense than their normal counterparts, and Parmley et al\textsuperscript{24} showed that the neutrophil primary granules from another affected individual retained immature staining characteristics. Defensins, major components of the neutrophil primary granule, were detected at significantly reduced levels in neutrophils from two affected patients.\textsuperscript{3} The defect resulting in specific granule deficiency may be manifest during a period of granulocyte maturation in which the synthesis of primary and specific granule proteins overlaps.

Cells from other myeloid lineages also show evidence of granule protein deficiency. Parker et al\textsuperscript{27} have described in this individual a partial deficiency of platelet von Willebrand factor, a component of the platelet \(\alpha\) granule.

It is not clear at this point whether the abnormalities described for eosinophils result from a defect in transcription, as has been shown for the neutrophil lineage.\textsuperscript{4} Although we are able to detect mRNA transcripts for the three absent granule proteins, we do not know whether these transcripts are present in the bone marrow at normal or at reduced concentrations.

The data presented indicate that specific granule deficiency is a disorder that involves the decreased production of numerous granule proteins in at least three distinct myeloid lineages. Delineation of the specific genetic abnormality will certainly provide information on the fundamental mechanism of granule protein biosynthesis.

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GRANULE-DEFICIENT EOSINOPHILS


Neutrophil-specific granule deficiency includes eosinophils

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