Localization and Quantitation of the Vitamin D Binding Protein (Gc-Globulin) in Human Neutrophils

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Plasma-derived vitamin D binding protein (DBP) is an important physiologic regulator of the neutrophil chemotactic response to activated complement. A cell-associated form of DBP has been observed in numerous cell types. We now report that mature, circulating human neutrophils also contain cell-associated DBP. Immunofluorescence studies of normal untreated neutrophils showed the presence of DBP on the cell surface. Western blotting of detergent-soluble neutrophil lysates with a polyclonal anti-DBP showed two major immunoreactive bands, one with an apparent molecular weight of 56 Kd (identical to purified plasma-derived DBP) and a second less prominent band at 12 to 14 Kd. Quantitation of the immunoreactive bands by video densitometry indicated that normal human neutrophils contain 1.5 ± 0.8 ng DBP/10^6 cells (n = 9). Immunoprecipitation of detergent-soluble lysates with the polyclonal anti-DBP showed only the 56-Kd form by Western blotting. In contrast, a monoclonal anti-DBP immunoprecipitated the 12 to 14 Kd form of DBP from lysates of surface-radioiodinated cells. Western blots of subcellular fractions showed that immunoreactive bands were found in the specific (secondary) granule and plasma-membrane fractions. In addition, pretreatment of neutrophils with 10 nmol/L phorbol myristate acetate (PMA) resulted in approximately a 50% reduction in the amount of DBP in both the specific granule and plasma-membrane fractions. Finally, analysis of the cell-free supernatants showed that DBP was spontaneously released into the extracellular milieu; moreover, this release was enhanced if the cells were first stimulated with C5a, formyl-norleucyl-leucyl-phenylalanine (fNLP) or PMA.

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THE VITAMIN D binding protein (DBP), also known as Gc-globulin, is a plasma protein that has the capacity to bind several diverse ligands including vitamin D sterols, unsaturated fatty acids, G-actin and the complement activation peptides C5a and C5a des Arg (reviewed by Cooke and Haddad). DBP is a highly polymorphic protein with three common isotypes and more than 120 minor isotypic variants. The protein is expressed as a single polypeptide chain with a molecular weight of approximately 56 Kd and circulates in plasma with a concentration of 6 to 7 µmol/L. DBP is a member of the albumin and β-lipoprotein gene family and shares considerable amino acid homology with these proteins. Besides functioning as a circulating vitamin D transport protein, it has been shown that plasma DBP effectively scavenges G-actin released at sites of cell injury and/or death and prevents polymerization of actin filaments in the circulation. Recently, DBP also has been shown to be the serum-derived macrophage-activating factor. It is interesting to note that homozygous deficiency of DBP has never been observed in nearly 100,000 individuals examined world-wide; such a deficiency is thought to be incompatible with life. Numerous investigators also have reported a cell-associated form of DBP. Using immunofluorescent or radioisotope techniques, these studies have shown that DBP is localized to the plasma membrane of human blood monocytes and lymphocytes, human placent al trophoblast and smooth muscle cells, rat pancreatic acinar cells, and porcine kidney tubule cells. Recently, Gouth et al have convincingly shown in the human monocytic cell line U937 that cell-surface DBP is derived from plasma (or serum) and not synthesized de novo. In addition to its cell-surface localization, intracellular DBP has been detected in normal human lymphocytes. In contrast, it has been shown that microinjection of purified DBP into the cytoplasm of nonmuscle cells will produce massive (but reversible) disruption of the microfilament organization. Although cell-associated DBP appears to be ubiquitously distributed, numerous questions remain concerning its cell surface binding site (receptor), metabolism, and function.

Previously, we and Perez et al have shown that DBP can bind C5a and its metabolite C5a des Arg and enhance their chemotactic activity for neutrophils. In addition, it was shown by immunologic cross-reactivity and amino acid sequence analysis that DBP is identical to a plasma-derived protein formerly known as cochemotaxin. Recently, several other groups have confirmed our observations that DBP can augment the neutrophil chemotactic activity of C5a and C5a des Arg and may modulate the recruitment of neutrophils to sites of inflammation. Although DBP appears to be a physiologically important regulator of neutrophil chemotactic activity for activated complement, the mechanism of chemotactic enhancement is not known. To address this question we have begun to investigate the interaction of DBP-C5a complexes with human neutrophils. However, these studies have been complicated by the observation that neutrophils, like many other cells, contain an endogenous DBP. Because little is known about cell-associated DBP in human neutrophils, we initiated an investigation to quantitate and localize neutrophil-associated DBP. We report here that human neutrophils contain approxi-
mately 1.5 ng DBP/10⁶ cells. The majority of the cell-associated DBP has a molecular weight identical to plasma-derived DBP (56 Kd) and is found in subcellular fractions that contain markers for the plasma membranes and specific (secondary) granules. Furthermore, it appears that stimulation of neutrophils causes upregulation of DBP from the intracellular pool to the cell surface and the extracellular milieu. We believe that this information is critical to begin to examine the potential role that DBP may play in neutrophil chemotaxis.

MATERIALS AND METHODS

Reagents. Purified human DBP was purchased from Calbiochem (San Diego, CA) and from Biodisign International (Kennebunkport, ME). The formylated chemotactically tripeptides formyl-methionyl-leucyl-phenylalanine (fMLP) and formyl-norleucyl-leucyl-phenylalanine (fNLP) were purchased from Bachem (Torrance, CA). Human serum was purchased from North Metro Diagnostics (San Diego, CA) and from Biodesign International (Kennebunkport, ME). The formylated chemotactic tripeptides formyl-methionyl-leucyl-phenylalanine (fMLP) and formyl-norleucyl-leucyl-phenylalanine (fNLP) were purchased from Bachem (Torrance, CA). Human serum was purchased from North Metro Diagnostics (San Diego, CA) and from Biodesign International (Kennebunkport, ME).

Antibodies. Goat antibodies to human DBP (IgG fraction) were purchased from Atlantic Antibodies (Incstar Corp, Stillwater, MN). Mouse monoclonal antibody (MoAb) (IgG1) to human DBP (MAK 89) was a generous gift from Dr John Haddad, University of Pennsylvania (Philadelphia, PA). Mouse MoAb to actin (KJ43A) and affinity-purified, alkaline phosphatase-conjugated secondary antibodies (goat antimouse IgG and rabbit antigoat IgG), preabsorbed against human serum, were purchased from Sigma Chemical Co (St Louis, MO). All other protease inhibitors were purchased from Bachem.

Isolation of human neutrophils. Neutrophils were isolated at room temperature (22°C) from the venous blood of healthy, medication-free, paid volunteers who gave informed consent. This procedure was approved by the Institutional Review Board of SUNY at Stony Brook, NY in accordance with assurance filed with, and approved by, the US Department of Health and Human Services. Briefly, heparinized blood (1 U/mL) was treated with an equal volume of 3% Dextran T-500 (Pharmacia-LKB, Piscataway, NJ), prepared in sterile, pyrogen-free saline, for 30 minutes to sediment the red blood cells (RBCs). After washing the cells in phosphate-buffered saline (PBS) the leukocyte-rich pellet was centrifuged through an endotoxin-free Lymphoprep (Accurate Scientific) gradient (400g for 30 minutes at 22°C) to separate the mononuclear leukocytes from the neutrophils. The neutrophil-rich pellet was resuspended in PBS and exposed to hypotonic conditions (75 mosM) using sterile, pyrogen-free water for 60 seconds to lyse any contaminating RBCs. Neutrophils were then washed twice in PBS, counted, and then placed on ice until used (usually within 30 minutes).

A typical cell preparation contained 98% neutrophils, 1% eosinophils, and 1% mononuclear cells and were 98% to 99% viable by trypan blue dye exclusion.

Detection of cell-surface DBP by immunofluorescence. Untreated human neutrophils (10⁶ cells) were allowed to adhere to poly-l-lysine-coated glass slides for 30 minutes at 4°C. Nonadherent cells were removed by washing 3 times with PBS. Neutrophils next were treated with 10 μg/mL of goat antihuman DBP or nonimmune goat IgG (both prepared in PBS + 1% bovine serum albumin [BSA]) for 60 minutes at 4°C. Cells were then washed 4 times with PBS-BSA and treated with 1 μg/mL FITC-conjugated rabbit F(ab')₂, antigoat IgG for 60 minutes at 4°C. After washing the cells 5 times with PBS-BSA, neutrophils were fixed in 4% paraformaldehyde in PBS for 30 minutes at 22°C. Finally, cells were washed with PBS and then mounted with 90% glycerol, 10% PBS containing 1 mg/mL p-phenylenediamine (to inhibit auto-fluorescence and enhance nuclear morphology), and examined at original magnification x1,000.

Preparation of neutrophil lysates and releasates. Detergent-soluble lysates of neutrophils were prepared by pelleting (400g for 10 minutes at 10°C) 50 x 10⁶ cells in a 1.5-mL microfuge tube. After the PBS supernate was removed, the cells were lysed by adding 100 μL of 2% Nonidet P-40 (NP-40) (Sigma), 50 mM/L HEPES (pH 7.5) containing 20 mM/L benzamidine, 10 mM/L EDTA, 10 mM/L Na₃, as well as the following inhibitors added fresh immediately before lysis: 2 mM/L PMSF, 2 mM/L 1,10-phenanthroline, 0.5 mM/L E-64, 0.1 mM/L leupeptin, and 0.1 mM/L pepstatin. Lysates were vortexed thoroughly until all particulate matter was solubilized (usually 5 to 10 seconds) and then placed on ice for 30 minutes. The detergent-insoluble material was then pelleted by spinning the lysates in a microfuge for 10 minutes at 15,000g at 4°C. The detergent-soluble lysate was carefully removed and prepared for electrophoresis by heating (100°C) for 7 minutes with an equal volume (approximately 100 μL) of electrophoresis sample buffer (0.125 mol/L Tris, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate [SDS]) containing 0.2 mol/L dithiothreitol (DTT) as the reducing agent.

Supernates of neutrophils stimulated to release the contents of their cytoplasmic granules (ie, releasates) were prepared as follows: cells (2.5 x 10⁷) were resuspended in 0.5 mL of Hanks' balanced salt solution (HBSS) and treated with either buffer, 1 μmol/L fNLP, 10 μmol/L C₅A, or 10 μmol/L PMA for 45 minutes in a 37°C water bath with constant shaking. The releasate (cell-free supernate) then was collected after centrifuging the cell preparations 400g for 10 minutes at 4°C. Aliquots of the releasate were prepared for electrophoresis by boiling with an equal volume of sample buffer (see above). The cell pellets were solubilized in 2% NP-40 lysis buffer containing the protease inhibitor cocktail and prepared for electrophoresis as described above.

Cell disruption and subcellular fractionation. Freshly isolated neutrophils were disrupted by nitrogen cavitation and subcellular fractions were then subsequently isolated by centrifuging through discontinuous Percoll gradients (Pharmacia-LKB). Briefly, 2.5 x 10⁷ cells were resuspended in 5 mL of disruption buffer that contained 100 mM/L KCl, 3 mM/L NaCl, 3.5 mM/L MgCl₂, 1 mM/L adenosine triphosphate (ATP), and 10 mM/L HEPES (pH 7.3). The following protease inhibitors were then added just before disruption: 2 mM/L PMSF, 2 mM/L 1,10-phenanthroline, 0.5 mM/L E-64, 0.1 mM/L leupeptin, and 0.1 mM/L pepstatin. Cells were pressurized to 350 psi using ultrapure nitrogen gas in a cell-disruption bomb (Parr Instrument Co, Moline, IL) for 20 minutes at 4°C with constant stirring. The disrupted cells were collected dropwise into a tube that contained EGTA (final concentration of 1.25 mM/L). Unbroken cells (typically <5% of total) and the nuclei were removed by centrifugation at 800g for 15 minutes at 4°C. The nuclear fraction was prepared by resuspending the nuclei in 1% NP-40, 25 mM/L HEPES (pH 7.4), adding 10 μg of mammalian DNAase I (Boehringer Mannheim, Indianapolis, IN), and incubating for 10 minutes at 37°C. The postnuclear supernate was then carefully layered on top of a discontinuous gradient of Percoll that consisted of 6 mL of 1.05 g/mL underlayered with 6 mL of 1.12 g/mL Percoll prepared in disruption buffer. Samples were spun for 20 minutes at 4°C at 48,000g using a Sorval RC2-B centrifuge (E.I. duPont de Nemours, Chadds Ford, PA) with an SS-34 rotor. The cytosol, plasma membrane, specific granules, and
azurophil granules were collected separately and Percoll was removed by spinning the samples at 210,000g for 30 minutes at 4°C using a Beckman TL-100 ultracentrifuge (Beckman Instruments, Palo Alto, CA). Granule fractions were disrupted by adding Triton X-100 (Sigma) to a final concentration of 0.5%. PMSF (2 mmol/L) and 2 mmol/L 1,10-phenanthroline were also added to the granules during the detergent lysis.

The five subcellular fractions (nuclear, cytosol, membrane, specific granule, and azurophil granule) were assayed for total protein using the Pierce Bicinchoninic acid (BCA) reagent (Pierce Chemical Co, Rockford, IL). Alkaline phosphatase (plasma-membrane marker), lactate dehydrogenase (cytosol marker), vitamin B$_{12}$ binding protein (specific granule marker), and myeloperoxidase (azurophil granule marker) were also measured in each sample to determine the purity of each fraction. Alkaline phosphatase and lactate dehydrogenase were measured using commercial diagnostic kits (Sigma Chemical Co). Vitamin B$_{12}$ binding protein was quantitated using $^{57}$Co vitamin B$_{12}$ (Amersham, Arlington Heights, IL) and myeloperoxidase was measured using o-dianisidine as previously described.

**Cell-surface radioiodination and immunoprecipitation.** Cell-surface proteins were radioiodinated using $^{125}$I-Na by a soluble glucose oxidase-lactoperoxidase method. Briefly, 50 to 100 neutrophils were resuspended in 1 mL of PBS containing 0.1% dextrose and placed on ice. The iodination reaction was initiated by adding 1 mCi of $^{125}$I-Na (Dupont-NEN, Boston, MA), 32-μg lactoperoxidase (Sigma), and 0.01 U glucose oxidase (Sigma) for 30 minutes on ice. The reaction was terminated by washing the cells three times in ice-cold PBS containing 10 mmol/L KI. After one wash in PBS, the cells were transferred to a 1.5-mL microfuge tube, pelleted, and lysed as above in NP-40 lysis buffer containing the protease inhibitor cocktail, except that the NP-40 concentration was 0.5%. The detergent-insoluble material was then removed by centrifuging the lysates in a microfuge for 10 minutes at 15,000g at 4°C.

Before immunoprecipitation, lysates first were precleared by incubating with 200 μg nonimmune IgG (either goat or mouse) for 4 hours at 4°C. The IgG was then removed by adding the lysate directly to 50 μL of packed Protein-G Sepharose (Pharmacia-LKB) for 2 hours at 4°C. DBP was immunoprecipitated from precleared detergent-soluble lysates by adding either 20 μg of the MAK-89 MoAb or 50 μg of the goat polyclonal antibody and incubating for 4 hours at 4°C. The immune complexes were then removed by adding the anti-DBP lysate mixture to 25 μL of pelleted Protein-G Sepharose and incubating for 2 hours at 4°C. The Protein-G beads were washed five times in 0.5% NP-40 lysis buffer, five times in 0.6 mol/L NaCl, 10 mmol/L NaPO$_4$ (pH 7.5), and twice in PBS. Electrophoresis sample buffer was added directly to the protein-G beads. After boiling the samples for 7 minutes the supernate was removed for electrophoretic analysis.

**Polyacrylamide gel electrophoresis (PAGE) and Western blotting.** The contents of the detergent-solubilized lysates were separated by PAGE in the presence of SDS (SDS-PAGE) using the discontinuous buffer system described by Laemmli. 32 To assure complete disulfide reduction of DBP (which contains 14 disulfide bonds), all samples were prepared for electrophoresis by boiling in an SDS-PAGE sample buffer (2% SDS) containing 0.2 mol/L DTT added fresh immediately before preparation. Samples were separated on a mini-gel apparatus (Bio-Rad, Richmond, CA). The separated proteins were transferred from the gel, at 12 V (constant voltage) for 30 minutes at 22°C, to 0.45 μm pore size polyvinylidene fluoride (PVDF) paper (Westran, Schleicher & Schuell, Keene, NH) using a semi-dry blotting apparatus (TransBlot SD; Bio-Rad). The blots were then incubated in a solution of 5% nonfat dry milk (BLOTTO) in PBS for 60 minutes at 22°C to block unretracted protein binding sites and washed briefly in PBS containing 0.1% Tween-20 (Sigma) and 0.025% NaN$_3$ (PBS-Tween). The primary antibody (1 to 10 μg/mL diluted in PBS-Tween) was then added and the blot was incubated for 16 hours at 22°C. After washing four times in PBS-Tween, the blot was added to an affinity-purified secondary antibody conjugated to alkaline phosphatase (1 μg/mL prepared in PBS-Tween) and incubated for 4 hours at 22°C. Finally, the blots were washed five times in PBS-Tween, twice in substrate buffer (0.1 mol/L Tris [pH 9.5], 0.1 mol/L NaCl, 5 mmol/L MgCl$_2$), and then incubated in a 1:5 dilution of a one component 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) phosphatase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 22°C until bands were visible (usually within 5 minutes).

**Quantitation by densitometry.** Blots and/or photographic positives of blots were subjected to densitometry using a Bio-Rad model 620 video densitometer linked to an Apple Macintosh IIcx computer (Apple Computers, Cupertino, CA). The quantity of cell-associated DBP was estimated by interpolation from the linear portion of standard curve generated using highly purified plasma-derived DBP (0.1, 0.5, 2.5, and 10 ng). The mean standard curve (r = 0.988) was generated from three separate western blots. The curve remained linear up to 20-ng DBP. Each analysis of cell lysates contained lanes with 10 ng and 2 ng purified DBP as internal standards to control for blot-to-blot variations in band intensity.

**RESULTS**

**Identification of DBP in human neutrophils.** Viable, intact human neutrophils show a strong cell-surface reactivity with a monospecific, polyclonal goat antihuman DBP (Fig 1 A). Greater than 95% of the cells displayed a prominent rim pattern of fluorescence. Furthermore, staining was greatly enhanced if the cells first were pretreated with purified plasma-derived DBP (data not shown). Control cell preparations treated with nonimmune goat IgG followed by FITC-labeled rabbit anti-goat IgG essentially showed no fluorescent staining (Fig 1B). The nuclear staining observed in both anti-DBP and nonimmune IgG treated cells was caused by p-phenylenediamine in the mounting media that was added to distinguish nuclear morphology. It is interesting to note that in contrast to the polyclonal anti-DBP, neutrophils reacted with a mouse monoclonal IgG to human DBP (MAK-89) displayed very poor cell-surface staining (data not shown).

To determine the molecular weight of the cell-associated DBP, detergent-soluble neutrophil lysates were subjected to SDS-PAGE followed by Western blotting with anti-DBP. Relatively similar amounts of immunoreactive DBP are present in the detergent-soluble lysate of neutrophils obtained from several healthy donors (Fig 2). No immunoreactive bands were noted in the detergent-insoluble cell pellet (data not shown). The major immunoreactive band in the detergent-soluble lysates comigrated at the same apparent molecular weight as purified plasma-derived DBP, ie, approximately 56 Kd under reducing conditions. In addition, a second less prominent immunoreactive band with a molecular weight of approximately 12 to 14 Kd usually was observed, even though the cells were lysed in the presence of a large quantity of protease inhibitors (Fig 2). The 12 to 14 Kd band was observed even in cells treated with 10 mmol/L...
Fig 1. Immunofluorescence detection of DBP on the neutrophil plasma membrane. Untreated normal human neutrophils adherent to poly-1-lysine-coated glass slides were treated with 10 μg/mL of goat antihuman DBP (A) or 10 μg/mL nonimmune goat IgG (B) for 60 minutes at 4°C. Cells were then washed four times with PBS-1% BSA and treated with 1 μg/mL FITC-conjugated rabbit F(ab')₂ antigoat IgG for 60 minutes at 4°C. After washing the cells five times with PBS-1% BSA, neutrophils were fixed in 4% paraformaldehyde in PBS for 30 minutes at 22°C. Finally, cells were washed with PBS and then mounted with 90% glycerol, 10% PBS containing 1 mg/mL p-phenylenediamine, and examined at original magnification ×1,000.
diisopropylfluorophosphate (DFP) before the detergent lysis step (data not shown). This minor immunoreactive band also was noted in neutrophil lysates electrophoresed under nonreducing conditions (data not shown), indicating that this fragment was not linked to the remainder of the DBP molecule by one of 14 disulfide bonds. Several minor immunoreactive bands occasionally were noted between the 12 to 14 and 56-Kd bands and may have been generated by proteolytic degradation of the 56-Kd band. Because the detection system for the Western blots used an alkaline phosphatase linked secondary antibody, there was the concern that the bands detected as neutrophil-associated DBP may be caused by either: (1) cell-derived alkaline phosphatase that reacted with the BCIP-NBT substrate and/or; (2) cell-associated human IgG that was detected by the secondary antibody (rabbit antigoat IgG). However, when neutrophil lysates from the equivalent of $10^7$ cells (twofold to fivefold over the amount typically examined) were separated by SDS-PAGE and blotted onto PVDF paper, no bands developed when the blot was reacted with either BCIP-NBT substrate alone or secondary antibody plus substrate, thereby largely discounting the concerns noted above (data not shown). Immunoprecipitation of DBP from neutrophil lysates using polyclonal goat anti-DBP showed a prominent band at 56 Kd (Fig 3, lane 2) and lysates depleted of DBP by prior immunoprecipitation showed essentially no immunoreactive bands (Fig 3, lane 1). In addition, the lack of immunoreactive bands in lysates depleted of DBP provides further evidence that the detection system specifically identifies DBP (Fig 3, lane 1). Because DBP can be readily immunoprecipitated from cell lysates, surface radioiodination followed by immunoprecipitation of detergent-soluble lysates was used to examine cell-surface DBP (Fig 4). DBP immunoprecipitated from surface-labeled untreated neutrophils using the MAK-89 monoclonal anti-DBP showed a 12- to 14-Kd-labeled band (Fig 4, lane 2). However, cells first pretreated with purified plasma-derived DBP before radioiodination displayed both the 56-Kd and 12- to 14-Kd forms (Fig 4, lane 1). There was no radiolabeling of intracellular proteins as indicated by lane 3 in which surface-labeled lysates were immunoprecipitated with anti-actin.

**Localization of DBP in human neutrophils.** To identify the subcellular distribution of DBP in human neutrophils, five subcellular fractions were isolated by centrifugation on discontinuous Percoll gradients after the cells were disrupted by nitrogen cavitation. Immunoreactive DBP at 56 Kd was primarily found in the plasma membrane fraction (Fig 5, lane 1) and in the fraction that contained the specific (secondary) granules (Fig 5, lane 5). Essentially identical results were obtained using neutrophil subcellular fractions prepared from two other donors (total n of 3). Although the subcellular fractions were 85% to 90% pure as assessed by the enzymatic and binding activity of the markers (data not shown), the cytosolic fraction (Fig 5, lane 3) shows some weak immunoreactive bands at and below 56 Kd. These bands could be caused by a slight contamination from the plasma membranes during the postcentrifugation fraction collection. Because DBP appeared to be localized to specific granules and cell membranes, we next investigated whether the granule-associated DBP could be upregulated to the cell surface on stimulation with the receptor-independent stimulus PMA. PMA is known to be an effective agent for the release of specific granule contents but a poor inducer of azurophil (primary) degranulation.33 Pretreatment of neutrophils with 10 nmol/L PMA reduced the relative amount of DBP in both the specific granule (Fig 6, lane 1) and plasma membrane fraction (Fig 6, lane 2) versus those from the untreated neutrophils (Fig 6, lanes 3 and 4). A separate experiment using PMA-pretreated neutrophils obtained from a different donor produced an identical pattern of intracellular and membrane DBP diminution (data not shown).

Finally, to determine if DBP was released into the extracellular milieu from specific granules and/or the plasma membrane, cell-free supernates (ie, releasates) of neutrophils were examined for the presence of immunoreactive DBP (Fig 7). DBP was released into the cell-free supernates on stimulation with either 10 nmol/L PMA (lane 2) or 10
than 45 minutes, levels of 56-Kd DBP decreased and the number of low molecular weight immunoreactive bands increased (data not shown). The releasates from PMA-stimulated- (Fig 7, lane 2) and C5α-stimulated- (Fig 7, lane 4) neutrophils also displayed a discrete immunoreactive band greater than 56 Kd. This band (molecular weight estimated at 75 Kd) was observed in four separate experiments in which neutrophil releasates were analyzed by Western blotting. However, this form of DBP has only been observed in releasates and never detected in detergent lysates of neutrophils.

Quantitation of DBP in human neutrophils. The amount of DBP detected by western blots of neutrophil lysates was quantitated by video densitometry. Only the 56-Kd DBP was quantitated because this band contained 80% to 90% of the immunoreactive material in the cell lysates. Detergent-soluble lysates of normal human neutrophils contained 1.5 ± 0.8 ng DBP/10⁶ cells (n = 9). Furthermore, this DBP was almost exclusively distributed between the specific granules (34% ± 3% of the total, n = 3) and plasma membrane (66% ± 4% of the total, n = 3) (see Fig 5). Stimulation of neutrophils with 10 nmol/L PMA for 15 minutes at 37°C did not alter the percent distribution of DBP but decreased the amount in both the specific granules and plasma membrane by approximately 50% (see Fig 6).

Total immunoreactive DBP released into the cell-free supernates was measured using a quantitative slot blot procedure (n = 3). DBP released by pretreatment of neutrophils (10⁶ cells) with either buffer, C5α (10 nmol/L), NLP (1 μmol/L), or PMA (10 nmol/L) was measured as a percent of the total DBP (100%) in a 2% NP-40 lysate. Results also were compared with the total amount of vitamin B₁₂ binding protein (B₁₂hp) in the same samples. Incubation of cells for 45 minutes at 37°C in the presence of buffer (HBSS) alone induced neutrophils to release 30% ± 4% DBP and 22% ± 4% B₁₂hp. Stimulation with C5α increased the release of DBP to 48% ± 5% of the total (64% ± 3% for B₁₂hp). NLP induced 46% ± 7% DBP release (66% ± 5% B₁₂hp), and treatment with PMA produced the most DBP in the releasate at 64% ± 8% (82% ± 4% release for B₁₂hp).

DISCUSSION

Neutrophils contain numerous cell-surface and intracellular molecules that serve various antimicrobial and proinflammatory functions. In this report, evidence is presented that normal human neutrophils contain DBP on the cell surface and in an intracellular pool. The majority of this cell-associated DBP migrated at the same apparent molecular weight (by SDS-PAGE under reducing and nonreducing conditions) as purified plasma-derived DBP (56 Kd). The origin of neutrophil-associated DBP is not yet known. However, it seems unlikely that mature circulating neutrophils synthesize DBP because B and T lymphocytes, both of which have a much greater capacity for protein biosynthesis than mature neutrophils, show no stable m-RNA transcripts for DBP when analyzed by reverse transcriptase polymerase chain reaction (PCR). Because neutrophils have a very limited capacity for de novo protein biosynthesis, and are short-lived end-stage cells, the bulk of neutrophil-
derived proteins are believed to be synthesized and packaged during myeloid development. Therefore, the most likely source for neutrophil-associated DBP is either (1) pre-existing pool formed during development in the bone marrow (BM), and/or (2) acquisition of DBP by endocytosis while the circulating neutrophil is bathed in plasma. Because of the limitations described above, these possibilities will be very difficult to test using mature neutrophils and investigations will probably require BM-derived precursors to define the origin of neutrophil-associated DBP.

Although the majority of the cell-associated DBP has a molecular weight of 56 Kd, the results clearly showed that immunoreactive DBP was not homogenous in size. Two major bands were observed in neutrophil lysates and numerous bands in releasates. Furthermore, it appeared that goat polyclonal anti-DBP and the mouse MoAb recognize different portions of neutrophil-associated DBP. Parallel western blots have shown that both antibodies equally recognized purified plasma-derived DBP as well as DBP in neutrophil lysates separated by SDS-PAGE. In contrast, the polyclonal antibody immunoprecipitated the 56-Kd form while the monoclonal precipitated only the 12- to 14-Kd fragment from untreated cells (compare lane 2 in Fig 3 v lane 2 in Fig 4). In addition, immunofluorescent studies of live, intact neutrophils showed that the polyclonal antibodies, but not the MoAb, can readily detect cell-surface DBP (see Fig 1). However, the MAK-89 monoclonal anti-DBP could immunoprecipitate purified plasma-derived DBP (56 Kd) added to cells before cell-surface labeling and detergent lysis (see lane 1 in Fig 4), and also endogenous 56-Kd DBP in cell lysates separated under denaturing conditions (data not shown). This indicates that the epitope recognized by the MoAb on the 56-Kd form of DBP (ie, the region of the intact protein containing the 12 to 14 Kd portion) is blocked on viable cells and in nondenaturing detergent lysates while it is accessible on the cell surface 12- to 14-Kd fragment. This could explain the observed differences between polyclonal and MoAb immunoprecipitation of neutrophil lysates and immunofluorescent staining of viable cells. Previously, it had been shown that MAK-89 monoclonal anti-DBP cannot detect cell-surface DBP on viable peripheral blood (PB) monocytes. However, DBP was observed on fixed, permeabilized cells, and moreover, 56-Kd DBP could be immunoprecipitated from surface-radioiodinated monocytes. The latter observation contrasts our results (Fig 4) and may reflect differences between leukocytes in cell-surface binding of DBP. In addition to the 12- to 14-Kd immunoreactive band, another unique form of DBP was found in neutrophil releasates (Fig 7). An immunoreactive band with an apparent molecular weight of 75 Kd (under reducing conditions) was always noted in releasates of stimulated neutrophils. This form of DBP may represent either a cell-derived precursor of DBP, or more likely, 56-
Neutrophil-associated DBP 28 1

1 2 3 4 5

-56 kD

-12 kD

Fig 5. Identification of DBP in subcellular fractions. Neutrophils from a normal donor were disrupted by nitrogen cavitation and the subcellular fractions isolated by centrifugation on a discontinuous Percoll gradient. Aliquots of each fraction were boiled in SDS-PAGE sample buffer containing 0.2 mol/L DTT, separated on a 12.5% polyacrylamide gel, blotted onto PVDF paper, and probed with goat anti-human DBP. Each sample lane contained 20 μg total protein. Lane 1: plasma membrane. Lane 2: nucleus. Lane 3: cytosol. Lane 4: azurophil (primary) granules. Lane 5: specific (secondary) granules.

Kd DBP covalently linked to a cellular macromolecule. To our knowledge, this is the first report of a form of human DBP with a molecular weight higher than intact plasma-derived DBP (ie, 56 kD). These alternate forms of DBP (12 to 14 kD and 75 kD) need to be explored more fully to ascertain their origin and function.

Although DBP was detected in subcellular fractions that contained markers for the specific granules and plasma membranes, neutrophil-associated DBP actually may be located in a subset of granules known as secretory vesicles. Borregaard et al. have shown that human neutrophils contain a subset of intracellular granules whose contents are rapidly mobilized to the cell surface and extracellular environment. Secretory vesicles (which band between the specific granule and plasma membrane fractions on discontinuous Percoll gradient centrifugation) appear to be an intracellular store for alkaline phosphatase, cytochrome b559, adhesion proteins, and chemotactic factor receptors. Recently, this group has shown that secretory vesicles also contain certain plasma proteins, in particular, albumin, transferrin, and IgG. Therefore, considering the above properties of secretory vesicles and the fact that neutrophil-associated DBP was detected in both the plasma membrane and specific granule fractions, and was readily mobilized to the cell surface and extracellular milieu, cell-associated DBP may be localized in secretory vesicles.

The amount of DBP calculated in normal human neutrophils (1.5 ng/10⁶ cells) appears trivial when compared with major cellular proteins like actin (6 μg/10⁶ cells) and leucocyte elastase (1 to 2 μg/10⁶ cells). However, when compared with other neutrophil-derived proteins, the levels of DBP seem considerable. For example, the vitamin B₃₂ binding protein, an often used marker for specific granules, has been measured at 300 pg/10⁶ cells. Therefore, considering the massive number of neutrophils that can migrate to a site of inflammation (>10⁹ cells), μmol/L quantities of DBP potentially could be released at an inflammatory site. Because of the multifunctional nature of plasma-derived DBP, the cell-associated form of DBP may play multiple roles in cell function. Neutrophil-associated DBP could have numerous physiologic roles in immune and inflammatory responses including (1) binding and sequestration of microbial actin released during phagocytosis, (2) autoregulation of actin polymerization during chemotaxis and phagocytosis, (3) binding and intracellular transport of vitamin D sterols, and (4) regulation of complement-derived chemotactic activity. Moreover, the subcellular distribution of neutrophil-associated DBP (cell surface and intracellular granules) and the kinetics of its upregulation and release during cell activation, further suggests that it may play a role in inflammation. Several neutrophil-derived molecules including Fc receptors, chemotactic factor receptors, and...
adhesion molecules are located on the cell surface and in an intracellular pool and are upregulated to the cell surface and/or shed into the extracellular microenvironment on stimulation. Although it has been shown that plasma-derived DBP regulates the chemotactic activity of complement peptides C5a and C5a des Arg, the role of neutrophil-associated DBP in this process remains unclear. In conclusion, this paper provides the first comprehensive and quantitative report of cell-associated DBP in human neutrophils, information that is essential for future studies designed to determine the role(s) of neutrophil-associated DBP.

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REFERENCES


4. Cooke NE, David EV: Serum vitamin D binding protein is a third member of the albumin and α-fetoprotein gene family. J Clin Invest 76:2420, 1985


42. Tosi MF, Zakem H: Surface expression of the Fcy receptor III (CD16) on chemotactant-stimulated neutrophils is determined by both surface shedding and translocation from intracellular storage compartments. J Clin Invest 90:462, 1992
Localization and quantitation of the vitamin D binding protein (Gc-globulin) in human neutrophils

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