Isolation, Characterization, and Immunological Detection of Neutrophil-Activating Peptide 2: A Proteolytic Degradation Product of Platelet Basic Protein (43343)

JOHN C. HOLT,* ZHANQING YAN,† WEIQI LU,† GWENDOLYN J. STEWART,† AND STEFAN NIEWIAROWSKI[1,†]

Rhône-Poulenc Rorer Biotechnology, Inc., King of Prussia, Pennsylvania 19406, and Department of Physiology and Thrombosis Research Center,† Temple University School of Medicine, Philadelphia, Pennsylvania 19140*

Abstract. **Neutrophil-activating peptide 2 (NAP-2), corresponding to platelet basic protein fragment 25–94, was prepared by chymotryptic digestion of its precursors, low affinity platelet factor 4 or β-thromboglobulin, followed by purification by high performance liquid chromatography. NAP-2 (0.1–1.5 μm) caused the release of human granulocyte elastase from cytochalasin B-treated neutrophils in a dose-dependent manner. In the same system, β-thromboglobulin, human platelet factor 4, S-pyridylethyl NAP-2, and platelet basic protein C-terminal fragment (77–94) were inactive, whereas platelet basic protein fragment 22–89 had low, but significant, activity. Sensitive immunological identification of NAP-2 based on nonequilibrium isoelectric focusing and immunoblotting is described.** [P.S.E.B.M. 1992, Vol 199]

Platelet basic protein (PBP) is a highly specific platelet α-granule protein that is the precursor of low affinity platelet factor 4 (LA-PF_4) and β-thromboglobulin (βTG) (1). These proteins differ only in NH_2-terminal amino acid sequence and isoelectric point. PBP is synthesized in megakaryocytes, contains 94 amino acid residues, and is converted to LA-PF_4 (which contains 85 amino acid residues) within megakaryocyte and platelet granules (1,2). βTG itself (81 amino acid residues), as originally described (3), cannot be detected in cell lysates prepared using trichloroacetic acid. It probably results from NH_2-terminal cleavage of LA-PF_4 or PBP after cell secretion or lysis. Production of βTG from PBP and LA-PF_4 can be demonstrated *in vitro* by incubating the platelet release supernatant at 37°C (4) or, in a purified system, by limited cleavage with plasmin or trypsin (5). The three forms of βTG antigen are immunologically identical when compared using rabbit polyclonal antibodies.

The biological activities of PBP and its derivatives are not well understood. Castor *et al.* (6) proposed that LA-PF_4 (referred to as connective tissue-activating peptide III [CTAP-III]) is a weak mitogen for connective tissue fibroblasts. Senior *et al.* (7) reported that βTG antigen promotes chemotaxis of fibroblasts. In recent studies, Walz and Baggiolini (8–10) isolated a cleavage product of βTG called neutrophil-activating peptide (NAP)-2, which was formed in cultures of stimulated mononuclear cells and appeared to be a potent activator of human neutrophils. NAP-2 was a 70-amino acid peptide corresponding to the major carboxy-terminal fragment of βTG. It showed 46% homology with neutrophil-activating peptide 1/interleukin 8. NAP-2 behaved as a typical chemotactic receptor agonist, inducing cytosolic free calcium changes, chemotaxis, and exocytosis, while PBP, LA-PF_4, and platelet factor 4 (PF4) had little, if any, such activity (9). There is evidence that NAP-2 interacts with NAP-1 receptor (11).

In a previous study (12), we identified a stable, highly basic fragment of βTG in lysed platelets that had

[1] To whom correspondence and requests for reprints should be addressed at Department of Physiology, 207 MRB, Temple University School of Medicine, 3400 North Broad Street, Philadelphia, PA 19140.

Received May 29, 1991. [P.S.E.B.M. 1992, Vol 199]
Accepted September 6, 1991.

0037-9727/92/1992-0171$3.00/0

been sedimented from anticoagulated blood at 1*g* and were, therefore, expected to be contaminated with neutrophils. Several lines of evidence suggested that cathepsin G from neutrophils was responsible for producing this highly basic species (referred to as βTG-F) by cleaving 15 NH_2-terminal residues from LA-PF_4. βTG-F appeared structurally identical to NAP-2. In the present paper, we describe a simple method for preparation of NAP-2 by means of chymotrypsin digestion of purified LA-PF_4 or βTG followed by isolation of NAP-2 by reverse phase high performance liquid chromatography (HPLC). NAP-2 was identified in platelet lysates by immunoblotting and was characterized by amino acid sequence and molecular weight. The effects of native PF4, βTG, reduced and alkylated derivative of NAP-2, and two other peptides derived from PBP on the release of specific human granulocyte elastase were compared.

Methods and Materials

Preparation of Human PF4, PBP, LA-PF_4, and βTG. Human platelet factor 4 was purified from outdated platelets supplied by the American Red Cross (Philadelphia, PA). Briefly, platelet-rich plasma was centrifuged and the supernatant was applied to a heparin-agarose column, washed with 0.5 *M* NaCl, and eluted with 1.5 *M* NaCl (13). For further purification, a new approach was used. Samples were collected, dialyzed against 0.01% trifluoroacetic acid (TFA), and lyophilized. Crude PF4 was dissolved in 0.1% TFA and applied on reverse phase HPLC using Vydac C-18 column (The Separation Group, Hesperia, CA), equilibrated in 0.1% TFA, and eluted with a gradient with 0–80% acetonitrile over 50 min. PF4 was eluted at 45% acetonitrile as a single peak. PF4 was identified by sodium dodecyl sulfate (SDS) gels and enzyme-linked immunosorbent assay using rabbit antihuman PF4 antibody.

LA-PF_4 was prepared from a suspension of disrupted human platelets. In brief, platelets from fresh, citrated, platelet-rich plasma were pelleted and resuspended in 0.05 *M* Tris-HCl buffer and 0.02 *M* Na_2H_2 EDTA (pH 7.2). Platelets were disrupted by three cycles of freezing (−70°C) and thawing (room temperature). Supernatant was lyophilized, dissolved in 0.1% TFA, and fractionated by reverse phase HPLC (Vydac C-18 column). LA-PF_4 was eluted as a single peak with 0–80% acetonitrile gradient over 50 min.

βTG was purchased from Bioprocessing Inc. (Durham, U.K.), and further purified by means of reverse phase HPLC. Both LA-PF_4 and βTG were eluted at 42% acetonitrile. In addition, all the proteins were identified by means of ELISA, SDS-polyacrylamide gel electrophoresis (PAGE) using PhastGel (Pharmacia, Uppsala, Sweden), NH_2-terminal amino acid sequencing, and amino acid composition. LA-PF_4 obtained from fresh human platelets showed NLAKGKEE at the NH_2 terminus, whereas βTG showed the sequence GKEE. PBP was obtained as described previously (1).

Preparation of PBP Fragments. PBP fragment 25–94 (NAP-2) was produced by digestion of βTG or LA-PF_4 with α-chymotrypsin (1/100 w/w) (Sigma, St. Louis, MO) at 37°C for 3 hr in 0.05 *M* Tris-HCl and 0.15 *M* NaCl (pH 7.4) and subsequently purified using reverse phase HPLC. PBP fragment 22–89 was generated by cleavage of βTG with endoproteinase Asp-N (Boehringer Mannheim, Chicago, IL) (1/100 w/w) at 37°C for 16 hr in 0.05 *M* sodium phosphate buffer (pH 7.8). The fragment was isolated from the digest by reverse phase HPLC.

S-Pyridylethyl NAP-2 was prepared by reducing purified NAP-2 (200 μg) in 0.1 Tris-HCl (200 μl, pH 8.5) containing 6 *M* guanidine HCl, 4 m*M* Na_2 EDTA, and 3.3 m*M* dithiothreitol (Sigma) over 0.5–3.0 hr, followed by incubation with 4-vinylpyridine (100%, 200 μl) for 30 min. The whole procedure was carried out in the dark, at 22°C, under a blanket of nitrogen. S-Pyridylethyl NAP-2 was purified from reaction mixture by reverse phase HPLC. Reduction and alkylation of NAP-2 resulted in cleavage of both disulfide bonds, which have been shown to be Cys 29-Cys 55 and Cys 31-Cys 71 (3).

PBP(Y) 77-94 was custom synthesized by Peninsula Laboratories (Belmont, CA). It was 95% pure, as determined by reverse phase HPLC. Amino acid sequences of the peptides derived from PBP are shown in Figure 1.

Identification of PF4 and NAP-2 by ELISA. Peaks from the HPLC column were identified by ELISA (14). Briefly, 96-well microplates (Becton Dickinson & Co., Lincoln Park, NJ) were coated overnight with HPLC fractions diluted in Voller's buffer (15 m*M* Na_2CO_3, pH 9.6). Plates were washed with 0.02 *M* phosphate buffer containing 0.05% Tween 20 (pH 7.2), and then blocked with 5% nonfat dry milk. Rabbit antihuman PF4 antibody or rabbit antihuman LA-PF_4 was used (1/1000) as the first antibody (14) and alkaline phosphatase-conjugated goat antirabbit IgG (Sigma) (1/800) was used as the second antibody. *p*-Nitrophenylphosphate in Zn-Mg buffer (13) was used as a substrate. The reaction was stopped by the addition of 2 *N* NaOH and the resulting color was quantitated at 405 nm in an automated microplate reader (Bio-tek Instruments, Inc., Winooski, VT).

SDS-PAGE. SDS-PAGE was performed using PhastGel (Pharmacia) with 5% stacking gel and 8–25% gradient separation gel. Molecular mass standards were rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa). They were run on each gel for molec-

ular mass calculation. Completed gels were stained by either Coomassie brilliant blue or silver reagent (15).

Nonequilibrium Isoelectrofocusing and Immunoblotting. Nonequilibrium isoelectrofocusing was performed using the method of Holt and Niewiarowski (12). Briefly, a vertical polyacrylamide slab gel was prepared containing 6% acrylamide, 0.16% methylenebisacrylamide, 1.6% (w/v) ampholytes (Ampholines, pH 3.5-10; LKB, Uppsala, Sweden), 0.6 mg/ml of ammonium persulfate, and 0.125% (v/v) TEMED. Electrolytes were prepared as follows: upper reservoir containing 9 m*M* phosphoric acid connected with anode; lower reservoir containing 20 m*M* sodium hydroxide connected with cathode. Gel and electrolytes were equilibrated at 4°C before use. Approximately 20 ng of purified protein or material corresponding to 10^7 platelets was loaded. Cytochrome c served as a visible marker and as a carrier protein when samples contained very low concentrations of protein. The gel was initially run under constant current conditions at 2.5 mA. Every 30 min, the current was increased in increments of 0.5 mA to an upper limit of 5 mA. The transfer of proteins from the completed gel to nitrocellulose was allowed to proceed by capillary action for 16 hr at 20–23°C. Proteins were detected by using rabbit antihuman LA-PF_4 antibodies (14), which recognize PBP and its degradation products, including LA-PF_4, βTG, and NAP-2.

Amino Acid Sequencing and Amino Acid Composition. Samples were subjected to NH_2-terminal sequencing performed on a gas phase sequencer (model 470; Applied Biosystems, Inc., Foster City, CA) with an online PTH analyzer (model 120; Applied Biosystems, Inc.). Amino acid composition was determined by the Protein Chemistry Facility at the Wistar Institute (Philadelphia, PA) under the direction of Dr. D. W. Speicher.

Preparation of Neutrophils and Human Granulocyte Elastase Release Assay. Human blood was obtained from healthy individuals with approval of the Institutional Human Experimentation Committee at Temple University (Philadelphia, PA). Neutrophils were isolated by layering citrated blood on a solution of sodium metrizoate and Dextran 500 (Polyprep; Nycomed, Oslo, Norway), followed by lysis of residual red blood cells with isotonic ammonium chloride (16). The viability of the cells was shown by trypan blue exclusion. Release of human granulocyte elastase (HGE) was measured by a modification of Janoff's method (17). Neutrophils were treated with 5 μg/ml of cytochalasin B (Sigma) for 20 min, followed by stimulation with selected agonists for 30 min. After centrifugation, elastase released to the supernatant was measured in 96-well microplates using the chromogenic substrate *N-t*-Boc-Ala-Ala-*p*-nitrophenyl ester (Sigma). The resulting color was quantitated at 405 nm in an automated microplate reader (Bio-tek). The amount of HGE released by 0.025% Triton X-100 or by 10 n*M* chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine was accepted as 100%.

Results

Neutrophil-activating peptide 2, previously referred to as βTG-F, was purified after digestion of βTG or LA-PF_4 with chymotrypsin. NAP-2 was identified in fractions eluted from HPLC column as a single immunoreactive peak with antihuman LA-PF_4 antibody in ELISA. Amino acid sequencing and amino acid composition confirmed the predicted primary structure of this peptide (Fig. 1). The recovery of NAP-2 was about 60% of the initial amount of βTG digested with chymotrypsin. Figure 2 shows that NAP-2 was separated from βTG when both proteins were applied to the HPLC column in equal concentrations. SDS-PAGE demonstrated that NAP-2 migrated with greater electrophoretic mobility than did βTG (Fig. 3). Nonequilibrium isoelectric focusing of platelet releasate and platelet lysate followed by immunoblotting demonstrated that NAP-2 was more basic than βTG, LA-PF_4, and PBP (Fig. 4).

The structural basis for the activity of NAP-2 was further investigated by isolating a natural peptide very similar to NAP-2, following limited proteolysis of LA-PF_4 with endoproteinase Asp-N. The major component obtained from the digest after reverse phase HPLC fractionation (Fig. 5) was analyzed by amino acid sequencing and compositional analysis. The main NH_2-terminal sequence was ^{22}Asp-Leu-Tyr-Ala. In two of three preparations analyzed, an additional sequence, ^{66}Asp-Gly-Arg-Lys, was present at approximately 25% of the level of the main sequence. Cleavage at Asp-66 would not be expected to release a cleaved peptide, because disulfide bond Cys 31-Cys 71 remained intact (Fig. 1). The composition of the Asp-N fragment matched residues 22–89 of PBP. Values for Asp, Glu, Ser, and Ala were all consistent with the loss of 5 COOH-terminal residues compared with the PBP sequence. Figure 6 compares the release of human granulocyte elastase from neutrophils by human PF4 and by three peptides derived from human PBP. NAP-2 caused release of HGE in a dose-dependent manner, with about 70% release of HGE at 1.5 μM NAP-2. By contrast, there was no significant release of HGE following incubation of neutrophils with similar concentrations of PF4, βTG, and S-pyridylethyl NAP-2. PBP 22–89 at 1.5 μM showed significant activity, but this was considerably lower than that of NAP-2. The C-terminal fragment of platelet basic protein (PBP 77–94) was inactive at a concentration of 18 μM (data not shown). It is noteworthy that the activity of the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine was about 100 times higher than that of NAP-2 (data not shown).

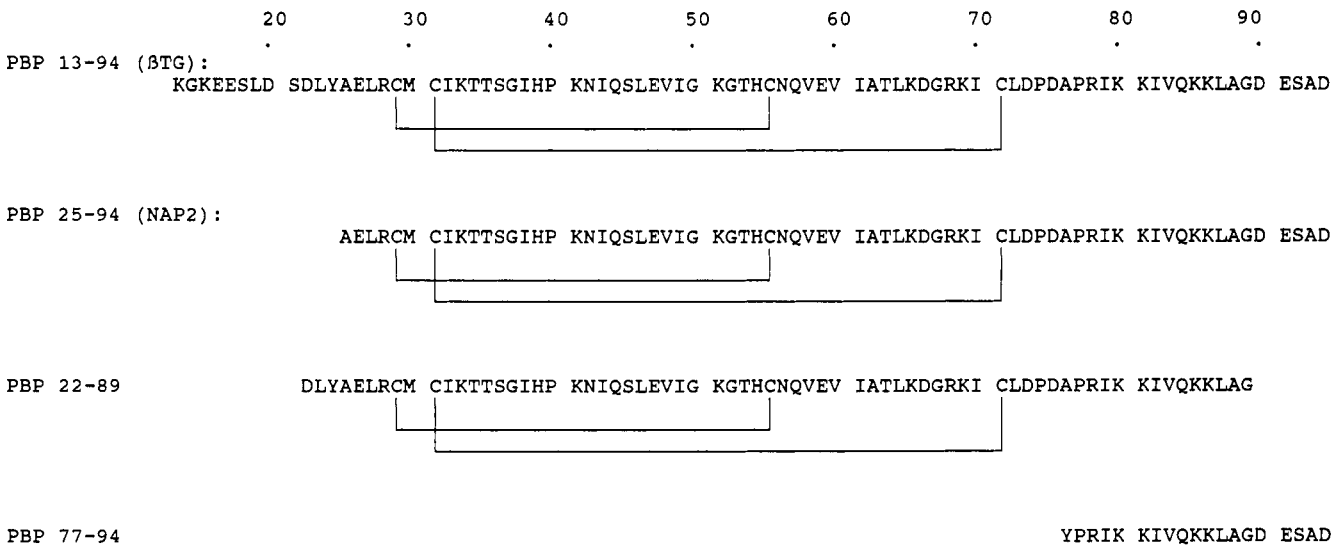

Figure 1. Amino acid sequence of peptides derived from platelet basic protein. Residues are numbered from the N terminus of PBP.

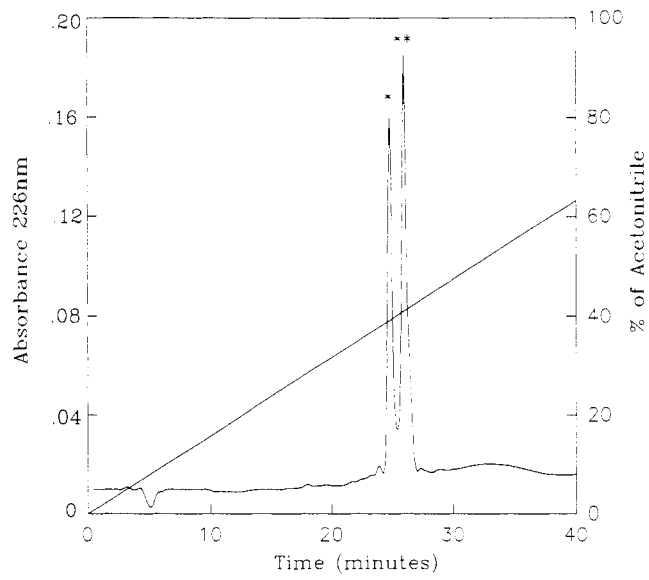

Figure 2. Separation of βTG and NAP-2 by means of reverse phase HPLC by applying a mixture of purified 50 μg of βTG and 50 μg of NAP-2. Collected samples of NAP-2 (*) and βTG (**) were identified by SDS-PAGE and ELISA.

Discussion

NAP-2, a proteolytic degradation product of PBP and βTG, is of considerable interest, since it appears to be a biologically important cytokine that may reflect the contribution of platelets to inflammatory processes. However, study of this peptide has been difficult because means for purification were laborious, involving preincubation of suspensions of platelets with macrophages or neutrophils (8–10,18). In this paper, we describe a simple method of purification of NAP-2 from the chymotryptic digest of LA-PF_4 or βTG. Chymotrypsin causes selective cleavage of one peptide bond in βTG or LA-PF_4 between Tyr 24 and Ala 25 of PBP, an observation consistent with the recent report by Castor *et al.* (19).

Walz *et al.* (9) reported that neither human PF4 nor PBP or LA-PF_4, the precursors of NAP-2, activate neutrophils. Our experiments confirm their report and define other requirements for the expression of biological activity of NAP-2. The removal of N-terminal amino acids appears to be important, since βTG was not active and the molecule obtained by proteolytic degradation of LA-PF_4 by endoproteinase Asp-N had low activity, suggesting that the active domain of NAP-2 might be hindered by the additional Asp-Leu-Tyr (D, L, Y) at the N terminus of the molecule. The mechanism by which these three amino acid residues hinders the activity of the molecule is not known. We assume that the removal of the five C-terminal amino acids by endoproteinase Asp-N may not have any effect. The C-terminal fragment of PBP containing the heparin-binding domain was not active, but it is not excluded that this region of the molecule may affect the conformation of the active site of NAP-2.

Loss of activity after reduction and alkylation suggests the importance of the secondary structure of the molecule determined by two pairs of cysteines linked by disulfide bridges. It is noteworthy that a number of low molecular weight proteins which are involved in inflammation, tissue repair, and cell growth (referred to as proteins encoded by small inducible genes or an intercrine cytokine family) show a high degree of homology with platelet basic protein and its derivatives, including identical alignment of four cysteines (20–23).

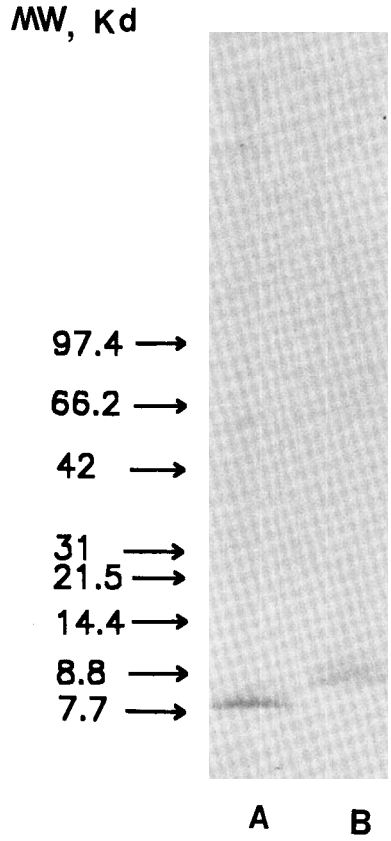

Figure 3. SDS-PAGE of βTG (lane A) and NAP-2 (Lane B). Each sample (0.5 μg) was mixed with sample buffer containing 0.0625 *M* Tris, 2% SDS, and 10% glycerine (pH 6.8), and run for 40 min on precast PhastGel gradient 8–25% (Pharmacia) using PhastSystem Separation and Control Unit (Pharmacia). Completed gels were developed with silver reagents using Development Unit (Pharmacia).

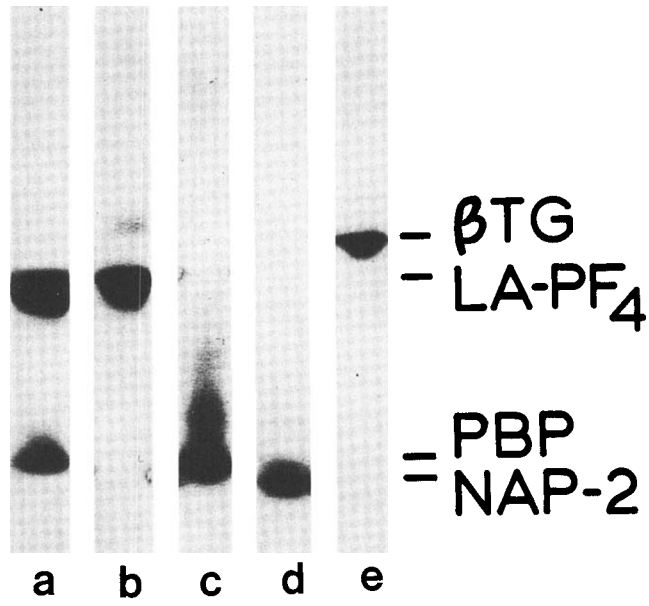

Figure 4. Immunoblot of nonequilibrium isoelectric focusing gel of (a) platelet release supernatant, (b) purified LA-PF_4, (c) purified platelet basic protein, (d) NAP-2 in lysed platelets sedimented at 1*g*, and (e) purified βTG. Bands from the focusing gel were transferred to nitrocellulose and probed with antiserum to LA-PF_4.

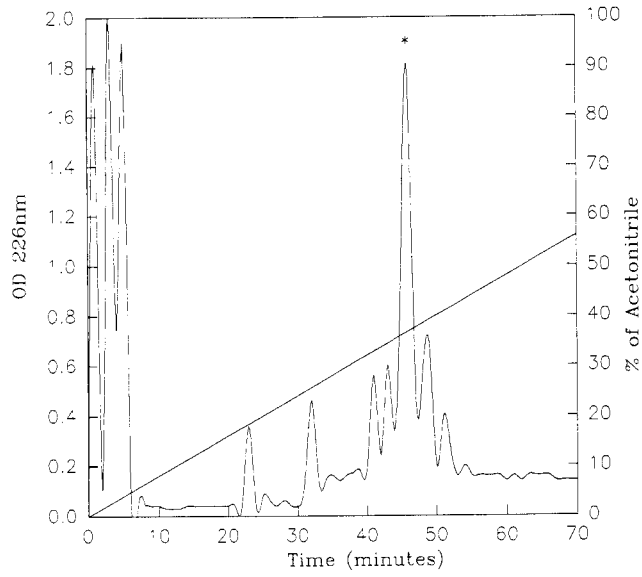

Figure 5. Separation of PBP 22–89 by reverse phase HPLC following incubation of 100 μg of βTG or LA-PF_4 with 1.0 μg of endoproteinase Asp-N at 37°C for 16 hr. Fragment PBP 22–89, identified by amino acid sequencing and amino acid analysis, is indicated by an asterisk.

These proteins include neutrophil-activating peptide 1/interleukin 8, RANTES, melanoma growth stimulatory activity, and macrophage inflammatory proteins 1 and 2 (20–23).

Our experiments indicate that NAP-2 has highly basic properties consistent with the loss of two Asp and two Glu residues during truncation of the N-terminal end of β-thromboglobulin. Accordingly, Castor *et al.* (24) estimated, by means of preparative isoelectric focusing, that the isoelectric point of NAP-2 is pH 9.2. The detection of NAP-2 by nonequilibrium isoelectric focusing and immunoblotting appears to be a highly specific method permitting detection of NAP-2 in plasma or inflammatory exudates.

We suggest that in certain disease states, platelets and neutrophils might interact to contribute to the inflammatory process. Both cell types accumulate in venous thrombi that are in close contact with the vein wall along part of the surface of thrombi (25). In the adult respiratory distress syndrome, platelet and leukocyte aggregates have been observed in pulmonary arterioles (26). Some degree of activation of platelets and of neutrophils is expected (27). The generation of NAP-2 from LA-PF_4 by cathepsin G would be expected in light of our observation of the formation of NAP-2 in a lysate of cells containing both platelets and neutrophils (12). NAP-2 so generated could induce secretion of more cathepsin G, which would, in turn, stimulate

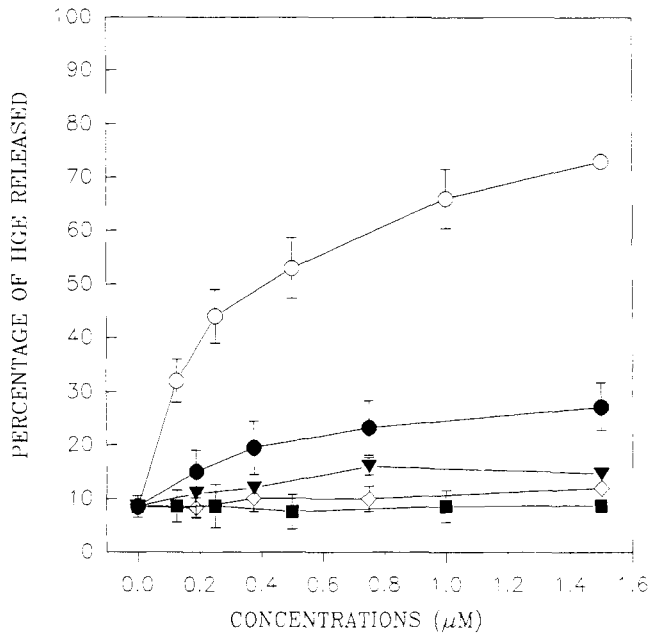

Figure 6. Dose-dependent release of HGE following stimulation of cytochalasin B-treated neutrophils by NAP-2 and related peptides. (○--○) NAP-2 induced significant ($P < 0.0001$) HGE release at all concentrations; (●--●) PBP 22–94 induced significant ($P < 0.01$) HGE release at all concentrations; (▼--▼) PF4, (◇--◇) βTG, and (■--■) reduced NAP-2 induced no significant HGE release at all concentrations. Data refer to mean values and SE from four experiments. Statistical analysis was performed by using Student's *t* test.

more platelet secretion (28,29), thereby providing more substrate for generation of more NAP-2. This would constitute a positive feedback loop.

The concentration of NAP-2 causing near maximal secretion of HGE from neutrophils was 0.5 μM, i.e., 3.0 μg/ml (Fig. 6). Platelets contain 0.8–2.4 μg of βTG per 10^8 platelets (30). At a platelet count of 2×10^8 per ml of blood, the release of all LA-PF_4 and its conversion to NAP-2 would give rise to 0.7–2.1 μg of NAP-2. However, the actual concentration of NAP-2 that might accumulate would be influenced by several factors. It would be reduced, because not all LA-PF_4 would be secreted and not all that was secreted would be converted to NAP-2. Furthermore, the concentration of NAP-2 that would be reached could be influenced by the site of release and cleavage. If NAP-2 were generated in flowing blood, dilution and clearance would be expected to greatly reduce the concentration of the agonist. If NAP-2 were generated in neutrophil-platelet aggregates that adhered to or were trapped in blood vessels, some of the NAP-2 might be trapped in intercellular space and be protected from washout. Since the number of platelets and neutrophils in a given volume would be increased over that in blood or plasma, the concentration of NAP-2 could reach a sufficiently high level to cause neutrophil activation. It might also affect nearby vessel wall cells. Van Osselaer *et al.* (18) found that intradermal injection of NAP-2 into rabbits caused increased vascular permeability. Other cellular effects of NAP-2 remain to be investigated.

The studies described have been supported by National Institutes of Health Grant HL36579.

1. Holt JC, Harris ME, Holt A, Lange E, Henschen A, Niewiarowski S. Characterization of human platelet basic protein, a precursor form of low-affinity platelet factor 4 and β-thromboglobulin. Biochemistry **25**:1988–1996, 1986.
2. Holt JC, Rabellino EM, Gewirtz AM, Gunkel LM, Rucinski B, Niewiarowski S. Occurrence of platelet basic protein, a precursor of low affinity platelet factor 4 and β-thromboglobulin, in human platelets and megakaryocytes. Exp Hematol **16**:302–306, 1988.
3. Begg GS, Pepper DS, Chesterman CN, Morgan FJ. Complete covalent structure of human β-thromboglobulin. Biochemistry **17**:1739–1744, 1978.
4. Niewiarowski S, Walz DA, James P, Rucinski B, Kueppers F. Identification and separation of secreted platelet proteins by isoelectric focusing. Blood **55**:453–456, 1980.
5. Holt JC, Niewiarowski S. Conversion of low-affinity platelet factor 4 to β-thromboglobulin by plasmin and trypsin. Biochim Biophys Acta **632**:284–289, 1980.
6. Castor CW, Miller JW, Walz DA. Structural and biological characteristics of connective tissue activating peptide (CTAP-III), a major human platelet-derived growth factor. Proc Natl Acad Sci USA **80**:765–769, 1983.
7. Senior RM, Griffin GL, Huang JS, Walz DA, Deuel TF. Chemotactic activity of platelet α-granule proteins for fibroblasts. J Cell Biol **96**:382–385, 1981.
8. Walz A, Baggiolini M. A novel cleavage product of β-thromboglobulin formed in cultures of stimulated mononuclear cells activates human neutrophils. Biochem Biophys Res Commun **159**:969–975, 1989.
9. Walz A, Dewald B, von Tscharner V, Baggiolini M. Effects of the neutrophil-activating peptide NAP-2, platelet basic protein, connective tissue-activating peptide III, and platelet factor 4 on human neutrophils. J Exp Med **170**:1745–1750, 1989.
10. Walz A, Baggiolini MA. Generation of the neutrophil-activating peptide NAP-2 from platelet basic protein or connective tissue-activating peptide III through monocyte proteases. J Exp Med **171**:449–454, 1990.
11. Leonard EJ, Yoshimura T, Rot A, Noer K, Walz A, Baggiolini M, Walz DA, Goetzl EJ, Castor CW. Chemotactic activity and receptor binding of neutrophil attractant/activation protein-1 (NAP-1) and structurally related host defense cytokines: Interaction of NAP1 with the NAP2 receptor. J Leukocyte Biol **49**:258–265, 1991.
12. Holt JC, Niewiarowski S. Platelet basic protein, low-affinity platelet factor 4, and β-thromboglobulin: Purification and identification. Methods Enzymol **169A**:224–232, 1989.
13. Rucinski B, Niewiarowski S, James P, Walz DA, Budzynski AZ. Anti-heparin proteins secreted by human platelets: Purification, characterization and radioimmunoassay. Blood **53**:47–62, 1979.
14. Tuszynski GP, Switalska HJ, Knudsen K. Methods of studying platelet-secreted proteins and the platelet cytoskeleton. Mod Methods Pharmacol **4**:267–286, 1987.
15. Merril CR, Goldman D, Sedman SF, Eber MH. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variations in cerebrospinal fluid proteins. Science **211**:1437–1438, 1981.
16. Eggleton P, Gargan R, Fischer D. Rapid method for isolation of

neutrophils in high yield without the use of dextran or density gradient polymers. J Immunol Methods **121**:105–113, 1989.
17. Janoff A. Alanine p-nitrophenyl esterase activity of human leucocyte granules. Biochem J **114**:157–159, 1969.
18. van Osselaer N, van Damme J, Rampart M, Herman AG. Increased microvascular permeability *in vitro* in response to intradermal injection of neutrophil-activating protein (NAP-2) in rabbit skin. Am J Pathol **138**:23–27, 1991.
19. Castor CW, Walz DA, Ragsdale CG, Hossler PA, Smith EM, Bignall MC, Aaron BP, Mountjoy K. Connective tissue activation XXXIII. Biologically active cleavage products of CTAP-III from human platelets. Biochem Biophys Res Commun **163**:1071–1078, 1989.
20. Kawahara RS, Deuel TF. Platelet-derived growth factor-inducible gene *JE* is a member of a family of small inducible genes related to platelet factor 4. J Biol Chem **264**:679–682, 1989.
21. Wolpe D, Cerami A. Macrophage inflammatory proteins 1 and 2: Members of a novel superfamily of cytokines. FASEB J **3**:2565–2573, 1989.
22. Zipfel PF, Balke J, Irving SG, Kelly K, Siebenlist U. Mitogenic activation of human t cells induces two closely related genes which share structural similarities with a new family of secreted factors. J Immunol **142**:1582–1590, 1989.
23. Oppenheim JJ, Zachariae COC, Mukaida N, Matsushima K. Properties of the novel proinflammatory supergene "intercrine" cytokine family. Annu Rev Immunol **9**:617–648, 1991.
24. Castor CW, Walz DA, Johnson PH, Hossler PA, Smith EM, Bignall MC, Aaron BP, Underhill P, Lazar J, Hudson DH, Cole LA, Perini F, Mountjoy K. Connective tissue activation. XXXIV. Effects of proteolytic processing on the biologic activities of CTAP-III. J Lab Clin Med **116**:516–526, 1990.
25. Aschoff L. Lectures on Pathology, New York: Paul B. Houser, Inc., pp253–279, 1974.
26. Fein A, Grossman RF, James JG, Overland E, Pitts L, Muraray JF, Staub NC. The value of edema fluid protein measurement in patients with pulmonary edema. Am J Med **67**:32–38, 1979.
27. Zimmerman GA, Renzetti AD, Hill HR. Functional and metabolic activity of granulocytes from patients with adult respiratory distress syndrome. Am Rev Respir Dis **127**:290–300, 1983.
28. Ferrer-Lopez P, Renesto P, Schattner M, Bassot S, Laurent P, Chignard M. Activation of human platelets by C5a-stimulated neutrophils: A role for cathepsin G. Am J Physiol **258**:C1100–C1107, 1990.
29. Evangelista V, Rajtar G, de Gaetano G, White JG, Cerletti C. Platelet activation by fMLP-stimulated polymorphonuclear leukocytes: The activity of cathepsin G is not prevented by antiproteinases. Blood **77**:2379–2388, 1991.
30. Niewiarowski S, Holt JC. Biochemistry and physiology of platelet secreted proteins. In: Colman RW, Hirsch J, Marder VJ, Salzman EW, Eds. Hemostasis and Thrombosis. Basic Principles and Clinical Practice, 2nd Edition. Philadelphia: J. B. Lippincott Co., p619, 1987.