Thrombocidins, microbicidal proteins of human blood platelets

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Chapter 3

Thrombocidins, Microbicidal Proteins from Human Blood Platelets, are C-terminal Deletion Products of CXC-Chemokines

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ABSTRACT

Antibacterial proteins are components of the innate immune system, found in many organisms and produced by a variety of cell types. Human blood platelets contain a number of antibacterial proteins in their α-granules, which are released upon thrombin activation. The present study was designed to purify these proteins obtained from human platelets and to characterize them chemically and biologically.

Platelet granule sonicate contained at least 10 different antibacterial proteins with diverse activity against *Escherichia coli*, *Streptococcus sanguis* and *Staphylococcus aureus*. The two major antibacterial proteins were purified in a 2-step protocol using cation exchange chromatography and continuous acid urea polyacrylamide gel electrophoresis, and were designated thrombocidin-1 (TC-1) and TC-2. Characterization of these proteins using mass-spectrometry and N-terminal sequencing revealed that TC-1 and TC-2 are variants of the CXC-chemokines neutrophil activating peptide-2 (NAP-2) and connective tissue activating peptide-III (CTAP-III), respectively. TC-1 and TC-2 differ from these chemokines by a C-terminal truncation of 2 amino acids. Both TCs, but not NAP-2 and CTAP-III, were bactericidal for *Bacillus subtilis*, *E. coli*, *S. aureus*, and *Lactococcus lactis*, and fungicidal for *Cryptococcus neoformans*. Killing of *B. subtilis* by either TC appeared to be very rapid. Since TCs were unable to dissipate the membrane potential of *L. lactis*, the mechansim of TC-mediated killing most probably does not involve pore formation.

INTRODUCTION

During the last decade, antibacterial proteins have been recognized as effectors in the innate immune system. The wide-spread presence of such proteins throughout the animal kingdom reflects their importance (4,25,27,40,49). The cationic nature of the vast majority of these proteins is thought to be crucial to target and disrupt microbial membranes (28). Based on their primary structure, antibacterial proteins are classified in four groups. The largest group found thus far is formed by the β-stranded proteins, containing 4-6 conserved cysteines linked by disulfide bridges. Defensins are probably the best-studied members of this group. Other classes are amphipatic α-helical proteins, proline-rich coiled proteins and looped or cyclic proteins (5,28).

The antibacterial proteins found in man are distributed over a variety of tissues and cell types. They have been found in leukocytes, most abundantly in polymorphonucleated neutrophils (PMNs[1]) where they are thought to be involved in the killing of engulfed bacteria (24). More recently, cationic antibacterial peptides have also been found in various epithelial tissues (54). Enteric defensins are produced and excreted by human (34,35) and mouse (32,50) Paneth cells. Beta-defensins, first isolated from bovine neutrophils (55) and epithelial tissue of tongue and trachea (15,16,48), have recently been identified in human airway (26,57) and urogenital epithelial tissue (61) as well as in plasma (2) and skin epithelial cells (29). Expression of some of the epithelial proteins was found to be elevated after injury or contact with LPS or bacteria (29,53,58,60), which indicates their relevance in non-specific host defense.

In addition to the cell types mentioned above, human and rabbit blood platelets are known to store antibacterial proteins (12,17,33,63,67,70,73). These antibacterial proteins are released from platelet α-granules *in vitro* after activation with thrombin (12). *In vivo*, direct contact of platelets with bacteria causes aggregation and activation of platelets (22). The subsequently released antibacterial proteins most likely are involved in the elimination of adherent bacteria (13). Dankert *et al* (12,13) showed that antibacterial proteins released from thrombin-activated platelets were involved in the clearance of viridans streptococci from cardiac vegetations in the rabbit experimental infective endocarditis (IE) model. Viridans streptococci with low susceptibility to these proteins persisted in vegetations, while highly susceptible bacteria were rapidly eliminated (13). Similarly, strains of *Staphylococcus aureus* and *Candida albicans* insusceptible to rabbit platelet microbicidal proteins (PMPs) caused more severe experimental IE than did PMP-susceptible strains (14,71). Furthermore, thrombocytopenic rabbits (59) or rabbits with antibodies neutralizing their platelet bactericidal proteins (39) were more susceptible to streptococcal IE than control rabbits.

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[1] The abbreviations used are: PMN, polymorphonuclear cell; LPS, lipopolysaccharide; PMP, platelet microbicidal protein; IE, infective endocarditis; TC, thrombocidin; NAP-2, neutrophil activating peptide-2; CTAP-III, connective tissue activating peptide-III; β-TG, β-thromboglobulin; PBP, platelet basic protein; PF-4, platelet factor-4; RP-HPLC, reversed phase high performance liquid chromatography; (C)AU-PAGE, (continuous) acid urea polyacrylamide gel electrophoresis; MW, molecular weight; TSB, tryptic soy broth; cfu, colony forming unit; HNP, human neutrophil protein (defensin); MBC, minimal bactericidal concentration; MFC, minimal fungicidal concentration; diS-C_3[5], 3, 3'-dipropylthiadicarbocyanine iodide.

The present study was undertaken to gain insight into the number, structure, activity, and mechanism of action of antimicrobial proteins present in human platelets.

EXPERIMENTAL PROCEDURES

Isolation of human blood platelets

Citrated human blood from healthy subjects was obtained from the Central Laboratory for Bloodtransfusion, Amsterdam, The Netherlands. Platelets were concentrated by the buffy coat method (20) and isolated using a protocol adapted from Fukami (23) and Kaplan *et al.* (37). Buffy coats were pooled in transfer bags (NPBI, Emmer-Compascuum, The Netherlands, 8 buffy coats per bag, approximately 550 ml), to which 200 ml of PBS + 0.38% trisodium citrate (w/v) was added. The bags were blown tight with air and centrifuged for 5 min at 600 × *g* at 20°C. The upper three quarters of the volume of each bag, containing platelets, were transferred into new bags. To this platelet concentrate, 1/9 volume of citrate solution [75 mM trisodium citrate; 38 mM citric acid] was added. The bags were blown tight again, and were centrifuged at 1,750 × *g* at 20°C for 10 min. The supernatants were removed and platelets were resuspended in Tris-citrate [63 mM Tris-HCl; 95 mM NaCl; 5 mM KCl; 12 mM citric acid; pH 6.5] by gentle massage. The platelet suspensions were collected in a siliconized flask. The bags were washed once with Tris-citrate, and this washing was added to the platelet suspension. Processing of 48 buffy coats routinely yielded approximately 75 ml of highly concentrated platelet suspension containing less than 0.05% leukocytes, as determined with a Coulter counter.

Isolation of platelet granules and preparation of platelet granule sonicate

Platelet concentrate was kept on ice and was cavitated 3 times for 15 min under nitrogen at 60 atm in a cavitation chamber (Parr Instrument Co, Moline, IL, USA). Cavitate was collected in siliconized polypropylene tubes (Becton-Dickinson, Leiden, The Netherlands). Cavitation resulted in 90% homogenization of the platelets as determined with a Coulter counter. Intact and disrupted platelets were removed by centrifugation (5,000 × *g*, 20 min). The supernatant was collected and centrifuged at 12,000 × *g* for 20 min to pellet the granules. The pellet was resuspended in 5% acetic acid and sonicated for 30 seconds on ice to disrupt the granules, using a Branson model B15 sonifier (Branson, Soest, The Netherlands). The granule sonicate was kept at 4°C for 24 hours to extract protein, and was subsequently centrifuged at 125,000 × *g* for 60 min to remove granule debris. The supernatant containing the extracted proteins was dialyzed against 5% acetic acid using 3,500 molecular weight cut-off dialysis tubing (Spectrum, Breda, The Netherlands). Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA).

Estimation of the number of antibacterial proteins in platelet granule sonicate

To obtain insight into the number and activities of antibacterial proteins in human platelet granules, granule protein was subjected to a pre-purification step, followed by C18 RP-HPLC separation of the proteins and screening for antibacterial activity. Pre-purification of platelet granular proteins obtained by cavitation and sonication was

performed using a Sephacryl S200 gelfiltration column (Pharmacia, Uppsala, Sweden, 2.5 × 90 cm). Fifteen ml of dialyzed granule sonicate containing 10-12 mg protein per ml was applied. Protein was eluted in 5% acetic acid under gravitational force (0.4 ml/min). Fractions of 2.5 ml were collected in glass tubes, and the absorbance at 280 nm was measured. Aliquots of 50 µl were analysed for antibacterial and lysozymal activity in radial diffusion assays (see below). Fractions containing antibacterial activity were pooled, lyophilized and dissolved in 5% acetonitrile. Subsequently, proteins were separated by RP-HPLC chromatography on an HS Hyper Prep C18 column (10 × 250 mm, Alltech, Deerfield, IL, USA). Water (purified on a water purification system, Millipore, Etten-Leur, The Netherlands) and acetonitrile (HPLC-grade, Baker, Deventer, The Netherlands), both supplemented with 0.1% trifluoroacetic acid (Baker, Deventer, The Netherlands) were used as eluents. Protein was eluted at 1 ml/min with an isocratic step of 5% acetonitrile for 10 min, followed by a shallow linear gradient from 5 to 35% acetonitrile (4% per h). Fractions of 2 ml were collected and 50 µl-aliquots were used for testing antibacterial and lysozymal activity in radial diffusion assays.

Purification of thrombocidins from platelet granule sonicate

To purify the major antibacterial proteins from human platelet granule sonicate to homogeneity, a rapid 2-step protocol was applied. As the first step we used a CM-Sepharose (Pharmacia, Uppsala, Sweden) ion exchange column (2.5 × 30 cm) equilibrated in phosphate buffer (50 mM, pH 7.0). Twenty-five ml of sonicate obtained from approximately 40 buffy coats and containing 3.5 mg protein per ml was applied to the column at 0.8 ml/min. The column was washed with phosphate buffer at 0.8 ml/min, and protein was eluted in a linear salt gradient from 0 to 1 M NaCl in phosphate buffer. Fractions of 4 ml were collected and dialyzed against 1% acetic acid. Cationic antibacterial proteins were detected using acid urea polyacrylamide gel electrophoresis (AU-PAGE) and gel overlay assays (see below). Fractions containing antibacterial proteins were pooled and lyophilized. Proteins were further purified using continuous acid urea (CAU-)PAGE as described by Harwig et al (30) with slight modifications. A cylindrical gel (3.7 cm in diameter, 7 cm heigh; 12.5% acrylamide, 5% acetic acid, 5M urea) was prepared in a Model 491 Prep Cell (BioRad, Veenendaal, The Netherlands). The gel was polymerized at 37°C and prerun at 200V for 2 h in 5% acetic acid. Protein was dissolved in sample buffer (3M urea in 5% acetic acid with methyl green as the tracking dye) and electrophorized at 40 mA with reversed polarity. Protein was eluted in 5% acetic acid at 0.8 ml/min and collected in fractions of 4 ml. Antibacterial proteins were detected by AU-PAGE and gel overlay assays (see below).

Purification of NAP-2, CTAP-III and PF-4

Since TC-1 and TC-2 appeared to be variants of the CXC-chemokines NAP-2 and CTAP-III, we tested the antibacterial activity of these proteins, as well as of platelet factor-4 (PF-4), another platelet CXC-chemokine. CTAP-III, NAP-2 and PF-4 were purified from release supernatants of thrombin-stimulated platelets, as previously described (6,8,43,51). Briefly, CTAP-III (together with other variants of β-thromboglobulin antigen) was absorbed by immunoaffinity chromatography and then purified to homogeneity using

sequential cation exchange (8) and reversed-phase chromatography (6). NAP-2 was then generated from CTAP-III by limited digestion with chymotrypsin and purified by reverse-phase chromatography (43). PF-4 was isolated from the flow-through of the immunocolumn obtained after absorption of beta-thromboglobulin antigen, and then further purified by sequential heparin-sepharose and reversed-phase chromatography (51). All chemokine preparations exceeded 99% purity and contained no detectable protein contaminants as judged from analysis by silver-stained SDS-PAGE and by automated N-terminal sequence analysis. The C-terminus of CTAP-III and NAP-2 was intact, as probed in Western blots by reactivity of the chemokines with an antiserum that required the ultimate aa-residue for binding to beta-thromboglobulin proteins (7). Furthermore, the full length of CTAP-III (85 aa), NAP-2 (70 aa) and PF-4 (70 aa) was verified by matrix-assisted desorption/ionization (MALDI) mass spectroscopy.

Protein sequencing and mass spectrometry

Sequencing of thrombocidins was performed at the Sequencing Unit of the University of Utrecht by automated Edman degradation (Applied Biosystems model 476A Protein Sequencer, San Jose, CA, USA). Electrospray ionization mass spectrometry was performed on a hybrid quadrupole time-of-flight mass spectrometer, a Q-TOF (Micromass, Manchester, UK), equipped with an on-line nanoelectrospray interface (capillary tip 20 µm internal diameter × 90 µm outer diameter) with an approximate flow rate of 250 nl/min. This flow was obtained by splitting of the 0.4 ml/min flow of a conventional high pressure gradient system 1 to 1000, using an Acurate flow splitter (LC Packings, Amsterdam, The Netherlands). Lyophilized samples were dissolved in water/methanol/acetic acid (50/50/1, v/v/v). Injections were done with a dedicated micro/nano HPLC autosampler, the FAMOS (LC Packings, Amsterdam, The Netherlands) in flow injection analysis mode. Thrombocidins were treated with trypsin (Difco, Detroit, MI, USA; 1:100, w/w) in ammonium hydrogen carbonate (50 mM, pH 8.0) for 18 h. Mass spectra of the tryptic digests were recorded from mass 50-2,000 Da every second with a resolution of 5000 full width half maximum. The resolution allows direct determination of the monoisotopic mass, also from multiple charged ions. In MS/MS mode ions were selected with a window of 2 Da with the first quadrupole, and fragments were collected with high efficiency with the orthogonal time-of-flight mass spectrometer. The collision gas applied was argon (4×10^{-5}mbar), and the collision voltage was approximately 30V.

Acid urea polyacrylamide gel electrophoresis (AU-PAGE)

CM-Sepharose and CAU-PAGE-purified proteins were analyzed using AU-PA slab-gels (12.5% acrylamide, 5% acetic acid, 5M urea). After polymerization at 37°C the gels were prerun in 5% acetic acid at 150 V until the current was constant (ca. 8 mA). Samples to be analyzed were lyophilyzed, dissolved in 8 µl of sample buffer (3M urea in 5% acetic acid with methyl green as the tracking dye) and electrophorized in 5% acetic acid at 150 V with reversed polarity. Gels were either stained with 0.1% Coomassie blue in 50% methanol and 10% acetic acid, or by silverstaining according to Blum et al (3). Gels run in parallel were used in overlay assays to localize antibacterial proteins (see below).

Detection of antibacterial proteins by gel overlay assay

Gel overlay assays to detect activity of antibacterial proteins in acid urea gels were performed according to Lehrer et al (41) with minor modifications. The test strain Escherichia coli ML35 was grown in Tryptic soy broth (TSB, Difco, Detroit, MI, USA) at 37°C overnight. This culture was diluted 50 times in fresh TSB and bacteria were grown to log-phase in 2.5 h. Bacteria were pelleted at 14,000 × g for 30 sec and washed twice with PBS (pH 7.4). For each assay an inoculum of 5×10^7 cfu was suspended in 15 ml of nutrient-poor agarose of 42°C (10 mM sodiumphosphate buffer pH 7.4; 0.06% [w/v] TSB; 1% [w/v] type I agarose [Sigma, St. Louis, MO, USA]). This suspension was poured into a square 12 × 12 cm dish (Hospidex, Nieuwkoop, The Netherlands). Immediately after electrophoresis, acid urea slab gels were washed 3 times for 12 min in phosphate buffer (10 mM, pH 7.4) and placed on top of the bacterial agarose bottom. After incubation at 37°C for 3 h, the gels were removed and a nutrient-rich agar (6% [w/v] TSB, 1% [w/v] agar noble [Difco, Detroit, MI, USA]) was poured over the bottom layer to allow growth of surviving bacteria. Clear zones after overnight incubation at 37°C indicated the presence of antibacterial proteins.

Radial diffusion assay

Chromatographic fractions of human platelet granule sonicates were assayed for antibacterial activity by radial diffusion assay (41). An agarose bottom containing either *E. coli* ML35, *Streptococcus sanguis* U108 (strain 2 in (13)) or *Staphylococcus aureus* 42D was prepared as described for the gel overlay assay. Evenly spaced 3 mm wells were punched in the agarose. Samples to be analyzed for antibacterial activity were lyophilized, dissolved in 5 µl of 0.01% acetic acid and transferred to the wells. Three µg of purified HNP 1-3 in 3 µl was included as a positive control. The dish was incubated at 37°C for 3 hours to allow diffusion of antibacterial protein, and subsequently 15 ml of nutrient-rich agar (6% [w/v] TSB; 1% [w/v] agar noble [Difco, Detroit, MI, USA]) was poured over the bottom layer. Plates were incubated overnight at 37°C to allow growth of surviving bacteria. Areas of clear zones were used as a measure of antibacterial activity.

Detection of lysozymal activity

Lysozymal activity in chromatographic fractions was determined by a radial diffusion assay in which lyophilized Micrococcus lysodeikticus cell walls (Sigma, St. Louis, MO, USA) were used as the substrate (final concentration 3 mg/ml). Cell walls were homogenized in 66 mM phosphate buffer pH 7.0, and mixed with low EEO agarose (Sigma, St. Louis, MO, USA) (1% [w/v] final concentration) in the same buffer. Fifteen ml of cell wall-containing agarose was poured into square dishes (12 × 12 cm), and evenly spaced 3 mm wells were punched. Samples (5 µl in 0.01% acetic acid) were pipetted into the wells. The dishes were incubated for 4-5 hours at 37°C after which diameters of clear zones were measured.

Microbicidal assay

Microbicidal activity of purified thrombocidins and of NAP-2, CTAP-III and PF-4 was quantified in a liquid microbicidal assay. Suspensions of logarithmically growing test

bacteria (*B. subtilis* ATCC6633, *E. coli* ML35 or *S. aureus* 42D) were prepared as described for the overlay assay. Two fungi, *Candida glabrata* and *Cryptococcus neoformans* (both clinical isolates) were maintained on Isosensitest agar plates (Oxoid, Unipath, Basingstoke, Hampshire, UK) and cultured for 48 h at 30°C in 0.7% [w/v] yeast nitrogen base (YNB, Difco, Detroit, MI, USA), supplemented with 0.15% [w/v] L-asparagine (Merck, Darmstadt, Germany) and 1% [w/v] glucose (Merck). Bacteria and fungi were diluted to 1-2 $\times$ 10^5 cfu/ml in 10 mM phosphate buffer, pH 7.0 + 0.06% [w/v] TSB. Two-fold serial dilutions of the protein to be tested were prepared in 0.01% acetic acid and 5 µl aliquots were transferred to a low protein binding polypropylene microtiter plate (Costar, Cambridge, USA). To each of the wells, 45 µl of the bacterial suspension was added. The plate was incubated on a rotary shaker (300 rpm) at 37°C. After 2 hours, aliquots of 0.5 µl and 10 µl were plated on blood agar plates (bacteria) or isosensitest agar plates (fungi) and incubated at 37°C. Alternatively, 10 µl-aliquots were spotted in duplicate on plates which had been dried for 1 h at 37°C. In some cases, 150 µl of TSB was added to the remainder of the incubations, and the microtiter plate was incubated at 37°C. Microbicidal activity was assessed the next day (bacteria) or after 48 h (fungi) after counting colonies on the agar plates and by visual inspection of growth in the microtiter plates. MBC and MFC were defined as the concentration of protein at which <0.1% of the inoculum survived after the 2 hours of exposure. All experiments were performed at least in duplicate.

Measurements of membrane potential ($\Delta\psi$) of *L. lactis*

The influence of TCs on membrane potential was assessed using *Lactococcus lactis* IL 1403 (46). This strain was grown at 30 °C in M17 broth (Oxoid) supplemented with 25 mM galactose plus 50 mM L-malate. The cells were harvested in the mid-exponential phase of growth, and washed and resuspended in 50 mM potassium phosphate, pH 6.5 or 5.0 The membrane potential ($\Delta\psi$) was measured using the $\Delta\psi$-sensitive fluorescent dye 3, 3'-dipropylthiadicarbocyanine iodide (diS-C_3[5]). The cells were diluted to a final concentration of 20 µg of protein / ml in 50 mM KPi of the indicated pH and equilibrated at 30 °C; the final diS-C_3(5) concentration was 3 µM. The excitation and emission wavelengths were 643 and 666 nm, respectively. The membrane potential was generated upon addition of either 25 mM of glucose or 25 mM of L-malate as source of metabolic energy.

RESULTS

Estimation of the number of antibacterial proteins in platelet granule sonicate

Platelet granule sonicate was subjected to S200 gel filtration chromatography and the antibacterial activity of the collected fractions was tested by radial diffusion assays using *E. coli* and *S. sanguis* as test organisms. Antibacterial activity against both test organisms largely coeluted, and also coeluted with the majority of the lysozymal activity (not shown). The fractions containing these activities were pooled, lyophilized and analyzed further by C18 RP-HPLC. A shallow gradient was applied to allow separation of

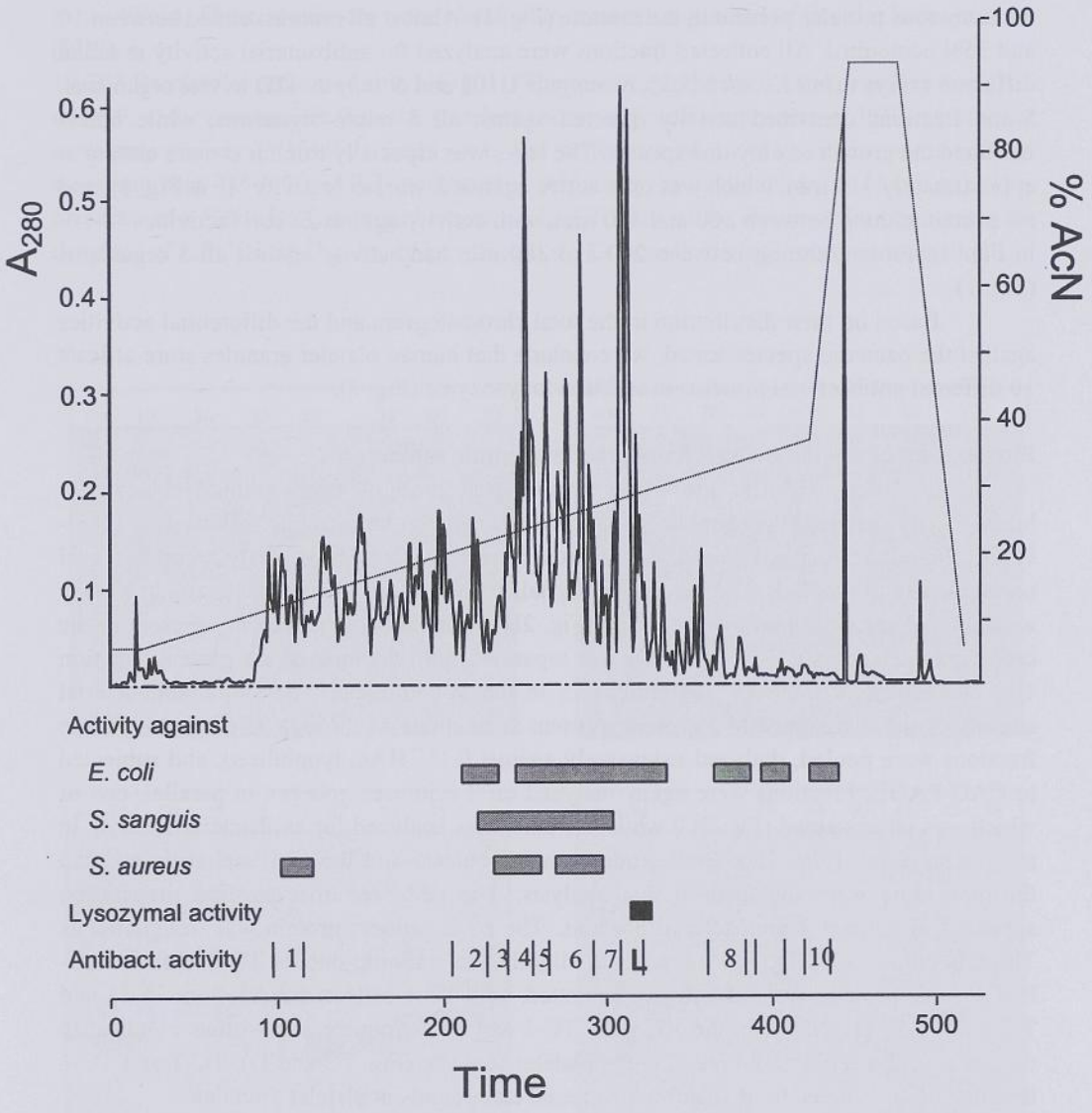

Figure 1. Survey of antibacterial activity present in human platelet granule sonicate. Platelet granule sonicate, pre-purified by S200 gel permeation chromatography, was subjected to C18 RP-HPLC. A shallow gradient (dashed line) of acetonitril in water was applied to elute protein. Absorbance at 280 nm (solid line) was recorded during elution. Presence of antibacterial activity in collected fractions was tested against *E. coli* ML35, *S. sanguis* U108 and *S. aureus* 42D in radial diffusion assays, and is indicated by gray bars. Lysozymal activity is represented by the black bar.

the numerous proteins present in the mixture (Fig. 1). Almost all proteins eluted between 10 and 35% acetonitril. All collected fractions were analyzed for antibacterial activity in radial diffusion assays using *E. coli* ML35, *S. sanguis* U108 and *S. aureus* 42D as test organisms. Some fractions contained activity directed against all 3 micro-organisms, while others inhibited the growth of only one species. The latter was especially true for protein eluting at approximately 110 min, which was only active against *S. aureus* (activity '1' in Fig. 1), and for protein eluting between 360 and 440 min, with activity against *E. coli* (activities '8-10' in Fig. 1). Protein eluting between 200 and 300 min had activity against all 3 organisms (Fig. 1).

Based on their distribution in the total chromatogram and the differential activities against the bacterial species tested, we conclude that human platelet granules store at least 10 different antibacterial proteins in addition to lysozyme (Fig. 1).

Purification of thrombocidins from platelet granule sonicate

In order to purify the major antibacterial proteins from granule sonicate to homogeneity, we used CM-Sepharose cation exchange chromatography followed by CAU-PAGE. Fractions obtained after CM-Sepharose chromatography were analysed on two acid urea gels run in parallel. One gel was silverstained (Fig. 2A), the other was used to assay antibacterial activity in an overlay assay (Fig. 2B). The antibacterial activity present in the crude granule sonicate (Fig. 2, "son") was separated from the bulk of the protein (fraction 10), and eluted in fractions 35 through 75 in the salt gradient. The major antibacterial activity could be assigned to 2 proteins present in fractions 45 through 75 (Fig. 2B). These fractions were pooled, dialyzed extensively against 0.1% HAc, lyophilized, and subjected to CAU-PAGE. Fractions were again analyzed on 2 acid urea gels run in parallel, one of which was silverstained (Fig. 2C) while the other was analyzed for antibacterial activity in an overlay assay (Fig. 2D). Both crude granule sonicate and the CM-Sepharose-purified thrombocidins were included in this analysis. The CM-Sepharose-purified preparation appeared to contain 2 antibacterial proteins. The most cationic protein was designated as Thrombocidin-1 (TC-1), and the second, slightly less cationic one as TC-2. After CAU-PAGE these proteins were effectively separated, with TC-1 collected in fractions 35-41 and TC-2 in 45-51 (Fig. 2C). In the AU gels, TC-1 and TC-2 migrate at positions identical to the main antibacterial activities in crude platelet sonicate (Fig. 2C and D). TC-1 and TC-2 thus can be considered to be major antibacterial compounds in platelet granules.

Characterization of TC-1

Several attempts to determine the N-terminal sequence of TC-1 by Edman degradation were not successful. Mass-spectrometrical techniques were used to elucidate the structure of TC-1. Analysis by MALDI spectrometry revealed that the purified TC-1 preparation contained a 7,435.9 Da protein, together with minor amounts of proteins of similar size (Table I). ES spectrometry revealed a mass of 7,436.3 Da, confirming the mass of the major protein in the preparation, identified by MALDI. This component will further be referred to as TC-1. Sequence data were obtained by trypsin digestion of TC-1 followed by mass-spectrometrical analyses in MS/MS mode of the resulting fragments. The sequences of 2 fragments, of 839.5 and 590.3 Da, were TTSGIHPK and LAGDES,

respectively. These sequences were identical to internal sequences of platelet basic protein (PBP), a
10,262 Da platelet protein (Table I, Fig. 3). The mass of undigested TC-1 was less than that of PBP, and even smaller than the smallest known degradation product of PBP, neutrophil activating protein-2 (NAP-2; 7,623 Da). The difference of 186 Da can be explained by assuming that TC-1 is NAP-2, truncated C-terminally by 2 amino acids (Ala-Asp). The presence of a 590.3 Da C-terminal fragment, LAGDES, and the absence of a fragment with the mass of LAGDESAD in the tryptic digest of TC-1 confirm the C-terminal truncation.

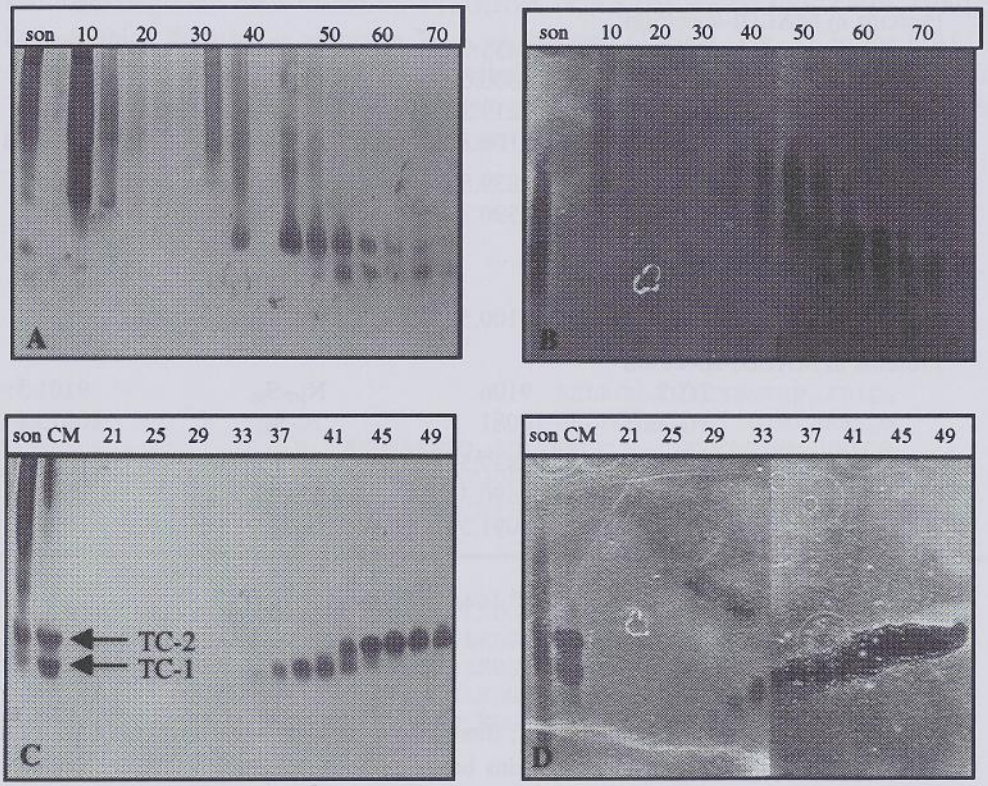

Figure 2. Purification of thrombocidins by CM-Sepharose chromatography and CAU-PAGE. Thrombocidins were pre-purified from platelet granule sonicate by CM-Sepharose chromatography (panels A and B). Of the indicated fractions, 50 µl aliquots were analyzed on 2 acid urea gels run in parallel, followed by silverstaining of one gel (panel A) and an overlay assay of the other gel (panel B). Fractions containing antibacterial protein were pooled and further purified by CAU-PAGE (panels C and D). Fractions collected in the second purification step were also analyzed on acid urea gels, followed by silverstaining (panel C) and overlay assay (panel D). Platelet granule sonicate (son) and CM-sepharose-purified thrombocidins (CM) were included. *E. coli* ML35was used as the test organism in the overlay assays (panels B and D).

Table I. Characterization of thrombocidins. Molecular weights (MWs) of TC-1 and TC-2 were determined by electrospray and MALDI-TOF mass-spectrometry. Trypsin-treated TCs were analyzed by electrospray mass-spectrometry and sequences of selected fragments were determined in MS/MS mode. Theoretical MWs of TCs are based on average masses, those of tryptic fragments on mono-isotopic masses.

Component	Experimental MW (Da)	Internal sequence of PBP matching experimental MW[a]	Theoretical MW (Da)
Thrombocidin-1			
Electrospray	7436.3	A_{25}-S_{92}	7437.5
Proteins in MALDI-spectrum			
TC-1	7435.9	A_{25}-S_{92}	7437.5
TC-1a[b]	7600.6	Y_{24}-S_{92}	7600.7
TC-1b[b]	7219.3	A_{25}-D_{90}	7220.9
TC-1c[b]	7106.2	A_{25}-G_{89}	7105.8
Tryptic fragments	839.5	T_{34}-K_{41}	839.5
	590.3	L_{87}-S_{92}	590.3
Thrombocidin-2			
Electrospray	9100.5	N_{10}-S_{92}	9101.5
Proteins in MALDI-spectrum			
TC-2	9106	N_{10}-S_{92}	9101.5
TC-2a[b]	10081	S_1-S_{92}	10075.6
Tryptic fragments	839.5	T_{34}-K_{41}	839.5
	590.3	L_{87}-S_{92}	590.3
	1091.5	G_{14}-L_{23}	1091.5

[a] *Amino acid numbering of PBP as in Fig. 3* [b] *Minor component*

In the MALDI spectrum of TC-1, three minor proteins were observed (TC-1a to 1c, Table I). The sequences of these proteins have not been determined directly, but their recorded masses can be explained by assuming that they also are derivatives of PBP, having N- and C-termini slightly different from TC-1. The N-terminus of TC-1 and of NAP-2 results from cleavage of PBP between Tyr_{24} and Ala_{25}, the cleavage site of chymotrypsin (8,9). TC-1a has an MW of 7,600.6, 164 Da larger than TC-1. This suggests the presence of an N-terminal tyrosine preceding Ala_{25}, possibly resulting from alternative cleavage between Leu_{23} and Tyr_{24} in PBP. Two other minor compounds were TC-1b and TC-1c with MWs of 7219.3 and 7106.2 Da, respectively. These values correspond to masses of proteins derived from TC-1 by further truncation by 2 (ES) or 3 (DES) C-terminal amino acids (Table I).

Characterization of TC-2

The N-terminal sequence of TC-2 was determined by Edman degradation to be NLAKGKEESLDSDLY, which is identical to the N-terminal sequence of connective tissue activating protein-III (CTAP-III). CTAP-III is a major platelet α-granule protein and, like NAP-2, a known degradation product of PBP (Fig. 3). The molecular weight of TC-2 was 9100.5 Da as determined by electrospray mass-spectrometry (Table I). This is less than the theoretical MW of CTAP-III (9287.7 Da), which can be explained by the absence of the two C-terminal amino acids (Ala-Asp) present in CTAP-III. The calculated mass of this molecule (9101.5) is in accordance with the mass found experimentally for TC-2. Analysis of tryptic fragments of TC-2 revealed the presence of a 590.3 Da fragment with the sequence LAGDES, confirming the C-terminal truncation as in TC-1 (Table I). Of TC-2, two other fragments were identified, TTSGIHPK (839.5 Da) and GKEESLDSDL (1091.5 Da) of which the latter is absent in TC-1, as expected (Table 1, Fig. 3). In the MALDI spectrum of TC-2 one minor peak was detected (TC-2a, Table I) with a MW of 10,081 Da. This value corresponds to the mass of PBP, truncated C-terminally by two amino acids (Ala-Asp). This molecule could be a precursor of TC-2.

```
TC-1                                          AELRCM30CIKTTSGIHP40KNIQS
NAP-2                                         AELRCM30CIKTTSGIHP40KNIQS
TC-2                    N10LAKGKEESLD20SDLYAELRCM30CIKTTSGIHP40KNIQS
CTAP-III                N10LAKGKEESLD20SDLYAELRCM30CIKTTSGIHP40KNIQS
PBP          SSTKGQTKRN10LAKGKEESLD20SDLYAELRCM30CIKTTSGIHP40KNIQS


TC-1         LEVIG50KGTHCNQVEV60IATLKDGRKI70CLDPDAPRIK80KIVQKKLAGD90ES
NAP-2        LEVIG50KGTHCNQVEV60IATLKDGRKI70CLDPDAPRIK80KIVQKKLAGD90ESAD
TC-2         LEVIG50KGTHCNQVEV60IATLKDGRKI70CLDPDAPRIK80KIVQKKLAGD90ES
CTAP-III     LEVIG50KGTHCNQVEV60IATLKDGRKI70CLDPDAPRIK80KIVQKKLAGD90ESAD
PBP          LEVIG50KGTHCNQVEV60IATLKDGRKI70CLDPDAPRIK80KIVQKKLAGD90ESAD
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Figure 3. Sequence alignment of thrombocidins and related chemokines. TC, thrombocidin; NAP-2, neutrophil activating peptide-2; CTAP-III, connective tissue activating peptide-III; PBP, platelet basic protein.

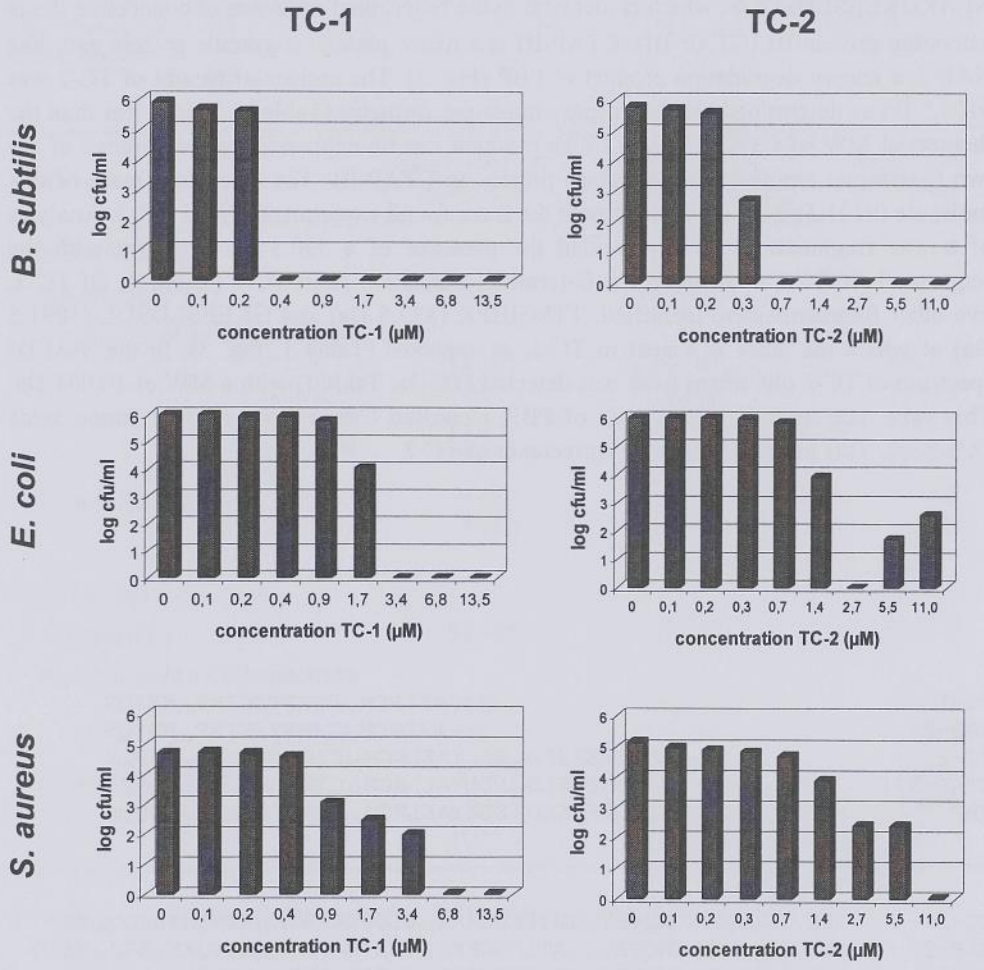

Fig. 4. Bactericidal activity of thrombocidins. Bacteria in logarithmic phase (*E. coli* ML35, *B. subtilis* ATCC6633 or *S. aureus* 42D, 1-2 × 10^5cfu/ml) were exposed to TC-1 and TC-2, serially diluted in 10mM phosphate buffer (pH 7.0) + 0.06% (w/v) TSB, for 2 h at 37°C. Colonies were counted after plating incubations on blood agar plates. Experiments were performed at least in duplicate; results from duplicate incubations never differed >20%. MBCs never differed more than one dilution step.

Bactericidal activity of thrombocidins

Bactericidal activity of purified TC-1 and TC-2 was investigated by determination of their MBC values for *S. aureus* 42D, *B. subtilis* ATCC6633 and *E. coli* ML35 (Fig. 4). *B. subtilis* was the most susceptible organism with MBCs of 0.4 and 0.7 μM of TC-1 and TC-2, respectively. *E. coli* ML35 was somewhat less susceptible, with MBC values of 3.4 and 2.7 μM of TC-1 and TC-2, respectively. MBCs of TC-1 and TC-2 for *S. aureus* 42D were 6.8 μM and 11 μM, respectively (Fig. 4). Incubations from which no bacteria could be recovered after 2 h of incubation never showed visible growth after addition of growth medium and overnight incubation (not shown).

Kinetics of bactericidal activity was investigated by exposure of *B. subtilis* to 3 μM of TC (Fig. 5). Killing by TC-1 appeared to be very rapid, causing a 3-log fold reduction of the inoculum within 1 min, and a 5-log fold reduction within 5 min. At 3 μM, killing by TC-2 was slower, reaching 3-log and 5-log fold reduction after 25 and 30 min, respectively (Fig. 5).

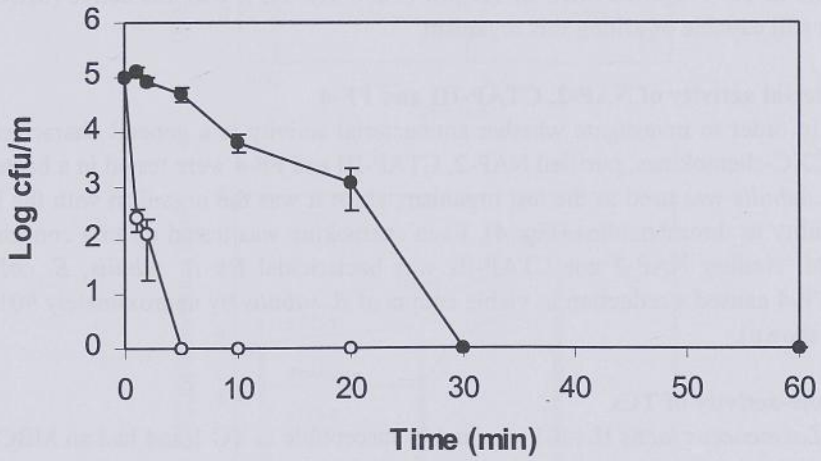

Fig. 5. Kinetics of bactericidal activity of TC-1 and TC-2 against *B. subtilis* ATCC6633. Bacteria (1×10^5cfu/ml) grown to log-phase were exposed to 3 μM TC-1 (○) or TC-2 (●) at 37°C. At given timepoints, aliquots were plated on blood agar plates and colonies were counted the next day. The average of three independent experiments (± SD) are given.

Table II. Fungicidal activity of thrombocidins. Fungi (1-2 x 10^5 cfu/ml) were exposed to TC-1 and TC-2 serially diluted in 10 mM phosphate buffer (pH 7.0) + 0.06% (w/v) TSB for 2 h at 37°C. MFCs were determined after plating incubations on SEN-agar plates. Duplicate experiments showed identical results.

Organism	MFC (μM)	
	TC-1	TC-2
Cryptococcus neoformans	1.9	30
Candida glabrata	>30	>30

Fungicidal activity of thrombocidins

Fungicidal activity of thrombocidins was tested using the same experimental set up as for the bactericidal activity testing. Both TC-1 and TC-2 appeared to be inactive against *Candida glabrata* up to 30 μM (Table II). *Cryptococcus neoformans*, however, was highly susceptible to TC-1 with an MBC of 1.9 μM (Table II). TC-2 was less active (MBC of 30 μM), but still capable of killing this organism.

Antibacterial activity of NAP-2, CTAP-III and PF-4

In order to investigate whether antibacterial activity is a general characteristic of platelet CXC-chemokines, purified NAP-2, CTAP-III and PF-4 were tested in a bactericidal assay. *B. subtilis* was used as the test organism, since it was the organism with the highest susceptibility to thrombocidins (Fig. 4). Each chemokine was tested up to a concentration of 30 μM. Neither NAP-2 nor CTAP-III was bactericidal for *B. subtilis*, *E. coli* or *S. aureus*. PF-4 caused a reduction in viable counts of *B. subtilis* by approximately 90% at 30 μM (not shown).

Membrane-activity of TCs

Lactococcus lactis IL1403 was highly susceptible to TC-1 and had an MBC of 0.5 μM. Since many antimicrobial peptides have been shown to act via $\Delta\psi$-dissipating processes (36,64,68), the $\Delta\psi$-sensitive fluorescent dye 3, 3'-dipropylthiadicarbocyanine iodide (diS-C_3[5]) was used to assess the effects of TC-1 and TC-2 on the membrane potential generated by glycolyzing or L-malate-metabolizing cells of *L.lactis* IL1403. The degree of fluorescence quenching of diS-C_3[5] is directly proportional to the membrane potential across the cytoplasmic membrane of the cells.

In glycolyzing cells of *L.lactis* the membrane potential is generated by proton extrusion via the F_0F_1-ATPase after sugar breakdown in the Embden-Meyerhof pathway, whereas in L-malate-metabolizing cells the membrane potential results from the electrogenic exchange of L-malate for L-lactate (52). The latter pathway, that is malolactic fermentation, only involves one enzyme, *i.e.*, malolactic enzyme, in addition to the L-malate/L-lactate exchanger. If the TC-induced killing results from the dissipation of the ion

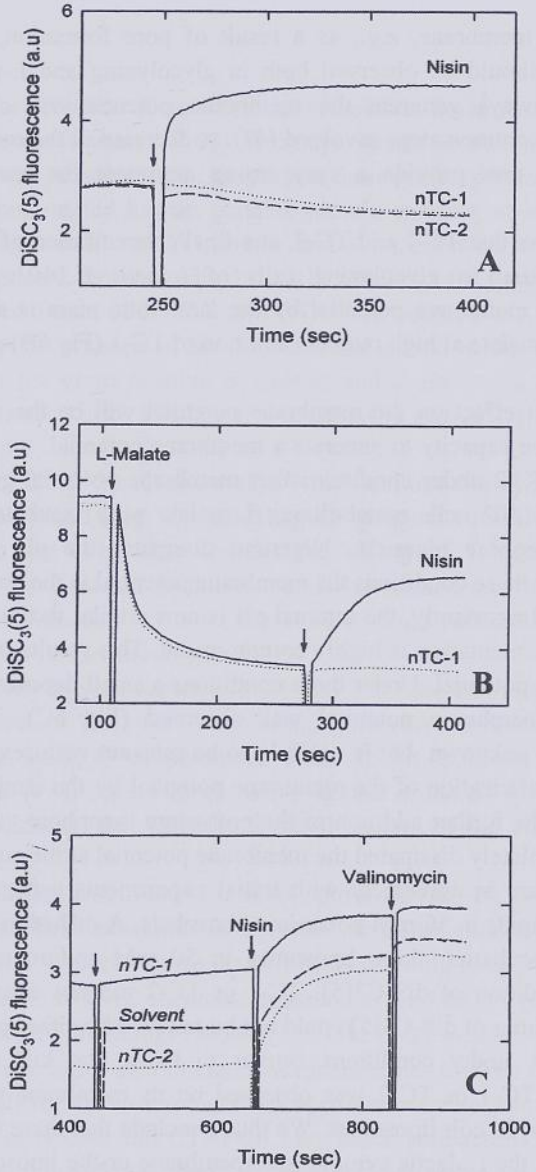

Figure 6. Effect of thrombocidins on the Δψ of glycolyzing and malate-metabolizing *Lactococcus lactis* IL 1403 cells. *Panel A*, *L. lactis* cells were resuspended to a final protein concentration of 20 μg of per ml in 50 mM potassium phosphate, pH 6.5, containing 3 μM $diSC_3[5]$. At time zero (not depicted), glucose was added to a final concentration of 25 mM which resulted in the generation of a membrane potential (observed as a decrease in fluorescence). After about 4 min, TC-1 (2 μM, final concentration), TC-2 (2 μM) or nisin (1 μM) was added. Further details are described under Materials and Methods. *Panel B*, experimental conditions were the same as the described for *panel A*, except that the pH was 5.0, and 25 mM L-malate was used to energize the cells. After about 2 min, TC-1 (1 μM) or nisin (0.5 μM) was added. *Panel C*, experimental conditions were the same as described for *panel B*, except that nigericin was present at 0.5 μM. The additions of TC-1 (1 μM), TC-2 (1 μM), solvent control, nisin (0.5 μM) and valinomycin (0.5 μM) are indicated.

gradients across the membrane, *e.g.*, as a result of pore formation, a lowering of the membrane potential should be observed both in glycolyzing and L-malate-metabolizing bacteria. These pathways generate the membrane potential via completely different mechanisms with no common steps involved (42). A decrease in the membrane potential in both systems would thus provide a very strong argument for pore formation in the membrane (46).

Fig. 6A shows that TC-1 and TC-2, at a final concentration of 2 μM, do not affect the membrane potential in glycolyzing cells of *L.lactis* IL1403. As a control, the depolarization of the membrane potential by the lantibiotic nisin is shown. Similarly, in cells metabolizing L-malate at high rate, the addition of TC-1 (Fig.6B) or TC-2 (not shown) had no effect.

Since the net effect on the membrane potential will be the resultant of putative pore formation and the capacity to generate a membrane potential, we also determined the effect of TC-1 and TC-2 under conditions that membrane potential generation is limited. For this, *L. lactis* IL1403 cells metabolizing L-malate were incubated at pH 5.0 in the presence of the ionophore nigericin. Nigericin dissipates the pH gradient across the membrane, and under these conditions the membrane potential is the only component of the proton motive force. Importantly, the internal pH is now similar to the external one, that is 5.0, and malolactic fermentation is highly compromised. This results in limited capacity to generate a membrane potential. Under these conditions a small depolarizing effect of TC-1 and TC-2 on the membrane potential was observed (Fig 6C). The nature of this depolarizing effect is unknown, but is unlikely to be relevant with respect to the observed cidal effect. The depolarization of the membrane potential by the lantibiotic nisin is again shown as a control; the further addition of the potassium ionophore valinomycin indicates that nisin nearly completely dissipated the membrane potential at the concentration tested.

These data are in agreement with initial experiments using liposomes prepared from *E coli* phospholipids in 50 mM potassium phosphate. A diffusion membrane potential was generated by incubating these liposomes in 50 mM sodium phosphate, and was monitored by the addition of diS-C3[5]. TC-1 or TC-2 did not dissipate the membrane potential since quenching of diS-C3[5] could not be relieved by either protein (not shown).

In summary, under conditions similar to those that kill L.lactis IL1403, no significant effect of TC-1 or TC-2 was observed on its membrane potential nor on the membrane potential of E. coli liposomes. We thus conclude that there is no direct evidence for pore formation in the L. lactis cytoplasmic membrane or the liposomes by either TC-1 or TC-2, and that it is unlikely that such a mechanism is the primary cause for the cidal activity of these compounds.

DISCUSSION

Although the presence of antibacterial proteins in human and rabbit platelets has been recognized for over 30 years (17,63), their identity has never been elucidated. The aim of the present study was to isolate and characterize these proteins from human platelets.

In HPLC-fractionated human platelet granule extracts, at least 10 proteins with differential activity against *E. coli*, *S. sanguis* and *S. aureus* were distinguished. The two major proteins, designated thrombocidin-1 (TC-1) and TC-2 for thrombocyte microbicidal proteins, are truncated forms of NAP-2 and CTAP-III, respectively, differing from these CXC-chemokines by the absence of the 2 C-terminal amino acids. Both TC-1 and TC-2 were bactericidal for the gram positive *B. subtilis* and *S. aureus* as well as for the gram negative *E. coli* test strain, with MBCs ranging from 0.4 μM (TC-1, *B.subtilis*) to 11 μM (TC-2, *S. aureus*).

The MBC of TC-2 for *E. coli* was 2.7 μM, but at 5.5 and 11 μM some bacteria were reproducibly recovered (Fig. 4), indicating an optimum concentration for activity. A similar phenomenon has been observed for the killing of certain staphylococcal and streptococcal strains by β-lactam antibiotics, and was termed the "paradoxical" response (21) or tolerance (47). Whether tolerance for TC-2 or other cationic antibacterial peptides exists in *E.coli* requires further investigation.

In their activity against bacteria, TC-1 and TC-2 were almost equally potent. The fungicidal concentration of TC-1 for *C. neoformans*, however, was over 10-fold lower than that of TC-2. Interestingly, preparations from rabbit platelets containing platelet microbicidal proteins (PMP) were more active against *Candida* species than against *C. neoformans* (69), indicating that the antimicrobial spectra of the human TCs and rabbit PMPs are different.

TC-1 only differs from NAP-2, and TC-2 from CTAP-III, by the absence of 2 C-terminal amino acids. This truncation is essential for bactericidal activity, since purified NAP-2 and CTAP-III at concentrations up to 30 μM did not kill *B. subtilis, E. coli* and *S. aureus*. The C-termini of all CXC chemokines extend as an α-helix (10,45). Other α-helical proteins like the cecropins are thought to insert into the outer membrane, thereby killing the bacteria (28). If thrombocidins also interact with membranes by their α-helical domain, the structural requirements for the C-terminal helix apparently are very strict. The 2 C-terminal amino acids present in NAP-2 and CTAP-III may block antibacterial activity possibly by altered charge distribution (65), as the C-terminal residue in NAP-2 and CTAP-III is the acidic aspartic acid.

At present, it is unclear how PBP is processed to finally yield TCs. The N-terminus of TC-1 and NAP-2 are identical. NAP-2 is formed extracellularly from PBP and CTAP-III reseased upon platelet activation, by neutrophil (31) or monocyte proteases (62) like cathepsin G (8,11,62). Since we have isolated TC-1 directly from platelets granules, at least some cathepsin G-like protease activity must be present inside the platelets. As TC-1 and TC-2 are C-terminal truncated NAP-2 and CTAP-III, respectively, carboxypeptidase activity most likely is also present within the platelet granules. Interestingly, we have identified a protein with the molecular weight of a C-terminally truncated PBP (TC-2a, Table I) which may be a precursor for TC-1 and TC-2.

NAP-2, like other CXC-chemokines activates neutrophils through the highly conserved Glu-Leu-Arg (ELR) sequence in the amino-terminal region (1,66). Although PBP, CTAP-III and β-TG also contain this sequence, they do not have neutrophil chemoattractant activity (9,56), presumably because the amino terminal part of these proteins folds back over the ELR sequence, thus hampering neutrophil receptor recognition (44). TC-1 is expected to have neutrophil-activating activity since its N-terminus is identical to that of NAP-2, and since several other C-terminally truncated isoforms of NAP-2 are even more active than NAP-2 itself (7,18,19). In view of potential use of TCs as antimicrobial agents in prevention and therapy of infections, neutrophil inductive activity may not always be desirable. Therefore, it would be useful to modify thrombocidins in order to dissociate microbicidal and neutrophil activating activity.

TC-1 and TC-2 killed the entire *B. subtilis* inoculum within 5 and 30 minutes, respectively (Fig. 5). These fast kinetics are characteristic for various bactericidal proteins, and are often associated with membrane disturbance (28). Dissipation of membrane potential by the formation of voltage-dependent channels has therefore been implicated as a general mechanism of peptide antibiotic-mediated killing activity (28), although not in all studies this assumption is supported by experimental data. Under the experimental conditions used, TCs did not dissipate the $\Delta\psi$ of whole *L.lactis* bacteria, nor of liposomes composed of *E. coli* lipids. Apparently, their target for microbial killing is located elsewhere, most likely intracellularly. Under conditions that the capacity of *L. lactis* to generate a membrane potential was limited, a small decline in $\Delta\psi$ was observed in the presence of TC (Fig. 6C). This suggests that, even though no dissipation of the membrane potential occurs, TCs do interact with the membrane. Whether they can passively cross the membrane and reach putative intracellular targets remains to be established.

Rabbit platelet microbicidal proteins (PMPs) dissipated the $\Delta\psi$ of *S. aureus* cells (38,68). The structures of these peptides have not been reported, but their amino acid composition (72) differs from those of TCs. Although test conditions were not identical, our studies indicate that human and rabbit microbicidal proteins do not only differ structurally, but also in their mode of action.

We have purified TC-1 and TC-2 from isolated platelet granules. It is well possible that *in vivo* they can also be formed extracellularly. In that case, proteases present in the (inflammatory) environment where platelets become activated, could process platelet-excreted PBP, CTAP-III, as well as NAP-2. This implies that the chemokines liberated to enhance defense reactions by attracting and activating neutrophils and initiating wound healing by activating fibroblasts, may also be a rich local source for the generation of potent antimicrobials, underscoring the importance of platelets in innate host defense.

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