CHAPTER 29

Flow Cytometric Determination of Cysteine and Serine Proteinase Activities in Living Cells with Rhodamine 110 Substrates

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I. Introduction

Proteases are of essential importance for the homeostasis of intra- and extracellular protein metabolism. They are distinguishable by their cutting mode as endo- and exopeptidases or by their reactive sites as serine, cysteine, aspartic, and metalloproteases (Powers *et al.*, 1993).

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Naturally occurring proteins like hemoglobin and casein or synthetic chromogenic or fluorogenic molecules are in use as experimental protease substrates (Bergmeyer, 1984). Chromogenic substrates are frequently used for cuvette assays. Fluorogenic substrates are one or two orders of magnitude more sensitive and can be used in cuvette or cellular assays.

Cellular assays are of particular interest for flow or image cytometrical measurements on single cells in heterogeneous suspensions of peripheral blood cells as well as of cells from hemato- and immunopoietic tissues, benign or malignant tumors, or normal body tissues.

Incubation of viable or fixed cells with 4-methoxy-2-napthylamine (MNA) amino acid or peptide derivatives as substrate, followed by *in situ* precipitation of the MNA reaction product by 5-nitrosalicylaldehyde, results in a greenish reaction product upon UV excitation at 365 nm as expression of intracellular protease activities (Dolbeare and Smith, 1977; Dolbeare and Vanderlaan, 1979). Protease activities in human gynecological malignancies have been determined in this way (Haskill and Becker, 1982; Haskill *et al.*, 1983a,b; Becker *et al.*, 1983).

7-Amino-4 methylcoumarin (Zimmerman *et al.*, 1976; Kanaoka *et al.*, 1977) or 7-amino-4-trifluoromethylcoumarin (Smith *et al.*, 1980; Cox and Eley, 1987) as alternative fluorophores are useful for cuvette assays. Their use for cytometric assays is, however, problematic due to overlapping substrate and product fluorescence spectra and diffusion of uncharged reaction product through cell membranes at physiological pH values (Waggoner, 1990).

A new class of fluorogenic rhodamine 110 (R110) substrates for cuvette assays was introduced by Leytus (Leytus *et al.*, 1983a,b). These substrates show a high potential for cytometric measurements due to practically complete fluorescence quenching in the substrates; high quantum yield of free R110, similar to fluorescein; excitation at 488 nm, e.g., by argon ion laser light; and practical independence of fluorescence intensity between pH 3 and 10. The slightly positive charge of the amphiphilic free R110 reaction product at physiological pH values causes autoaccumulation in cytoplasm and mitochondria due to the internally negative transmembrane and mitochondrial electrical potentials (Rothe *et al.*, 1992; Assfalg-Machleidt *et al.*, 1992).

II. Materials and Methods

A. Synthesis of R110 Substrates

1. Chemicals

Amino acid derivatives were purchased from Bachem (Heidelberg, Germany), rhodamine 110 from Exciton Chemical Company (Dayton, OH), and 33% HBr in acetic acid and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) from E.Merck (Darmstadt, Germany), and all other reagents and anhydrous solvents were from Aldrich (Steinheim, Germany).

2. Synthesis of $(Z\text{-}Arg)_2$-R110

$(Z\text{-}Arg)_2$-R110 [compound (COMP) No. 1, MW, 983.9] was synthesized according to Leytus *et al.* (1983b), using a 10-fold instead of a 25-fold molar excess of protected amino acid and EDC in relation to R110. The reaction was complete within 10 hr as judged by RP-HPLC. Reaction progress and purity of all products was checked by RP-HPLC, ^{1}H-NMR, and FAB mass spectroscopy. The HPLC analysis was performed on an two-pump LKB Bromma system (Pharmacia, Freiburg, Germany) with UV detector (254 nm) and a Shimadzu fluorescence detector RF 535 (Latek GmbH, Germany) with excitation monochromator at 480 nm and emission monochromator at 525 nm. The HPLC column (4 × 250 mm) was filled with Nucleosil 100 C_{18} (M.Grom, Herrenberg, Germany). Arginine containing substrates were analyzed in acetonitril (MeCN)/0.25*M* phosphate buffer, pH 3.5 (gradient : 0–3 min 15% MeCN, 3–18 min to 60% MeCN). A MeCN/0.2 *M* triethylammonium acetate, pH 7.0 (gradient : 0–3 min 50% MeCN, 3–18 min to 80% MeCN) buffer system was used for all other substrates.

3. Synthesis of *N,N'*-Bis-L-arginyl-rhodamine 110 tetrahydrobromide, COMP No. 2 [$(Arg)_2$-R110 ⋆ 4 HBr]

Chilled 33% HBr in acetic acid (30 ml) is dropped into a stirred solution of COMP No. 1 (1.0 g; 1 mmol) in methanol (10 ml) at −15°C. The resulting brown suspension is diluted after 2 hr at room temperature with acetone (300 ml) and centrifuged at 1000*g* for 5 min. The pellet is redissolved in methanol (30 ml) and reprecipitated with acetone (300 ml). Following centrifugation the pellet is redissolved three times in methanol (30 ml), reprecipitated with diethyl ether (400 ml), recentrifuged, and finally dried *in vacuo* to yield 0.93 g of a yellow powder (90%, MW, 966.4) which appears homogeneous upon HPLC analysis.

4. Synthesis of *N,N'*-Bis-(N_α-benzyloxycarbonyl-L-arginyl)-rhodamine 110 tetrahydrochloride, COMP No. 3 [$(Z\text{-}Arg\text{-}Arg)_2$-R110 ⋆ 4 HCl;]

N_α-Benzyloxycarbonyl-L-arginine hydrochloride (2 g, 5.8 mmol) is dissolved in an anhydrous 1 : 1 mixture of dimethylformamide and pyridine (50 ml) at 0°C. Five minutes after addition of EDC (1.0 g, 5.2 mmol) a solution of COMP No. 2 (500 mg; 0.5 mmol) in the same solvent (5 ml) is added. After 15 hr the solvent is reduced *in vacuo* to about 20 ml and the product is precipitated by addition of ethyl acetate (200 ml). The substrate is further purified by precipitation with 1 *N* HCl (150 ml) from dimethylformamide (10 ml; two times) and diethyl ether (100 ml) from methanol (10 ml). The product is collected by centrifugation and dried *in vacuo* to give 0.52 g of a light orange powder (80%) with a purity of over 98% (MW, 1368.22) as controlled by HPLC.

5. Synthesis of *N,N'*-Bis-(benzyloxycarbonyl-L-alanyl)-rhodamine 110, COMP No. 4 [(Z-Ala)$_2$-R110]

Benzyloxycarbonyl-L-alanine (1.5 g, 6.7 mmol) is dissolved at 0°C in an anhydrous 1 : 1 mixture of dimethylformamide and pyridine (25 ml). Five minutes after the addition of EDC (1.2 g, 6.3 mmol) a solution of R110 hydrochloride (0.25 g, 0.68 mmol) in the same solvent mixture (2 ml) is added. After 10 hr at room temperature the solvent is removed *in vacuo* and the resulting orange oil is redissolved in a mixture of ethyl acetate (100 ml) and water (50 ml). The organic phase is washed (30-ml portions each) two times with 10% sodium carbonate solution, one time with water, three times with 1 *N* HCl, and again three times with water. The organic phase is dried over anhydrous sodium sulfate. After evaporation of the solvent *in vacuo* the crude orange product is dissolved in ethyl acetate (5 ml) and precipitated with hexane (80 ml). After 1 hr at 4°C the suspension is centrifuged at 2000*g* for 10 min and the product is dried *in vacuo* to yield 0.47 g of a pale pink powder (93%; MW, 740.78).

6. Synthesis of *N,N'*-Bis-L-alanyl-rhodamine 110 dihydrobromide, COMP No. 5 [(Ala)$_2$-R110 * 2HBr]

To a solution of COMP No. 4 (0.45 g, 6.1 mmol) in methanol (10 ml) is added 33% HBr in acetic acid (10 ml) with stirring at −15°C. As controlled by HPLC the reaction is complete after 2 hr at room temperature and the suspension is diluted with ethyl acetate (200 ml). The precipitate is collected by centrifugation at 1000*g* for 5 min. The pellet is redissolved in methanol (5 ml) and precipitated with diethyl ether (100 ml). After centrifugation (1000*g*, 5 min) the precipitate is washed three times with diethyl ether (50 ml) and dried in an evacuated desiccator over sodium hydroxide yielding 0.32 g analytical pure orange product (84%; MW, 634.34).

7. Synthesis of *N,N'*-Bis-(benzyloxycarbonyl-L-alanyl-L-alanyl)-rhodamine 110, COMP No. 6 [(Z-Ala-Ala)$_2$-R110]

Benzyloxycarbonyl-L-alanine (1.5 g, 6.7 mmol) is dissolved in an ice bath in an anhydrous 1 : 1 mixture of dimethylformamide and pyridine (25 ml). Five minutes after the addition of EDC (1.2 g, 6.3 mmol) a solution of COMP No. 5 (0.25 g, 0.28 mmol) in the same solvent mixture (2 ml) is added. After 12 hr the solvent is removed *in vacuo* and the resulting orange oil is redissolved in a mixture of ethyl acetate (100 ml) and water (50 ml). The organic phase is washed (30-ml portions each) two times with 10% sodium carbonate solution, one time with water, three times with 1 *N* HCl, and again three times with water. The ester phase is dried over anhydrous sodium sulfate. After evaporation of the solvent *in vacuo* the red crude product is dissolved in ethyl acetate (5 ml)

and precipitated with hexane (80 ml). After 1 hr at 4°C the precipitate is collected by centrifugation and dried *in vacuo* to yield 0.17 *g* (70%; MW, 882.94) of final product.

B. Cell Preparation

1. Cell Culture Cells

Cell culture cells are washed twice in 50 ml saline, buffered to pH 7.35 with 10 m*M* Hepes (*N*-[2-hydroxyethyl]-piperazine-*N'*-[2-ethanesulfonic acid]) and resuspended in the same buffer (HBS) to a concentration between 1 and 10×10^6 cells/ml.

2. Organ Tissue Cells

A total of 50–100 mg organ tissue is covered by 0.1 ml HBS buffer to avoid drying and minced several times with an electrical tissue chopper (McIlwain, Gomshall, UK) equipped with five parallel razor blades at 0.7 mm distance. The sample support is turned each time by 90° to achieve maximum cutting. The chopped tissue is transferred from the Teflon chopping table with 3–5 ml HBS buffer into a 50-ml Falcon tube with conical buttom. The suspension is sucked back and forth with moderate speed using an Eppendorf-type pipette with a plastic tip previously cut to obtain an opening between 1.5 and 2 mm. Bubble formation is carefully avoided. The cell suspension is filled up to 50 ml with HBS, filtered through a steel sieve with 60-μm wire distance, followed by two centrifugal washes for 10 min at 200*g* and 0–4°C. The cells are finally resuspended in HBS buffer to a concentration between 1 and 10×10^6 cells/ml and stored at 0–4°C until staining.

3. Blood Cells

A total of 2–3 ml of heparinized whole blood (10–30 IU/ml) is carefully layered from the syringe on top of 5 ml Ficoll-Hypaque solution (Sigma Chemicals, St. Louis, MO) in a 10-ml glass or polycarbonate test tube, followed by 30-45 min cell sedimentation at room temperature. Erythrocytes aggregate at the blood/Ficoll-Hypaque interface and sediment rapidly leaving thrombo-, lympho-, mono-, and granulocytes behind. The upper 1–1.5 ml blood plasma is removed, washed in 15 ml HBS buffer by centrifugation immediately or prior to staining, and resuspended in HBS buffer to a concentration between 1 and 10×10^6 cell/ml. Besides relative ease, this procedure most importantly avoids any contact of nonerythrocytic cells with the separation medium which otherwise, e.g., significantly stimulates mono- or granulocytes and thereby potentially influences subsequent enzyme activity measurements.

C. Protease Assays

A total of 250 μl cells (1–10 $\times$ 10^6/ml) is incubated with 5 μl dye cocktail containing between 0.02 and 0.2 m*M* (Z-Arg-Arg)$_2$R110, (Z-Phe-Arg)$_2$R110 or (Z-Ala-Ala)$_2$R110 together with 3 m*M* propidium iodide (PI, Sigma) in dimethylformamide (DMF) for 15 to 45 min at 20 or 37°C.

The incubation temperature and substrate concentration depends of the cell type. Blood cells require 0.2 m*M* substrate concentration, while, e.g., 0.02 m*M* is sufficient for guinea pig kidney cells. The plateau phase of substrate cleavage is usually reached after 15 to 30 min at 37°C depending again on the cell type.

The specificity of the intracellular R110 substrate cleavage is controlled by preincubation of the cells for 15 min at 20 or 37°C with 5 μl of a 0.5 m*M* solution of (Z-Phe-Ala)-diazomethylketone (DMK) cysteine proteinase inhibitor (Bachem, Heidelberg, Germany) in dimethyl sulfoxide (DMSO) while serine proteinases are inhibited by preincubation with 5 μl of a 50 m*M* diisopropylfluorophosphate (DFP, Aldrich) solution in DMSO. Extreme care has to be taken when working with DFP because of volatility and high neurotoxicity. (Proceed according to manufacturer's recommendations: hood, gloves, inactivation in 4–6 *N* NaOH, antidote: atropine).

D. Flow Cytometry

The assays can be directly measured in the flow cytometer. Blood cells require 488 nm argon ion laser light (5–50 mW) for sufficient fluorescence excitation while high-pressure mercury arc lamp (HBO-100) light using a 460- to 500-nm band pass excitation filter is sufficient in the case of larger cells especially for measurements with the serine proteinase substrate (Z-Ala-Ala)$_2$R110. R110 fluorescence is collected in the F1 (FITC) channel between 512–542 nm while the signals of the PI-stained DNA of dead cells are registered in the F3 (PI,PERCP,CY5) channel between 600 and 700 nm. Simultaneous forward (FSC) and sideward (SSC) scatter signals or alternatively electric volume signals (ECV) from hydrodynamically focused measuring orifices should be additionally collected. Three or four decade logarithmically amplified fluorescence, FSC or ECV signals are stored together with linearily amplified SSC signals in list-mode data files. A long-term standardization of the flow cytometer by monosized yellow/green (YG) fluorescent particles (Polysciences, Warrington, PA) permits later automated classification of the data based results (Valet *et al.*, 1993).

III. Results

The peptide residues of the R110 substrates (Fig. 1) are sequentially cleaved by proteases. Single peptide cleavage on either side of the R110 substrate results

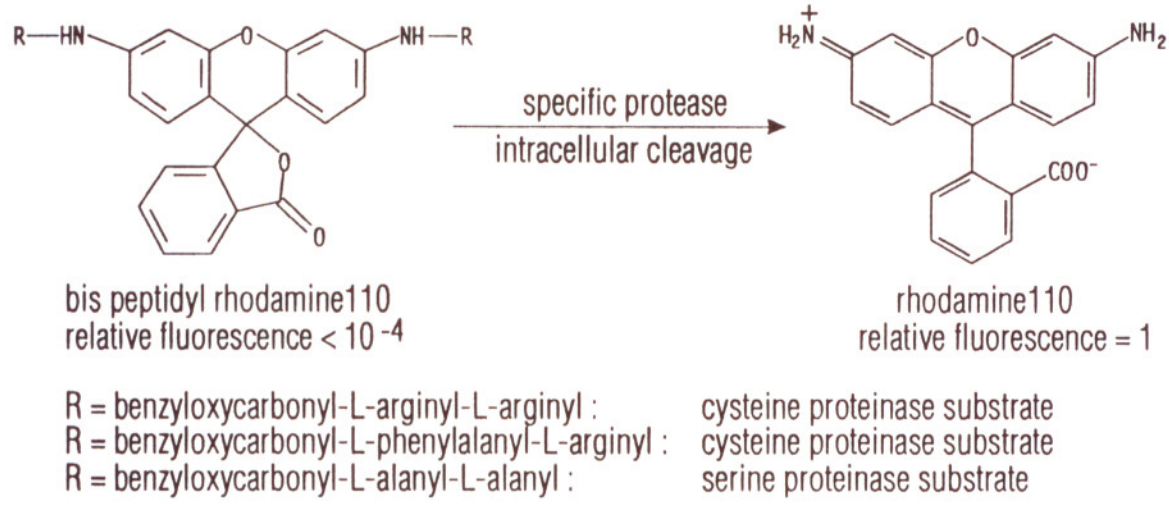

Fig. 1 Schematic representation of R110 protease substrate cleavage.

in 1/10 of the fluorescence as compared to cleavage of both peptide residues. While complete substrate cleavage in cuvette assays, where many substrate molecules have to be cleaved by comparatively few enzyme molecules, sometimes requires several hours incubation at 37°C, HPLC analysis of blood cell lysates following 15 to 45 min incubation at 37°C with R110 protease substrates shows that intracellular proteinases *in situ* completely cleave R110 substrate molecules resulting in full R110 fluorescence.

The fluorescence excitation and emission spectra of R110 virtually superimpose the fluorescein spectra (Fig. 2, left) i.e., 488-nm fluorescence excitation

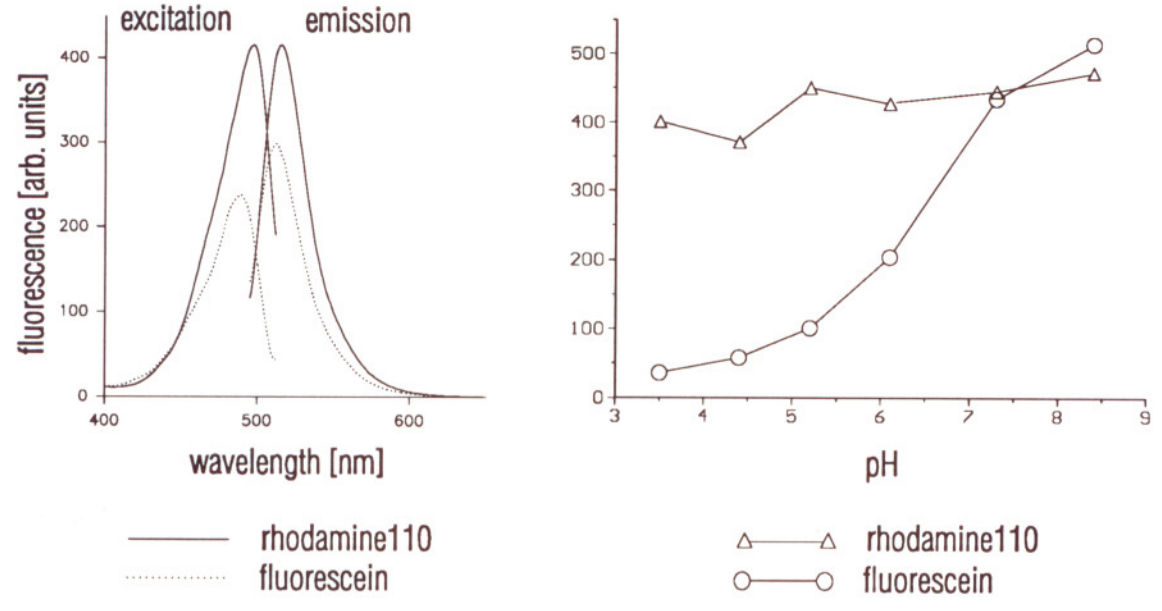

Fig. 2 Fluorescence excitation and emission spectra of R110 and fluorescein (left) and of their pH-dependent fluorescence (right).

is optimal. As a further advantage R110 fluorescence unlike fluorescein fluorescence is practically independent of pH values in the range pH 3.5–9 (Fig. 2, right).

Cysteine proteinase activity in human blood cells is practically only observed in monocytes (Fig. 3A). This activity increases during inflammatory processes as they occur, e.g., 24 hr following liver tranplantation (Fig. 3B).

Serine proteinase activity, in contrast, is observed in lympho-, mono-, and granulocytes (Fig. 3C). Lymphocytes frequently consist of two clusters of high- and low-activity cells (Fig. 3D,) in diseased as well as in apparently healthy persons (Fig. 4C). Monocyte serine proteinase activity is also increased following transplantation (Fig. 3D). The activity of high-activity granulocytes remains practically constant (Figs. 3C and 3D) but a new population of low-activity granulocytes containing approximately 1/3 of the serine proteinase activity of high-proteinase granulocytes is observed 24 hr after liver transplantation (Fig. 3D) with the low-activity cells carrying an increased amount of CD63 antigen on the cells surface (not shown).

The R110 cysteine and serine proteinase are specifically cleaved since preincubation of the cells with DMK or DFP protease inhibitors practically com-

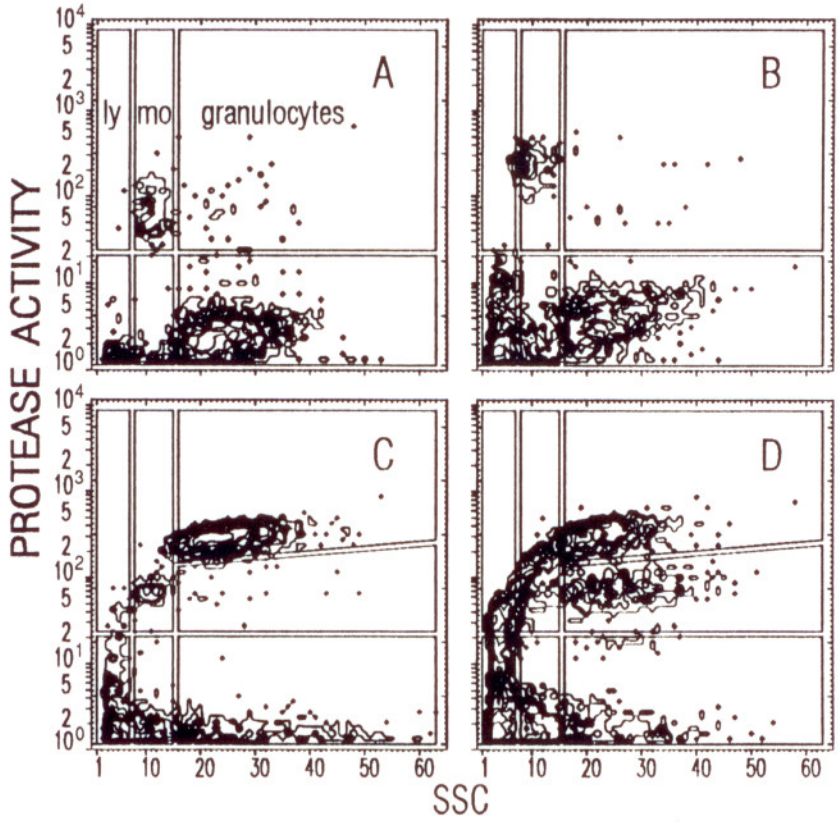

Fig. 3 SSC against R110 fluorescence of peripheral blood lympho-, mono-, and granulocytes in HBS buffer following 30 min 37°C incubation with 4 μM (Z-Arg-Arg)$_2$R110 (A,B) or (Z-Ala-Ala)$_2$R110 (C,D) substrate before (A,C) and 24 hr after liver transplantation (B,D) as determined in a FACScan (Becton–Dickinson) flow cytometer.

pletely inhibits substrate cleavage (Figs. 4B and 4D) in comparison to the uninhibited assays (Figs. 4A and 4C).

IV. Discussion

R110 protease substrates are advantageous for cytometric single-cell protease activity measurements because of specific cleavage, low toxicity, absent fluorescence background, autoaccumulation due to positive electrical charge at physiological pH, and pH-independent fluorescence.

R110 protease substrates will permit, e.g., detailed investigations of protease activity regulation during cell cycle and differentiation, in lymphocyte subpopulations (Fig. 3d), during monocyte/macrophage activation (Rothe *et al.*, 1992) and apoptosis (Elsherif *et al.*, 1994), in premalignant and malignant tumors or in aquatic microplankton cells (Sieracki *et al.*, 1993). Monocyte activation can be followed from an increase of the ratio of $(Z\text{-Phe-Arg})_2R110$ over $(Z\text{-Arg-Arg})_2R110$ cleavage due to preferential cleavage of $(Z\text{-Phe-Arg})_2R110$ by cathepsin L and $(Z\text{-Arg-Arg})_2R110$ by cathepsin B and H (Rothe *et al.*, 1992).

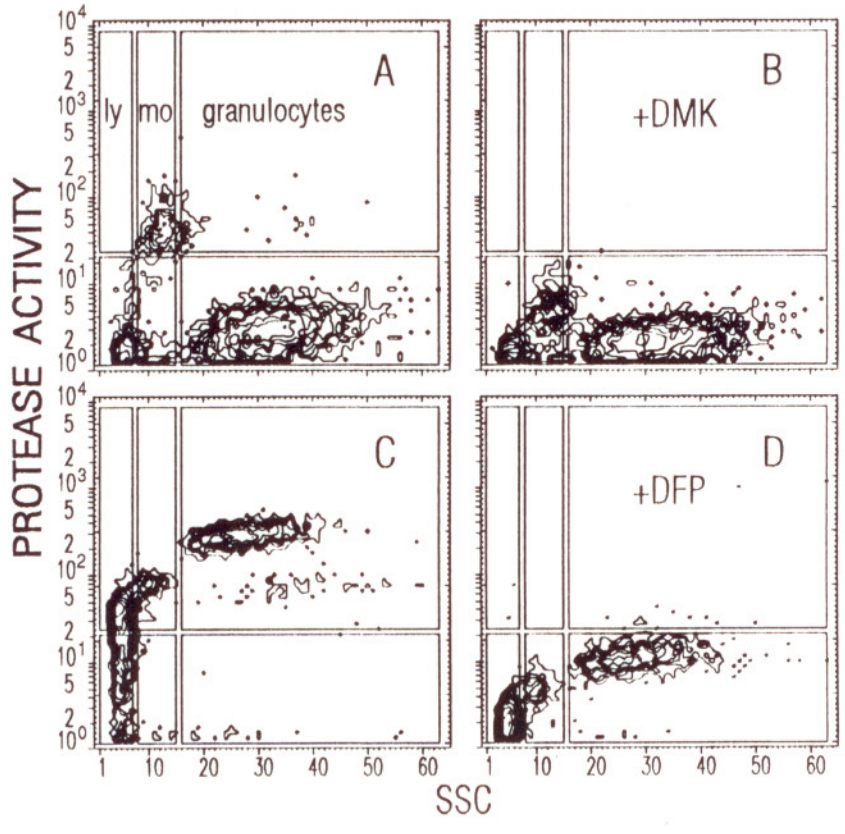

Fig. 4 SSC against R110 fluorescence of peripheral blood lympho-, mono-, and granulocytes preincubated for 15 min at 37°C with HBS buffer (A,C), 10 μM DMK cysteine proteinase inhibitor (B), or 1 mM DFP serine proteinase (D) inhibitor, followed by 30 min 37°C incubation with 4 μM $(Z\text{-Arg-Arg})_2R110$ (A,B) or $(Z\text{-Ala-Ala})_2R110$ (C,D) protease substrate.

The appearance of a distinct low protease granulocyte population raises the question whether circulating granulocytes upon stimulation secrete in a discrete step approximately 2/3 of their serine proteinase activity, whether a newly produced granulocyte population appears in circulation due to a general stimulus, or whether these granulocytes represent transfused blood granulocytes. Although the mechanism of low-activity granulocyte formation is presently unclear, the increased expression of CD63 antigen on the cell surface points toward involvement of a secretory mechanism.

Besides cysteine and serine proteinase substrates, additional R110 substrates for the determination of leucine and phenylalanine aminopeptidases (metalloproteinase) and cathepsin D (aspartic proteinase) have been recently synthesized (Klingel *et al.*, 1994). The intracellular cathepsin D measurement is performed as the coupled enzyme reaction with cathepsin D endopeptidase cuts followed by aminopeptidase digestion of remaining residues until liberation of free R110. Coupled protease reaction, at least in principle, will allow the specific determination of many other intracellular endopeptidases.

Altogether, the cytometric use of R110 proteinase substrates will open up a new area of studies on the mechanism and regulation of cellular protein and peptide catabolism.

Note: The authors will be glad to provide substrate samples for research purposes. Very recently $(Z\text{-Ala-Ala})_2R110$ (R-6504) has become commercially available through Molecular Probes (Eugene, OR).

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