Biol. Chem. Hoppe-Seyler
Vol. 373, pp. 547–554, July 1992

Flow Cytometric Analysis of Protease Activities in Vital Cells*

GREGOR ROTHE[a], SVEN KLINGEL[a], IRMGARD ASSFALG-MACHLEIDT[b], WERNER MACHLEIDT[b], CHRISTOPH ZIRKELBACH[a], RICHARD B. BANATI[c], WALTER F. MANGEL[d] AND GÜNTER VALET[a]

[a] Arbeitsgruppe Zellbiochemie, Max-Planck-Institut für Biochemie, W-8033 Martinsried, FRG
[b] Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Goethestr. 33, W-8000 München 2, FRG
[c] Abteilung Neuromorphologie, Max-Planck-Institut für Psychiatrie, W-8033 Martinsried, FRG
[d] Biology Department, Brookhaven National Laboratory, Upton, NY 11973, USA

Summary

The analysis of lysosomal proteases in cell lysates is complicated by pH-dependent and oxidative changes of their activity and complex formation with cytosolic inhibitors. Therefore, new flow cytometric methods were developed for the intracellular measurement of protease activities in viable cells. Intracellular cleavage of substrates such as Z-Arg-Arg-4-trifluoromethylcoumarinyl-7-amide to green fluorescent 7-amino-4-trifluoromethylcoumarin (AFC) in viable neutrophils and monocytes was only detected following phagocytosis of *Escherichia coli*. A measurement of the cysteine or serine proteinase activities in resting human leukocytes was, however, not possible with AFC derivatives as the overlapping blue fluorescence of the substrates reduces sensitivity. Nonfluorescent bis-substituted peptide derivatives of rhodamine 110 (R110), which are intracellularly cleaved to green fluorescent mono-substituted R110 and free R110 proved to be more sensitive substrates. The activity of the lysosomal cysteine proteinases of human monocytes or rat macrophages, i.e. cathepsin B and L, was specifically measured with $(Z\text{-Arg-Arg})_2$-R110, $(Z\text{-Phe-Arg})_2$-R110, or $(Z\text{-Ala-Arg-Arg})_2$-R110. Fluorescence generation was completely inhibited by Z-Phe-Ala-diazomethane or E-64. The serine proteinases of human neutrophils were analyzed with Elastase-substrates such as $(Z\text{-Ala-Ala})_2$-R110 or $(Z\text{-Ala-Ala-Ala})_2$-R110. Specificity was shown by inhibition with diisopropylfluorophosphate.

Introduction

High plasma levels of the lysosomal proteases elastase or cathepsin B are detected during acute inflammatory disorders such as sepsis or posttraumatic shock [1,2]. These proteases together with the oxidants, released by neutrophils, are mediators of the inflammatory tissue destruction [3]. The cellular expression of lysosomal proteases, the mechanism of their intracellular processing, and the mechanisms of their extracellular release are, therefore, of major interest for the development of new thera-

---

*Presented in part at the XIV meeting of the Society for Analytical Cytology, Asheville, NC, USA, March 1990 (Cytometry Suppl. 4: 77, 1990)

Enzymes: Cathepsin B (EC 3.4.22.1); Elastase (EC 3.4.21.37)

Abbreviations: AFC, 7-amino-4-trifluoromethylcoumarin; DFP, diisopropylfluorophosphate; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; HBS, HEPES buffered saline (5 mM HEPES, 0.15 M NaCl, pH 7.35); R110, rhodamine 110; Z, benzyloxycarbonyl; -AFC, -4-trifluoromethylcoumarinyl-7-amide; $-CHN_2$, -diazomethane.

peutic approaches. Methods for the analysis of lysosomal proteases in cell lysates depend on the preparation of homogenous cell populations. Furthermore, during cell lysis the activity of proteases may be altered by the interaction with cytosolic inhibitors, pH-changes, or oxidative inactivation [4]. Earlier methods for the intracellular analysis of protease activities were based on the intracellular cleavage of *N*-acyl derivatives of 4-methoxy-2-naphthylamine. The highly diffusible product 4-methoxy-2-naphthylamine was trapped inside the cell by coupling with 5-nitrosalicylaldehyde yielding a yellow fluorescent, crystalline product [5-8]. These methods are restricted to the analysis of proteases with an acidic pH optimum, as incubation at acidic pH is necessary to promote intracellular coupling of the 5-nitrosalicylaldehyde to the product. Furthermore, a high background is induced by the staining of proteins with the coupling reagent. A new method for the intracellular analysis of protease activities in viable cells at physiological pH was developed using either the intracellular conversion of blue fluorescent peptide-AFC substrates [9] to 365-nm excitable green fluorescent AFC or the intracellular cleavage of nonfluorescent R110 derivatives to 488-nm excitable green fluorescent R110 [10,11].

Material and Methods

Cells

Heparinized human peripheral blood was depleted of erythrocytes by 1g sedimentation upon Histopaque-1077 (Sigma, Deisenhofen, FRG) for 30 min at room temperature. The upper half of the supernatant plasma containing 0.4-1.2 x 10^7 leukocytes/ml and thrombocytes was collected and stored on ice.

Peritoneal macrophages were obtained from male Wistar rats either as resident cells or as elicited cells 4 d after intraperitoneal injection of 3 ml of thioglycolate broth solution (Serva, Heidelberg, FRG) by peritoneal lavage with HBS supplemented with 2 mM EDTA. The cells were sedimented at 200g for 5 min, resuspended in HBS with EDTA at 1 x 10^7 cells/ml, and stored on ice.

Fluorescence spectrometry

Fluorescence emission spectra of AFC, Z-Arg-Arg-AFC, R110, Z-Arg-R110, and (Z-Arg-Arg)$_2$-R110 (all stock solutions 20 mM in DMF) in 0.1-M Tris HCl (pH 7.2) were recorded in quartz cuvettes of 10 mm path length on a Perkin-Elmer LS-5 luminescence spectrometer (Bodenseewerk Perkin-Elmer, Überlingen) connected to a VAX 8550 computer (Digital Equipment, Maynard, MA, USA) at 365 nm or 488 nm excitation and with a nominal bandwidth of 5 nm.

Synthesis of bis-substituted rhodamine 110 peptide derivatives

The bis-substituted R110 peptide derivatives (Z-Phe-Arg)$_2$-R110, (Z-Arg-Arg)$_2$-R110, (Z-Ala-Arg-Arg)$_2$-R110, (Z-Ala-Ala)$_2$-R110, (Z-Ala-Ala-Ala)$_2$-R110, and (Z-Ala-Ala-Val)$_2$-R110 were synthesized in analogy to Leytus et al. [10,11] and dissolved 4-mM in DMF. Structure and purity were confirmed by thin layer chromatography, high-resolution mass spectroscopy and NMR spectroscopy.

Cell staining

The human leukocyte or rat peritoneal macrophage cell suspensions (20 µl) were preincubated in 1 ml HBS with 1 µl of DMSO or 1 µl of inhibitor stock solutions (DFP (Aldrich, Steinheim, FRG): 1 M in DMSO; E-64 (Sigma): 100 mM in DMSO; 1,10-phenanthroline (Sigma): 1 M in DMSO; Z-Phe-Ala-CHN$_2$ (Bachem, Heidelberg, FRG): 100 mM in DMSO; Z-Phe-Phe-CHN$_2$ (Bachem): 10 mM in DMSO; pepstatin A (Sigma): 10 mM in DMSO) for the measurement of the intracellular proteolytic activity. The cells were then incubated for 20 min at 37°C with 1 µl of the 10-mM stock solution of Z-Arg-Arg-AFC (Serva), or 1 µl of the 4-mM stock solutions of the R110 peptide derivatives. The incubations were stopped on ice and the samples were analyzed following counterstaining of dead cells with propidium iodide (Sigma).

Phagocytosis

Escherichia coli K12 (Sigma) (*E. coli*) were grown as a stationary culture in RPMI-1640 (Gibco BRL, Eggenstein, FRG). The bacteria were washed and resuspended in HBS at 7 x 10^9 bacteria/ml. For the measurement of proteolytic processes during phagocytosis the leukocyte suspension in autologous plasma (20 µl) was incubated with the *E. coli* suspension (5 µl) at 37°C. After 10 min the cells were diluted with 1 ml of HBS, incubated with the proteinase inhibitors or DMSO, and stained with the fluorogenic substrates as described above.

Flow cytometry

The cellular AFC green fluorescence (420-530 nm) and the red fluorescence of propidium iodide-stained dead cells (550-700 nm) excited (300-400 nm) by a HBO-100 high-pressure mercury arc lamp were analyzed together with electrical determination of the cell volume in a FLUVO-II flow cytometer (Heka Elektronik, Lambrecht/Pfalz, FRG). The cellular R110 green fluorescence (515-545 nm) and the red fluorescence of propidium iodide-stained dead cells (> 650 nm), the cellular forward and side scatter were measured with excitation by an 488-nm argon laser on a FACScan flow cytometer (Becton Dickinson, San José, CA, USA). The list-mode data of 2,000 to 10,000 leukocytes per sample were analyzed with the DIAGNOS1 program system [12].

Results and Discussion

Measurement of the proteolytic digestion of E. coli with Z-Arg-Arg-AFC

Incubation of human peripheral blood leukocytes with Z-Arg-Arg-AFC did result in a low AFC green fluorescence of lymphocytes, neutrophils, and monocytes (Fig. 1a). This was unexpected as monocytes contain signficiantly higher amounts of cysteine proteinases [13,14] compared to neutrophils. Incubation with *E. coli* for 10 min resulted in an 4.36-fold (± 0.45, SEM, n = 3) increase of the fluorescence of neutrophils (Fig. 1b) (Table 1). This was not due to the ingestion of bacterial proteases as no detectable cleavage of Z-Arg-Arg-AFC occurred in *E. coli*, alone. Soluble stimulation by the protein kinase C agonist phorbol 12-myristate 13-acetate (150 nM), the lectin concanavalin A (100 µg/ml), or the bacterial peptide *N*-formyl-Met-Leu-Phe (10^{-6} M) induced similar activation of neutrophils as observed by their oxidative burst response [15] but did not lead to detectable AFC green fluorescence (data not shown). This suggests that in human leukocytes, a high increase of proteolytic acti-

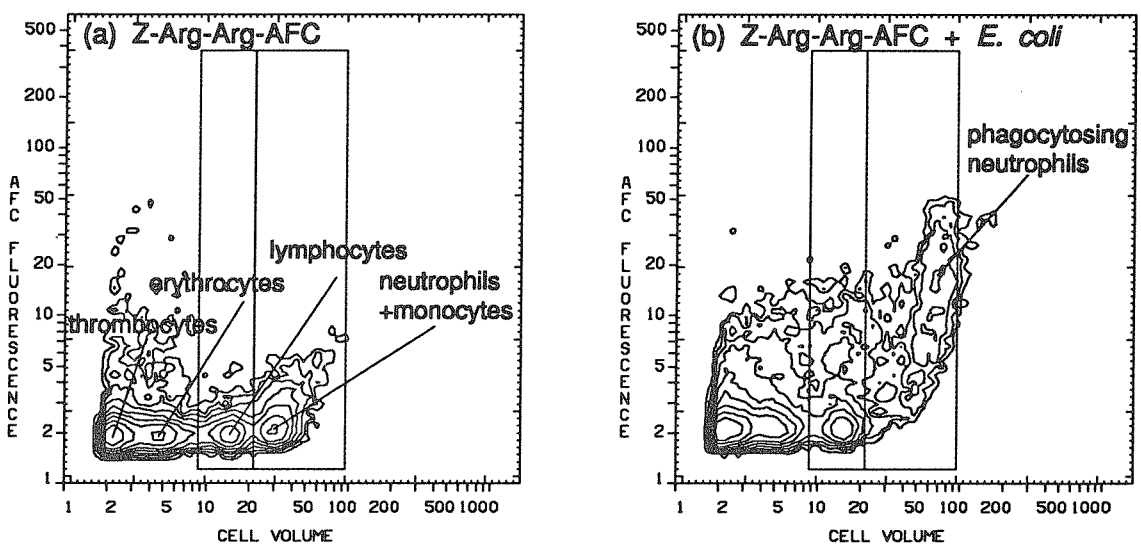

Fig. 1. Intracellular cleavage of Z-Arg-Arg-AFC. Human peripheral blood leukocytes were incubated with Z-Arg-Arg-AFC without stimulation (a) or following preincubation with *E. coli* for 10 min (b).

vity is correlated to the discharge of lysosomal proteases into the phagosome [16,17]. The intracellular generation of AFC was not inhibited by preincubation of the cells with either of the inhibitors E-64, DFP, 1,10-phenanthroline, or pepstatin A (Table 1) [4], alone, suggesting that the activity of neither the serine proteinases, cysteine proteinases, metalloproteinases, or aspartic proteinases was limiting for the observed protease activity.

Table 1. Specificity of the intracellular cleavage of Z-Arg-Arg-AFC by human neutrophils

	Cellular fluorescence during incubation with Z-Arg-Arg-AFC (arbitrary fluorescence units)	
Inhibitor	Nonstimulated cells	Phagocytosing cells
None	.0415 ± .0054	.1847 ± .0154
E-64	.0414	.1905
DFP	.0356	.2131
Pepstatin A	.0439	.1089
1,10-phenanthroline	.0402	.1404

Cells were preincubated without or with *E. coli* for 15 min, followed by an incubation with the inhibitors for 5 min, and 10 μM Z-Arg-Arg-AFC for 15 min. (n = 4 for controls, n = 2 for inhibitor experiments).

The low sensitivity of Z-Arg-Arg-AFC and other *N*-acyl-AFC substrates in an assay of lysosomal cysteine or serine protease activity in non-stimulated human leukocytes may be due to the significant background fluorescence caused by the spectral overlap of the blue fluorescence of the *N*-acyl-AFC substrates (Fig. 2a). This is especially a problem in intracellular assays, where a discrimination between kinetic intracellular substrate accumulation from enzymatic product formation is required.

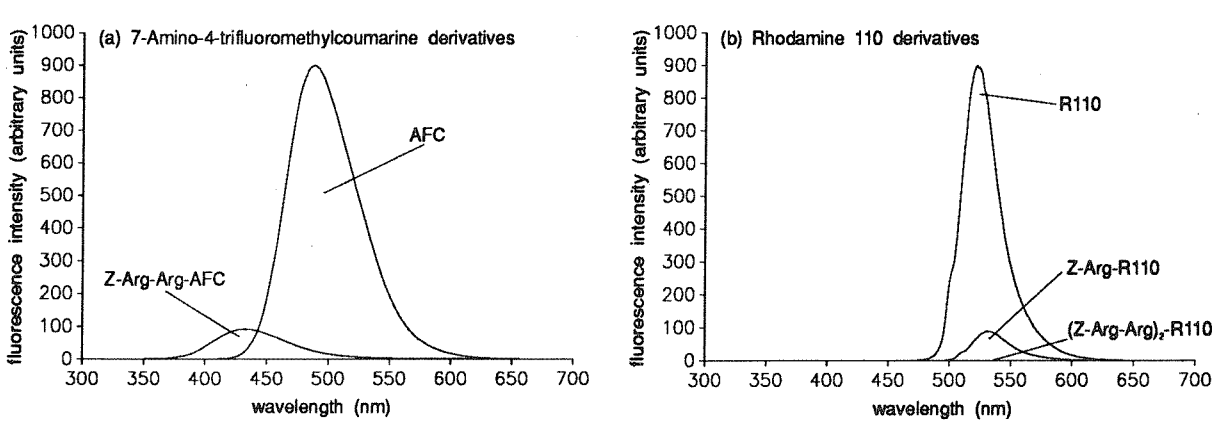

Fig. 2. Fluorescence emission spectra of AFC (a) and R110 (b) protease substrates. Spectra were recorded with 365 nm excitation for 1 μM Z-Arg-Arg-AFC and 1 μM AFC (a) and 488 nm excitation for 200 nM each of $(Z\text{-Arg-Arg})_2$-R110, Z-Arg-R110, and R110 (b). This excitation corresponds to the flow cytometric experiments.

Measurement of cathepsin B and L activity with (Z-Phe-Arg)$_2$-R110 or (Z-Arg-Arg)$_2$-R110

N,N'-bis-substituted derivatives of R110 have a more than 10^{-4} lower fluorescence than free R110 [10,11] (Fig. 2b). The 488-nm argon laser excitable green fluorescence generated during the sequential cleavage of bis-substituted R110 to mono-substituted R110 and R110 (Fig. 3) can be, therefore, measured without spectral overlap of the virtually non fluorescent substrates.

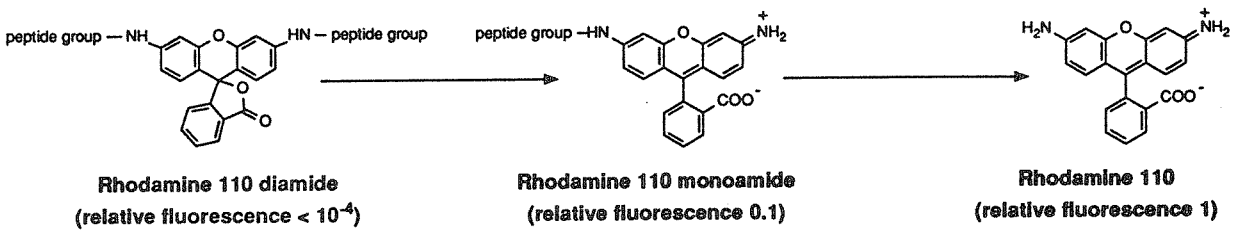

Fig. 3. Sequential generation of fluorescence during cleavage of bis-substituted R110 peptide derivatives.

Incubation of human peripheral blood leukocytes with (Z-Arg-Arg)$_2$-R110 resulted in a 23.6-fold (± 2.6, n=9) higher fluorescence in monocytes than in neutrophils (Fig. 4a), corresponding to the high cysteine proteinase activity in cell lysates of monocytes [13,14]. A specificity of the fluorescence generation for cysteine proteinases was shown by the inhibition of 95.6 % (± 0.5, n=4) of the fluorescence by preincubation of cells with Z-Phe-Ala-CHN$_2$ (Fig. 4b) [19]. Data generated with purified enzymes show that (Z-Arg-Arg)$_2$-R110, in contrast to the cathepsin B substrate Z-Arg-Arg-4-methyl-coumaryl-7-amide [18], has a 360-fold higher sensitivity for cleavage by cathepsin L as compared to cathepsin B [13]. A similar specific intracellular cleavage by cysteine proteinases inside human monocytes was found for (Z-Phe-Arg)$_2$-R110 and (Z-Ala-Arg-Arg)$_2$-R110 (data not shown).

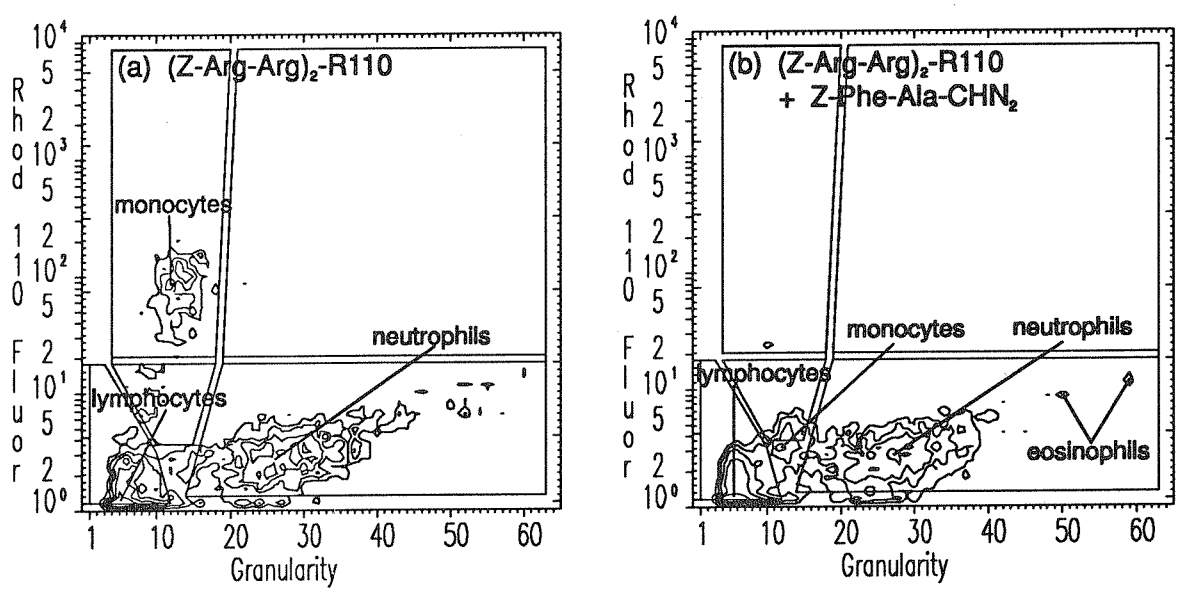

Fig. 4. Intracellular cleavage of the cathepsin B and L substrate (Z-Arg-Arg)$_2$-R110. Human peripheral blood leukocytes were incubated with (Z-Arg-Arg)$_2$-R110 without (a) or with a preincubation with the cysteine proteinase inhibitor Z-Phe-Ala-CHN$_2$ (b).

Resident rat peritoneal macrophages showed a high accumulation of fluorescence during incubation with (Z-Arg-Arg)$_2$-R110 in contrast to the low fluorescence of other intraperitoneal cells, e.g. lymphocytes. (Z-Arg-Arg)$_2$-R110 was cleaved in the resident macrophages at a significant higher rate of 2.05-fold (± 0.24, n=5) over (Z-Phe-Arg)$_2$-R110 (Table 2). Thioglycolate-elicited peritoneal macrophages as macrophages in an activated state, showed a reverse ratio of the intracellular cleavage of the substrates with a decreased intracellular cleavage of (Z-Arg-Arg)$_2$-R110 (53.7 ± 5.0 % of resident cells, n=5) and an increased cleavage of (Z-Phe-Arg)$_2$-R110 (215.8 ± 2.5 % of resident cells, n=5). This suggests that the differential intracellular cleavage of both substrates represents differences in intracellular enzymatic activities rather than a different membrane permeability of the substrates. This may correlate to the differential expression of cathepsin L and cathepsin B during the maturation of mononuclear phagocytes [13,20-24]. Further attempts to differentiate between cathepsin B activity and cathepsin L activity by the selective inhibition of cathepsin L by low amounts of Z-Phe-Phe-CHN$_2$ [25] were unsuccessful since the lower inhibition by Z-Phe-Phe-CHN$_2$ was independent of the substrate or cell type (Table 2).

Table 2. Intracellular cleavage of cysteine proteinase substrates by rat peritoneal macrophages

		Cellular fluorescence (arbitrary fluorescence units)	
Inhibitor	Substrate	Resident macrophages	Elicited macrophages
None	(Z-Arg-Arg)$_2$-R110	.9861	.5341
Z-Phe-Ala-CHN$_2$	(Z-Arg-Arg)$_2$-R110	.2982	.1251
Z-Phe-Phe-CHN$_2$	(Z-Arg-Arg)$_2$-R110	.4586	.2032
None	(Z-Phe-Arg)$_2$-R110	.5563	1.7190
Z-Phe-Ala-CHN$_2$	(Z-Phe-Arg)$_2$-R110	.1295	.1746
Z-Phe-Phe-CHN$_2$	(Z-Phe-Arg)$_2$-R110	.1484	.2034

Cells were preincubated with 10 µM of the inhibitors for 10 min or with 0.1 % DMSO (v/v) as a solvent control, followed by an incubation with the fluorogenic substrates (4 µM) for 20 min at 37°C. (data representative for 2 to 4 experiments).

Measurement of elastase activity with (Z-Ala-Ala)$_2$-R110 or (Z-Ala-Ala-Ala)$_2$-R110

An attempt to measure the lysosomal serine proteinase elastase, which is present in high amounts in the azurophilic granules of neutrophils [26] and in lower amounts in monocytes [27,28], was made by the synthesis of the substrates (Z-Ala-Ala)$_2$-R110, (Z-Ala-Ala-Ala)$_2$-R110, and (Z-Ala-Ala-Val)$_2$-R110 using typical peptide substitutions [29]. (Z-Ala-Ala)$_2$-R110 was cleaved intracellularly at a high rate in human neutrophils while monocytes developed only 25.0 % (± 0.8, n =5) of the fluorescence of neutrophils (Fig. 5a). A selective fluorescent staining of the cellular granules was documented by confocal laser scanning microscopy. Specificity was shown by an inhibition by 97.7 % (± 0.1, n=5) in the presence of DFP. Neutrophils incubated with the tripeptide elastase substrate (Z-Ala-Ala-Ala)$_2$-R110 developed only 4.4 % (± 0.3, n=4) of the fluorescence of (Z-Ala-Ala)$_2$-R110 incubated neutrophils

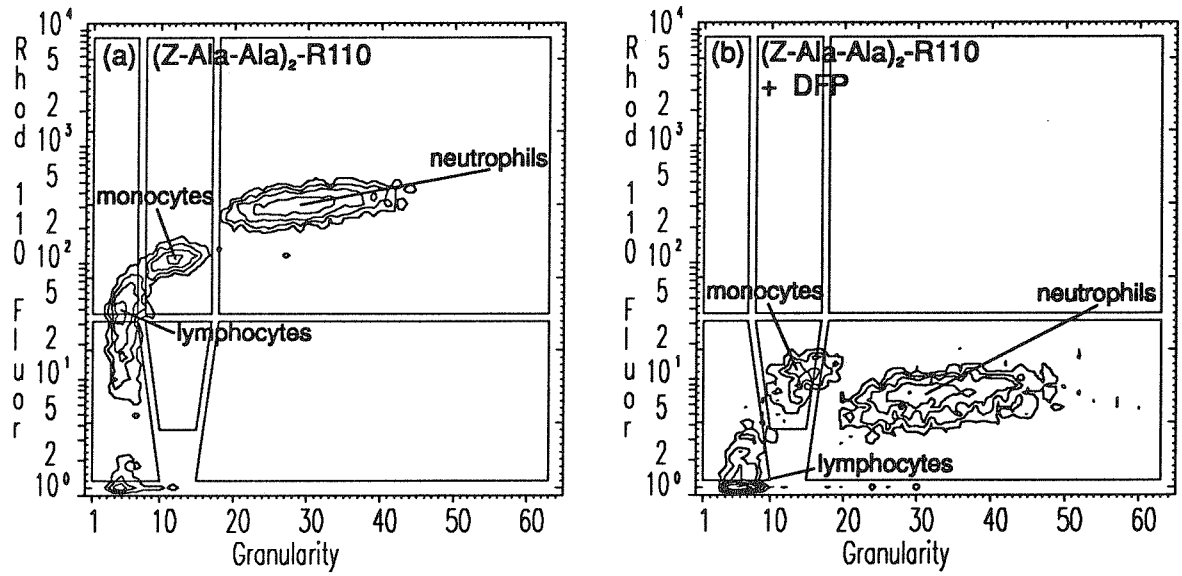

Fig. 5: Intracellular cleavage of the elastase substrate (Z-Ala-Ala)$_2$-R110. Human peripheral blood leukocytes were incubated with (Z-Ala-Ala)$_2$-R110 without (a) or with an preincubation with the serine proteinase inhibitor DFP (b).

and (Z-Ala-Ala-Val)$_2$-R110 did not lead to a significant increase of the cellular fluorescence, suggesting that the very lipophilic substrates do not reach the lysosomes due to trapping in the outer plasma membrane.

In conclusion, bis-substituted R110 dipeptide and tripeptide protease substrates are sensitive substrates for the specific intracellular measurement of lysosomal cysteine and serine proteinases. These methods should be useful for the study of the physiological regulation of the intracellular processing of proteases. Furthermore, they should allow the evaluation of synthetic, membrane-permeable proteinase inhibitors in new therapeutic approaches against inflammatory tissue destruction [3,29-32].

Acknowledgements

We thank Prof. W. Schäfer for high-resolution mass spectroscopy and Prof. J. Sonnenbichler and Dr. I. Zetl for NMR spectroscopy. This work was supported by the Sonderforschungsbereich 207, Universität München (project G-6 and G-1).

References

1. Jochum, M. & Fritz, H. (1989) in: Immune Consequences of Trauma, Shock, and Sepsis (Faist, E., Ninnemann, J. & Green, D. eds.) pp. 165-172, Springer-Verlag, Berlin.
2. Assfalg-Machleidt, I., Jochum, M., Nast-Kolb, D., Siebeck, M., Billing, A., Joka, T., Rothe, G., Valet, G., Zauner, R., Scheuber, H.P. & Machleidt, W. (1990) Biol. Chem. Hoppe-Seyler **371** (Suppl.), 211-222.
3. Weiss, S.J. (1989) N. Engl. J. Med. **320**, 365-376.
4. Kirschke, H. & Barrett, A.J. (1987) in: Lysosomes: Their Role in Protein Breakdown (Glaumann, H. & Ballard, F.J. eds.) pp. 193-238, Academic Press, London.

5. Dolbeare, F.A. & Smith R.E. (1977) Clin. Chem. **23**, 1485-1491.
6. Murphy, R.F. (1985) Proc. Natl. Acad. Sci. USA **82**, 8523-8526.
7. Krepela, E., Bártek, J., Skalková, D., Vicar, J., Rasnick, D., Taylor-Papadamitriou, J. & Hallowes, R.C. (1987) J. Cell Sci. **87**, 145-154.
8. Van Noorden, C.J.F., Vogels, I.M.C. & Smith, R.E. (1989) J. Histochem. Cytochem. **37**, 617-624.
9. Smith, R.E., Bissell, E.R., Mitchell, A.R. & Pearson, K.W. (1980) Thromb. Res. **17**, 393-402.
10. Leytus, S.P., Melhado, L.L. & Mangel, W.F. (1983) Biochem. J. **209**, 299-307.
11. Leytus, S.P., Patterson, W.L. & Mangel, W.F. (1983) Biochem. J. **215**, 253-260.
12. Valet, G., Warnecke, H.H. & Kahle, H. (1987) in: Clinical Cytometry and Histometry (Burger, G., Ploem, J.S. & Goerttler, K. eds.) pp. 58-65, Academic Press, London.
13. Assfalg-Machleidt, I., Rothe, G., Klingel, S., Banati, R.B., Mangel, W.F., Valet, G. & Machleidt, W. (1992) this volume.
14. Davies, M., Hughes, K.T., Andrew, P.W., Smith, G.P., and Peters, T.J. (1984) Cell. Mol. Biol. **30**, 337-343.
15. Rothe, G., Oser, A. & Valet, G. (1988) Naturwissen. **75**, 354-355.
16. Henson, P.M., Henson, J.E., Fittschen, C., Kimani, G., Bratton, D.L., & Riches, D.W.H. (1988) in: Inflammation: Basic Principles and Clinical Correlates (Gallin, J.I., Goldstein, I.M. & Snyderman, R. eds.) pp. 363-390, Raven Press, New York.
17. Jaconi, M.E.E., Lew, D.P., Carpentier, J.L., Magnusson, K.E., Sjögren, M. & Stendahl, O. (1990) J. Cell Biol. **110**, 1555-1564.
18. Barrett, A.J. & Kirschke, H. (1981) Methods Enzymol. **80**, 535-561.
19. Green, G.D.J. & Shaw, E. (1981) J. Biol. Chem. **256**, 1923-1928.
20. Reilly, J.J., Mason, R.W., Chen, P., Joseph, L.J., Sukhatme, V.P., Yee, R. & Chapman, H.A. (1989) Biochem. J. **257**, 493-498.
21. Burnett, D., Crocker, J., Afford, S.C., Bunce, C.M., Brown, G. & Stockley, R.A. (1986) Biochim. Biophys. Acta **887**, 283-290.
22. Murnane, M.J., Phipps, P.M. & Denekmap, D.S. (1988) Exp. Hematol. **16**, 33-37.
23. Ward, C.J., Crocker, J., Chan, S.J., Stockley, R.A. & Burnett, D. (1990) Biochem. Biophys. Res. Comm. **167**, 659-664.
24. Tsukahara, T., Ishiura, S., Kominami, E. & Sugati, H. (1990) Exp. Cell Res. **199**, 111-116.
25. Crawford, C., Mason, R.W., Wikstrom, P. & Shaw E. (1988) Biochem. J. **253**, 751-758.
26. Cramer, E.M., Beesley, J.E., Pulford, K.A.F., Breton-Gorius, J. & Mason, D.Y. (1989) Am. J. Pathol. **134**, 1275-1284.
27. Jensen, H.S. & Christensen, L.D. (1990) Clin. Exp. Rheumatol. **8**, 535-539.
28. Fujisawa, T., Kephart, G.M., Gray, B.H. & Gleich, G.J. (1990) Am. Rev. Respir. Dis. **141**, 689-697.
29. Powers, J.C. (1986) Adv. Inflamm. Res. **11**, 145-157.
30. Van Noorden, C.J.F., Smith, R.E. & Rasnick, D. (1987) J. Rheumatol. **15**, 1525-1535.
31. Baricos, W.H., O'Connor, S.E., Cortez, S.L., Wu, L.T. & Shah, S.V. (1988) Biochem. Biophys. Res. Commun. **155**, 1318-1323.
32. Shaw, E. (1990) Adv. Enzymol. & Related Areas Mol. Biol. **63**, 271-347.