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# INDEPENDENT REGULATION OF ENDOPEPTIDASE ACTIVITY AND RESPIRATORY BURST ACTIVITY OF NEUTROPHILS ANALYZED BY FLOW CYTOMETRY

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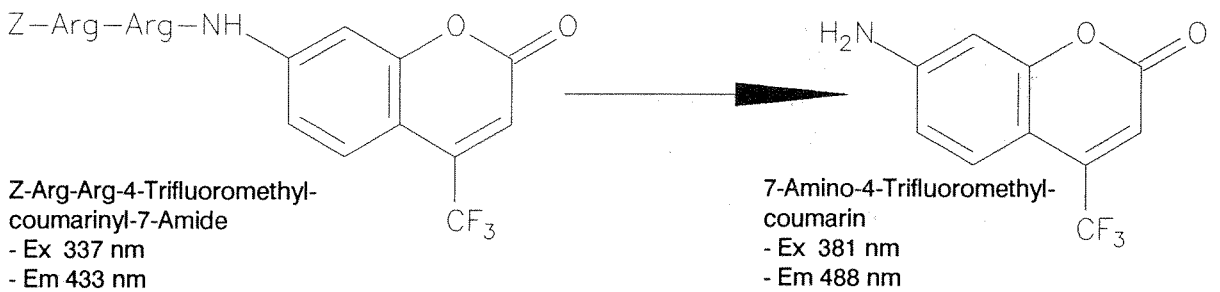
## INTRODUCTION

Degradation of ingested bacteria, or tissue destruction by neutrophils or monocytes are achieved by reactive oxygen metabolites produced during the respiratory burst and by proteinases from the azurophilic granules. While the heterogeneous activation of neutrophils for oxidative product formation by soluble stimuli like the chemotactic peptide *N*-formyl-Met-Leu-Phe (FMLP) or particulate stimuli like *E. coli* can be well characterized with fluorogenic substrates such as dihydrorhodamine 123,[1] hydroethidine,[2] or 2',7'-dichlorofluorescin diacetate[3] the intracellular detection of proteinase activity is so far complicated by an incubation of cells at pH 6.0 to promote coupling of the highly diffusible reaction product 4-methoxy-2-naphthylamine with 5-nitrosalicylaldehyde.[4-6] A new method was developed to investigate cellular endopeptidases in viable cells at physiological pH values.

## METHODS AND RESULTS

The fluorogenic substrate *N*α-Z-L-arginyl-L-arginine-4-trifluoromethyl coumarinyl-7-amide (Z-Arg-Arg-AFC) (Fig. 1) was used for the flow cytometric measurement of endopeptidase activity in neutrophils and monocytes. Leukocytes were suspended in 5 mM Hepes-buffered saline (0.15 M NaCl, pH 7.35) and incubated for 15 min at 37°C with 10 μM Z-Arg-Arg-AFC (Serva Feinbiochemica, Heidelberg, FRG, stock 5 mM in *N*,*N*-dimethylformamide). Dead cells were counterstained with 60 μM propidium iodide (PI). The fluorescence of the intracellular cleavage product 7-amino-4-trifluoromethylcoumarin (AFC) was measured with a FLUVO-II flow cytometer (HEKA-Elektronik, Lambrecht, FRG) (excitation: 300 - 400 nm, AFC /PI emission: 420 - 530 nm / 550 - 700 nm).




Z-Arg-Arg-4-Trifluoromethyl-coumarinyl-7-Amide
- Ex 337 nm
- Em 433 nm

7-Amino-4-Trifluoromethyl-coumarin
- Ex 381 nm
- Em 488 nm

Fig. 1. Hydrolysis of blue fluorescent Z-Arg-Arg-AFC to green fluorescent AFC

Dim fluorescence developed in resting lymphocytes, neutrophils and monocytes while bright AFC fluorescence was measured in dead leukocytes indicating storage of the proteinases in an inactive form in vital cells. A six-fold increase in fluorescence occurred in neutrophils after 10 min of incubation with *E. coli* (Fig. 2) but unlike induction respiratory burst activity no activation of proteinases was induced by soluble stimuli like phorbol 12-myristate 13-acetate (150 nM, 30 min), FMLP ($10^{-6}$ M, 15 min, ± 5 μg/ml cytochalasin B) or concanavalin A (100 μg/ml) indicating that proteinase activation occurred only following phagosome-lysosome fusion. The intracellular cleavage of Z-Arg-Arg-AFC following phagocytosis of *E. coli* was not due to bacterial proteinases since *E. coli* did not accumulate fluorescence

when incubated with Z-Arg-Arg-AFC, in contrast to bright fluorescence of bacteria incubated with other proteinase substrates like Z-Phe-Arg-AFC.

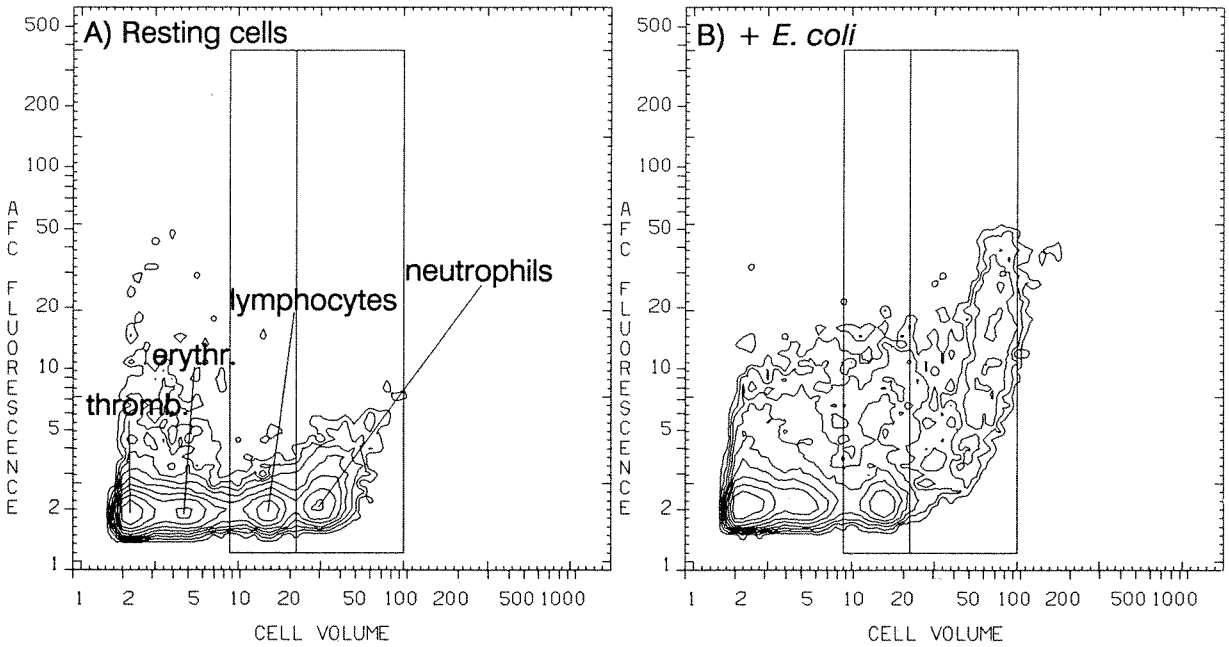



Fig. 2. Intracellular cleavage of Z-Arg-Arg-AFC in resting human peripheral blood leukocytes (A) or in human leukocytes preincubated with *E. coli* for 10 min (B).

The intracellular cleavage of Z-Arg-Arg-AFC by phagocytosing or dead leukocytes was neither substantially inhibited by preincubation of the cells with diisopropyl fluorophosphate (2 mM), E-64 (200 $\mu$M), 1,10-phenanthroline (2 mM) or pepstatin A (20 $\mu$M) (reviewed in [7]) indicating that the activity of neither the serine proteinases, cysteine proteinases, metalloproteinases or aspartic proteinases alone was limiting for the reaction.

## CONCLUSIONS

Z-Arg-Arg-AFC is a useful substrate for the study of endopeptidase activation in phagocytes following cell death or phagocytosis of bacteria. It should be of value for the functional characterization of disease-related alterations of neutrophil function. Z-Arg-Arg-AFC does, however, not allow the specific measurement of lysosomal cysteine proteinases like cathepsin B inside living neutrophils or monocytes. This can be achieved, however, by fluorogenic proteinase substrates of higher specificity such as $(Z\text{-Phe-Arg})_2$-rhodamine 110.[8]

REFERENCES

1. Rothe G, Oser A, Valet G (1988) Naturwissenschaften 75:354-5
2. Rothe G, Valet G (1990) J Leukocyte Biol *in press*
3. Bass DA, Olbrantz P, Szejda P, Seeds MC, McCall CE (1983) J Immunol 130:1910-7
4. Dolbeare FA, Smith RE (1977) Clin Chem 23:1485-91
5. Van Noorden CJF, Vogels IMC, Everts V, Beertsen W (1987) Histochem J 19:483-7
6. Krepela E, Bártek J, Skalková D, Vicar J, Rasnick D, Taylor-Papadimitriou J, Hallowes RC (1987) J Cell Sci 87:145-54
7. Kirschke H, Barrett AJ (1987) In: Lysosomes: Their Role in Protein Breakdown. Academic Press, London, pp 193-238
8. Rothe G, Oser A, Assfalg-Machleidt I, Machleidt W, Mangel WF, Valet G (1990) Cytometry Suppl. 3: *abstract in press*

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