(19) Measurement of Mononuclear Phagocyte Cathepsin B/L Activity with (N-Benzyloxycarbonyl-Arg-Arg)$_2$-Rhodamine 110

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Outline

Cathepsin B and L are lysosomal cysteine proteinases contained in monocytes, but not in neutrophils or lymphocytes, which degrade a large variety of biological substrates at acidic pH. Higher levels of cathepsin B/L are found in tissue macrophages compared to monocytes and in inflammatory macrophages compared to resident macrophages. The nonfluorescent (N-benzyloxycarbonyl-Arg-Arg)$_2$-rhodamine 110 is intracellularly cleaved by cathepsin B and L in a sequential way to the green fluorescent N-benzyloxycarbonyl-Arg-Arg-rhodamine 110 and free rhodamine 110. The specificity of the cellular fluorescence for cathepsin B/L is shown by inhibition with the cysteine proteinase inhibitor N-benzyloxycarbonyl-Phe-Ala-CHN$_2$.

Specimen: 3 ml heparinized human blood (10 U heparin/ml)

Reagents

HBSS without phenol red or bicarbonate, supplemented with 10 mM HEPES (pH 7.35)

(N-benzyloxycarbonyl-Arg-Arg)$_2$-rhodamine 110 (MW 1369.22)
stock solution: 4 mM in DMF (5.477 mg/ml)

propidium iodide (PI) (MW 668.4)
stock solution: 3 mM (2 mg/ml) in 5 mM HEPES-buffered saline (0.15 M NaCl, pH 7.35)

specific inhibitor
N-benzyloxycarbonyl-Phe-Ala-CHN$_2$ (MW 394.4): 10 mM in DMSO (3.944 mg/ml)

Procedure

1. Layer 3 ml heparinized blood carefully on top of 3 ml lymphocyte separation medium. Allow erythrocytes to sediment for 40 minutes at room temperature.
2. Withdraw the upper 800 µl supernatant plasma and store on ice. This will contain platelets and approximately 2 x 10^7/ml unseparated leukocytes.
3. **For lysosomal cathepsin B/L activity**, put 1.00 ml HBSS, 20 µl cell suspension, and 1 µl (N-benzyloxycarbonyl-Arg-Arg)$_2$-rhodamine 110 solution in a 12 x 75 mm polypropylene test tube (final (N-benzyloxycarbonyl-Arg-Arg)$_2$-rhodamine 110 concentration 4 µM). Incubate at 37°C. Take 250 µl aliquots at 10, 20, and 30 minutes.
4. **For specific inhibition of cathepsin B/L activity**, put 1.00 ml HBSS, 20 µl cell suspension, and 1 µl N-benzyloxycarbonyl-Phe-Ala-CHN$_2$ stock in a 12 x 75 mm polypropylene test tube. Incubate for 10 minutes at 37°C. Add 1 µl (N-benzyloxycarbonyl-Arg-Arg)$_2$-rhodamine 110 solution. Continue incubation, taking 250 µl aliquots at 10, 20, and 30 minutes after addition.
5. Counterstain dead cells by incubating 250 µl stained cell suspension with 5 µl 3mM PI for 3 minutes on ice (final PI concentration 60 µM).
6. Run on flow cytometer.

Excitation: 488 nm (argon laser) or high pressure mercury arc lamp with 470-500 nm bandpass filter
Filters: 510-530 bandpass for rhodamine green fluorescence
600 nm longpass for PI (dead cells) red fluorescence

Reagents

HBSS	Sigma Chemical Co.	# H-1387
Propidium iodide	Serva	# 33671
N-Benzyloxycarbonyl-Phe-Ala-CHN_2	Bachem	# N-1040
(N-Benzyloxycarbonyl-Arg-Arg)$_2$-rhodamine 110 synthesized in analogy to Leytus *et al.*		

References

Leytus, S.P., W.L. Patterson, and W.F. Mangel New class of sensitive and selective fluorogenic substrates for serine proteinases. Amino acid and dipeptide derivatives of rhodamine. *Biochem. J.* **215**:253-260, 1983.

Green, D.J., and E. Shaw Peptidyl diazomethyl ketones are specific inactivators of thiol proteinases. *J. Biol. Chem.* **256**:1923-1928, 1981.

Rothe, G., A. Oser, I. Assfalg-Machleidt, W. Machleidt, W.F. Mangel, and G. Valet Cathepsin B activity measured with (Z-Phe-Arg)$_2$-rhodamine 110 as a new flow cytometric marker of monocyte/macrophage activation. *Cytometry suppl.* **4**:77, 1990.

Klingel, S., Diplomarbeit, Ludwig-Maximilians-Universität München, 1991.

Rothe, G., I. Assfalg-Machleidt, W. Machleidt, and G. Valet Independent regulation of endopeptidase activity and respiratory burst activity of neutrophils analyzed by flow cytometry. In **Advances in Analytical Cellular Pathology**. G. Burger, M. Oberholzer, and G.P. Vooijs, editors. Elsevier, 1990. pp.119-120.

Assfalg-Machleidt, I., M. Jochum, D. Nast-Kolb, M. Siebeck, A. Billing, T. Joka, G. Rothe, G. Valet, R. Zauner, H.P. Scheuber, and W. Machleidt Cathepsin B - indicator for the release of lysosomal cysteine proteinases in severe trauma and inflammation. *Biol. Chem. Hoppe-Seyler* **371** (suppl.):211-222, 1990.

NOTES:

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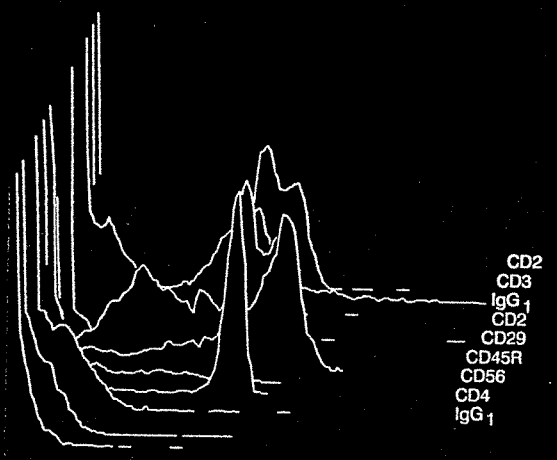

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