(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-CHN₂.

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-CHN₂ (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 10⁷/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-CHN₂ 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 μ l stained cell suspension with 5 μ l 3mM PI for 3 minutes on ice (final PI concentration 60 μ M).
- 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.				

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