DIRECT ASSAY OF PHAGOLYSOSOMAL HYDROLASE BY
FLUOROGENIC SUBSTRATE-BINDING MICROSPHERES


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ABSTRACT

A new method of directly measuring phagolysosomal hydrolase has been
developed. 4-methylumbelliferyl-β-D-glucuronide(4MUGL)-binding micro-
spheres and decanoyl fluorescein(DF)-binding microspheres were prepared.
The microspheres were phagocytosed by human peripheral neutrophils, then
within phagolysosomes immobilized 4MUGL and immobilized DF were hydro-
lyzed by β-glucuronidase and lipase respectively. These reactions were
confirmed by the facts that 4MU, which is fluorescent, was released from
4MUGL-binding microspheres and DF binding microspheres turned fluores-
cent, when the microspheres were phagocytosed. Each enzyme activity
within phalysosomes was examined by measuring the increase of fluores-
cence intensity without cell rupture.

INTRODUCTION

Phagocytes enclose foreign substances (microorganisms, lipids,
wastes, etc.) into phagolysosomes, where to kill and digest them with
highly reactive oxygen and lysosomal hydrolase. Among many kinds of
hydrolase in lysosomes, β-glucuronidase is one of the representative
enzymes of lysosomes. Accordingly, the measurement of the β-glucuronidase
activity within phagolysosomes would be useful for examining the digestive
activity of phagocytes. Esterase is also an important enzyme within
phagolysosomes, because the enzyme scavenges lipid on the arterial wall.
Therefore, the measurement of the lipase activity within phagolysosomes
could be useful for the study of the prevention and diagnosis of athero-
sclerosis.

There have been, however, some difficulties in direct assay of enzymes
released into the phagolysosomes of the cells, while the activities of
phagocytic enzymes released into the medium by exocytosis have been measured
in vitro system (1-8). We have recently developed a new method, which
makes possible directly to measure highly reactive oxygen within phagosomes,
using luminol-binding microspheres (9). In this paper, we report a new
method of directly measuring the activity of hydrolase (β-glucuronidase
(10) and lipase (11), within phagolysosomes using fluorogenic substrate-
binding microspheres.
MATERIALS AND METHODS

Preparation of 4MUGL-binding microspheres

Carboxylated hydrophilic microspheres (2 μm in diameter) were prepared in the following method (9): glycidyl methacrylate, 2-hydroxyethyl methacrylate, methacrylic acid and triethylene glycol dimethacrylate were mixed at a molar ratio 65:20:10:5. Twenty four grams of the monomer mixture were dissolved in 76 g of ethyl propionate, and 0.13 g of 2,2'-azobis (2,4-dimethyl-4-methoxyvaleronitril) were then added to the resultant solution. Polymerization was carried out at 40°C for 3 hr. The precipitated particles were aminated by ammonia and hydrolyzed by dilute sulfuric acid.

One ml of an aqueous solution of NaI04 (2 mg/ml) and 1 ml of dimethylsulfoxide solution containing 4MUGL (2 mg) were mixed and stirred at 25°C for one hour. After adjusting pH of the reaction solution to 8.5 with NaOH, an equal volume of the aminated microsphere suspension (1%) was added to the solution and allowed to react for two hours at 25°C.

Preparation of DF-binding microspheres

Preparation method of the microspheres was similar to that of 4MUGL-binding microspheres: 2-hydroxyethyl methacrylate, methyl methacrylate, glycidyl methacrylate, methacrylic acid and triethylene glycol dimethacrylate were mixed at a molar ratio of 40:35:10:10:5. Polymerization and amination were carried out in the same way as above-mentioned, to prepare hydrophilic microspheres with amino groups (1.5 μm in diameter). 14 mg of dacanoyl chloride, 10 mg of dichloro-triazinyl fluorescein and 100 μl of triethylamine were dissolved in 900 μl of dimethylformamide. The mixture was stirred at 25°C for 6 hr and triazinyl-decanoyl fluorescein was produced. Five ml of the suspension of aminated microspheres (1%) was mixed with 62.5 μl of dichloro-triazinyl decanoyl fluorescein solution and the pH of the mixture was adjusted to 8.0. The suspension was stirred at 40°C for 2 hr and decanoyl fluorescein (DF)-binding microspheres (1%) was obtained. One ml of DF-microspheres (1%) was mixed with 100 μl of fresh human serum and incubated at 37°C for 30 min. Thus opsonized DF-microspheres were obtained.

Separation of neutrophils

Mononuclear cells were removed from normal human peripheral blood by Ficoll-Hypaque centrifugation. Erythrocytes were lysed with ammonium sulfate solution, and neutrophils were resuspended in PBS.

Assay of phagolysosomal S-glucuronidase

100 μl of neutrophil suspension (4 x 10⁶ /ml) and 100 μl of microsphere suspension (2 x 10⁸ /ml) were mixed and incubated at 37°C. After incubation for various periods, the mixture was centrifuged at 250 g for 3 min. The supernatant was used to determine the amount of extracellular 4MU in the medium. The cell pellet was solubilized by 50 μl of a mixture of 1 % Triton X - 100 and ethylene glycol monomethyl ether (1:1), and used to determine the amount of intracellular 4MU. The fluorescence intensities of the samples were measured at 450 nm by use of exciting light of 365 nm.