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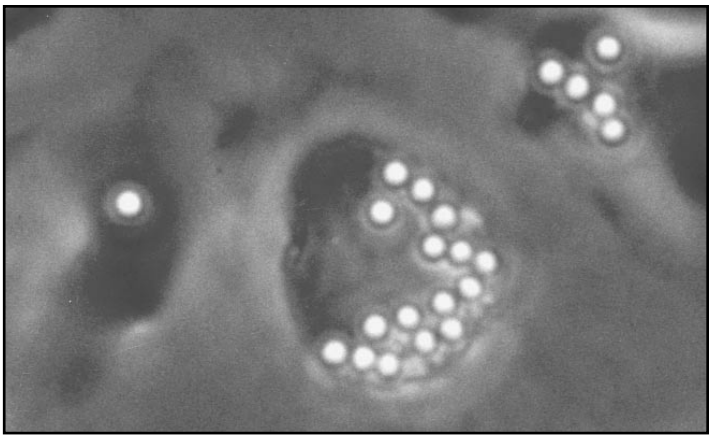
Phagocytosis and Microparticles



Overview:

Phagocytosis is one of the first lines of defense against invading microorganisms. It is also important for the processes of tissue remodeling and removal of senescent cells. In the first instance studies are directed at understanding how living systems defend themselves from foreign bodies and the development of effective therapies against organisms that resist this natural defense system. In the second instance, studies are directed at understanding tissue remodeling during development and repair. While most cells have some capability for phagocytosis, i.e. the need to phagocytose apoptotic cells, the "professional" phagocytes are the phagocytic leukocytes (granulocytes, monocytes, and macrophages). Granulocytes are found in the peripheral blood and actively travel to the site of infection by adhering to the vascular endothelium and chemotaxis. Monocytes migrate from the blood stream into the tissues where they differentiate into macrophages. The macrophages extend pseudopods into the lumen of the capillaries to clear the circulation of invading microorganisms.

In general, phagocytosis is initiated by the stimulation of specific receptors on the phagocyte by a ligand on the surface of a particle or invading microorganism. There are three main types of phagocytic receptors; complement receptors, Fc receptors which recognize the Fc portions of immunoglobulins, and scavenger receptors which recognize "non-self" compounds. The complement and Fc receptors recognize the opsonines that are attached to the invading microorganisms by the host, whereas the scavenger receptors recognize microbes directly. Stimulation of the receptors then causes internalization of the particle via an actin-based polymerization mechanism and a phagosome is formed. The phagosome then joins the endocytic pathway to form the mature phagolysosome.

While this general scheme is common to phagocytosis, to date no single model can adequately describe this extremely complex process. This is due in part to the wide variety of receptors that can stimulate phagocytosis and the ability of the microorganisms them selves to influence the process. Added to this is the fact that invading organisms and particles can be recognized by more than one type of receptor and that the receptors can mediate both particle adhesion and internalization. In their review, Aderem and Underhill (1999) noted that the study of phagocytosis requires an understanding of the mechanisms of signal transduction, actin-based motility, membrane trafficking, and infectious disease.

Polysciences' dyes match popular filter settings:
YG = FITC NYO = Rhodamine PC RED = Phycoerythrin

Dye	Excitation Max.	Emission Max.
	(nm)	(nm)
BB	365	435
YG	445	500
YO	535	570
PC RED	535	588

Excitation and emission data listed are for dyes only. The exact excitation and emission maxima of the particles have not been determined.

Synthetic polymeric particles have been used extensively to study phagocytosis. Some of these are styrene-acrylamide copolymer latices (Kawaguchi et al., 1988), polyacrolein cellulose, and polylactic/glycolic acid copolymers (Tabata and Ikada, 1988, 1991). Polystyrene particles are the most often used due to their ready availability, uniform size, stability, and non-toxic properties. When a small particle that has a narrow coefficient of variation for size is used, as few as one particle per cell can be used and the number of particles ingested per cell can be determined (Steinkam et al., 1981, Parod and Brain 1983). Piskin et al. (1994) conducted studies of phagocytosis by blood cells and mouse peritoneal macrophages using polystyrene microspheres of various sizes and surface properties. Their results illustrate several important points. The first is that

the number of microbeads phagocytosed per cell was greatest for the smallest particle they used (0.9 micron). Secondly the number of particles phagocytosed declined as particle size increased to 6.0 microns. Still, both leukocytes and macrophages were able to internalize one or two particles of 4 to 6 microns. Thirdly, the more hydrophobic the particles, the more readily they were phagocytosed. The presence of positively charged amino groups on the less hydrophobic particles increased their uptake. Conversely, the presence of negatively charged carboxyl groups lowered the number of particles internalized. Fourth, coating particles with bovine serum albumin significantly reduced the number of particles internalized whereas coating with fibronectin dramatically increased the phagocytosis of particles.

The use of polystyrene microparticles for phagocytosis studies requires that the particles induce the complex series of events involved in phagocytosis. To elicit this response, the particles are first coated with serum (opsonization) or a specific IgG. After opsonization the particles and the cells are continuously mixed at 37°C during which phagocytosis occurs. The reaction is then stopped by the addition of ice cold medium and the cells washed to remove the any free particles in the medium. The cells are then resuspended in cold medium and analyzed for the number of particles internalized. The quantification of phagocytic activity can be done a number of ways; direct microscopic examination, spectrophotometric evaluation, fluorometric evaluation, and flow cytometry. The procedure outlined below gives general guidelines for the preparation of particles and cells and flow cytometric analysis.

Procedure:

The following procedure is essentially that reviewed in Harvath and Terle, 1999. This generalized procedure is intended to be a starting point for the development assays directed at the interests of the investigator and all components of the assay should be optimized to address the specific experimental objectives.

Materials Needed:

- Acid citrate dextrose solution-A (ACD-A): 22.0 g/L sodium citrate ($Na_3C_6H_5O_7.H_2O$), 8.0 g/L citric acid, and 24.5 g/L dextrose
- Blood sample mixed with the ACD-A anticoagulant (15 ml ACD-A/100 ml blood).
- Dextran, pyrogen free, average molecular weight, 100,000 to 200,000 Daltons.
- Ficoll-hypaque lymphocyte separation medium
- Hanks balanced salt solution
- Phosphate-buffered saline
- Krebs' Ringers PBS (PBS with 1.0 mM calcium, 1.5 mM magnesium, and 5.5 mM glucose, pH 7.3)
- 3.5% NaCl solution
- Sterile water
- 50 ml Polypropylene conical centrifuge tubes
- Fluorescent carboxy particles
  See List of Fluoresbrite Carboxylate Microspheres under "Products for Phagocytosis" on page 4.
- Normal sera or IgG for opsonization
- Crystal violet solution, 2 mg/ml in 0.15 M NaCl

Cell isolation:

Granulocytes

The following method is according to Boyum (1968) as modifed by Harvath et al. (1991).

1. Prepare a solution of 5% dextran in PBS and a 3.5% solution of NaCl.
2. Use a fresh blood sample anticoagulated with ACD-A. The blood sample should be maintained at room temperature, do not refrigerate. Perform the cell isolation as soon after blood collection as possible.
3. Add the 5% dextran solution to the blood, 3 ml of dextran solution/10 ml of blood. Gently mix and let stand at room temperature for 45 min to allow the red cells to sediment.
4. Aspirate the plasma layer taking care to not disturb the sedimented red blood cells.
5. Isolate the lymphocytes in the plasma by centrifuging through ficoll-hypaque, using a ratio of 2 parts ficoll-hypaque to 3 parts plasma. For this, add the ficoll-hypaque to a conical centrifuge tube and then carefully layer the plasma on to the ficoll-hypaque such that a sharp interface is visible.
6. Centrifuge the tubes at 500 x g for 35 min with the centrifuge brake off.
7. Carefully remove the platelets and mononuclear cells which are concentrated at the plasma-ficoll-hypaque interface and the rest of the supernatant. Resuspend the pellet containing the granulocytes in 2 - 3 ml of PBS.
8. To the resuspended cells add 24 ml of sterile water and gently mix by inversion several times. Add 8 ml of 3.5% NaCl and mix gently.
9. Add Hank's balanced salt solution to the mixture to bring the total volume to 50 ml. Mix gently and centrifuge 500 x g for 10 min.
10. Remove the supernatant and wash the cells two times in 25 to 30 ml of Hank's balanced salt solution.
11. Resuspend the cells in 5 to 10 ml of Hank's balanced salt solution and count. The resuspended cells should contain greater than 98% granulocytes.

Whole Blood

1. Fresh blood anticoagulated with ACD-A should be used. The blood should be maintained at room temperature and the assay performed within 5 h of collection.
2. In general, plan on using 100 - 200 µl of whole blood per assay.

Opsonization:

1. For opsonization with normal serum add particles to serum that has been diluted to 50% with Krebs' Ringers PBS. Gently mix and let incubate 30 min at 37°C. After incubation the particles are added to the phagocytosis assay mixture at concentration equlivent to ≃ 5% serum. Adjust the particle density to 10^8 particles/ml.
2. For opsonization with a specific immunoglobulin, it is important to determine the concentration of immunoglobulin that does not cause aggregation of the particles. The immunoglobulin is incubated with the particles for 60 min at 37°C. Following incubation the particles are washed three times with PBS and the particle density adjusted to 10^8 particles/ml.

Phagocytosis Assay:

Isolated Cells

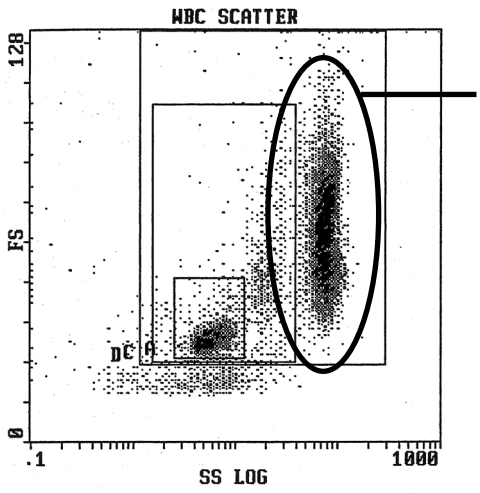
1. Add 100 µl of granulocytes (10^7 cells/ml in Krebs' Ringers phosphate buffer) to a polypropylene tube.
2. Add 10 µl of opsonized particles (10^8 particles/ml) to the tube and incubate with gentle shaking for 30 min at 37°C.
3. As a control prepare an identical sample that is incubated at 4°C.
4. At the end of the 30 min incubation, stop the phagocytosis by adding 2 ml of ice cold PBS, mix and then wash the cells twice ice cold PBS.
5. Resuspend the cells in 500 µl of cold PBS, keep the samples at 4°C, and analyze as soon as possible.

Whole Blood

1. Add 200 µl of anticoagulated whole blood to a polypropylene tube.
2. Add 10 µl of opsonized particles (108 particles/ml) to the tube and incubate with gentle shaking for 30 min at 37°C.
3. As a control prepare an identical sample that is incubated at 4°C.
4. At the end of the 30 min incubation stop the phagocytosis by adding 2 ml of ice cold PBS and mixing. Wash the cells once with ice cold PBS.
5. Resuspend the cell pellet in 3 ml of sterile water and gently mix for 20 to 30 seconds. Add 1 ml of 3.5% NaCl to make the suspension isotonic and pellet the cells by centrifuging at 500 x g for 5 min.
6. Resuspend the cells in 500 µl of cold PBS, keep the samples at 4°C, and analyze as soon as possible.

Flow Cytometric Analysis:

1. Adjust the forward and right-angle scatter detectors so that the granulocyte population is clearly visible and gate on the granulocyte population for analysis.



2. The experimental sample is analyzed by setting the appropriate fluorescence detectors so that several distinct population peaks are easily distinguished. The different peaks should correspond to cells that do not contain particles and those that have internalized 1, 2, 3, or more particles.
3. Once the optimal fluorescence gain settings have been established, analyze 10,000 cells.
4. To quench the fluorescence of extracellular particles add 500 µl of crystal violet solution to the reaction mixture and analyze another 10,000 cells. This fluorescence represents the signal from the internalized particles.

Products for Phagocytosis:

Fluoresbrite™ Carboxylate Microspheres

Fluoresbrite Carboxylate Microspheres are fluorescent monodisperse polystyrene microspheres that have carboxylate groups on their surfaces. They can be coated passively or the carboxyl groups can be activated for covalent coupling of proteins. Polysciences' Fluoresbrite particles are used world wide in phagocytosis and neural retrograde transport studies, and as markers for cell bound antigens. These microspheres are packaged as 2.5% aqueous suspensions.

Fluoresbrite™ Bright Blue (BB) Carboxylate Microspheres

Catalog #	Description	Size/Format
19773	0.05 µ	10 ml
19774	0.10 µ	10 ml
18339	0.50 µ	10 ml
17458	1.00 µ	10 ml
17686	1.75 µ	5 ml
18340	4.50 µ	5 ml
19102	6.00 µ	2 ml
19103	10.0 µ	2 ml

Fluoresbrite™ Yellow Green (YG) Carboxylate Microspheres

Catalog #	Description	Size/Format
16661	0.05 µ	10 ml
16662	0.10 µ	10 ml
09834	0.20 µ	10 ml
24051	0.30 µ	10 ml
24052	0.35 µ	10 ml
24053	0.40 µ	10 ml
15700	0.50 µ	10 ml
07766	0.75 µ	10 ml
15702	1.00 µ	10 ml
09719	1.50 µ	10 ml
17687	1.75 µ	5 ml
09847	2.00 µ	5 ml
17147	3.00 µ	5 ml
16592	4.50 µ	5 ml
18141	6.00 µ	2 ml
18142	10.0 µ	2 ml

Fluoresbrite™ Yellow Orange (YO) Carboxylate Microspheres

Catalog #	Description	Size/Format
19775	0.05 µ	10 ml
18719	0.10 µ	10 ml
19391	0.20 µ	10 ml
18720	0.50 µ	10 ml
18449	1.00 µ	10 ml
19392	1.75 µ	5 ml
19393	3.00 µ	5 ml
19394	4.50 µ	5 ml
19395	6.00 µ	2 ml

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