Measurement of Bacterial Ingestion and Killing by Macrophages

This unit presents fairly simple assays for measuring the binding of bacteria to macrophages, internalization of bacteria (also called ingestion or phagocytosis; Fig. 14.6.1), and bacterial killing by macrophages. The first basic protocol describes how to measure the ability of macrophages to ingest bacteria. Because it is critical to remove residual extracellular organisms, the protocol presents two alternative steps to accomplish this: a washing procedure and a more stringent method in which cells are sedimented through sucrose. In addition, it is important to distinguish those bacteria truly ingested by a macrophage from those that are bound to, but not internalized by, the cell. A simple but effective way to do this is described as the first alternate protocol.

The unit also presents two ways to measure the ability of a macrophage to kill bacteria it has internalized. The second basic protocol describes a straightforward assay in which bacterial colonies are enumerated before and after a killing period; a subsequent colony count will indicate whether the bacteria grew within or were killed by the macrophage. The second alternate protocol describes a way to measure bacterial viability based on bacterial metabolism, in which the ability of bacterial dehydrogenases to mediate the reduction of a tetrazolium salt to purple formazan is monitored by measuring absorbance spectrophotometrically. The advantage of the basic assay is that it is simple and accurate, measuring bacterial growth directly. The alternate method relies on a colorimetric measurement of surviving bacteria, but has the advantage of requiring significantly fewer cells so that more parameters and/or replicates can be run.

The assays in this unit can be performed with human or mouse monocytes or macrophages, obtained fresh or from culture (see also UNIT 14.1). Cells from other species may also be used. Human bronchoalveolar lavage macrophages and peripheral blood monocytes (PBMC) often express good bactericidal activity, and mouse peritoneal exudate cells elicited with proteose peptone are quite bactericidal. Interestingly, not all macrophage populations kill all bacteria equally well. For instance, macrophages elicited with thioglycollate medium do not kill *Listeria monocytogenes*, a facultative intracellular organism used widely by immunologists to study cell-mediated immune responses and host defense mechanisms against bacteria.

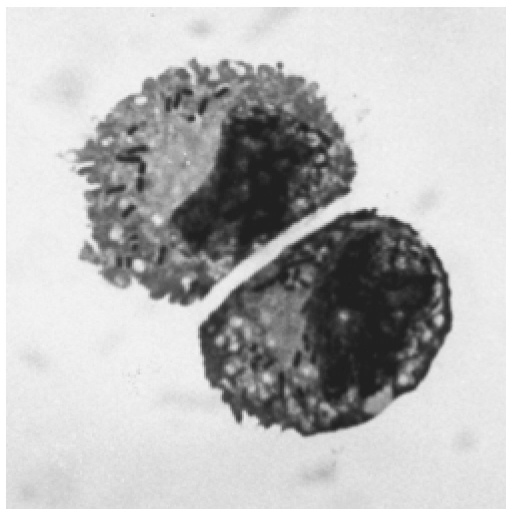

Figure 14.6.1 Mouse J774A.1 macrophages phagocytosed *L. monocytogenes* for 30 min at 37°C. Cells were washed, and a cytocentrifuge preparation was made, stained with Diff-Quik, and photographed. Magnification is ~1000×.

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NOTE: Keep Amphyl (Baxter Scientific; 1/20 dilution in water) or some other sterilizing solution handy while performing these protocols. All materials that come into contact with live bacteria, including pipets and glass tubes, should be soaked in the solution immediately after use. Glass tubes should be soaked in the solution, then washed carefully and rinsed even more carefully to remove residual Amphyl. Amphyl solution should also be used to wipe down lab benches and to kill bacteria prior to disposal. Plastics should be autoclaved prior to disposal. Sterile technique must be used throughout all protocols to prevent introduction of exogenous bacteria, which can contaminate the assay.

NOTE: Even small amounts of antibiotics can interfere with these assays. Therefore, it is critical that antibiotics not be used at any stage of these assays; likewise, if the monocytes or macrophages to be analyzed have been cultured in antibiotics, they should be washed three times and cultured antibiotic-free for at least 24 hr before harvest to prevent carryover of antibiotics. For example, adding even small amounts of gentamicin after phagocytosis has been shown to cause significant intracellular killing of *Listeria* (see Critical Parameters).

NOTE: C-3 sufficient serum from the same species should be used in all phagocytosis assays, unless a source of complement is not needed. Serum should be prepared in the laboratory (not purchased) and each lot should be tested for its ability to support phatocytosis and bacterial killing.

BASIC PROTOCOL 1

MEASUREMENT OF BACTERIAL PHAGOCYTOSIS BY MACROPHAGES

This protocol describes a simple assay to measure the ability of macrophages to bind and internalize, or phagocytose, bacteria. Bacteria and cells are mixed in suspension and rotated to give optimal interaction. Extracellular bacteria are then removed by washing alone, or by washing and centrifugation through sucrose. The amount of phagocytosis is determined by examining stained cells under oil-immersion microscopy.

Materials

Balanced salt solution (BSS; see recipe)
Monocytes/macrophages: e.g., cultured macrophage cell line, murine peritoneal exudate macrophages (*UNIT 3.15* or *UNIT 14.1*), or human PBMC (*UNIT 7.1*)
Overnight bacterial culture (e.g., *Listeria monocytogenes* EGD), live or heat-killed (see Support Protocol)
Normal serum (see recipe), fresh or freshly thawed and kept on ice
30% (w/v) sucrose in PBS (filter sterilize and store for months at 4°C unless contaminated)
PBS (*APPENDIX 2*) containing 5% (v/v) FCS, ice-cold
Diff-Quik (Baxter Healthcare)

Centrifuge
10 × 75–mm polypropylene or polystyrene snap-top tubes
Microscope slides and cover slips
Labquake shaker (Labindustries)
Cytospin 2 cytocentrifuge (Shandon/Lipshaw)

1. Wash monocyte/macrophage sample by adding 10 ml BSS, centrifuging 2 min at 250 × *g*, 4°C, and discarding supernatant. Repeat wash, then resuspend cells in BSS to a final concentration of 2.5×10^7 cells/ml.

   Monocytes/macrophages can be prepared as in UNIT 3.15 (support protocol, steps 1 to 4) or UNIT 14.1 (but using 10% proteose peptone or 4% thioglycollate medium).

2. Add 0.1 ml macrophage suspension (2.5×10^6 cells) to 10 × 75–mm snap-top tube.

   Set up one tube for each condition (group) to be studied.

3. Vortex *L. monocytogenes* culture and dilute 1/10 in BSS.

   If the goal of the experiment is to measure the effects of specific opsonins, it is a good idea to wash the bacteria free of residual culture medium before diluting.

4. Vortex again, then transfer 0.1 ml of bacteria (2.5×10^7 cells) to snap-top tube.

   Vortexing is important so that the bacteria are added as single cells. For this assay, a ratio of ten bacteria to one macrophage is used to allow easy visualization and quantitation of phagocytosis. However, bacteria are well-phagocytosed and readily detectable at 1:1 ratios.

5. Add 50 µl fresh or freshly thawed ice-cold normal serum. Add BSS to 1 ml. Cap tightly and seal cap to tube with Parafilm.

6. Place tubes on a Labquake shaker and rotate end-over-end for 20 to 30 min at ~8 rpm, 37°C.

   Time periods >30 min should not be used, because significant killing of many bacteria can occur quite rapidly and dead bacteria degraded by extensive exposure to 37°C may fail to be stained and detected as phagocytosed.

7a. *Basic method:* Centrifuge tubes 8 min at 250 × *g* (1000 rpm in IEC 269 rotor), 4°C. Remove supernatant, add 2 vol ice-cold BSS, and resuspend cells gently using a Pasteur pipet. Repeat wash twice more to eliminate most residual extracellular bacteria. Resuspend cells to desired concentration in ice-cold PBS/5% FCS.

7b. *For more stringent removal of extracellular bacteria:* Wash cells three times in BSS as described in step 7a and resuspend in 1 ml ice-cold BSS. Using a 1 ml plastic pipet, underlay with 1 ml of 30% sucrose. Centrifuge 8 min at 250 × *g*, 4°C. Carefully remove BSS and sucrose with a Pasteur pipet. Resuspend pellet to desired cell density in ice-cold PBS/5% FCS.

   Cells are usually resuspended to 2.0 ml so that cells are at a density of ~10^6 cells/ml for cytocentrifugation.

8. Cytocentrifuge 0.1 ml cells (~1×10^5 cells) onto a microscope slide by spinning 5 min at 650 rpm, room temperature, in Cytospin 2.

   The number of cells loaded into the cytocentrifuge may have to be varied with cell size—if the macrophages are large, as some cell lines are, use fewer cells.

9. Stain slide with Diff-Quik as per supplier's instructions.

10. Quantitate phagocytosis under oil-immersion microscopy (1000×), examining at least 200 cells and counting the number of internalized bacteria in each one. Calculate amount of phagocytosis according to the following formula: phagocytic index = (percentage of macrophages containing at least one bacterium) × (mean number of bacteria per positive cell).

    This calculation takes into consideration not only the number (percentage) of cells that are phagocytic, but also how phagocytic they are—i.e., how many bacteria are internalized by each cell.

ALTERNATE PROTOCOL 1

DISCRIMINATION BETWEEN EXTRACELLULAR AND INTRACELLULAR BACTERIA USING FITC LABELING

Using oil-immersion microscopy it is usually possible to visually distinguish internalized bacteria from those that are merely adherent if they are attached to the periphery of the cell. The few non-cell-associated bacteria remaining after the washing procedures are also easily distinguished. However, it is difficult to discriminate extracellular bacteria that lie over or under the cell from those truly internalized. This distinction can be achieved using fluorescence microscopy. Bacteria (at least gram-positive bacteria; see APPENDIX 3O) can be easily stained by FITC. Following phagocytosis of FITC-labeled bacteria, addition of ethidium bromide causes extracellular bacteria to fluoresce red-orange, while internalized organisms remain green.

Additional Materials *(also see Basic Protocol 1)*

Heat-killed bacteria (e.g., *Listeria monocytogenes* EGD; see Support Protocol), 10^9 cells/ml
FITC/$NaHCO_3$: 0.1 mg/ml FITC (fluorescein isothiocyanate) isomer 1 (Sigma) in 0.1 M $NaHCO_3$, pH 9.0
PBS containing 5% FCS and 5 mM glucose
Ethidium bromide (Sigma)

70°C water bath
Fluorescence microscope equipped with standard fluorescein optics using a long-pass filter and oil immersion

CAUTION: Ethidium bromide is a strong teratogen; it should be handled with gloves and disposed of properly.

1. Centrifuge bacteria 3 min at 12,000 × *g*, room temperature, and discard supernatant. Resuspend pellet in 1 ml FITC/$NaHCO_3$.
2. Incubate 60 min at 25°C. Wash bacteria by centrifuging and resuspending as described in step 1, but using PBS. Repeat until the supernatant is clear of residual FITC.

   This usually takes four washes, the last of which will contain no green color.

3. Use labeled bacteria in phagocytosis reaction (see Basic Protocol 1, steps 7a or 7b). Resuspend cells in 1 ml PBS/5% FCS/5 mM glucose.

   Final concentration should be ~2.5×10^6 cells per ml.

4. Remove 100-µl aliquots and mix with ethidium bromide to 50 µg/ml final.
5. Immediately place a 10- to 20-µl drop of the suspension on a microscope slide and overlay with a coverslip. Keep the slide in the dark until viewed by fluorescence microscopy, but not longer than ~2 hr.
6. Use a fluorescence microscope with a long-pass FITC filter to view the cells under oil immersion (1000×). Examine at least 60 consecutive individual macrophages containing bacteria, distinguishing internalized bacteria from those bound to the cell surface. Calculate phagocytic index (see Basic Protocol 1, step 10).

   Internalized bacteria can be differentiated from those bound to the cell surface because following exposure to ethidium bromide, internal bacteria remain green whereas external organisms fluoresce red-orange. Ethidium bromide will slowly enter cells and stain internalized bacteria green as well, but there is a substantial time window beofre this occurs.

   If the microscopist is red-green color blind, as are nearly 10% of American males, then red and green bacteria can be distinguished by using two different filters. First use the FITC long-pass filter, which passes red and green light. Next use a standard rhodamine filter, which passes red light only. Red (external) bacteria can be counted using the rhodamine

filter, and green (internal) bacteria can be detected by subtracting those counted with the rhodamine filter from those counted with the long-pass fluorescein filter (total bacteria).

MEASUREMENT OF MACROPHAGE KILLING OF BACTERIA

BASIC PROTOCOL 2

The ability of macrophages to kill phagocytosed bacteria is measured by quantitating cell-associated bacteria after a brief phagocytosis period, then determining how many organisms remain following a longer incubation. Killing of most bacteria occurs very quickly; measuring surviving bacteria after a short period of time lessens the chance of measuring residual extracellular bacteria or bacteria that grow in and kill the macrophage, then grow extracellularly. After macrophages phagocytose bacteria and extracellular organisms are removed, bacterial colonies are quantitated by plating and counting before and after a 90- to 120-min incubation.

Materials

Live log-phase bacterial culture (e.g., *L. monocytogenes*, *E. coli,* or *Staphylococcus* sp.), cultured overnight after inoculation of liquid medium from frozen stock (see Support Protocol)
Balanced salt solution (BSS; see recipe)
Monocytes/macrophages: e.g., cultured macrophage cell line, murine peritoneal exudate macrophages (*UNIT 13.5* or *UNIT 14.1*), or human PBMC (*UNIT 7.1*)
Normal serum (see recipe), fresh or freshly thawed and kept on ice
BSS/5% normal serum, ice-cold
Appropriate culture plates: e.g., tryptic soy agar (TSA) plates (Remel; store at 4°C and prewarm to 37°C before use)
Sterile water

2.0-ml conical polypropylene tubes with O-ring screw cap (Sarstedt)
10 × 75–mm snap-top polypropylene tubes
Labquake shaker (Labindustries)
13 × 100–mm Pyrex glass tubes with screw caps (Fisher or Corning), sterile

1. Vortex overnight culture of *Listeria* and prepare a 1/300 dilution in BSS. In a 10 × 75–mm snap-top tube or a 2-ml polypropylene screw-cap tube, mix the following (setting up two or three tubes for each condition to be tested and systematically labeling tubes):

   2.5×10^6 macrophages in 0.1 ml BSS
   0.3 ml vortexed dilute bacterial culture (2.5×10^6 bacteria)
   50 µl ice-cold normal serum
   BSS to 1 ml.

   Close caps tightly, using Parafilm on the snap-top tubes.

   Both screw-cap tubes with O-rings and snap-top tubes are suitable for this assay. Screw-cap tubes are easier to seal, whereas snap-top tubes have a larger volume and are more convenient for washing cells.

   When working with bacteria, vortex cultures before removing sample to ensure that bacteria are added as single cells.

   Monocytes/macrophages can be prepared as in UNIT 3.16 (support protocols, steps 1 to 4) or UNIT 14.1 (but using 10% proteose peptone or 4% thioglycollate medium). If there is reason to suspect macrophages could be contaminated with bacteria (e.g., for human bronchoalveolar lavage macrophages), it is important to include a control without added Listeria.

2. Place tubes in Labquake shaker and rotate end-over-end at ~8 rpm 15 to 20 min at 37°C. Wash away extracellular bacteria using either a simple wash or sucrose centrifugation (see Basic Protocol 1, steps 7a and 7b, respectively). Resuspend cells in 1 ml BSS/5% serum.

3. Set up four screw-cap glass tubes containing 0.9 ml sterile water each. Starting with 0.1 ml of cell mixture, make four 1/10 serial dilutions into these tubes, using a new pipet for each dilution to eliminate carryover of bacteria and vortexing briefly between dilutions.

   Diluting cells in water causes the macrophages to lyse and release the bacteria.

4. Briefly vortex each tube and plate 0.1 ml, in duplicate, on TSA plates prewarmed to 37°C.

   This group of plates will be the time 0 (T-0) control, indicating how many live bacteria were in the macrophages after phagocytosis but before killing occurred.

   Agar plates can be divided into quarters by marking the bottom with a permanent marker. This way a single plate can be used for two dilutions (two replicates each). Plastic 1-ml pipets work well for diluting and plating. Dilution tubes can be set up a day in advance and kept at 4°C.

   Before use, culture plates should be warmed at 37°C, overnight if they are new or for a few hours if they are 2 to 3 weeks old, so that the samples will soak into the agar. Plates can be used after the expiration date, but should be left at room temperature overnight rather than at 37°C so they do not dry out.

5. Cap and tightly seal undiluted sample tubes. Incubate samples 90 to 120 min at 37°C.

   Samples can be incubated rotating, as in the phagocytosis assay (see Basic Protocol 1) or not; good killing is obtained in either case.

6. Place tubes on ice to stop bacterial growth. Prepare serial dilutions and plate samples as described in step 4.

7. Once plated samples have been absorbed into agar, invert plates and incubate 24 to 48 hr at 37°C. Count the colonies and compare the number of colonies at T-0 to the number of colonies after incubation: bactericidal activity has occurred if there are fewer bacteria in the tube after the 90- to 120-min incubation than at T-0.

   Use a standard 37°C incubator rather than a water-jacketed CO_2 incubator, which provides too much moisture.

   Plates should be autoclaved before disposal.

ALTERNATE PROTOCOL 2

COLORIMETRIC DETERMINATION OF MACROPHAGE KILLING OF BACTERIA

Bacteria and cells are mixed in a microtiter plate and extracellular organisms are removed by washing. The numbers of surviving bacteria are determined before and after a 90-min incubation by measuring their ability to reduce the yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) to a purple formazan product. This reaction, which is mediated by bacterial dehydrogenases, can be detected colorimetrically by reading the plates on a microtiter plate reader at 570 nm and comparing the results to a standard curve. This assay is a modification of the one originally described by Peck (1985).

Additional Materials *(also see Basic Protocol 2)*

RPMI-5: RPMI 1640 (GIBCO/BRL) without phenol red, supplemented with 5% autologous normal serum (see recipe)
Amphyl (Baxter Scientific) diluted 1/20 in water
5% (w/v) saponin (Sigma; filter sterilize and store 3 to 6 months at room temperature in ≤10-ml aliquots to minimize contamination)
2.95% (w/v) tryptose phosphate broth (Difco; autoclave and store in 5-ml aliquots in glass screw-cap tubes ≤1 year at 4°C)
5 mg/ml MTT in PBS (Sigma; filter sterilize and store in light-resistant container 3 to 6 months at 4°C)
1 N HCl (optional)

Flat-bottom 96-well microtiter plates (Fisher or Falcon)
37°C, 10% CO_2 incubator
Microtiter plate centrifuge carriers (e.g., Reagents International for IEC #276 rotor)
Microtiter plate reader (e.g., BioTek 312e)

CAUTION: MTT is a carcinogen and teratogen. Use appropriate precautions as suggested on the bottle.

1. Harvest macrophages by centrifuging 10 min at 250 × *g*, 4°C, and discarding supernatant. Resuspend them at 10^6 cells/ml in RPMI-5.

2. Place 100-µl aliquots (10^5 macrophages) into microtiter plate wells, in quadruplicate, preparing two parallel plates designated T-0 and T-90. Add 10 µl bacteria (at a concentration of 10^7 cells/ml in BSS) to each well. Incubate plates 20 min at 37°C in 10% CO_2 incubator to allow phagocytosis to occur.

   This gives a bacterium/cell ratio of ~1:1.

3. Centrifuge plates 5 min at 250 × *g* (1000 rpm in IEC 276), 4°C. Carefully aspirate supernatants with a Pasteur pipet to remove extracellular bacteria; avoid removing any cells. Discard supernatants in Amphyl solution.

   Tilting the plates to remove the supernatants works best. The pipet can be put directly into the "corner" of the well to aspirate the supernatant without disturbing cells.

4. Add 100 µl RPMI-5 to each sample well and to four empty wells in each plate (as blanks). Centrifuge plates 10 min at 250 × *g* to increase interaction between cells and any residual bacteria.

   If using a BioTek 312e microtiter plate reader, be sure to designate the corresponding wells on both plates as blank wells; the reader will automatically read the same wells as blanks from plate to plate.

5. Add 20 µl of 5% saponin to all wells in the T-0 plate. Incubate plate 1 min at room temperature to lyse the cells and release the bacteria. Add 100 µl tryptose phosphate broth to each well. Store plate at 4°C until needed (step 7).

   It may be necessary to check that saponin does not kill the bacterium being used.

6. Incubate the T-90 plate for 90 min at 37°C in 10% CO_2 incubator to allow killing or growth of the bacteria. Remove plate from incubator and repeat step 5, except do not store.

   Kinetics experiments show 90 min to be optimal for this procedure, although times up to 180 min give comparable results. A 90-min incubation was selected for convenience. This may vary with different bacteria and cells.

   If scheduling is a problem, at this point the plates can be held overnight at 4°C to prevent or minimize growth of bacteria. The next day, one can proceed to step 7.

7. Incubate both T-0 and T-90 plates 4 hr at 37°C in 10% CO_2 incubator to allow growth of remaining viable bacteria.

   A 4-hr growth period is optimal for L. monocytogenes, which doubles approximately every 30 min. Other bacteria may require a longer or shorter period depending on the rate of multiplication and bacterial metabolism.

8. Add 15 µl of 5 mg/ml MTT in PBS and incubate 20 min at 37°C in 10% CO_2 incubator. Optionally, add 10 µl of 1 N HCl to each well to stop the reaction. Measure absorbance at 570 nm on a microtiter plate reader.

   If the machine does not automatically subtract the blank wells, be sure to do so to obtain the net absorbance.

9. Generate a standard curve by incubating known numbers of bacteria with MTT in a microtiter plate and measuring absorbance as in step 8. Determine the number of bacteria (cfu) in T-0 and T-90 samples by extrapolation from standard curve: bacterial killing has occurred when there is a significant decrease (≥0.2 logs) in the number of bacteria per well between 0 and 90 min.

   Once a standard curve has been generated, this need not be redone for every experiment.

   Bacterial cfu should be determined by growing aliquots of bacteria on TSA plates and counting colonies.

10. Add Amphyl solution to plates prior to disposal. Follow special disposal requirements for MTT according to guidelines provided by safety offices at individual institutions.

SUPPORT PROTOCOL

PREPARATION OF *LISTERIA MONOCYTOGENES*

Virulent *Listeria monocytogenes* can be obtained from the ATCC (strain 15313). Other virulent strains, obtained for instance as patient isolates, can also be used. Grow bacteria to log phase by inoculating tryptose phosphate broth with bacteria (amount depends on the state of the bacteria: e.g., frozen, on a slant, or in broth), then incubating in a shaking water bath at 37°C until culture reaches log phase (4 to 6 hr). Place 0.5-ml aliquots into 10 × 75–mm polypropylene or polystyrene snap-top tubes and store at −80°C until use (cells can be used for at least 1 year). Before use thaw tube and inoculate one Pasteur pipet drop (~30 µl) into 5 ml liquid medium (e.g., tryptose phosphate broth). Grow culture overnight to give late-log- to stationary-phase cultures (~2×10^9 live organisms/ml).

If bacteria in early log phase are required: Inoculate 1 ml of the above culture into fresh bacterial culture medium, incubate in a shaking water bath at 37°C, and harvest log-phase bacteria 4 to 6 hr later

To prepare heat-killed bacteria: Heat log-phase bacteria 60 min at 70°C in water bath. Measure volume, then wash bacteria by centrifuging 20 min at 900 × *g*, 4°C, and discarding supernatant. Resuspend in ≥10 ml PBS and repeat wash. Resuspend in PBS to ~10^{10} bacteria/ml final concentration (based on 2×10^9 bacteria/ml in overnight culture).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2;*** *for suppliers, see* ***APPENDIX 5.***

Balanced salt solution (BSS; phosphate-, not carbonate-, buffered)

Stock I (10×):
10 g dextrose *or* 11 g dextrose monohydrate
0.6 g KH_2PO_4
3.58 g $Na_2HPO_4 \cdot 7H_2O$ *or* 1.85 g anhydrous Na_2HPO_4
20 ml 5% phenol red (GIBCO/BRL)
H_2O to 1 liter
Divide into 500-ml aliquots
Store at 4°C (stable ~6 months)

Stock II (10×):
1.86 g $CaCl_2 \cdot 2H_2O$
4 g KCl
80 g NaCl
2 g $MgCl_2 \cdot 6H_2O$ *or* 1.04 g anhydrous $MgCl_2$
2 g $MgSO_4 \cdot 7H_2O$
H_2O to 1 liter
Divide into 500-ml aliquots
Store at 4°C (stable ~6 months)

For 1× BSS: Mix 1 part stock I, 8 parts distilled water, and 1 part stock II (be certain to dilute one stock with water before adding the other to avoid formation of a precipitate). Filter sterilize and store up to 1 month at 4°C, as long as the pH (color) does not change and the solution does not become contaminated. Be sure that pH is ~7.0 and conductivity ~16.0 at room temperature (check when making first 1× stock in a batch).

Normal serum

Obtain blood from C3-sufficient individual(s) of appropriate species (e.g., if using mouse monocytes/macrophages, obtain from mouse by cardiac puncture, *UNIT 1.7*). Immediately place blood on ice, allow to clot at least 1 hr, then centrifuge 25 min at 600 × *g*, 4°C. Collect serum and store in 0.5-ml aliquots at −80°C until use. Test each lot before use for its ability to support phagocytosis and killing. Once thawed, serum should not be refrozen and reused.

Normal mouse serum may be conveniently obtained from retired breeders, which are relatively old and large.

COMMENTARY

Background Information

The ability to phagocytose and kill a variety of microbes, including bacterial pathogens, is a major effector function of macrophages. The importance to the host of these functions cannot be overestimated. This is emphasized by the fact that there are no known genetic defects specific for macrophage functions in the mammalian world. These properties of macrophages are particularly important for host defense against facultative intracellular organisms, which often can survive and replicate within macrophages. In fact, pathogenesis of facultative intracellular bacteria such as *M. tuberculosis, Legionella pneumophila,* and *Salmonella* sp. is determined by their ability to survive within macrophages. Facultative intracellular organisms constitute one of the main classes of microbes that infect AIDS patients.

Macrophages can be heterogeneous with regard to their ability to kill facultative intracellular bacteria. Thus it is important that good assays be available that allow careful measurement of each step in this process (Leenen and

Campbell, 1993). Moreover, bactericidal activity of macrophages is a culmination of their ability to bind, ingest, and kill bacteria. These activities need not be linked but can occur as independent steps in the sequence. That is, a macrophage may bind, or bind and internalize a bacterium, yet not be able to kill it. It is therefore important to distinguish these activities experimentally.

Surprisingly little is known regarding how the receptors used by macrophages to bind and phagocytose bacteria initiate bacterial killing, or about the mechanisms by which macrophages actually kill bacteria. It is known that bacteria bind complement components, and the bacterium:complement complexes bind complement receptors on macrophage surfaces. Although this sequence of events has not yet been demonstrated for all bacteria, it has been well-described for *L. pneumophila, L. monocytogenes*, and *M. tuberculosis* (Payne and Horwitz, 1987; Schlesinger et al., 1990; Drevets and Campbell, 1991a). For many organisms, including these three, complement receptors type 3 (CR3) and type 1 (CR1) seem to be the major receptors. Yet binding to these receptors does not necessarily lead to internalization of bacteria—complement receptors must be activated before they can internalize microorganisms. It is for this reason that it is important to be able to distinguish external bacteria that have merely adhered to macrophages from those that have been internalized (phagocytosed), as permitted by the first alternate protocol (which uses a method that was described in detail by Drevets and Campbell, 1991b). Interestingly, for *Listeria* to be phagocytosed and killed by a macrophage, phagocytosis must be mediated primarily through CR3. Macrophages that phagocytose this bacterium primarily through other receptors cannot kill it (Drevets et al., 1992). This suggests that the receptor a macrophage uses to internalize a bacterium may influence the intracellular fate of the organism. Phagocytosis may also be mediated by specific antibodies that function as opsonins. These bind the bacterium, and the bacterium:antibody complex binds the cell via the Fc receptor. Bacteria may thus be opsonized in antibodies or in heat-inactivated serum, in which case phagocytosis is not mediated by, or dependent upon, complement.

Even less is known regarding how macrophages kill bacteria. Many organisms are susceptible to reactive oxygen intermediates (ROI), such as the hydroxyl radical and hydrogen peroxide (see Klebanoff, 1992, for review). Recent data suggest that products of reactive nitrogen metabolism, particularly the short-lived intermediate nitric oxide, may play an important role in macrophage bactericidal activity (Green et al., 1990; Flesch and Kaufmann, 1991). Because so little is known about killing mechanisms, it is important that bactericidal assays be simple and accurate and exclude artifacts that may confound measurement of macrophage activity.

The ability of bacteria to become associated with macrophages is easily measured. Macrophages and bacteria are incubated for a short period of time—long enough to allow binding and ingestion, but not so long as to allow the macrophage to degrade organisms it has phagocytosed. The trick is then to put the macrophages onto a surface on which they and the bacteria can be stained and intracellular organisms can be counted. Conventionally this is done in one of two ways. In many assays, macrophages are made adherent to a coverslip or Labtek chamber, then bacteria are added and often centrifuged onto the macrophages. Measuring the ability of adherent macrophages to phagocytose bacteria has two disadvantages: first, macrophages may be so busy adhering to, or perhaps attempting to phagocytose, the surface that they are not very efficient at phagocytosing bacteria. Second, it is more difficult to remove residual extracellular bacteria because it is more difficult to wash adherent cells. For these reasons, there are advantages to an assay in which macrophages and bacteria are mixed in suspension, as in this unit. Only in a suspension assay can extracellular bacteria be removed as described in these protocols; attempting to wash extracellular bacteria from adherent macrophages is more difficult. The first alternate protocol provides an easy way to visualize bacteria either by preparing a cytosmear or by examining them under fluorescence microscopy. This protocol can also be used to distinguish extracellular and intracellular organisms.

These assays can be used to measure constitutive phagocytic and bacterial properties of various macrophage populations, including human blood monocytes and pulmonary macrophages. In addition, the ability of cytokines and other reagents to induce phagocytosis and/or bactericidal activity in non-phagocytic and/or non-bactericidal cell populations can also be studied. If cells are or become strongly adherent when cultured with certain reagents, cultures often can be done in Teflon containers, such as Teflon petri dishes. These methods can

also be used to study whether particular drugs are able to kill bacteria within macrophages. Which receptor a macrophage uses to phagocytose certain bacteria can be studied by asking whether internalization of bacteria is inhibited by antibodies to the receptor. Finally, whether bacteria are merely bound to the surface or truly internalized by a cell can also be determined.

Macrophages can be stimulated either in vitro (as in this unit) or by in vivo injection prior to studying their functions. For example, resident mouse peritoneal macrophages are poorly phagocytic and nonbactericidal. One can inject a mouse intra-peritoneally with certain cytokines and ask whether they induce bactericidal activity. However, these experiments are difficult to interpret for two reasons. First, it is not clear whether the cytokines act on resident macrophages or recruit inflammatory macrophages. Second, it is not clear whether the cytokines affect macrophages directly or induce some other cell to secrete yet other mediators which then act on macrophages. For these reasons, in vitro experiments may be easier to interpret.

Alternate Protocol 1, for distinguishing extra- and intracellular bacteria using FITC labeling, could perhaps be adapted for fluorescence-activated cell sorting (FACS); it is certainly possible to measure FITC-labeled bacteria using FACS, and flow cytometry has been used to distinguish extra- and intracellular yeast (Fattorossi et al., 1989). However, two problems have prevented us from using flow cytometry. First, our goal is to identify individual phagocytic cells and determine whether the bacteria are truly internalized (green) or simply bound to the surface (red-orange) of the cell. This could not be done at the single-cell level using FACS, as the cell sorter would count bacteria, not cells. A second, more difficult problem is that although external bacteria stain bright red-orange, there is some uptake of ethidium bromide by the cells in general, and this might be hard to distinguish (see color photographs included in Drevets and Campbell, 1991b). Thus, we think it unlikely that this method could easily be adapted to flow cytometry.

Critical Parameters and Troubleshooting

It is essential that fresh or fresh-frozen C3-sufficient normal serum be used as a source of opsonins for the phagocytosis and killing assays. We find that commercially purchased serum generally lacks active complement components and therefore serum should be prepared in the laboratory (see Reagents and Solutions). It is critical that new serum preparations be tested against a positive control for their ability to support phagocytosis and bactericidal activity. Other reagents need not be tested, with the following exception: if macrophages are to be stimulated with a particular mediator, it is necessary to be sure it is not directly bactericidal, and this should be tested by incubating the mediator with bacteria in the absence of macrophages.

It is surprising how important it is to ensure that tubes are rotated end-over-end during the phagocytic assay. If one forgets to turn the rotator on, little or no phagocytosis occurs, hence there is little bactericidal activity. The mixing provided by end-over-end rotation allows maximal interaction between bacteria and macrophages. The most efficient anti-microbial cells in vivo are those recruited to a site of infection or injury, which are mobile and not adherent or fixed in tissues. Thus a suspension assay rather than an assay in which macrophages are adherent may more closely mimic bacteria-cell contact in vivo.

The protocols described in this section can be used for many bacteria, particularly those that have short doubling times (30 min to 2 hr). Most can be grown in tryptose phosphate broth or another conventional bacterial culture medium. The phagocytosis assay (Basic Protocol 1) requires only that the bacteria be able to be stained with Diff-Quik—they can be either dead or alive. For organisms that cannot be stained with Diff-Quik, other dyes may be used. Alternative Protocol 1 requires that the bacteria take up FITC. We find that Lucifer yellow can also be used, and that Lucifer yellow–stained bacteria are green and quenched to red-orange by ethidium bromide just as with FITC. For the bactericidal assay (Basic Protocol 2), the bacteria must be able to be phagocytosed by macrophages in order to measure killing, which occurs only intracellularly. In addition, the assay requires a relatively short doubling time and should be measured after a short interval (90 to 180 min) to minimize the problems caused by growth of any residual extracellular bacteria. This is of particular concern when adherent macrophages must be used, because these cannot be washed free of extracellular bacteria as well as can macrophages in suspension. Thus the assays describee can easily be used with bacteria of the genuses *Listeria,*

Escherichia, Salmonella, Staphylococcus, and *Streptococcus*, among others.

The most critical single element required for the success, and more importantly the interpretation, of the bactericidal assay is that it be done in the complete absence of antibiotics. Nearly 70% of the experiments reported in the literature in which macrophages are claimed to have bactericidal activity are seriously flawed because the assays were done in the presence of antibiotics. Antibiotics are widely used to rid macrophages of residual extracellular bacteria. However, most extracellular bacteria will be phagocytosed and killed by macrophages during the assay, as they would be in vivo. Moreover, washing bacteria three times routinely leaves very few extracellular bacteria (Drevets and Campbell, 1991b)—too few to significantly influence the assay results in any case. Therefore, bacteria pose a less significant problem than antibiotics. Antibiotics produce false positive results—that is, they make a nonbactericidal macrophage appear to be bactericidal. This is particularly worrisome because phagosomes, the organelles into which bacteria are phagocytosed, may concentrate antibiotics. Antibiotics can also become a hidden nemesis when, for instance, cells are cultured antibiotic-free, but in the presence of 20% conditioned medium from L929 cells that were grown with antibiotics. On one occasion we thought bone marrow macrophages cultured in such a fashion were bactericidal, but they lost their "bactericidal activity" when the L929 supernatant was obtained from cells grown without antibiotic. Even antibiotics such as gentamicin that cannot permeate the cell membrane can accumulate within macrophages by pinocytosis of the extracellular milieu. Experiments demonstrating the effect of small amounts of gentamicin on the ability of macrophages to kill *L. monocytogenes* have recently been described (Drevets et al., 1994).

Because most bacteria are killed quite quickly by macrophages, bactericidal assays are best measured within a few hours after co-incubation of cells and bacteria. If the assay is delayed for several hours after mixing, the few residual extracellular bacteria can grow rapidly and confound the results. Furthermore, if bacteria have killed a macrophage and escaped into the extracellular milieu, this can also cause a problem. Hence bactericidal activity is best measured 90 to 120 min after phagocytosis, though similar results are obtained as late as 180 min after phagocytosis.

Anticipated Results

A macrophage with good phagocytic capability will yield a phagocytic index of 500 to 1500 at a cell/bacteria ratio of 1:10. This generally means that ~80% of the cells are phagocytic and the phagocytic cells contain 10 to 20 bacteria each. The assays described here allow one to estimate macrophage functional heterogeneity by determining what percentage of macrophages are phagocytic, and estimate levels of phagocytic activity by determining how many bacteria each phagocytic cell internalizes. Even cells that only bind but cannot internalize bacteria can be distinguished by counting external versus internal organisms using fluorescence microscopy.

Four kinds of results can be obtained from the bactericidal assays described here. First, macrophages may have no inhibitory effect on bacterial growth. Second, bacteria may grow in the presence of macrophages treated with a particular mediator, but less than in its absence, indicating that the treated macrophages only slowed growth of the bacteria—which is likely to be of little help to the host. Third, the number of bacteria at T-0 and T-90 may be the same in the presence of macrophages (i.e., neither an increase nor decrease in bacteria is observed), indicating that the macrophages were bacteriostatic. Bacteriostatic macrophages could later allow the bacteria to grow and colonize the host. Fourth, the number of bacteria may decrease $\geq 0.2 \log_{10}$ between T-0 and T-90 in the presence of macrophages, indicating that the macrophages were bactericidal. Good bactericidal activity is considered to be $>0.4 \log_{10}$, and even in 90 min can reach $\geq 1.0 \log_{10}$.

Time Considerations

The time required to complete any of the assays described is not extensive, although it will depend on the number of groups of cells being assayed on a given day. A typical phagocytosis assay in itself can be easily completed in about a half day or less. The greatest amount of time required for this assay is the counting of the cells and bacteria to obtain the data, which need not be done on the same day as the remainder of the assay.

The colony plating assay can be completed easily within one working day. However, the plates must be incubated overnight before the bacterial colonies can be counted and the data obtained. Doing some preparation, such as filling the glass tubes to be used for dilutions with 0.9 ml sterile water, marking the TSA plates

and assay tubes, and warming the TSA plates the day prior to the assay is encouraged to save time on the day of the experiment.

The MTT assay, if done from start to finish, will take an entire working day because of the number and length of incubations throughout the assay. The advantage is that the data can be obtained in a single working day. Marking the 96-well plates and all the tubes that will be used to dilute the bacteria and cells for the assay ahead of time will help get things going more quickly on the day of the experiment. One should also keep in mind that this assay may be stopped following lysis of the cells and addition of tryptose phosphate broth to the wells, and then completed the next day.

In reference to all of the assays described, one problem that can frequently arise and cause great irritation is forgetting to grow up the bacteria to be used in the experiment overnight. Omitting this preparation will add a minimum of 2.5 hr to the estimated time required for the assay, because bacteria will have to be grown on the day of the assay.

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Key Reference

Drevets, D.A. and Campbell, P.A. 1991b. See above.

Describes method for determining critically whether bacteria are merely bound to the surface of bacteria, or have been internalized—a question clearly important for bactericidal activity as well, as bacteria bound to macrophage surfaces are not killed by the macrophage unless internalized.

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