

A Simple, Reproducible, Inexpensive, Yet Old-Fashioned Method for Determining Phagocytic and Bactericidal Activities of Macrophages

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Macrophages (M ϕ) play a pivotal role in the protection system by recognizing and eliminating invading pathogenic bacteria. Phagocytosis and the killing of invading bacteria are major effector functions of M ϕ . Although the phagocytic and bactericidal activities of M ϕ have been analyzed via several methods using a light microscope, a fluorescence microscope, or a fluorescence-activated cell sorter, expensive materials and equipment are usually required, and the methods are rather complicated. Moreover, it is impossible to determine both the phagocytic and bactericidal activities of M ϕ simultaneously using these methods. In this review, we describe a simple, reproducible, inexpensive, yet old-fashioned method (antibiotic protection assay) for determining the phagocytic and bactericidal activities of M ϕ .

Key Words: Anti-bacterial agent, gentamicin, macrophage, phagocytosis

INTRODUCTION

Mammalian cells usually uptake macromolecules from the extracellular microenvironment through their receptor(s) or by penetration.¹ Professional phagocytes such as macrophages (M ϕ) are not exceptional. However, M ϕ have another unique uptake process called phagocytosis, in which M ϕ actively uptake not only macromolecules but also large particles such as bacterial pathogens.¹

M ϕ recognize and engulf invading bacteria, and specific vacuoles called phagosomes are then formatted therein.² The phagosomes mature into phagolysosomes, in which bacterial pathogens encounter various antimicrobial agents such as lysozymes by which bacterial pathogens are digested.³ Reactive oxygen intermediates and nitrogen oxide synthesized in the

cytosol participate in the killing of bacterial pathogens by penetrating into phagolysosomes.⁴

Since M ϕ are particularly important for protection against bacterial infection, analysis of the phagocytic and bactericidal activities of M ϕ is essential for the determination of their functional activities. Bacterial pathogens are categorized into at least two groups on the basis of their kinetics in M ϕ :⁵ extracellular bacteria and intracellular bacteria. Extracellular bacteria are easily killed by M ϕ , whereas intracellular bacteria show resistance to digestion by M ϕ .⁵ However, the majority of intracellular bacteria are also killed by M ϕ , although this depends on the activation status of M ϕ .⁶⁻¹⁰

Several methods are employed to determine the phagocytic and bactericidal activities of M ϕ . Yet, in most cases, expensive materials and equipment are usually required, and the methods are rather complicated. Therefore, this review focuses on a simple, reproducible, inexpensive, yet old-fashioned method for determining the phagocytic and bactericidal activities of M ϕ .

WHAT IS AN ANTIBIOTIC PROTECTION ASSAY?

An antibiotic protection assay is traditionally employed to determine the phagocytic and bactericidal activities of M ϕ .⁶⁻¹³ This

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assay system is based on counting colony-forming units (CFU) in M ϕ after phagocytosis (Fig. 1). After incubating M ϕ with bacteria for a short period of time, the bacteria are engulfed by M ϕ . The phagocytic activity of M ϕ can be determined by counting the CFU in M ϕ at this time point [CFU (P)]. After further incubation for a short period of time, the engulfed bacteria are killed by M ϕ . The number of viable bacteria in M ϕ can be counted at this time point [CFU (B)]. The bactericidal activity of M ϕ can thus be calculated by comparing CFU (P) with CFU (B). Thus, both the phagocytic and bactericidal activities of M ϕ can easily be determined. It is important that bacteria not engulfed by M ϕ must be killed to avoid bacterial growth outside the M ϕ . As antibiotics are essential for killing bacteria that are not engulfed by M ϕ , this assay is known as an antibiotic protection assay.

ANTIBIOTICS RECOMMENDED FOR AN ANTIBIOTIC PROTECTION ASSAY

In an antibiotic protection assay, selection of an antibiotic that can effectively kill extracellular (i.e., bacteria not engulfed by M ϕ) yet not intracellular (i.e., bacteria engulfed by M ϕ) bacteria is quite important. As the sensitivity against antibiotics differs for each bacterium, an antibiotic that can effectively kill bacteria must be employed. Antibiotics with low molecular weight pass through plasma membrane of M ϕ . Therefore, the bacteria engulfed by M ϕ are killed by this type of antibiotic (Fig. 2A). In contrast, antibiotics with high molecular weight are unable to penetrate into cytosol. Therefore, the bacteria engulfed by M ϕ are not killed by this type of antibiotic (Fig. 2B). Thus, an antibiotic with a high molecular weight must be used in an antibiotic protection assay.

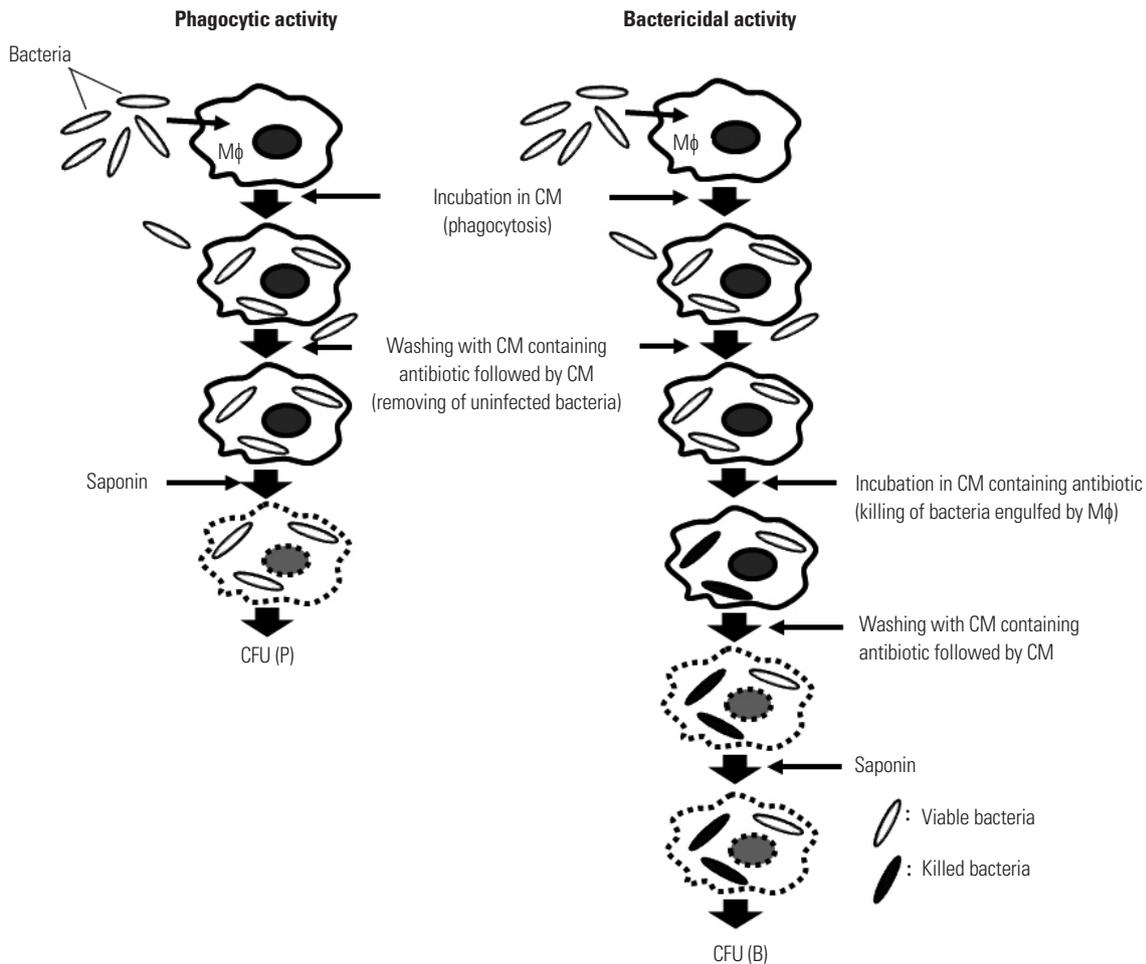


Fig. 1. An antibiotic protection assay. Two sets of M ϕ (one for the determination of phagocytic activity and the other for the determination of bactericidal activity) are incubated for a short period of time with bacteria in CM to be engulfed by M ϕ . To determine phagocytic activity, M ϕ are washed with CM containing antibiotic to kill extracellular bacteria followed by CM. They are then treated with saponin to release bacteria from M ϕ , and CFUs are determined [CFU (P)]. To determine bactericidal activity, M ϕ infected with bacteria are further incubated for a short period of time in CM containing antibiotic to kill intracellular bacteria followed by CM. They are then treated with saponin, and the number of viable bacteria in M ϕ is determined by counting the CFU after washing with CM [CFU (B)]. Bactericidal activity of M ϕ can be calculated by comparing CFU (P) with CFU (B). CM, complete medium; CFU, colony-forming units; M ϕ , macrophages.

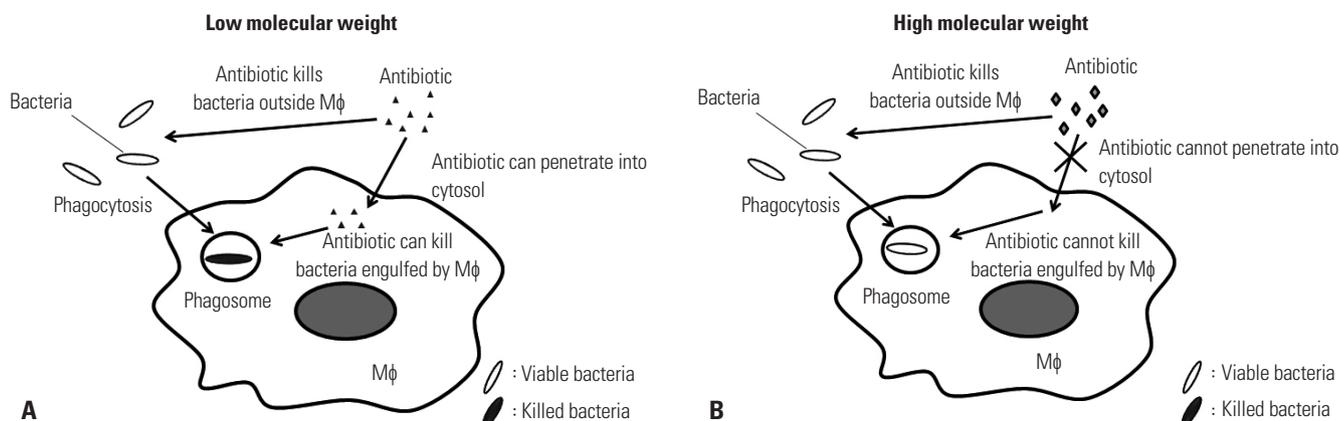


Fig. 2. Influence of antibiotics with high and low molecular weight on bacteria in Mφ. Mφ engulf bacteria by forming specific vacuoles called phagosomes. An antibiotic with a low molecular weight penetrates into the cytosol and kills both extracellular and intracellular bacteria (A). An antibiotic with a high molecular weight is unable to penetrate into the cytosol and thus kills only extracellular bacteria (B). Mφ, macrophages.

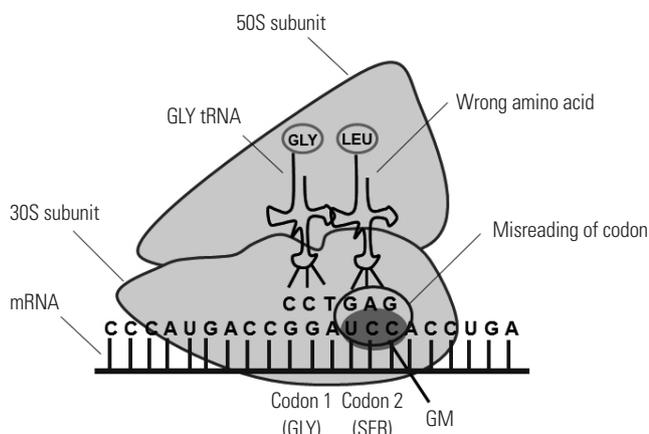


Fig. 3. Killing mechanism of GM. GM inhibits translation of mRNA by binding to the 30S subunit of the ribosome. The irreversible binding of GM to the ribosome causes the misreading of the codons, which in turn causes an error in the proofreading process of translation, leading to incorrect protein expression and bacterial cell death. GM, gentamicin.

GENTAMICIN (GM) IS WIDELY USED FOR DETERMINING PHAGOCYTTIC AND BACTERICIDAL ACTIVITIES OF Mφ IN AN ANTIBIOTIC PROTECTION ASSAY

Numerous antibiotics with high molecular weight have been identified previously.¹⁴ Among these, gentamicin (GM) is widely employed for an antibiotic protection assay. The reasons are as follows: 1) GM is an aminoglycoside antibiotic that binds to the bacterial ribosome 30S subunit and induces the misreading of a wide range of RNAs (Fig. 3).¹⁵ 2) GM has a broad-spectrum (Table 1).¹⁶⁻²⁰ 3) GM is considered to be unable to penetrate into the cytosol of Mφ due to its high molecular weight (Table 2).¹⁴ It is generally accepted that molecules with a molecular weight of less than 400 g/mol are able to pass through the plasma membrane.²¹ Therefore, antibiotics with a molecular weight of more than 400 g/mol are recommended for determining the phagocytic and bactericidal activities of Mφ.

There are several antibiotics with a higher molecular weight than that of GM (Table 2). However, these antibiotics are not recommended by several reasons. For example, the molecular weight of erythromycin is markedly higher than that of GM, yet erythromycin expresses bacteriostatic, but not bactericidal, activity.¹⁴ Similarly, the molecular weights of kanamycin and streptomycin are higher than those of GM (Table 2).¹⁴ However, *Pseudomonas* spp. show resistance to these antibiotics.^{22,23} It is needless to say that antibiotics other than GM with high molecular weight can nevertheless be employed for determining the phagocytic and bactericidal activities of Mφ against particular bacterial pathogens. However, GM is recommended for determining these activities, as this antibiotic has a broad spectrum and kills extracellular, but not intracellular, bacteria. Therefore, we focus on an antibiotic protection assay using GM (GM protection assay) in the following section.

EXPERIMENTAL PROCEDURE FOR DETERMINING PHAGOCYTTIC ACTIVITY OF Mφ USING A GM PROTECTION ASSAY

An experimental procedure for determining the phagocytic activity of Mφ using a GM protection assay is shown in Fig. 4. Mφ are incubated in RPMI 1640 containing 10% fetal calf serum [designated as complete medium (CM)] for 120 min to adhere to the bottom of tissue culture plates. Cells are incubated with bacteria for a given length of time to ingest bacteria. Subsequently, cells are washed three times with CM containing the optimal concentration of GM to remove non-ingested bacteria. Note that pre-warmed, but not cold, CM should be used throughout the experiment so as not to detach the Mφ from the bottom of tissue culture plates. After washing with CM, a portion of cells is treated with saponin, which is plated on agar plates after sonication, and the CFUs are determined. Phagocytic activity is calculated as follows: {number of viable bacteria ingested by Mφ [CFU (P)]/total number of viable bacteria

incubated with Mφ [CFU (T)]×100 (%). Thus, the percentage of bacteria engulfed by Mφ (phagocytic activity) can be quantitated.

Table 1. MIC of GM Against Various Bacteria

Organism	MIC (mg/mL)	Reference
<i>Bacillus cereus</i>	1.6	Klein, et al. ¹⁶
<i>Corynebacterium</i> spp.	3.0–37.5	Waitz and Weinstein ¹⁷
<i>Enterobacter</i> spp.	0.3–3.0	Waitz and Weinstein ¹⁷
<i>Escherichia coli</i>	0.3–0.75	Waitz and Weinstein ¹⁷
<i>Haemophilus influenzae</i>	7.5	Waitz and Weinstein ¹⁷
<i>Lactobacillus</i> spp.	0.08	Waitz and Weinstein ¹⁷
<i>Listeria monocytogenes</i>	0.03–4.0	Espaze and Reynaud ¹⁸
<i>Mycobacterium tuberculosis</i>	1.0–4.0	Ho, et al. ¹⁹
<i>Mycoplasma</i> spp.	0.75–1.4	Waitz and Weinstein ¹⁷
<i>Neisseria gonorrhoeae</i>	0.8–1.6	Klein, et al. ¹⁶
<i>Neisseria meningitidis</i>	6.3–25	Klein, et al. ¹⁶
<i>Pasteurella multocida</i>	3.0–7.5	Waitz and Weinstein ¹⁷
<i>Pseudomonas aeruginosa</i>	0.3–3.0	Waitz and Weinstein ¹⁷
<i>Pseudomonas pseudomallei</i>	17.5–75.0	Waitz and Weinstein ¹⁷
<i>Proteus</i> spp.	0.75–3.0	Waitz and Weinstein ¹⁷
<i>Salmonella</i> spp.	0.08–0.3	Waitz and Weinstein ¹⁷
<i>Shigella</i> spp.	4.0–16.0	Wilson, et al. ²⁰
<i>Staphylococcus aureus</i>	0.4–3.1	Klein, et al. ¹⁶
<i>Staphylococcus mastitis</i>	0.01	Waitz and Weinstein ¹⁷
<i>Streptococcus agalactiae</i>	0.3–3.0	Waitz and Weinstein ¹⁷
<i>Vibrio</i> spp.	0.7	Waitz and Weinstein ¹⁷

MIC, minimal inhibitory concentration; GM, gentamicin.

Table 2. Molecular Weights of Representative Antibiotics

Antibiotic	Molecular weight (g/mol)	Reference
Amoxicillin	365.4	Bryskier ¹⁴
Ampicillin	349.4	Bryskier ¹⁴
Cefaclor	367.8	Bryskier ¹⁴
Cefalexin	347.4	Bryskier ¹⁴
Cephalosporin C	415.4	Bryskier ¹⁴
Chloramphenicol	323.1	Bryskier ¹⁴
Ciclacillin	341.4	Bryskier ¹⁴
Ciprofloxacin	331.3	Bryskier ¹⁴
Erythromycin	733.9	Bryskier ¹⁴
Fosfomycin	138.1	Bryskier ¹⁴
Gentamicin	477.6	Bryskier ¹⁴
Imipenem	299.3	Bryskier ¹⁴
Kanamycin	484.5	Bryskier ¹⁴
Minocycline	457.5	Bryskier ¹⁴
Penicillin G	334.4	Bryskier ¹⁴
Spectinomycin	332.4	Bryskier ¹⁴
Streptomycin	581.6	Bryskier ¹⁴
Tetracycline	444.4	Bryskier ¹⁴

EXPERIMENTAL PROCEDURE FOR DETERMINING BACTERICIDAL ACTIVITY OF Mφ USING A GM PROTECTION ASSAY

An experimental procedure for determining the bactericidal activity of Mφ using a GM protection assay is also shown in Fig. 4. Mφ incubated in CM for 120 min are incubated with bacteria and then washed three times with CM containing the optimal concentration of GM to remove non-ingested bacteria. Subsequently, cells are further incubated in CM containing the optimal concentration of GM for a given length of time to kill non-ingested bacteria followed by CM. Cells are then treated with saponin, plated on agar plates after sonication, and the CFUs are determined. Bactericidal activity is calculated as follows: 100–{number of remaining viable bacteria in Mφ [CFU (B)]/CFU (P)}×100 (%). Thus, the percentage of bacteria killed by Mφ (bactericidal activity) can be quantitated.

AN ANTIBIOTIC PROTECTION ASSAY FOR BACTERIA SHOWING RESISTANCE TO GM

As described above, GM is widely used to determine the phagocytic and bactericidal activities of Mφ against various bacteria. However, certain bacteria show resistance to GM (Table 3).^{15,17} Hence, GM cannot be used to determine the phagocytic and bactericidal activities of Mφ against these bacteria. In such cases, an antibiotic other than GM with a high molecular weight must be used.

PRELIMINARY EXPERIMENTS THAT SHOULD BE PERFORMED BEFORE A GM PROTECTION ASSAY

Although the GM protection assay is quite simple, preliminary experiments must be performed. One of the most important points is to determine the optimal conditions (i.e., concentration and length of effectiveness) of GM. Although GM has been considered to be unable to kill bacteria in Mφ,^{12,13,24–26} several studies have reported that GM, even in high concentrations, kills bacteria in Mφ.^{27,28} Therefore, the optimal concentration and length of effectiveness for GM should be determined with care. After determining the minimal inhibitory concentration (MIC), the optimal concentration of GM is then determined. Specifically, Mφ infected with bacteria are incubated with different concentrations of GM (higher than MIC; e.g., 2.5-fold MIC), and verification that GM does not penetrate into Mφ should be performed. In addition, further verification as to whether Mφ are damaged by the concentration of GM should also be performed, as Mφ are commonly destroyed by high concentrations (e.g., 100 µg/mL) of GM (Fig. 5).

Saponin facilitates the destruction of plasma and phagosomes

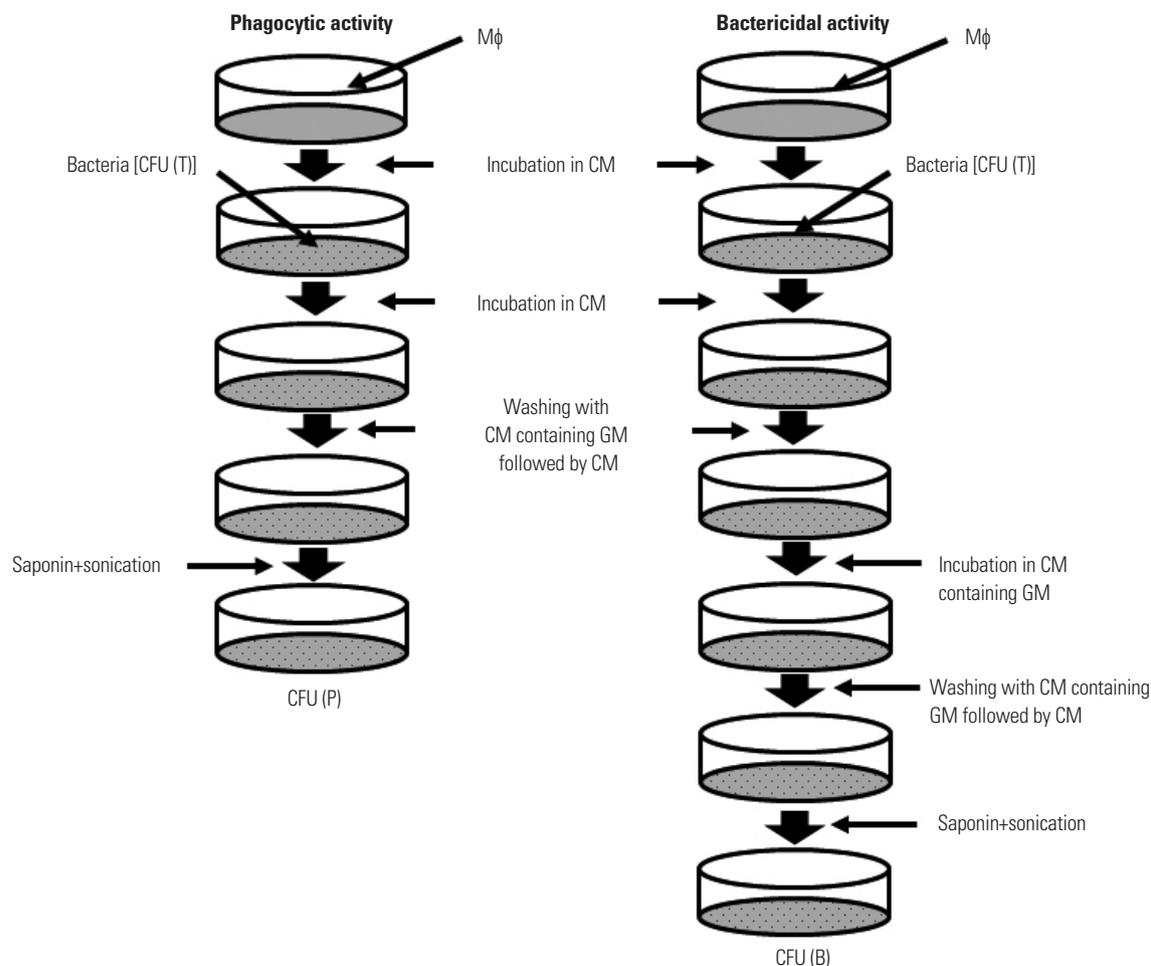


Fig. 4. Experimental procedure for determining the phagocytic and bactericidal activities of M ϕ using a GM protection assay. Two tissue culture plates are prepared; one to determine phagocytic activity and the other to determine bactericidal activity. M ϕ are incubated in CM for 120 min to adhere to the bottom of tissue culture plates and then incubated with bacteria (M ϕ :bacteria=1:10) for a short period of time in CM to ingest bacteria. To remove non-ingested bacteria, cells are washed three times with CM containing the optimal concentration of GM followed by CM, and CFUs are then determined [CFU (P)]. Infected M ϕ incubated in another plate are further incubated for a short period of time in CM containing the optimal concentration of GM. During this period, engulfed bacteria are killed by M ϕ . Cells are washed three times with CM, and the number of viable bacteria in M ϕ can also be determined by counting CFU after washing with CM [CFU (B)]. Bactericidal activity of M ϕ can be calculated by comparing CFU (P) with CFU (B) after saponin treatment and sonication. GM, gentamicin; CM, complete medium; CFU, colony-forming units; M ϕ , macrophages.

Table 3. Bacteria Showing Resistance to GM

Organism	Reference
<i>Aeromonas liquefaciens</i>	Waitz and Weinstein ¹⁷
<i>Bacteroides</i> spp.	Vakulenko and Mobashery ¹⁵
<i>Burkholderia cepacia</i>	Vakulenko and Mobashery ¹⁵
<i>Clostridium</i> spp.	Waitz and Weinstein ¹⁷
<i>Stenotrophomonas maltophilia</i>	Vakulenko and Mobashery ¹⁵
<i>Streptococcus pneumoniae</i>	Vakulenko and Mobashery ¹⁵

GM, gentamicin.

al membranes of M ϕ by interacting with cholesterol, which are abundant in their plasma membranes (Fig. 6).²⁹ Therefore, before counting CFU in M ϕ , M ϕ must be treated with saponin. We can confirm that M ϕ are completely destroyed by 0.5% saponin.

In order to determine the phagocytic and bactericidal activ-

ities of M ϕ , the CFUs in M ϕ are counted at different time points after infection. In most cases, the number of viable bacteria engulfed by M ϕ is highest at 45–60 min after incubation with bacteria, and the bacteria are usually killed within 90–120 min after being engulfed by M ϕ . It is needless to say that there are some exceptions; for example, *Mycobacterium* spp. are not killed within 120 min after being engulfed by M ϕ .^{30,31}

SEVERAL METHODS RECENTLY EMPLOYED FOR DETERMINING PHAGOCYTTIC AND BACTERICIDAL ACTIVITIES OF M ϕ

Bacteria engulfed by M ϕ can be detected using a light microscope, a fluorescence microscope, or a flow cytometer.^{32–36} However, it is difficult to distinguish bacteria engulfed by M ϕ

from those merely attached to the plasma membranes of M ϕ by these methods. The problem can be solved by using ethidium bromide.^{32,33,35,36} Although the phagocytic activity of M ϕ can be determined using these methods, another experiment must be performed to determine the bactericidal activity of M ϕ

using MTT and an absorption spectrometer in each case.^{33,36-39} Thus, it is possible to determine the phagocytic and bactericidal activities of M ϕ using these methods. However, expensive materials and equipment are required and the methods are rather complicated. Moreover, it is impossible to deter-

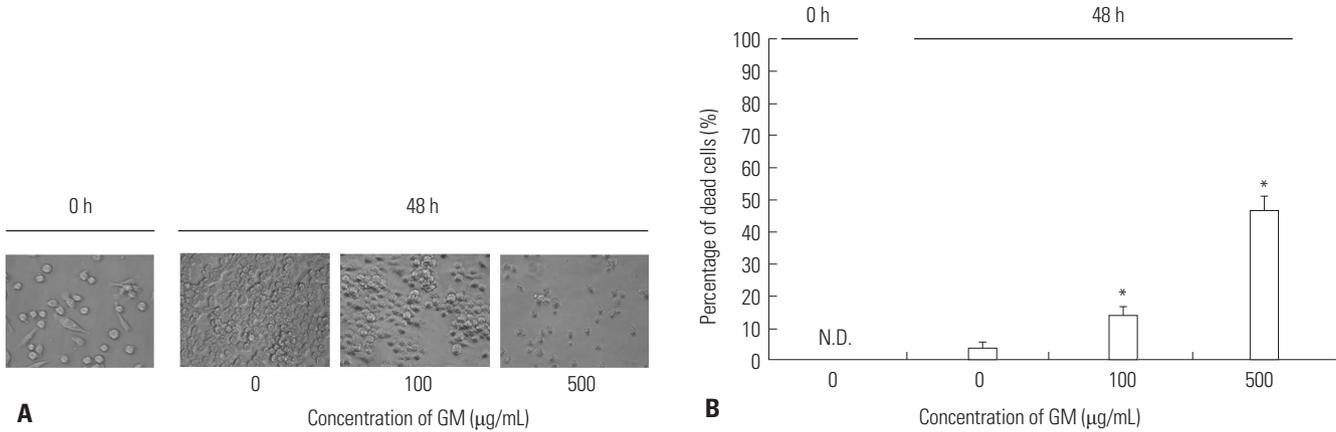


Fig. 5. Influence of GM on M ϕ . (A) RAW264 were incubated with CM containing various concentrations of GM for 48 h, and the morphological changes were observed under phase contrast microscope. Representative data from two independent experiments are shown. (B) RAW264 were incubated with CM containing various concentrations of GM for 48 h, and their viabilities were enumerated via trypan blue exclusion test. Data are presented as mean \pm SD of two independent experiments. * p <0.05: 0 vs. 100 or 500. N.D., not detectable; GM, gentamicin; CM, complete medium; M ϕ , macrophages.

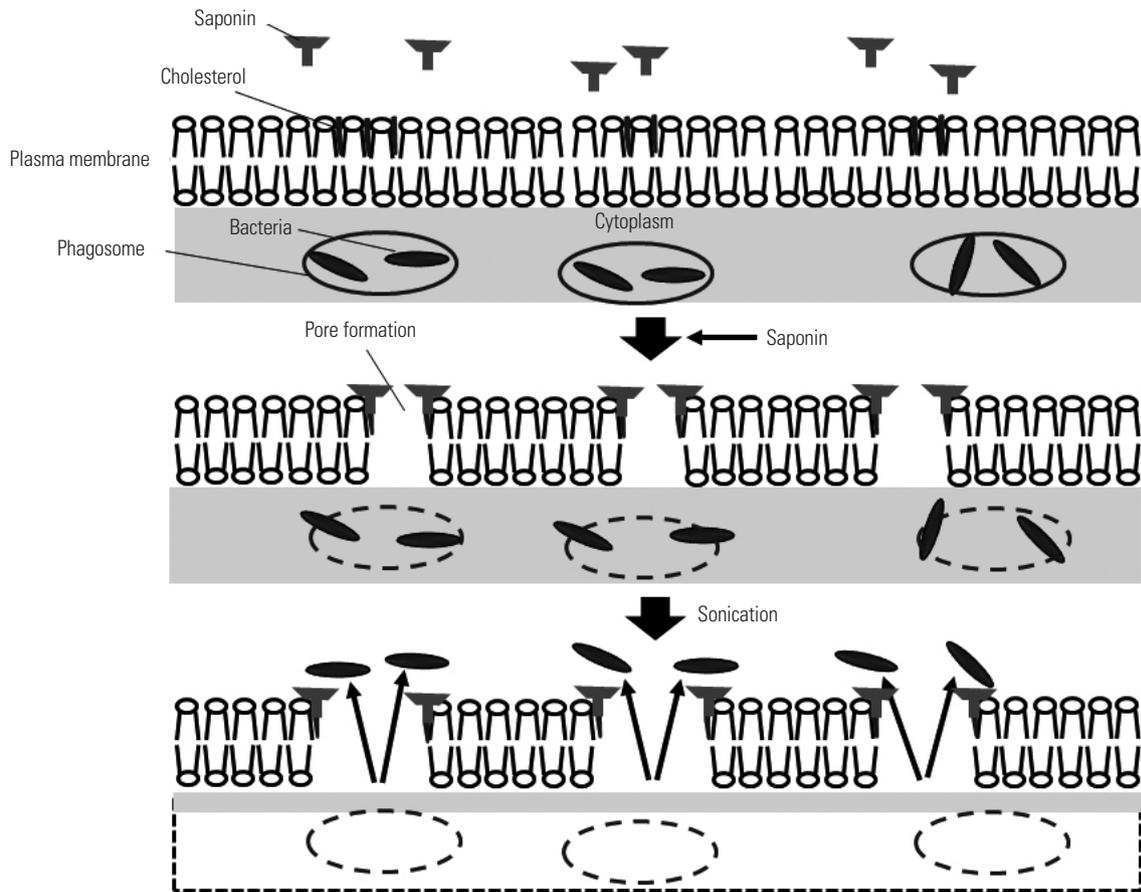


Fig. 6. Effects of saponin and sonication on plasma and phagosomal membranes of M ϕ . Saponin interacts with cholesterol residues in plasma and phagosomal membranes and forms pores in lipid bilayers. After sonication, internalized bacteria are released. M ϕ , macrophages.

mine both the phagocytic and bactericidal activities of M ϕ simultaneously using these methods.

CONCLUSION

This review describes a simple, reproducible, inexpensive, yet old-fashioned method for determining the phagocytic and bactericidal activities of M ϕ . Although the phagocytic and bactericidal activities of M ϕ against various bacterial pathogens can be determined by using methods that have been recently employed, it is impossible to determine both activities simultaneously. In addition, these methods require expensive materials and equipment and complicated methods. In contrast, both the phagocytic and bactericidal activities of M ϕ can be determined simultaneously by using an antibiotic protection assay for which expensive materials and equipment are not required. We therefore recommend investigators to reevaluate the antibiotic protection assay.

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