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Opsono-Adherence Assay to Evaluate Functional Antibodies in Vaccine Development against Bacillus anthracis and other Encapsulated Pathogens

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TITLE:

Opsono-Adherence Assay to Evaluate Functional Antibodies in Vaccine Development Against *Bacillus anthracis* and Other Encapsulated Pathogens

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KEYWORDS:

Opsonization, adherence, antibodies, serum, macrophages, capsule, vaccine development, innate immunity, non-human primates, RAW 264.7 cells, automated fluorescence microscopy, correlate of immunity

SUMMARY:

The opsono-adherence assay is an alternative method to the opsono-phagocytic killing assay to evaluate the opsonic functions of antibodies in vaccine development.

LONG ABSTRACT:

The opsono-adherence assay is a functional assay that enumerates the attachment of bacterial pathogens to professional phagocytes. Because adherence is requisite to phagocytosis and killing, the assay is an alternative method to the opsono-phagocytic killing assay. An advantage of this assay is the option of using inactivated pathogens and mammalian cell lines, which allows standardization across multiple experiments. The use of an inactivated pathogen in the assay also facilitates work with biosafety level 3 infectious agents and other virulent pathogens. In our work, the opsono-adherence assay was used to assess the functional ability of antibodies, from sera of animals immunized with an anthrax capsule-based vaccine, to induce adherence of fixed *Bacillus anthracis* to a mouse macrophage cell line, RAW 264.7. Automated fluorescence microscopy was used to capture images of bacilli adhering to macrophages. Increased adherence was correlated with the presence of anti-capsule antibodies in the serum. Non-human primates that exhibited

high serum anti-capsule antibody concentrations were protected from anthrax challenge. Thus, the opsono-adherence assay can be used to elucidate the biological functions of antigen specific antibodies in sera, to evaluate the efficacy of vaccine candidates and other therapeutics, and to serve as a possible correlate of immunity.

INTRODUCTION:

Recognition, adherence, internalization, and degradation of a pathogen are integral to phagocytosis[1], a salient pathway in the host innate immune response first described by Ilya Metchnikoff in 1883[2,3]. Phagocytic leukocytes, as well as other cells of the immune system, are highly discriminatory in their selection of target; they are able to distinguish between "infectious non-self" and "non-infectious self" through pathogen associated molecular patterns by their repertoire of pattern recognition receptors (PRRs)[4,5]. Host recognition of a pathogen may also occur with the binding of host generated opsonins, such as complement and antibodies[6]. This process, called opsonization, coats the pathogen with these molecules, enhancing internalization upon binding to opsonic receptors (e.g., complement and Fc receptors) on phagocytic cells[6]. For a pathogen to adhere to a phagocyte, collective binding of multiple receptors with their cognate ligands is necessary. Only then can adherence trigger and sustain signaling cascades inside the host cell to initiate internalization[6].

Due to the importance of phagocytosis in the clearance of pathogens and prevention of infection, extracellular pathogens have developed numerous ways to subvert this process to prolong their survival. One strategy of importance is the production of an anionic polymeric (e.g., polysaccharide or polyamino acid) capsule which is anti-phagocytic by virtue of its charge, is poorly immunogenic, and shields molecules on the bacterial envelope from PRRs[6,7]. Pathogens such as *Cryptococcus neoformans* and *Streptococcus pneumoniae* have capsules composed of saccharide polymers, whereas *Staphylococcus epidermidis* and some *Bacillus* species produce a poly-ɣ-glutamic acid (PGGA) capsule[7,8]. Yet other pathogens produce capsules that resemble the non-infectious self. For example, *Streptococcus pyogenes* and a pathogenic strain of *B. cereus* have a hyaluronic acid capsule that is not only anti-phagocytic but which also may not be recognized as foreign by the immune system[9,10].

Conjugation of capsule to carrier proteins converts them from poor, T-independent antigens into highly immunogenic T-dependent antigens that can induce high serum anti-capsule antibody titers[11,12]. This strategy is employed for licensed vaccines against *S. pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitides*[11]. The opsonic activities of anti-capsule antibodies have commonly been evaluated by the opsono-phagocytic killing assay (OPKA)[13-16]. This assay shows whether functional antibodies can trigger phagocytosis and killing[14]. However, the use of OPKA with infectious pathogens, such as Tier 1 Biological Select Agents and Toxins (BSAT), including *B. anthracis*[17], is hazardous and presents security risks; these assays necessitate extensive handling of a select agent. Select agent handling can only be done in restricted biosafety level 3 (BSL-3) laboratories; work in these areas demands protracted operating procedures due to the numerous safety and security precautions that must be followed. BSL-3 laboratories are also typically not equipped with the specialized equipment used for OPKA work, such as microscopes and cytometers. Thus, we developed an alternative assay based on the use of inactivated

bacteria[18,19]. We refer to this as an opsono-adherence assay (OAA) that is not dependent on internalization and killing as assay outputs; instead, adherence of opsonized inactivated pathogens is used as an index of phagocytosis. Mechanistically, OAA is a suitable substitute because adherence occurs *a priori* and is intimately intertwined with internalization and intracellular killing. From a biosafety perspective, OAA is preferred because it requires minimal handling of an infectious agent, is experimentally of shorter duration than OPKA, and can be performed in BSL-2 laboratories after a stock of the inactivated pathogen has been produced and transferred.

We demonstrate the utilization of OAA to examine the opsonic function of anti-capsule antibodies found in sera of non-human primates (NHPs) vaccinated with a capsule conjugate [i.e. PGGA from *B. anthracis* conjugated to the outer membrane protein complex (OMPC) of *Neisseria meningitides*][20]. Serum opsonized bacilli were incubated with an adherent mouse macrophage cell line, RAW 264.7. After fixation, the cell monolayer and adherent bacilli were imaged by fluorescence microscopy. Bacterial adherence increased when the bacilli were incubated with serum from NHPs vaccinated with the capsule conjugate[20]. Adherence correlated with survival of the anthrax challenge[20,21]. Thus, the use of OAA characterized the function of anti-capsule antibodies and greatly facilitated testing of our vaccine candidate.

PROTOCOL:

In compliance with the Animal Welfare Act, Public Health Service policy, and other federal statutes and regulations pertaining to animals and experiments involving animals, the research described here was conducted under an Institutional Animal Care and Use Committee–approved protocol. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011[24].

1. Culture and maintenance of the cell line, RAW 264.7

NOTE: The following procedures must be performed with aseptic techniques.

1.1. Heat fetal bovine serum (FBS) in a 56 °C water bath for 30 min to inactivate complement.

NOTE: The inactivation time of 30 min begins when FBS reaches 56 °C. To monitor the temperature, heat a similar volume of water in a beaker in the same water bath. Once the water reaches 56 °C, begin the 30 min countdown for FBS. Alternatively, FBS that have been heat-inactivated are also commercially available.

1.2. Prepare medium for RAW 264.7 cells by combining 90% DMEM with high glucose and 10% heat inactivated FBS (v/v). Add L-glutamine to a final concentration of 6 mM.

NOTE: DMEM with high glucose is formulated with 4 mM L-glutamine. Supplementing L-glutamine to 6 mM promotes consistent cell growth. 100 U/mL penicillin and 100 µg/mL

streptomycin may be added to prevent contamination. Another common murine cell line, J774A.1, may also be used and is grown under similar conditions.

1.3. Thaw a frozen vial of cells in a 37 °C water bath and remove from bath as soon as it melts. Add content aseptically to 5 mL complete medium in a conical tube. Invert conical tube twice and spin at 300 x *g* for 5 min to pellet cells.

1.4. Aspirate medium using a sterile Pasteur pipet attached to a vacuum to remove freezing agent. Resuspend cell pellet in 5 mL of fresh medium.

1.5. Transfer suspension to a tissue culture treated flask or dish. Add enough medium to completely cover the bottom of the flask and swirl to evenly distribute cells. Indicate passage number starting with "1".

1.6. Incubate cells at 37 °C in the presence of 5% CO_2 and humidity until 95% to 100% confluency is reached. Check cells daily under the microscope beginning with the second day of incubation. Do not allow the cells to reach >100% confluency as this will cause the cells to lift off and die.

NOTE: The time needed to reach confluency depends on how many live cells were plated and the growth area of the flask. Thus, it is prudent to check the cells daily.

1.7. Subculture cells by scraping and diluting the cell stock in fresh medium allowing at least two days of growth between scrapings. Increase the passage number by 1 with each subculture.

NOTE: Passing and immediate scraping the following day will result in faster loss of general cell adherence over time. RAW 264.7 cells should only be used up to approximately passage 15.

1.8. To plate RAW 264.7 cells for the OAA assay, replace with fresh medium and use a cell scraper to gently scrape cells off the flask. Pipette up and down to break up the cell clumps for easier cell counting.

NOTE: The traditional method for subculturing RAW 264.7 is by scraping. In our experience, the use of trypsin causes RAW 264.7 cells to lose adherence over time faster than with scraping.

1.9. To count cells, dilute equal volumes of cells and trypan blue solution. Load 10 µL onto a hemocytometer. Observe and count the number of live cells that exclude the dye using an inverted compound microscope with a 10x objective lens.

1.10. Plate 1.4 x 10^4 cells per well in a 96 well 0.15 µm-thick glass flat bottom plate (Plate #1). Allow cells to grow for 3 days. Replace spent medium one day before performing OAA.

NOTE: After 3 days of incubation, the number of cells should be approximately 5 x 10^4/well.

2. Bacterial Culture and Preparations

NOTE: The bacterial species used in this protocol is an encapsulated virulent strain, *B. anthracis* Ames. All manipulations with this species require appropriate biosafety and security clearances and must be performed in a Class II or Class III biological safety cabinet located in a BSL-3 laboratory. Follow institutional operating procedures for BSL-3 work. Follow institutional guidelines for use of personal protective equipment when handling bacteria.

2.1. Prepare Brain Heart Infusion (BHI) broth by dissolving 37 g BHI powder in 1 L water and autoclaving at 121 °C for 15 min. Store broth at ambient temperature.

2.2. Prepare 8% sodium bicarbonate solution in water and sterilize using a 0.20 µm filter syringe. Store solution at 4 °C and use within 1 day.

2.3. Dissolve 8 g Nutrient Broth, 3 g yeast extract and 15 g agar in 1 L water to prepare NBY agar plates. Autoclave at 121 °C for 15 min. Pour into petri dishes and allow to solidify. Store plates at 4 °C.

NOTE: NBY agar plates may be made with or without sodium bicarbonate. The addition of sodium bicarbonate to broth and agar plates induces the growth of mucoid colonies consisting of encapsulated bacilli. Final concentration of sodium bicarbonate in both liquid and solid media is 0.8%.

2.4. Prepare culture broth for *B. anthracis* by mixing 50% autoclaved BHI broth, 40% FBS and 10% sodium bicarbonate solution (v/v).

NOTE: This broth is formulated to produce short chains of encapsulated bacilli.

2.5. Prepare a master plate of *B. anthracis* by streaking it for isolation on an NBY agar plate from a spore stock one day before culturing in broth. Incubate at 37 °C.

2.6. Inoculate 30 mL of culture broth in a vented 250 mL flask with several colonies of *B. anthracis*.

NOTE: A specific volume to surface ratio of broth, as specified here, is needed to produce encapsulated bacilli.

2.7. Shake the broth culture at 125 rpm, 37 °C with 20% CO_2 and humidity for 18–24 h.

NOTE: Growing *B. anthracis* at temperatures greater than 37 °C may inhibit capsule production.

2.8. Use negative staining with India ink to ensure sufficient encapsulation and that bacilli are in short chains (1–5 bacilli/chain) prior to fixation (see section 3).

2.9. To determine colony forming units (CFU), serially dilute 100 µL of culture 1:10 in Phosphate Buffered Saline (PBS) solution and plate culture dilutions on sheep blood agar plates. Incubate for 12–18 h at 37 °C. Count CFUs and determine number of bacteria per mL of culture.

NOTE: Counting can also be performed using a hemocytometer under the microscope once the bacteria have been fixed. However, this is not recommended because the bacilli are difficult to see under low magnification.

2.10. Add 16% paraformaldehyde (PF) to the entire volume of bacterial culture at a final concentration of 4% PF to fix the bacilli. Slowly agitate on a shaker at ambient temperature for 7 days.

NOTE: Seven days of incubation in 4% PF is based on the USAMRIID institutional standard operating procedure for fixing vegetative bacilli.

2.11. To remove the fixative and test for sterility, thrice wash 4 mL (10% volume) of fixed bacterial suspension in a 50 mL conical tube by centrifugation at ≥3000 x *g* for 45 min and using a 30 mL/wash with PBS. Store the remaining sample in 4% PF at 4 °C.

NOTE: After pelleting, the bacterial pellet is fluffy due to the presence of capsule. Take care not to disturb the pellet during washing. It is necessary to remove all fixative so that it does not interfere with the viability testing. If necessary, increase the number of washes.

2.12. For viability testing, inoculate 40 mL of a bacterial growth medium (e.g. BHI broth) with 4 mL of washed suspension (≤ 10% of broth volume) and incubate at 37 °C for 7 days. Check optical density at 600 nm before and after 7 days to measure turbidity.

2.13. After 7 days of incubation in broth culture, plate ≥100 µL of broth onto an agar plate and incubate for another 7 days. If no increase in turbidity and no growth on plates are seen, the original culture is considered sterile and may be transferred to a BSL-2 laboratory.

NOTE: The Federal Select Agent Program[22] mandates that inactivated *B. anthracis* can only be transferred from BSL-3 laboratories after demonstrating complete sterility of sample. Viability testing for inactivated *B. anthracis* consists of culturing 10% of the sample in broth for 7 days followed by culturing on solid medium for another 7 days[22,23]. Follow institutional guidelines to receive transfer approval once sterility is established.

3. **Negative staining and light microscopy to observe encapsulation and bacilli chains**

3.1. Mix 5 µL bacterial suspension with 2 µL of India ink on a microscope slide. Place a 1 or 1.5 µm thick cover glass on top of the suspension without creating bubbles.

NOTE: Nail polish may be used to seal the cover glass onto the slide.

3.2. Observe the bacterial suspension using a 40x to 100x oil objective lens on a light microscope. Ensure that the bacilli are encapsulated by observing a zone of clearance (capsule excludes India ink) surrounding each bacterium and that the bacilli chains are short.

4. FITC-labeling of bacteria

4.1. Dissolve fluorescein isothiocyanate (FITC) in dimethyl sulfoxide at a concentration of 2 mg/mL.

4.2. Measure the volume of inactivated bacterial stock.

4.3. Wash the stock 3x in PBS to remove fixative.

4.4. Add FITC solution at a final concentration of 75 µg/mL. Slowly agitate mixture for 18–24 h at 4 °C.

4.5. Thrice wash the bacteria by pelleting at ≥3000 x g, 45 min and using a 30 mL PBS/wash to remove excess FITC. Ensure that the fluffy pellet is not disturbed during washing. Resuspend the bacilli in PBS in the same start volume.

4.6. Aliquot and store the bacilli in microtubes at -20 °C until use. Do not freeze/thaw bacilli multiple times as the bacilli may lose capsule.

5. **Bacterial Opsonization**

5.1. Heat inactivate a small volume of test sera (from NHPs) at 56 °C for 30 min in a heat block.

5.2. Thaw and wash FITC-labelled bacteria with PBS 2x by pelleting at 15000 x *g* for 3–5 min. Resuspend in PBS in the same start volume.

5.3. In a 96 well round bottom plate (plate #2), serially dilute test sera in cell medium. In another 96 well round bottom plate (plate #3), add 10 µL of diluted test sera into each well.

NOTE: In every plate, PBS and normal monkey sera were included in place of diluted test sera as negative controls. We also chose one test serum as a positive control to generate a standard curve to normalize all assays done on different days. The positive control test serum was arbitrarily chosen because it was plentiful.

5.4. To plate #3, add 10 µL freshly reconstituted baby rabbit complement.

NOTE: Once reconstituted, discard remaining baby rabbit complement; do not refreeze and thaw.

5.5. To plate #3, add the appropriate volume of FITC-labeled bacilli for a multiplicity of infection of 1:20. For example, add the volume that contains 5 x 10^4 x 20 bacilli for 5 x 10^4 cells. Top off each well in plate #3 with the appropriate volume of cell medium to total 105 µL.

NOTE: Plate #1, which contains cells, receives 100 µL of opsonized bacteria with the remaining 5 µL as the void volume. We tested each dilution of the test sera in duplicate.

5.6. Incubate plate #3 at 37 °C for 30 min in the presence of 5% CO_2 with humidity to opsonize bacilli.

6. Adherence Assay and Fluorescent Labeling of Mammalian cells

6.1. Maintain all reagents at 37 °C prior to use.

6.2. Place plate #1, which contains adherent cells, on a 37 °C heat block.

6.3. Use a multichannel pipettor to remove spent medium from wells and quickly replace with 100 µL of opsonized bacteria from plate #3. Ensure no bubbles have been generated in the wells during pipetting. Incubate plate #1 at 37 °C with 5% CO_2 and humidity for 30 min for adherence.

6.4. Wash each well 5x with 150 µL PBS on top of a 37 °C heat block to remove unattached bacilli.

NOTE: Care must be taken to ensure that the cells are not accidentally dispersed during washing.

6.5. Dilute 16% PF with PBS 1:4 to make 4% PF. Fix cells with 4% PF for 1 h by adding 150 µL to each well. Wash cells 2x with PBS.

6.6. Mix 2 µL HCS (high-content screening) Orange Cell Stain in 10 mL PBS. Add 150 µL of this staining solution to fixed cells and incubate for 30 min at ambient temperature.

6.7. Wash wells 2x with PBS.

6.8. Store at 4 °C until microscopy.

7. Microscopy

NOTE: In general, a high-throughput automated fluorescence microscope will facilitate imaging for the OAA. If an automated system is not available, fluorescent images of adherent bacilli can be taken manually as exemplified in our previous publication [21]. In our current study [20], adherent bacilli and cell monolayers were imaged using the Zeiss 700 laser scanning microscopy system with specialized equipment; our system was composed of the Zeiss Axio Observer Z1 inverted microscope equipped with an automated stage, the Definite Focus module, 40 x NA 0.6 objective, Axio Cam HRc camera and Zen 2012 (Blue Edition) software. The images acquired are widefield images, not confocal images. As this system configuration is likely not available in other

laboratories, the following protocol is intended as a basic microscope setup that is applicable to a variety of automated microscopy systems.

7.1. Incubate plate #1 at ambient temperature for 30 min. Turn on the microscope and related equipment.

NOTE: Acclimation at ambient temperature prevents condensation from forming on the glass plate during imaging. In addition, it prevents defocusing due to temperature shift.

7.2. Place plate on the stage and focus on the cells using bright field or phase contrast.

7.3. Select 96 well format as the plate setting. Select channel settings.

NOTE: FITC's peak excitation and emission wavelength are 495/519 nm; the HCS orange cell stain are 556 and 572 nm. Other fluorophores may be used depending on the filters that are available on the microscope.

7.4. Select setting to Tile mode. Indicate the number of images to be acquired.

NOTE: The tiling function allows the capture of multiple locations in a sample well and can be set to acquire image in a tiling or a random format. We imaged 10 random areas per well.

7.5. Change acquisition mode to "black & white" or "monochrome" and binning ≥ 2x2.

NOTE: Setting the binning from 1x1 to 2x2 or 4x4 would increase microscopy speed but would also result in lower resolution of the image. For OAA, it is sufficient to use these settings as the assay enumerates both the macrophages and the adherent bacteria.

7.6. Turn on autofocus or focusing module. Indicate how often autofocusing function is to be performed. Turn on Z stack function, if available. Indicate the number of Z stacks that will capture the thickness of the cell monolayer and adherent bacilli.

NOTE: The number of Z stacks chosen will increase microscopy time but will ensure an image with the correct focus is taken at each Z stack.

7.7. Designate a file folder to save images to. Run the automated imaging program.

8. Data Collection and Analysis

8.1. Count the number of adherent bacilli and mammalian cells per image. Count each chain of bacilli as one bacterium.

8.2. Plot the bacilli/cells of the positive control test serum and titer (e. g., 1:10 dilution is 10 titer units) to generate a standard curve in an appropriate statistics program.

8.3. Analyze the positive control test serum using non-linear regression curve fitting. Then analyze the remaining samples using the standard curve of the positive control test serum to determine corresponding titers.

NOTE: It is usually most accurate to choose the sample dilutions near the middle of the standard curve when determining titers.

8.4. Calculate the geometric mean of the samples from each vaccine group. Calculate the P-values of log transformed titers by least significant difference ANOVA analysis.

REPRESENTATIVE RESULTS:

This section shows representative micrographs collected during an OAA experiment along with results that show that the OAA can be used to examine the biological function of antibodies. Here, the assay was successfully used to evaluate the efficacy of an anthrax vaccine candidate. It is critical to verify the state of encapsulation on the bacilli as little to no encapsulation causes them to adhere to host cells, producing a high background. **Figure 1** is an image of *B. anthracis* Ames negatively stained with India ink to examine encapsulation. OAA requires multiple adjacent fields of view to be illuminated and if confocal microscopy is used, multiple stacks or sections. Thus, it is necessary to verify that the bacilli are neither too dim nor photo-bleaches too quickly. **Figure 2** is an image of the bacilli labeled with FITC. **Figure 3** shows maximum projection images of *B. anthracis* Ames attached to the cell monolayers. These are representative fields of view that were counted and scored for the OAA. The increase in attachment of bacilli incubated with PGGA-OMPC antiserum is due to the presence of anti-capsule antibodies that opsonized the bacilli. **Figure 4** shows that the serum titers of NHPs vaccinated with 10 and 50 µg PGGA-OMPC are significantly higher than the titers for OMPC and PGGA alone.

FIGURE LEGENDS:

Figure 1. An India ink image of fixed *B. anthracis* Ames. Note the zone of clearance surrounding the bacilli due to the presence of capsule. Bar = 10 µm. The image was taken on the EVOS FL automated microscopy system with 100 x 1.4 numerical aperture (NA) oil objective lens.

Figure 2. Images of FITC labeled fixed *B. anthracis* Ames. (Left) Differential interference contrast image; fluorescent image pseudo-colored in green (middle); merged (right). Bar = 10 µm. Confocal images were taken on the Zeiss 700 LSM Confocal Microscopy with 40 x 1.3 NA oil objective lens.

Figure 3. Fluorescent images of *B. anthracis* Ames adhered to RAW 264.7 cell monolayers. The bacilli were opsonized with test serum from NHPs vaccinated and boosted with (**A**) 10 µg PGGA-OMPC, (**B**) 50 µg PGGA-OMPC, (**C**) 50 µg PGGA alone, or (**D**) OMPC alone. Green = *B. anthracis* Ames, red = RAW 264.7 cells. Bar = 20 µm. Wide field images were taken on the Zeiss Axio Observer Z1 microscope with 40 x 0.6 objective lens and the Axio Cam HRc camera.

Figure 4. Opsono-adherence titers of NHPs vaccinated and boosted with 10 µg PGGA-OMPC, 50 µg PGGA-OMPC, PGGA alone, or OMPC alone. The geometric means of 5 animals per group are graphed. Error bars indicate SEM. *p > 0.001. The p values were calculated using ANOVA with LSD posthoc test. The data were originally published in Wang et al. 2004[20].

DISCUSSION:

Capsule based vaccines have been shown to be efficacious against numerous bacterial pathogens, and many are licensed for use in humans[25-27]. These vaccines work by generating antibodies targeting the capsule and many of these studies use the OPKA to show the opsono-phagocytic functions of the antibodies[13,14,16,28,29]. Due to biosafety concerns and for ease of work, we developed an OAA to evaluate antibodies from animals vaccinated with *B. anthracis* capsule conjugates. The presence of capsule on *B. anthracis* prevents adherence and phagocytosis[30], but opsonization of bacilli with sera from vaccinated animals induces attachment to macrophages[20,21].

For OAA, the adherence activity, not internalization and killing, is used to quantitate opsonic activity. This afforded the OAA several advantages. We were able to utilize paraformaldehyde killed, fluorescently labeled encapsulated bacilli instead of live organisms. Changes to the bacterial surface from aldehyde fixation and fluorescent labeling did not change the bacteria's overall adherence to the macrophages; fixed and labeled bacilli were no more adherent than live bacilli (data not shown). The degree of encapsulation can vary from culture to culture and affect overall adherence despite similar growth conditions. Thus, the use of a single inactivated bacterial stock allows standardization across experiments performed on different days and across different sets of experiments. This is valuable for comparing sera from the 20 NHPs used in this work and sera that will be generated in our upcoming studies. Last, most macrophages, including RAW 264.7 cells, are sensitive to the anthrax lethal toxin produced by the live pathogen[31], making the use of live bacilli inherently problematic.

The use of RAW 264.7 cells also facilitated standardization. We initially used a commonly used cell line in OPKA, HL-60 cells. However, we found difficulties in differentiating them to granulocytes with dimethyl-formamide, as reported by others[28,32]. RAW 264.7 cells have the advantage that they do not need to be chemically differentiated and are adherent and thus more amenable to microscopy-based OAA. This cell line has been used in numerous phagocytosis studies with intracellular pathogens[33,34], including *B. anthracis*[31]. The use of RAW 264.7 cells, which are derived from mouse, is also advantageous because mouse Fc receptors are promiscuous in their binding specificity for IgG derived from other species[35]. This allowed us to evaluate the anti-capsule antibodies from NHPs with a murine cell line.

Anthrax, although very rare, is endemic in some regions of the world and the U.S. and a concern is that our FBS lot, sourced from the U.S., may already contain anti-capsule antibodies as a result of the animal being exposed to *B. anthracis* spores in the soil. To address this issue, we tested the FBS used. The addition of heat inactivated FBS does increase adherence compared to DMEM

alone (data not shown). However, FBS did not increase adherence compared with heat inactivated normal sera from guinea pig, monkey, mouse or human (data not shown). Thus, the FBS lot we used does not contain anti-capsule antibodies as a result of exposure. In addition, it would also not contain anti-capsule antibodies as a result of the animal being vaccinated with the unencapsulated *B. anthracis* Sterne strain, which lacks the pXO2 plasmid necessary for capsule production. After testing, the particular FBS lot was used for all subsequent OAA.

A limitation of the technique is the task of counting bacilli and cells by laboratory personnel, which took an enormous amount of time and effort. This can be rectified with the use of software that has this type of analysis; this software was not available to us at the time. However, because manual counting was necessary, a critical step was the removal or washing off of extraneous and unattached bacilli to facilitate the task. It was also necessary to test reagents that led to lower background noise. OAA requires a source of complement because it enhances opsonization[36]. Therefore, we tested a variety of complement sources including guinea pig, human and baby rabbit complement. We chose baby rabbit complement for our assay because we found that it does not have weak, multi-specific activities against heterophile antigens, it is commonly used in OPKA studies[14,15,37], and it is readily available commercially.

We find the OAA to be quantitative and highly reproducible. We use it to complement studies involving ligand binding assays such as an ELISA, which only quantifies total IgG levels but does not distinguish between functional and non-functional antibodies. OAA can be used to complement other functional assays (e.g., serum bactericidal activity and toxin neutralization assays) to show the different functionality of vaccine generated antibodies[16,38]. The development of OAA has increased our repertoire of non-clinical immunogenicity studies to evaluate our vaccines and therapeutics.

DISCLOSURES:

The authors do not have a commercial or other association that might pose a conflict of interest.

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J. Chua, D. Chabot and A. Friedlander designed the procedures described in the manuscript. J. Chua and T. Putmon-Taylor performed the experiments. D. Chabot performed data analysis. J. Chua wrote the manuscript.

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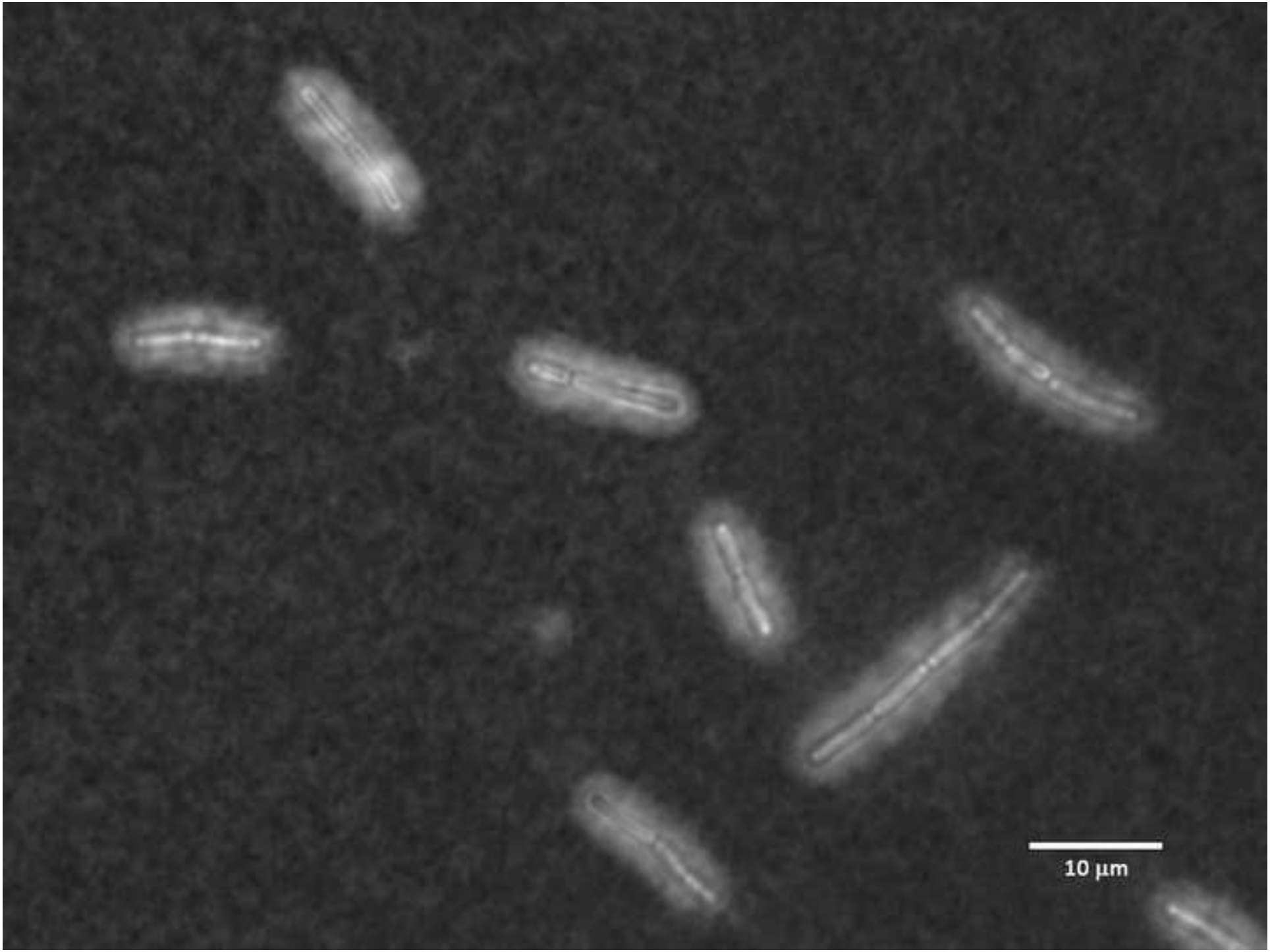

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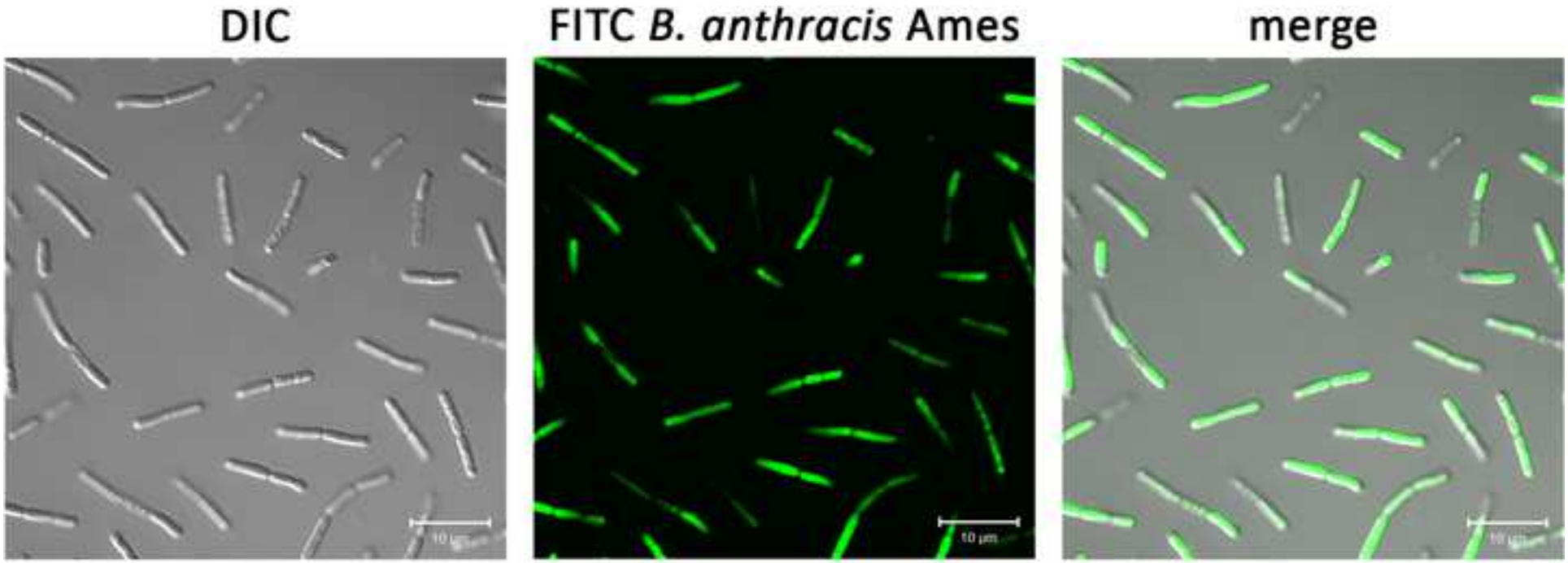

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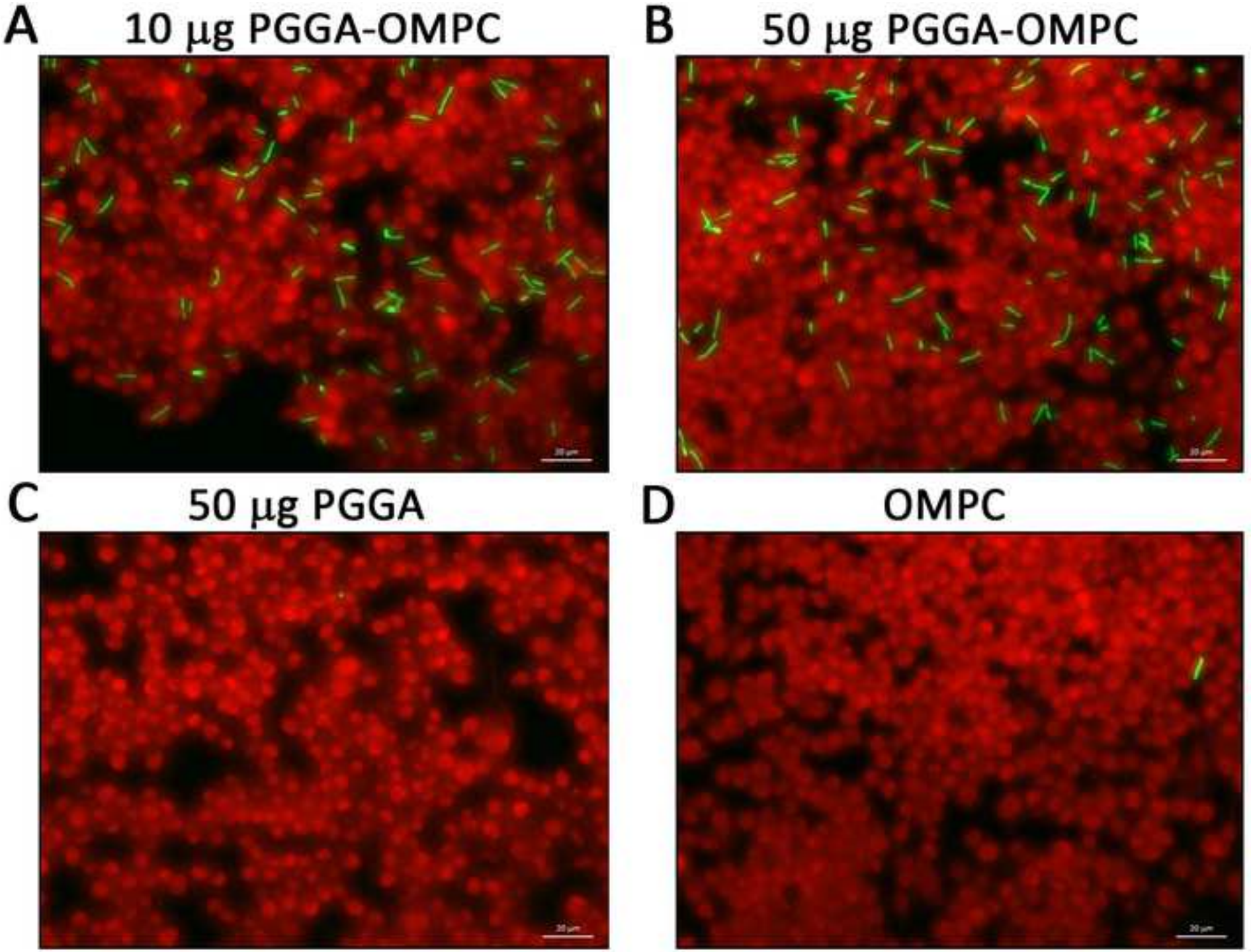

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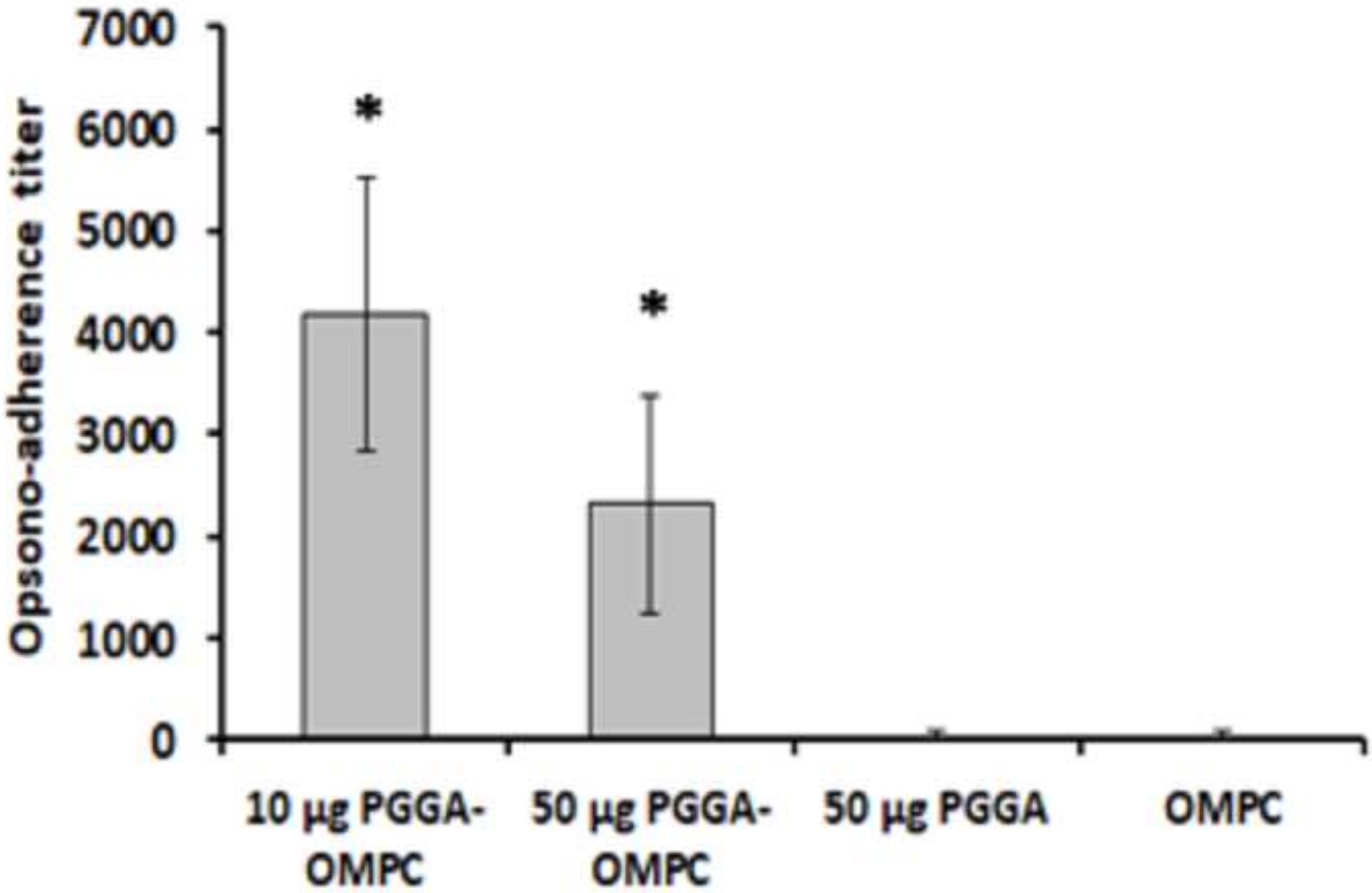

NAME

0.20 µm syringe filter (25mm, regenerated cellulose)
10 mL syringe (Luer-Lok tip)
15µ 96 well black plates (plate #1 for imaging)
16% paraformaldehyde
75 cm sq. tissue culture treated flask
Agar (powder)
Baby Rabbit Complement
Bacto Yeast Extract
BBL Brain Heart Infusion (BHI)
Blood Agar (TSA with Sheep Blood) plates
Cell scraper
Costar 96 well cell culture plates (plates #2 & 3 for dilutions)
Cover glass
Difco Nutrient Broth
Dulbecco's Modified Eagle Medium (DMEM), high glucose
EVOS FL Auto Cell Imaging System (fluorescence microscope)
Fetal Bovine Serum
Fluorescein isothiocyanate
HCS Cell Mask Orange Cell Stain
hemocytometer (Improved Neubauer)
India Ink solution
L- glutamine (200 mM)
Nikon Eclipse TE2000-U (inverted compound microscope)
PBS without Calcium or Magnesium
Penicillin-Streptomycin Solution, 100x
petri dishes (100 x 15 mm)
RAW 264.7 macrophage cell line (Tib47)
Slides
Sodium Bicarbonate
Trypan Blue Solution (0.4%)
Zeiss 700 Laser Scanning Microscopy (confocal microscope)

COMPANY	CATALOG #
Corning, Corning, NY	431222
BD, Franklin Lakes, NJ	302995
In Vitro Scientific, Sunnyvale, CA	P96-1-N
Electron Microscopy Science, Hatfield, PA	15710
Corning, Corning, NY	430641
Sigma-Aldrich, St. Louis, MO	A1296
Cedarlane Labs, Burlington, NC	CL3441
BD, Sparks, MD	288620
BD, Sparks, MD	211059
Remel, Lenexa, KS	R01198
Sarstedt, Newton, NC	83.183
Corning, Corning, NY	3596
Electron Microscopy Science, Hatfield, PA	72200-10
BD, Sparks, MD	234000
Gibco, Thermo Fisher Scientific, Waltham, MA	11965-092
Life Technologies, Thermo Fisher Scientific, Waltham, MA	AMAFD1000
Hyclone, GE Healthcare Life Sciences, South Logan, UT	SH30071.03
Invitrogen, Thermo Fisher Scientific, Waltham, MA	F143
Invitrogen, Thermo Fisher Scientific, Waltham, MA	H32713
Hausser Scientific, Horsham, PA	3900
BD, Sparks, MD	261194
Gibco, Thermo Fisher Scientific, Waltham MA	25030081
Nikon Instruments, Melville, NY	TE2000
Lonza, Walkersville, MD	17-516F
Hyclone, GE Healthcare Life Sciences, South Logan, UT	SV30010
Falcon, Corning, Durham, NC	351029
ATCC, Manassas, VA	ATCC TIB-71
VWR, Radnor, PA	16004-422
Sigma-Aldrich, St. Louis, MO	S5761
Sigma-Aldrich, St. Louis, MO	T8154
Carl Zeiss Microimaging, Thornwood, NY	4109001865956000

COMMENT

contains 4500 mg/L glucose, 4 mM L-glutamine, Phenol Red

not gamma irradiated, not heat inactivated

supplement medium with additional 2mM L-glutamine

for agar plates

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