2010

A flow cytometric assay to quantify *in vivo* bacterial uptake by alveolar macrophages

Adam M. Pitz
Creighton University School of Medicine

Greg A. Perry
Creighton University School of Medicine, gperry@creighton.edu

Heather C. Jensen-Smith
Creighton University School of Medicine

Martha J. Gentry-Nielsen
Creighton University School of Medicine, mgentry@creighton.edu

Journal of Microbiological Methods 81 (2010) 194–196

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Note

A flow cytometric assay to quantify *in vivo* bacterial uptake by alveolar macrophages

Adam M. Pitz [a], Greg A. Perry [a], Heather C. Jensen-Smith [b], Martha J. Gentry-Nielsen [a,c,*]

[a] Department of Microbiology and Immunology, Creighton University School of Medicine, 2500 California Plaza, Omaha, NE 68178, USA
[b] Department of Biomedical Sciences, Creighton University School of Medicine, 2500 California Plaza, Omaha, NE 68178, USA
[c] Research Service, Omaha Veterans Affairs Medical Center, 4101 Woolworth Avenue, Omaha, NE 68105, USA

ARTICLE INFO

Article history:
Received 11 September 2009
Received in revised form 8 February 2010
Accepted 11 February 2010
Available online 18 February 2010

Keywords:
Animal Models
Host Defense
Human
Lung
Phagocytosis

ABSTRACT

Our laboratory has developed a flow cytometric assay to quantify alveolar macrophage (MΦ) phagocytosis of bacteria within a live animal. MΦs collected by bronchoalveolar lavage from rats infected transtracheally with Syto 9-labeled bacteria are fluorescently labeled for identification and analyzed by flow cytometry to quantify their bacterial uptake.

Recent studies have utilized flow cytometry to measure macrophage (MΦ) phagocytosis of microorganisms (Stojkovic et al., 2008; Luo et al., 2006; Gille et al., 2006; Shida et al. 2006; Utaisincharoen et al., 2003). However, quantifying alveolar MΦ phagocytosis by flow cytometry is problematic due to their high autofluorescence. Additionally, MΦ phagocytosis assays performed *in vitro* with pre-opsonized or modified (e.g. heat-killed) bacteria to facilitate uptake (Medeiros et al., 2009; Fitzpatrick et al., 2008; Brown et al., 2007; Kudo et al., 2004) are limited by not adequately mimicking the biological environment within the infected lung. We therefore developed an *in vivo* assay using live, non-opsonized organisms that allows examination of uptake within the lungs as well as detection of underlying host immune defects leading to reduced opsonization and/or phagocytosis during pulmonary infections. Our assay described herein has the potential for adaptation to other pathogens, other animal models, and even *in vitro* human studies.

Staphylococcus aureus 29213, *Streptococcus pneumoniae* 6303, *Klebsiella pneumoniae* 35657, and *Escherichia coli* 25922 (American Type Culture Collection, Rockville, MD, USA) were grown on sheep blood agar plates (Remel, Lenexa, KS, USA) for 16 hours at 37 °C in 5% CO_2. Organisms suspended in sterile water to an optical density of 1.0 at 540 nm (~3×10^8 colony forming units (cfu)/ml) were incubated at room temperature for 15 min in the dark with 10 nM Syto 9 (Molecular Probes, Eugene, OR, USA). They were washed twice to remove excess dye and resuspended in sterile water. A FACSAria cytometer (Becton Dickinson, San Jose, CA, USA) with a 530/30 band pass filter was utilized to detect Syto 9 fluorescence. Syto 9 labeled staphylococci effectively, causing them to fluoresce more intensely than the autofluorescence of rat alveolar MΦs (Fig. 1A). Syto 9 did not alter the viability of the bacteria, as shown by comparison of plate counts performed before and after staining (7×10^8 cfu/ml vs. 5×10^8 cfu/ml, respectively). To demonstrate that our assay could be adapted to measure phagocytosis of other common bacteria, we used Syto 9 to directly stain *S. pneumoniae*, *K. pneumoniae*, and *E. coli*, all of which stained more brightly than rat MΦs (Fig. 1B-D). Several other fluorescent dyes were evaluated, but failed due to leakage (DAPI and Hoechst 33258), low fluorescent intensity (CT Blue CMAC and Qdot 800) or the need for species-specific reagents (APC-Cy7).

Pathogen-free, male Sprague-Dawley rats (Charles River Laboratories, Kingston, NY, USA) were infected transtracheally with 1×10^8 cfu of Syto 9-labeled staphylococci under light anesthesia as described previously (Vander Top et al., 2006). To prevent the loss of bacterial fluorescent intensity due to bacterial lysis within the MΦs, the rats were euthanized exactly 15 minutes post-infection by an intraperitoneal injection of 75 mg/kg body weight of pentobarbital (Nembutal, Abbott Laboratories, Abbott Park, IL, USA) followed by exsanguination. *Ex vivo* bronchoalveolar lavage was performed to collect pulmonary cells from the rats' lungs as described previously (Preheim et al., 1991). The recovered cells were stained with phycoerythrin (PE)-conjugated antibody (RP-1; BD Pharmingen, San Diego, CA, USA) to label neutrophils and a biotinylated antibody

* Corresponding author. Department of Microbiology and Immunology, Creighton University School of Medicine, 2500 California Plaza, Omaha, NE 68178, USA. Tel.: +1 402 280 2346; fax: +1 402 280 1875.
E-mail address: mgentry@creighton.edu (M.J. Gentry-Nielsen).

0167-7012/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2010.02.005

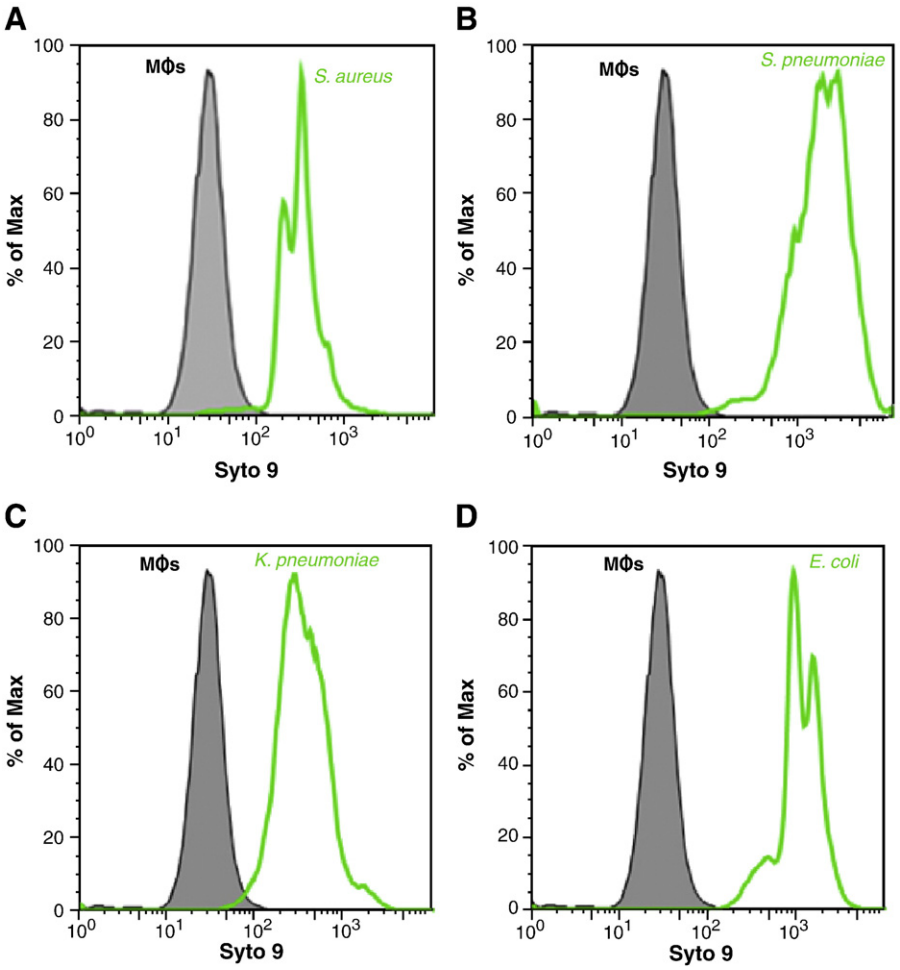

Fig. 1. Syto 9-labeled bacteria. *S aureus* (A), *S. pneumoniae* (B), *K. pneumoniae* (C), and *E. coli* (D) were successfully labeled with Syto 9 and flouresced more intensely than MΦ autofluorescence.

(1C7; BD Pharmingen) followed by streptavidin-allophycocyanin (APC) (BD Pharmingen) to label MΦs (Vander Top et al., 2006).

Three-color flow cytometric analysis was performed using dual laser excitation (488 nm and 637 nm). On each assay day, MΦs collected from a control rat infected with non-fluorescent staphylococci were used to exclude autofluorescence in the Syto 9 channel. Based on scatter characteristics, a minimum of 10,000 cells were analyzed in the APC (660 nm) channel for MΦs and PE (575 nm) channel for neutrophils. The percentage of MΦs with associated bacteria was determined as the percentage of APC positive cells fluorescing brighter in the Syto 9 (530 nm) channel than MΦs from the control rat, as analyzed using FlowJo Software (Tree Star, Ashland, OR, USA).

Confocal microscopy was performed to ensure that staphylococci in positive MΦs had been internalized rather than bound to the cell surface. Cytospin slides prepared from lavage samples of each rat were examined with a scanning confocal microscope (Zeiss Micro-Imaging, Thornwood, NY, USA). Syto 9-labeled staphylococci were imaged using 488 nm excitation and a band pass filter of 500-530 nm. A Chameleon ULTRA titanium:sapphire laser (Coherent, Santa Clara, CA, USA) was used for two-photon excitation (760 nm) of DAPI-stained MΦ nuclei (435-485 nm band pass filter). Syto 9-labeled staphylococci were observed throughout MΦs that were optically sectioned at 0.82 μm intervals. Individual MΦ images in the same focal plane as MΦ nuclei indicated the presence or absence of cytoplasmic bacteria as depicted in Figs. 2A and B, respectively. Fewer than 2% of the positive cells demonstrated 1-2 surface-associated bacteria, verifying that the uptake measured was from phagocytosed organisms.

Manual counts of cytospin slides stained with hematoxylin and eosin also were performed to confirm the MΦ phagocytosis values determined by flow cytometry. Two hundred cells from each sample were counted independently by two technicians using a light microscope to quantify the percentage of MΦs containing phagocytosed staphylococci (Fig. 2C) vs. those without internalized bacteria (Fig. 2D). The flow cytometry and light microscopy phagocytosis values from 17 rats were compared by regression analysis (Fig. 3). The mean percent of MΦ phagocytosis determined by flow cytometry was 23.5% and by manual counting was 16.6%, with a Spearman's rank order correlation coefficient of 0.63 ($p = 0.007$). Manual counts of MΦs from control rats infected with unstained bacteria also were similar, indicating that Syto 9 did not affect staphylococcal virulence or adherence.

In conclusion, we have developed a flow cytometric assay to quantify bacterial uptake by MΦs *in vivo*. The *in vivo* assay allows the determination to be made within the lungs of experimental animals during an actual infection. Although the assay was developed with *S. aureus* in rat lungs, successful staining of additional bacterial species indicates it can be adapted to other microorganisms and alternative *in vivo* animal models, as well as to quantification of uptake by human alveolar MΦs *in vitro*.

The authors would like to thank Mary Snitily for her skilled technical assistance in development of the assay. This research was supported by revenue from Nebraska cigarette taxes awarded to

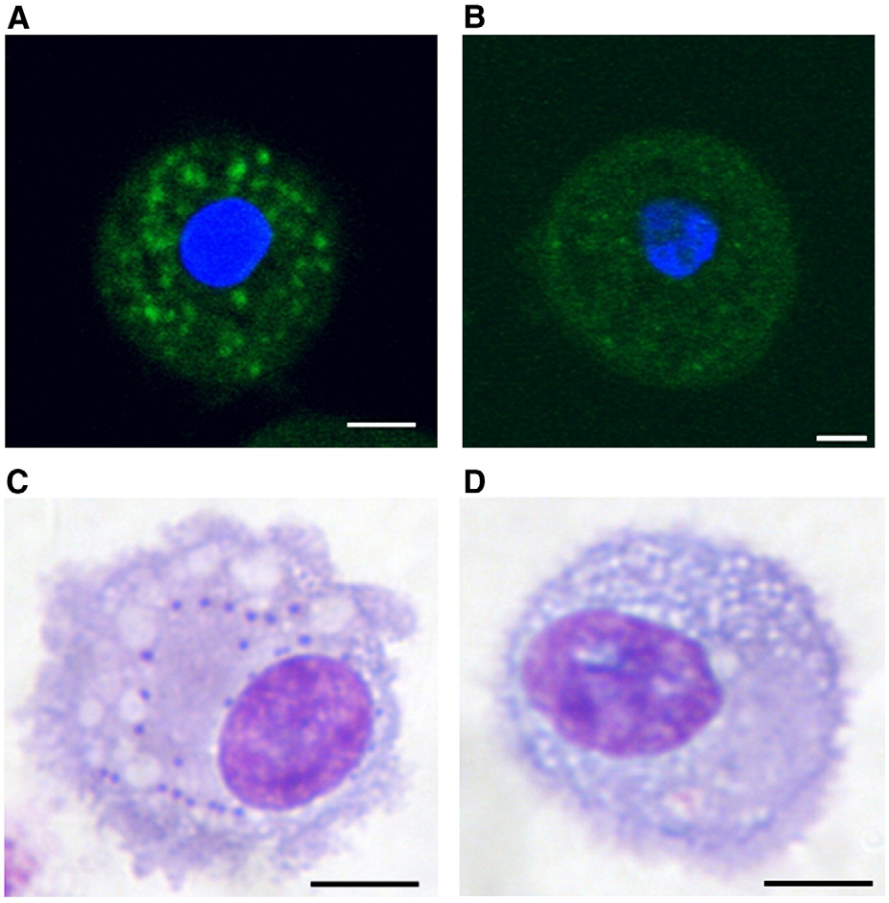

Fig. 2. Microscopic images of MΦs with and without bacteria. A 0.82 μm thick optical slice of a MΦ displays fluorescent puncta of varying sizes indicating the presence of internalized Syto-9-labeled bacteria in multiple cytoplasmic foci (A) compared to a MΦ without bacteria (B). As a comparison to flow cytometry, two hundred cells from each rat were counted by light microscopy to determine the percentage of MΦs that phagocytosed bacteria. A MΦ that ingested bacteria (C) was easily identified from a MΦ that had no bacteria (D). Scale bar = 5 μm.

Creighton University by the Nebraska Department of Health and Human Services. It was performed with resources and the use of facilities at the Omaha Veterans Affairs Medical Center and was approved by the VA Animal Use and Care Committee. Confocal microscopy was conducted at the Integrative Biological Imaging Facility at Creighton University, which is supported by Creighton University School of Medicine and constructed with support from C06 Grant RR17417-01 from the NCRR, NIH.

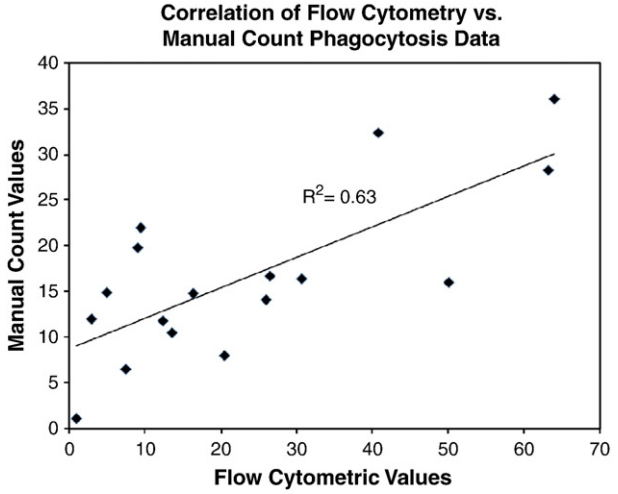

Fig. 3. Comparison of MΦ phagocytosis values as determined by flow cytometry and light microscopy. A strong correlation exists between both values ($p = 0.007$).

References

Brown, L.A., Ping, X.D., Harris, F.L., Gauthier, T.W., 2007. Glutathione availability modulates alveolar macrophage function in the chronic ethanol-fed rat. Am. J. Physiol. Lung. Cell. Mol. Physiol. 292, L824–L832.

Fitzpatrick, A.M., Holguin, F., Teague, W.G., Brown, L.A., 2008. Alveolar macrophage phagocytosis is impaired in children with poorly controlled asthma. J. Allergy. Clin. Immunol. 121, 1372–1378.

Gille, C., Spring, B., Tewes, L., Poets, C.F., Orlikowsky, T., 2006. A new method to quantify phagocytosis and intracellular degradation using green fluorescent protein-labeled *Escherichia coli*: comparison of cord blood macrophages and peripheral blood macrophages of healthy adults. Cytometry 69, 152–154.

Kudo, K., Sano, H., Takahashi, H., Kuronuma, K., Yokota, S., Fujii, N., Shimada, K., Yano, I., Kumazawa, Y., Voelker, D.R., Abe, S., Kuroki, Y., 2004. Pulmonary collectins enhance phagocytosis of *Mycobacterium avium* through increased activity of mannose receptor. J. Immunol. 172, 7592–7602.

Luo, Y., Cook, E., Fries, B.C., Casadevall, A., 2006. Phagocytic efficacy of macrophage-like cells as a function of cell cycle and Fcgamma receptors (FcgammaR) and complement receptor (CR)3 expression. Clin. Exp. Immunol. 145, 380–387.

Medeiros, A.I., Serezani, C.H., Lee, S.P., Peters-Golden, M., 2009. Efferocytosis impairs pulmonary macrophage and lung antibacterial function via PGE2/EP2 signaling. J. Exp. Med. 206, 61–68.

Preheim, L.C., Gentry, M.J., Snitily, M.U., 1991. Pulmonary recruitment, adherence, and chemotaxis of neutrophils in a rat model of cirrhosis and pneumococcal pneumonia. J. Infect. Dis. 164, 1203–1206.

Shida, K., Kiyoshima-Shibata, J., Nagaoka, M., Watanabe, K., Nanno, M., 2006. Induction of interleukin-12 by Lactobacillus strains having a rigid cell wall resistant to intracellular digestion. J. Dairy. Sci. 89, 3306–3317.

Stojkovic, B., Torres, E.M., Prouty, A.M., Patel, H.K., Zhuang, L., Koehler, T.M., Ballard, J.D., Blanke, S.R., 2008. High-throughput, single-cell analysis of macrophage interactions with fluorescently labeled *Bacillus anthracis* spores. Appl. Environ. Microbiol. 74, 5201–5210.

Utaisincharoen, P., Kespichayawattana, W., Anuntagool, N., Chaisuriya, P., Pichyangkul, S., Krieg, A.M., Sirisinha, S., 2003. CpG ODN enhances uptake of bacteria by mouse macrophages. Clin. Exp. Immunol. 132, 70–75.

Vander Top, E.A., Perry, G.A., Gentry-Nielsen, M.J., 2006. A novel flow cytometric assay for measurement of *in vivo* pulmonary neutrophil phagocytosis. BMC. Microbiol. 6, 61.