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Research paper

A new flow-cytometry-based opsonophagocytosis assay for the rapid measurement of functional antibody levels against Group B Streptococcus

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ABSTRACT

Opsonophagocytosis is the primary mechanism for the clearance of Group B *Streptococcus* (GBS) by the host, and levels of opsonic antibodies may correlate with protection in preclinical models. A killing-based opsonophagocytosis assay (OPA), can be used to determine the functional activity of vaccine-induced GBS-specific antibodies. The assay, which measures the number of bacterial colonies surviving phagocytic killing in the presence of specific antibodies and complement, is rather expensive, time-consuming and poorly standardized.

Here we describe a rapid, sensitive and reproducible fluorescent OPA assay (fOPA) based on flow cytometry analysis (FACS), which allows internalized bacteria to be distinguished from those associated to the plasma membrane of phagocytic cells. Fixed GBS were labeled with pHrodo™, a fluorescent dye which dramatically increases the emitted fluorescence at the acidic conditions present in the phagocytic endosomal compartment. Labeled bacteria were incubated with HL-60 cells differentiated to phagocytes, antibodies and complement, and then analyzed by FACS. A further improvement to our method, allowing to reduce assay variability, consisted on a step of selection of effector cells among the HL-60 population.

Analysis of sera from mice immunized with different GBS vaccines revealed comparable sensitivity and specificity with the traditional killing OPA assay (kOPA), and a good correlation between the fluorescent signal of bacteria internalized by HL-60 phagocytes and killing. Remarkably, the pHrodo-based approach reduced the variability observed with other fOPA assays. The obtained data indicate the proposed fOPA as a reliable and useful tool for functional antibody assessment.

1. Introduction

Streptococcus agalactiae also referred to as Group B *Streptococcus* (GBS), is one of the most common causes of life-threatening bacterial infections in infants. Neonatal GBS infections can result in pneumonia, sepsis, meningitis and, in some cases, death (McCracken, 1973; Ferrieri, 1985; Gibbs et al., 2004). The introduction in the United States of guidelines for neonatal GBS disease prevention, first issued in 1996 and updated in 2002 and 2010, recommending universal screening of pregnant women and intrapartum antimicrobial prophylaxis to carriers, was associated with a decline in the incidence of the Early Onset Disease, (Boyer et al., 1983a,b; Gibbs et al., 1994; Moore et al., 2003; Law et al., 2005), but has not eradicated GBS disease in infants (Schuchat, 2000; Phares et al., 2008). GBS is still a public health concern and the introduction of additional prevention and therapeutic strategies is highly desirable. During the last two decades, polysaccharide-protein conjugate vaccines against GBS have been extensively studied in preclinical and human clinical studies (Baker et al., 1999, 2000, 2003a,b, 2007; Lancaster et al., 2011; Heath, 2011).

An obstacle to the development of vaccines against GBS is the difficulty of conducting clinical efficacy trials, because of the relatively low incidence of neonatal GBS disease. A

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possible solution to overcome this difficulty may come from the development of an effective functional antibody assay as *in vitro* correlate of protection. The most commonly used method to assess functional antibodies to GBS in post-immunization sera is the *in vitro* killing-based opsonophagocytosis assay (kOPA) that mimics the *in vivo* process of killing by host effector cells, following opsonization by specific antibodies (Baltimore et al., 1977; Edwards et al., 1979; Guttormsen et al., 2008). This assay can constitute a viable surrogate of the effectiveness of a GBS vaccine, as passive protection of mice by sera from individuals immunized with GBS polysaccharide-based vaccines correlated with high functional antibody titers measured by OPA (Baltimore et al., 1981; Kasper et al., 1996; Brigtsen et al., 2002). However, bacterial growth, colony plating and counting are time and resource consuming steps and standardization presents challenges due to the source and quality of effector cells and to the variability associated with plating and colony counting. Although cultured phagocytes (differentiated HL-60 cells) can be used in place of human peripheral polymorphonuclear leukocytes (PMNs), as a less variable neutrophil source (Romero-Steiner et al., 1997; Guttormsen et al., 2008,), the fraction of HL-60 cells differentiated to active phagocytes varies, representing a further source of variability.

Fluorescence based OPAs can limit the effort and variability associated with plating and counting of surviving bacteria (Plested and Coull, 2003; Guttormsen et al., 2009; Simons, 2010,). These assays use bacteria labeled with fluorophores, such as fluorescein-derivatives (dicarboxyfluorescein, dihydrodichlorofluorescein, Oregon green), rhodamine or Alexa Fluor derivatives (Rodríguez et al., 2001; Guttormsen et al., 2009) and are rapid and efficient for large scale testing of sera. However, these approaches do not distinguish between adherent and internalized bacteria.

We developed a new flow cytometry based OPA that allows discriminating between adherent and internalized bacteria through the use of pHrodo™, a new pH sensitive fluorogenic dye. Bacteria conjugated to pHrodo™ show a very low fluorescent signal at the neutral pH present on the cell surface, but emit a bright red fluorescence in the acidic environment of phago-lysosomes. This level of discrimination eliminates washing and quenching steps that are necessary with other non pH-dependent indicators of bacterial uptake. Moreover the fOPA here described takes advantage of the introduction of specific markers of HL-60 differentiation to neutrophils, which allow keeping under control the variability of effector cells.

The method was evaluated for sensitivity and specificity, by testing a panel of sera from mice immunized with different GBS glycoconjugate vaccines against polysaccharide Ia. kOPA titers were compared with fOPA titers, and a confocal microscopy analysis was conducted to study bacterial localization inside neutrophils, in the presence or in the absence of specific antibodies and complement.

2. Materials and methods

2.1. Bacterial strains

GBS strains 515 (serotype Ia) (Baker et al., 1982) and COH1 (serotype III) (Wessels et al., 1992) were used in this work. Bacteria were grown in Todd–Hewitt Broth (THB) to an optical density at 600 nm ($OD_{600\ nm}$) of 0.45. Ten percent glycerol was added to the culture before dispensing 1 ml aliquots in cryo-vials for flash freezing in a 95% ethanol-dry ice bath. Frozen cultures were kept at −70 °C until use.

2.2. Antisera

OPAs were performed with rabbit and mouse sera. Rabbit sera were raised by immunizing one animal with three doses of monovalent CRM_{197}-conjugated polysaccharide Ia, Ib and III in presence of aluminum hydroxide (Alum). Mouse sera were pooled from animals immunized with a GBS vaccine composed by polysaccharide Ia, Ib and III conjugated to CRM_{197}, formulated with Alum or MF59 (Podda, 2001).

Animal treatments were performed in compliance with the Italian laws and approved by the institutional review board (Animal Ethical Committee) of Novartis Vaccines and Diagnostics, Siena, Italy.

2.3. Bacterial labeling with pHrodo™

Bacteria were grown in THB to $OD_{600\ nm} = 0.6$, washed twice with Phosphate Buffered Saline (PBS, pH 7.2–7.4,Gibco) and suspended in half volume of PBS-0.08% paraformaldehyde (PFA, Sigma). Cells were incubated at 37 °C for 30 min and kept at 2–8 °C in PBS-0.08%PFA. Immediately before labeling, cells were washed with PBS, suspended at 20 mg (wet weight)/ml using a freshly prepared 100 mM Sodium Hydrogen Carbonate solution pH 8.5 (Merck) and split into aliquots of 750 μl.

A 10 mM stock solution of PHrodo™ Succinimidyl Ester (Invitrogen) in dimethyl sulfoxide (Sigma) was diluted in the bacterial suspension at a final concentration of 0.1 mM. Each sample was incubated for 45 min at room temperature in the dark and then added with 750 μl of Hank's Balanced Salt Solution with Ca^{2+} and Mg^{2+} (HBSS, Gibco), then spin down with a bench top centrifuge for 60 s at 14,000×*g*. The supernatant was aspirated and the pellet suspended in HBSS and stored in the dark at 4 °C for two months. Bacterial labeling was evaluated by FACS. The maximal fluorescence emission of pHrodo™ labeled GBS was 585 nm. The absolute concentration of labeled bacteria was determined by using TruCOUNT tubes (BD pharmingen). The beads contained in each tube were suspended in 100 μl of PBS and added to 100 μl of bacteria diluted 1/100 in PBS. The absolute cell count (N) was calculated using the following equation: N = (number of events in region containing bacteria) (number of beads per test)/(no. of events in absolute count beads region), where the number of beads per test was provided by BD Pharmingen together with TruCOUNT Absolute Count Tubes.

2.4. Binding assays

Labeled bacteria were counted by FACS using truCount Tubes and dispensed in 96 microtiter plates at 5×10^5 cells/well. When live bacteria were tested, 1 ml aliquot of frozen cells ($OD_{600\ nm}$: 0.45–0.5) was thawed at room temperature, diluted in 9 ml of PBS and centrifuged at 3000 rpm for 10 min. The pellet was suspended in 20 ml of HBSS and dispensed in plates (100 μl/well) in order to obtain 5×10^5 bacteria/well. The plate was centrifuged; the pellet was suspended in 100 μl of HBSS-1% normal rabbit serum and incubated for

20 min at room temperature. Cells were then washed and incubated for 1 h at 4 °C in 100 μl of preimmune or immune sera previously diluted 1/50 up to 102,400 in HBSS. After centrifugation and washing with 200 μl of PBS-0.1% Bovine Serum Albumine (BSA, Sigma), samples were incubated for 1 h at 4 °C with 50 μl of Alexa Fluor 647 F(ab′)$_2$ fragment of goat anti mouse IgG (H+L) (Invitrogen) diluted 1/200 in PBS-0.2% BSA. Cells were spun down by centrifugation, washed twice with PBS and suspended in 130 μl of PBS. Fluorescence in the 96 well plates was measured with FACS CantoII flow cytometer (BD Biosciences, San Jose, CA), equipped with a 96-well plate loader.

2.5. Growth and differentiation of HL-60 cells

HL-60, a promyelocytic leukemia cell line, was obtained from the American Type Culture Collection (CCL-240) and was maintained in RPMI 1640 glutamax (Invitrogen), supplemented with 10% heat inactivated Fetal Bovine Serum (FBS, HyClone). Cells were grown and differentiated to neutrophils in growth medium supplemented with 0.78% Dimethyl Formamide (DMF, Sigma), according to Romero-Steiner et al. (1997).

2.6. Killing-based opsonophagocytosis assay (kOPA)

The reaction was performed in 96 well polypropylene microtiter plates (Nunc), in a total volume of 125 μl HBSS. For each reaction mixture, heat inactivated (56 °C for 30 min) test serum (12.5 μl), GBS bacteria (25 μl), differentiated HL-60 cells (75 μl) and baby rabbit complement (12.5 μl, Cederlane) were added using a multichannel pipette. Control reactions were performed in the presence of heat inactivated baby rabbit complement or in the absence of antibodies or effector cells. Further negative controls were performed with preimmune or mock immunization sera.

For each serum sample, six dilutions were tested. The bacterial suspension was prepared by directly diluting frozen aliquot stocks. One ml aliquot of frozen bacteria ($OD_{600\ nm}$: 0.45–0.5) was thawed at RT, diluted in 9 ml of PBS and centrifuged at 3000 rpm for 10 min. The pellet was suspended in an appropriate volume of HBSS in order to obtain a final concentration of ~600 CFU/μl (~6×10 exp 4 CFU/well"). Bacteria were then diluted 1/2 in HBSS + % normal rabbit serum (Sigma) and dispensed in plates. The effector cells to GBS cells ratio varied from 25:1 to 40:1. The reaction plate was incubated for 1 h at 37 °C with shaking at 300 rpm by a Thermomixer (Eppendorf). T0 reactions were diluted 1/100 in sterile water by the aid of an electronic multichannel pipette. T60 reactions were diluted 1/20 and 1/200 in sterile water. Ten microliters of each dilution were then plated in trypticase soy agar plate + 5% blood sheep (Particle Measuring Systems) and plates were incubated over night at 37 °C + 5% CO_2 in order to determine bacterial–counts at T0 and T60.

The OPA titer was expressed as the reciprocal serum dilution leading to 50% killing of bacteria, and percent of killing was calculated as follows: killing (%) = [(mean CFU at T0 − mean CFU at T60)/mean CFU at T0] 100.

2.7. Fluorescent-based opsonophagocytosis assay (fOPA)

The reaction was performed in 96 well polypropylene microtiter plates (Nunc) in a total volume of 125 μl. Heat inactivated serum samples (12.5 μl), 25 μl of pHrodo™ labeled bacteria (1×10^7 bacteria/well) 75 μl of differentiated HL-60 cells (1×10^6 cells/well) and 12.5 μl of 10% baby rabbit complement were mixed. Positive and negative controls followed the same scheme as for the kOPA. The plate was incubated at 37 °C for 30 min and shaking (600 rpm). After incubation, the plate was centrifuged at 1300 rpm for 5 min at 4 °C, the supernatant was discarded and the pellet was washed with 200 μl of PBS. A mixture of LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit for 405 nm excitation (Invitrogen) (final concentration 0.5 μg/ml), V450-anti-human CD11b (BD Horizon, final concentration 4 μg/ml) and FITC-anti-human CD35 (BD Pharmingen, final concentration 2.5 μg/ml) were added to each well for a total volume of 50 μl. The plate was incubated for 30 min at 4 °C in the dark. After washing with PBS, cells were suspended in 130 μl of PBS and samples were analyzed by FACS Canto II flow cytometer equipped with High Throughput System custom refrigerated at 2–8 °C.

Phagocytosis was expressed as: A) Phagocytic activity: mean fluorescent intensity (MFI); B) Percentage of phagocytosis: (number of cells taking up particles)/(total cell number analyzed).

fOPA titers were calculated as the reciprocal of the serum dilution corresponding to the cut off value (twice the mean phagocytic activity in negative controls).

2.8. FACS analysis

Samples were acquired on FACS Canto II flow cytometer equipped with 3-laser system (405, 488, 633 nm), eight color configuration and BD FACS Diva™ v6.1.3 software. The cytometer was checked daily by the Rainbow set up beads (BD Biosciences). The compensation matrix was performed by anti-mouse Ig_K/negative control (FBS) Compensation Particles Set (BD Biosciences) following manufacturer recommendations. BD CompBeads were stained as compensation controls for V450 anti-human CD11b and for FITC anti-human CD35, while pHrodo™ labeled bacteria were used as phycoerythrin (PE) fluorescence to calculate the compensation matrix. The compensation values were calculated automatically by DiVa™ software. The BD High Throughput Sampler (HTS) System was used to run the plate samples. A total of 10,000 events were collected from each sample gated on live cells.

Forward scatter and Side scatter were acquired on a linear scale and fluorescence was acquired on a logarithmic scale. PE and fluorescein isothiocyanate (FITC) were excited using 488 nm laser and the emission of fluorescence was collected using 585/42 nm and 530/30 nm filters, respectively. V450 and LIVE/DEAD Fixable Aqua were excited by 405 laser and fluorescence emission was collected with 450/50 nm and 510/50 nm DF filters. After acquisition, all data were exported as Flow Cytometry Standard format 3.0 files (FCS files) and analyzed by FlowJo (Mac-Version 9.1; Treestar US, Ashland, OR).

2.9. Confocal microscopy

Differentiated HL-60 cells were dispensed in 96 microtiter plates and incubated with labeled bacteria for 30 min in the presence of specific or unrelated serum and baby rabbit complement, under the same conditions and using the same

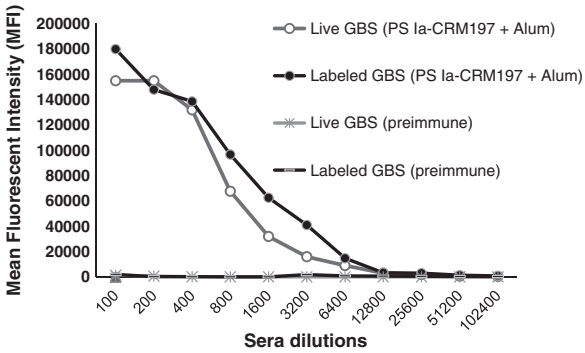

Fig. 1. Flow cytometry analysis of labeled GBS bacteria. PHrodo™ labeled or live-unlabeled GBS Ia cells were incubated with mouse sera against GBS Capsular polysaccharide Ia-CRM_{197} conjugate and a secondary anti-mouse antibody Alexa Fluor 647 conjugate, followed by FACS analysis.

concentration described for the fOPA. After incubation, cells were washed twice with PBS (centrifuging the plate at 900 rpm for 5 min at 2–8 °C) and fixed with 4% PFA in PBS for 5 min at 2–8 °C. After washing, bacteria were pelleted by centrifugation at 900 rpm for 5 min. The plasma membrane was then stained by incubating cells for 30 min at 4 °C with 100 µl of Alexa Fluor 488-phalloidin (0.16 µM, Molecular Probes) solution or concanavalinA-FITC (Sigma) solution in PBS (2 µg/ml). After washing, cells were suspended in 10 µl of SlowFade Antifade kit (Molecular Probes) and mounted on a glass slide. Images were acquired on a Zeiss LSM 710 laser scanning confocal microscope.

2.10. Data analysis

Each experiment was performed in triplicate. Data are represented as mean ± SD. Correlations were analyzed by a linear regression model. Fitting was analyzed with the support of a statistical software (GraphPad Prism 5).

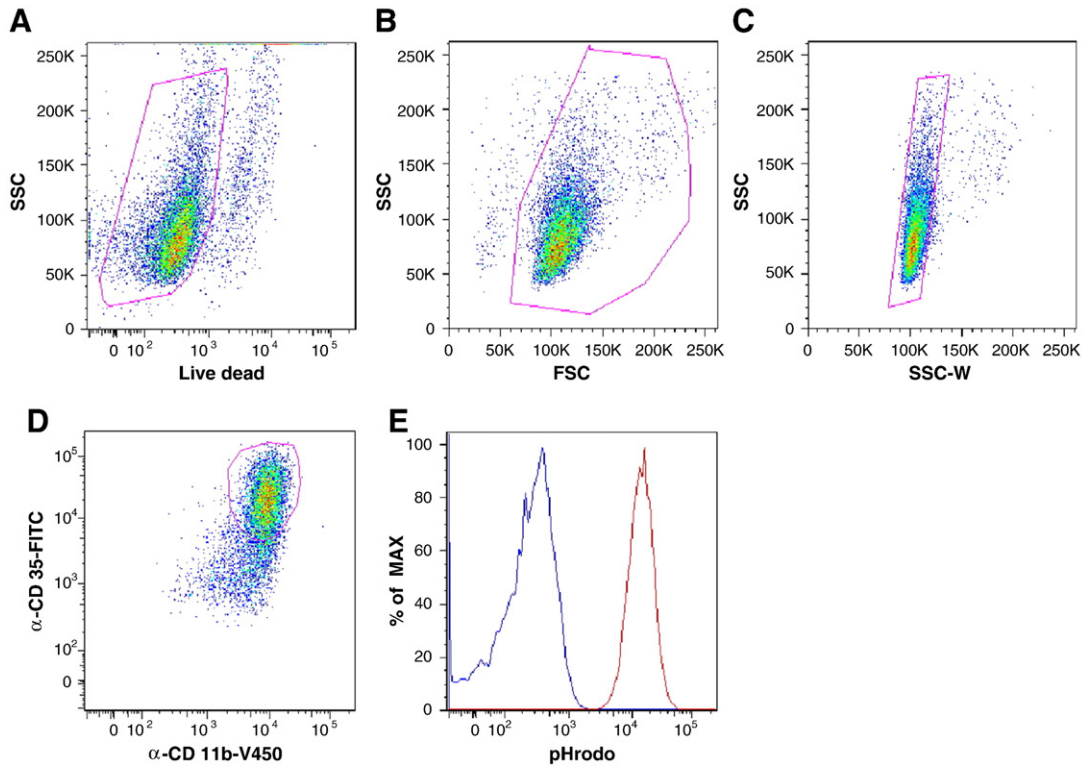

Fig. 2. Gating strategy to analyze fluorescent OPA by FACS. (A) Discrimination between live (gated region) and dead HL-60 cells. (B) Selection of HL-60 cells using forward scatter(FSC) and side scatter (SSC). (C) Discrimination of aggregates from singlets using side scatter-W versus side scatter (SSC-W). (D) Neutrophil identification with specific markers of HL-60 cell differentiation (anti-CD35 and anti-CD11b). (E) Histogram overlay showing the fluorescence shift between the experimental (in red) and negative control (in blue) samples.

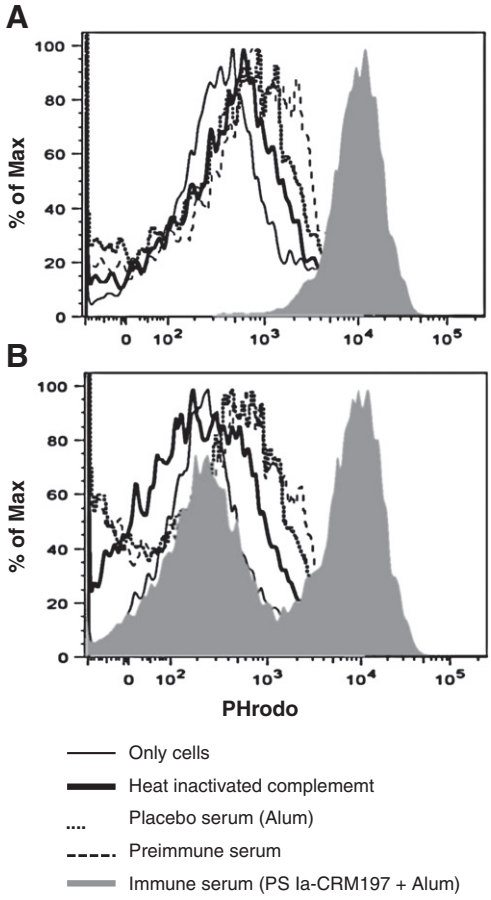

Fig. 3. Improvement of assay performance by introducing markers of HL-60cell differentiation. Histograms show the fluorescent intensity of negative and positive samples collected from fOPA performed with (A) and without (B) antibodies against CD11b and Cd35 receptors.

3. Results

3.1. Fluorescent labeling of bacteria

The amine-reactive succinimidyl ester of pHrodo™ dye was used to label paraformaldehyde (PFA) fixed bacteria via amine groups present on the bacterial cell wall. To optimize bacterial labeling, PFA fixed bacteria were first incubated with 0.1 mM up to 0.9 mM concentrations of pHrodo™. A dye concentration of 0.1 mM, yielded the highest ratio between the mean fluorescence intensities of the positive and the negative controls (data not shown) was chosen for further use.

To assess whether the fixation or conjugation steps altered the integrity of target antigens, labeled GBS Ia bacteria were compared with live bacteria for reactivity with a pool of mouse sera specific for polysaccharide Ia using flow cytometry analysis. As shown in Fig. 1, comparable dose dependent antibody binding was observed for live and labeled bacteria, while incubation with pre-immune sera did not result in any binding. A correlation coefficient (R^2) of 0.95 was obtained for the linear regression derived by plotting the dose dependent fluorescent signals measured for live and labeled bacteria. The data indicated that the integrity of target antigens was maintained after pHrodo™ labeling.

3.2. Flow cytometry-based assay set up

Labeled bacteria were pre-incubated with heat inactivated rabbit serum specific for polysaccharide Ia, followed by HL-60 derived neutrophils and baby rabbit serum as source of complement, as described in the Materials and methods section. To reduce assay variability, fluorescently labeled anti-CD35 and anti-CD11b antibodies were introduced as specific markers of HL-60 cell differentiation to phagocytes. Furthermore, the amine reactive dye LIVE/DEAD® was used to discriminate between live and dead HL-60 cells. This dye can permeate compromised membranes of necrotic cells and

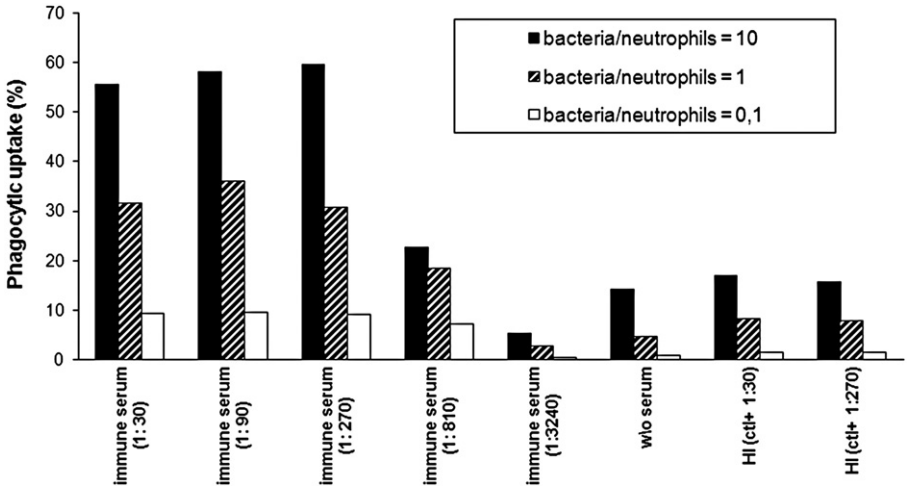

Fig. 4. Opsonophagocytosis results using pHrodo™ labeled GBS type Ia. Differentiated HL-60 cells were incubated for 30 min at 37 °C with different concentrations of pHorodo™ labeled GBS cells preincubated with buffer (w/o serum) or with a rabbit serum against GBS capsular polysaccharide Ia-CRM conjugate formulated with Alum (immune serum). Reaction was performed in the presence of 10% active or heat inactivated (HI) rabbit serum as source of complement.

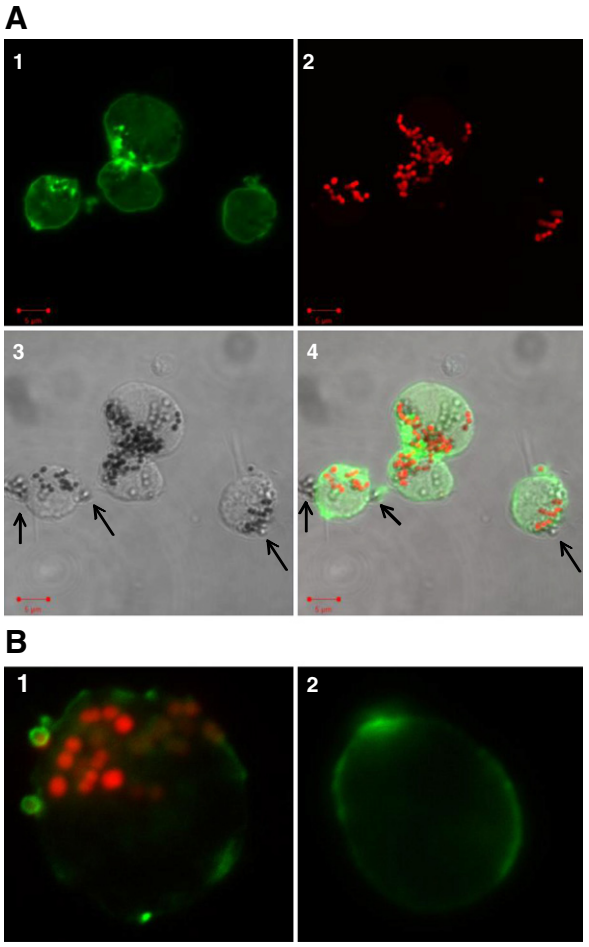

Fig. 5. A) Confocal microscopy of neutrophils interacting with pHrodo™ labeled bacteria. Differentiated HL-60 cells were incubated for 30 min at 37 °C in the presence of baby rabbit complement with GBS Ia pHrodo™ labeled bacteria pre-treated with a mouse serum against GBS PS Ia-CRM$_{197}$. After washing, cells were fixed with 4% paraformaldeyde and plasma membrane of phagocytes was stained with Alexa Fluor 488-phalloidin. Panels A1 and A2 show respectively the plasma membrane of neutrophils in green and internalized bacteria in red. The bright field image in panel A3 show bacteria internalized and outside neutrophils (arrows) and merged images are shown in panel A4. B) Differentiated cells were incubated for 30 min at 37 °C in the presence of baby rabbit complement with GBS Ia pHrodo™ labeled bacteria pretreated with a mouse serum against GBS PS Ia-CRM$_{197}$ (B1) or uncorrelated serum (B2). After washing, cells were fixed with 4% paraphormaldeyde and cellular and bacterial wall were stained with Alexa Fluor 488-phalloidin.

react with internal and surface exposed free amines, resulting in a more intense fluorescent staining of dead cells compared to live cells where only surface free amines are available.

After incubation, samples were analyzed by flow cytometry. Live HL-60 cells were first gated based on LIVE/DEAD® (Fig. 2A) and then based on forward scatter versus side scatter cytogram (Fig. 2B). The percentage of live cells shown in Fig. 2A was 79% of whole cells and this number varied from 72 to 85% in experiments performed in different days. Doublets were eliminated using SSC-W versus SSC-A plot (Fig. 2C). Moreover HL-60 positive to CD35 and CD11b receptors were gated to identify the neutrophil effector cell population (Fig. 2D), which corresponded to 62.5% of total live cells (from 45 to 78% in the different experiments).

Finally, a phycoerythrin (PE) fluorescence histogram was used to evaluate phagocytic activity, which was expressed as MFI and calculated by setting a Log 4 range over the whole scale in the PE channel (Fig. 2E).

Focusing on effector cells allowed cleaning off, from the read out, the fluorescent signal of undifferentiated HL-60, as demonstrated by the disappearance in the immune serum histogram shown in Fig. 3A of the double peak present in Fig. 3B. In this way, enhanced assay sensitivity could be attained. We believe that the high variability in the number of live effector cells in the HL-60 population contributes to the low reproducibility encountered in the classical kOPA. This variability does not affect the phagocytic activity measurement of our fOPA method, as the fluorescent intensity derived from undifferentiated cells does not contribute to the read out of the assay. Indeed, the MFI values obtained for each dilution of a particular test serum were comparable irrespective of the proportion of live effector cells.

Several assay conditions were tested to optimize the method: particularly, different bacteria to neutrophil ratios (Fig. 4), incubation times and complement concentrations were tested. Highest phagocytosis rates were achieved using a ten fold excess of bacteria over phagocytes, 30 min incubation and 10% baby rabbit complement.

3.3. Confocal microscopy analysis

To assess whether pHrodo™ labeled GBS Ia bacteria became brighter once internalized into neutrophils, differentiated HL-60 cells were incubated with pHrodo™ labeled bacteria in the presence of an hyperimmune specific serum and complement for 30 min at 37 °C, and the plasma membrane of neutrophils was stained with Alexa Fluor 488-phalloidin. Z stacks images of the sample were taken by confocal microscopy.

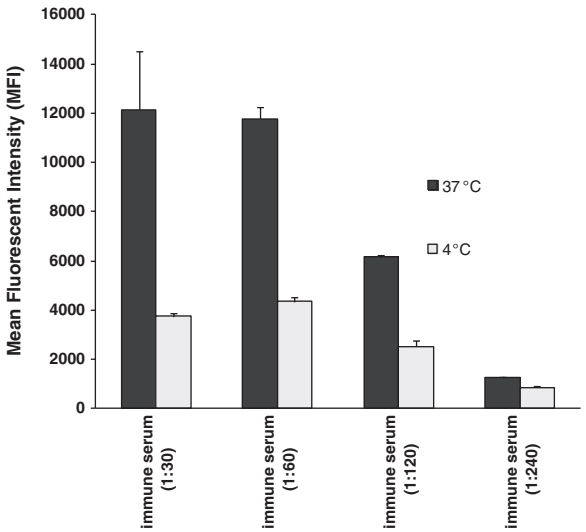

Fig. 6. Effect of temperature on the opsonic activity. Differentiated HL-60 cells were incubated for 30 min at 37 °C or 4 °C with different concentrations of pHrodo labeled GBS Ia bacteria preincubated with a rabbit immune serum against GBS capsular polysaccharide Ia-CRM conjugate (formulated with Alum) in the presence of 10% active rabbit serum as source of complement.

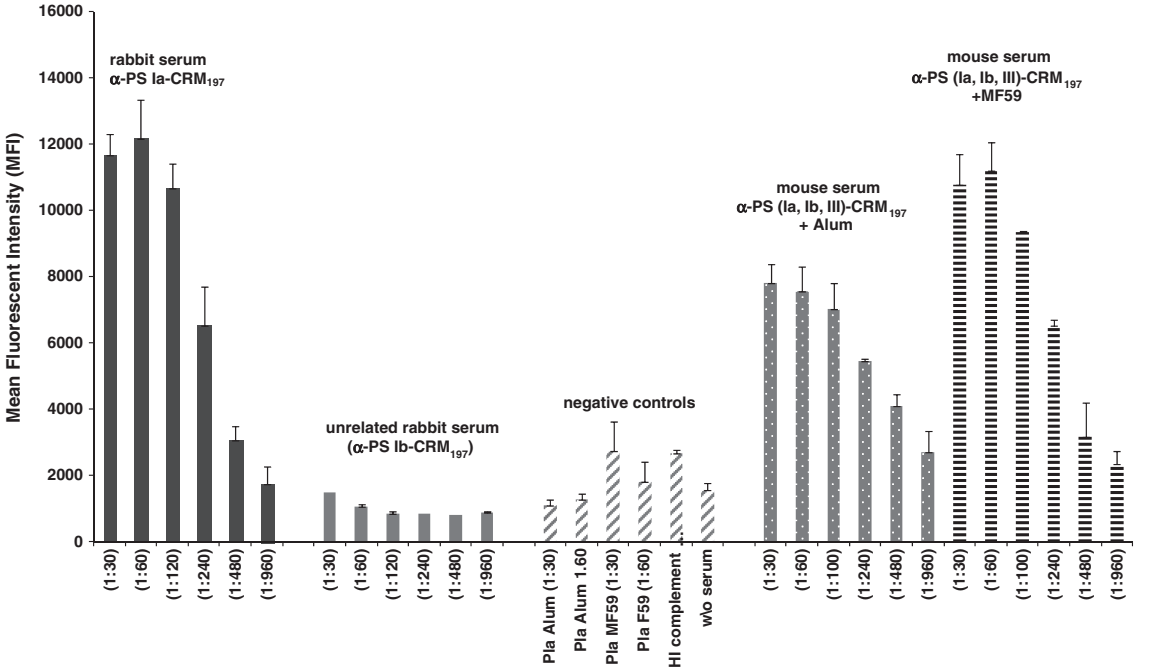

Fig. 7. Opsonic activity of mouse sera against GBS capsular polysaccharide CRM_{197}-conjugate. Differentiated HL-60 cells were incubated with pHrodo™ labeled GBS serotype Ia in the presence of baby rabbit complement and a pool of sera from rabbits immunized with CRM_{197}-conjugated polysaccharides Ia or Ib plus Alum and pools of sera from mice immunized with trivalent vaccines consisting of CRM_{197}-conjugated polysaccharides Ia, Ib and III (PS (Ia, Ib, III)-CRM197) formulated either in Alum or MF59. Negative controls were represented by pooled sera from a mock immunization, the positive control serum combined with heat inactivated baby rabbit complement and a mixture containing all components without the serum.

Neutrophil plasma membrane (green) and pHrodo labeled bacteria (red) are shown respectively in panels A1 and A2 of Fig. 5A. The bright field panel (A3) shows the presence of internalized and non internalized (arrows) bacteria, adhering to the neutrophil plasma membrane. Finally, the red and green images merged with the bright field image (panel A4) clearly confirmed that only internalized bacteria were brightly fluorescent.

Further analyses by confocal microscopy confirmed that labeled GBS bacteria were internalized by differentiated HL-60 cells in the presence of specific antibodies and complement (Fig. 5B1), whereas no bacteria were found inside the cells of negative control samples tested with unrelated serum (Fig. 5B2). These results clearly indicate that bacteria internalization depends both on the presence of functional antibodies and active rabbit complement.

3.4. Assay sensitivity and specificity

To test the specificity of the assay, the effect of the temperature on GBS Ia internalization was examined by testing different dilutions of specific rabbit serum at 4 °C and 37 °C. Fig. 6 shows the MFI values obtained for each serum dilution at the two different temperatures. A dramatic reduction of the phagocytic activity at 4 °C was observed compared to 37 °C, indicating that the pHrodo-based assay was able to specifically detect internalized GBS bacteria.

Assay specificity and sensitivity were also assessed by testing sera from rabbits immunized with CRM_{197}-conjugated polysaccharides Ia or Ib plus Alum and pools of sera from mice immunized with two trivalent vaccines consisting of CRM_{197}-conjugated polysaccharides Ia, Ib and III formulated either in Alum or in MF59. Negative controls comprised sera from placebo immunized mice and reactions without serum or containing heat inactivated complement.

As shown in Fig. 7, very high signal-to-background ratios were obtained for all specific immune sera compared to negative controls, confirming high specificity of the assay. Remarkably, MFI values were inversely proportional to increasing sera dilutions, indicating that the method can be used for quantitative determination of functional antibodies in test sera.

An arbitrary threshold was established, corresponding to two times the MFI of negative controls (reaction without serum, reaction with heat inactivated complement, reaction

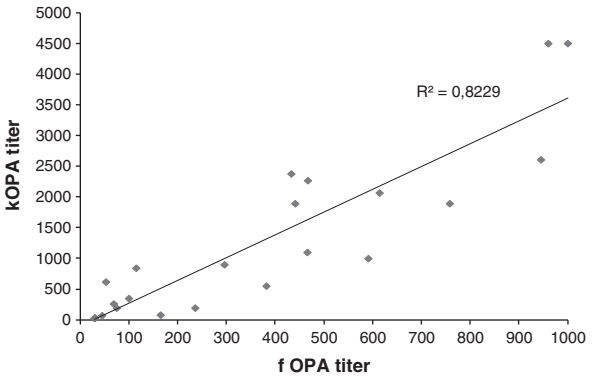

Fig. 8. Correlation between fOPA titer and OPA titers. Sera of mice immunized with different formulation of vaccine were tested by fOPA and kOPA on pHrodo labeled or live GBS Ia bacteria.

with the placebo or pre-immune sera), and the fOPA titer was calculated as the reciprocal serum dilution yielding threshold fluorescence values.

To investigate the correlation between the data that can be obtained using the classical kOPA test and the newly developed fOPA method, we measured fOPA titers in a panel of sera displaying a wide range of kOPA titers to GBS Ia. Remarkably, a good correlation ($R^2 = 0.82$, $p < 0.05$) between fOPA and kOPA read outs was observed (Fig. 8). A subset of sera was also tested against GBS serotype III using the isolate COH1 and a good correlation between the two methods ($R^2 = 0.85$, $p < 0.05$) was obtained also in this case (data not shown). The data indicate that the fOPA method can be used to test functional antibodies against different serotypes.

4. Discussion and conclusions

We developed an opsonophagocytosis assay for GBS using pHrodo™ labeled bacteria. Our method offers several advantages over both killing-based and other fluorescence-based opsonophagocytic assays.

The most commonly-used fluorophores in OPA assays are fluorescein (fluorescein, dicarboxyfluorescein, oregon green, dihydrodichlorofluorescein) or Alexa Fluor derivatives. Flow cytometry based on those fluorophores can detect cell-associated fluorescence but cannot distinguish between internalized and adhering bacteria, necessitating quenching steps with trypan blue or ethidium bromide to clean out the background fluorescence of externally bound bacteria. The pHrodo™-based assay provides sensitive detection without the need for quenching or washings steps, saving time and eliminating measurement uncertainty. Indeed, pHrodo™ is a pH sensitive fluorophore showing a very low fluorescent signal at the neutral pH of extracellular and cytoplasmic environment and a bright fluorescent signal in acidic compartments, such as phago-lysosomes, deriving from the fusion of phagosome-containing bacteria with lysosomes which occurs immediately after internalization.

As shown by confocal microscopy images, GBS bacteria labeled with pHrodo™ exhibit low fluorescence outside the cell, yet emit a bright red fluorescence after internalization into the acidic environment of the phagocyte. By determining whether phagosome containing bacteria mature to phago-lysosome acidic compartments, the pHrodo™ assay is predictive of phagocytic killing. Several different mechanisms can lead to bacterial survival after phagocytosis, rendering the phagocytosis measurement non strictly indicative of pathogen clearance. For instance, it has been observed that certain mycobacteria (e.g. *Mycobacterium avium*, *Mycobacterium tubercolosis*) are not always killed even when enclosed in phagocytic cells, because the phagosome-lysosome fusion is not accompanied by the normal acidification that creates the appropriate conditions for killing (Hornef et al., 2002; Bellaire et al., 2005; Huynh and Grinstein, 2007). Further, the phagosome-lysosome fusion may not occur or the phagosome may not close. OPA using pHrodo™ for bacterial labeling permits the detection of efficiently phagocytosed and killed bacteria, a finding that is further supported by the correlation observed between kOPA and fOPA titers.

The fOPA also presents some improvements over the reference, killing-based assays. Operational costs related to HL-60 differentiation are reduced, as the absolute number of effector cells is much lower than in kOPA (Guttormsen et al., 2008). Assay components, such as bacteria and effector cells, can be more effectively controlled by FACS, immediately before each experiment. The absolute number of pHrodo labeled bacteria can be determined by using BD TruCount Tubes. When such a count is done by comparing biological events to standardized beads events, it is not affected by bacterial aggregation, as instead occurs in spectrophotometer measurements, usually used for OPAs. Moreover the use of specific markers of cell differentiation allows selecting and analyzing only effective phagocytes among the whole HL-60 cell population eliminating one of the major causes of assay variability. Our method promises to be more easily standardized in comparison with kOPA methods. It provides a quantifiable read out recorded as MFI that dramatically reduces the variability due to the operator and associated with viable bacterial counts, as measurement of killing titers. Finally the fOPA method is faster, i.e. results are obtained in a single day.

In conclusion, the flow cytometry-based opsonophagocytosis assay described in the present study is a rapid and sensitive method for testing the functionality of serum antibody responses to GBS and shows specificity and correlation with killing. The method has the potential, therefore, to become a viable alternative to the standard killing-based assays, used as correlate of protection for GBS vaccines.

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