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Opsonophagocytic Assay To Evaluate Immunogenicity of Nontyphoidal *Salmonella* Vaccines

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Nontyphoidal *Salmonella* (NTS) invasive infections are an important cause of morbidity and mortality in sub-Saharan Africa. Several vaccines are in development to prevent these infections. We describe an NTS opsonophagocytic killing assay that uses HL-60 cells and baby rabbit complement to quantify functional antibodies elicited by candidate NTS vaccines.

Invasive nontyphoidal *Salmonella* (NTS) infections are increasingly being recognized as an important cause of morbidity and mortality in infants and HIV-infected adults and children in sub-Saharan Africa (1). Multiple vaccines are currently in development, including live-attenuated vaccines, conjugate vaccines, and generalized modules for membrane antigens against invasive *S.* Typhimurium and *S.* Enteritidis (2–7). As these vaccines enter clinical development, it is important to establish their immunogenicity through relevant, well-characterized immunological assays. There is a growing realization within the vaccinology community that in addition to determining antibody levels through binding assays, it is important to determine the functional, antimicrobial capacity of these antibodies.

Although many studies have evaluated anti-*Salmonella* serum bactericidal and opsonophagocytic antibody activity (2, 8–15), there is a need for reproducible assays that can be used routinely to characterize functional antibody responses to *Salmonella* in a standardized manner. We previously developed a complement-dependent serum bactericidal antibody (SBA) activity assay that quantifies serum antibody responses to typhoidal (*S.* Typhi and *S.* Paratyphi A) and nontyphoidal (*S.* Typhimurium and *S.* Enteritidis) *Salmonella* (16). Here, we describe an assay that measures the opsonophagocytic capacity of NTS antibodies based on the well-characterized pneumococcus opsonophagocytic activity (OPA), which employs HL-60 phagocytic cells and a standardized source of complement, i.e., baby rabbit complement (BRC) (17, 18).

The *Salmonella* OPA assay was evaluated using serum samples from mice that had been vaccinated with live-attenuated NTS vaccines (7). BALB/c mice were orally immunized with 10^9 CFU of *S.* Typhimurium CVD 1931 or *S.* Enteritidis CVD 1944 suspended in phosphate-buffered saline (PBS) or given PBS alone on days 0, 28, and 56, as previously described (7). *S.* Typhimurium CVD 1931 and *S.* Enteritidis CVD 1944 are vaccine strains derived from invasive disease-associated clinical isolates (*S.* Typhimurium D65 and *S.* Enteritidis R11, respectively), which harbor deletions in *guaBA* and *clpX*. Serum samples from vaccinated mice taken 83 days postimmunization were designated immune, and serum samples from mice that received PBS were designated nonimmune.

HL-60 cells were maintained in RPMI 1640 complete medium (RPMI 1640 [Corning CellGro, Manassas, VA] supplemented with 10% [vol/vol] fetal bovine serum [FBS; HyClone, Waltham, MA] and 1% GlutaMAX, 1% sodium pyruvate, and 1% penicillin-streptomycin [Life Technologies]) and differentiated for 6 days in RPMI 1640 complete medium, 0.8% dimethylformamide (Fisher Scientific, Waltham, MA). Prior to use, cells were washed once in Hanks balanced salt solution (HBSS) (Life Technologies) and resuspended to 1×10^6 cells/ml in opsonization buffer (OPB) (HBSS, 0.1% gelatin [Sigma-Aldrich, St. Louis, MO], 10% FBS). *Salmonella* strains (*S.* Typhimurium D65 and *S.* Enteritidis SO1 [19]) from overnight cultures were diluted 1 in 1,000 in Hy-Soy medium (0.5% sodium chloride, 1% Hy-Soy [Kerry, Clackmannshire, United Kingdom], and 0.5% Hy-Yest [Kerry]), grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.3, and then diluted to 3×10^4 CFU/ml in OPB. Immune and nonimmune sera were heat-inactivated at 56°C for 20 min, and 2-fold serial dilutions in OPB (25 μl/well final volume) were performed in a U-bottomed 96-well microplate (Sigma-Aldrich, St. Louis, MO). The bottom row was left without sera to act as a negative control. To each well, 3×10^2 CFU of bacterial suspension in 10 μl OPB was added. Opsonization of bacteria was allowed to occur for 15 min at 37°C with 5% CO_2. After incubation, 25 μl of BRC (lot 31235; Pel-Freez Biologicals, Rogers, AZ) was added to the wells at a final concentration of 12.5% (vol/vol), and 40 μl of differentiated HL-60 cells was added at a concentration of 4×10^4 cells/well. Plates were incubated at 37°C with shaking at 160 rpm for 45 min. Bacteria were enumerated by mixing the contents of each well by pipetting vigorously and performing viable counts in triplicate for each well. OPA titer was defined as the reciprocal of the highest serum dilution that produced >50% killing compared with the killing observed in the negative-control wells. Titers were determined from the mean bacterial counts in duplicate wells.

First, we determined the components required to demonstrate antibody-mediated OPA. Serum samples from mice immunized with CVD 1931 (*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*) were

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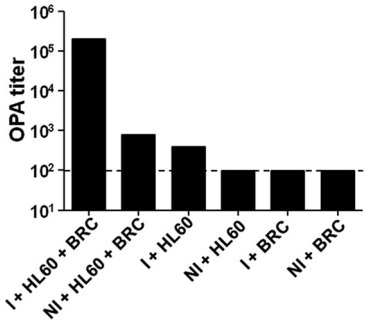

FIG 1 Optimization of OPA assay. To determine all of the components required for optimal opsonophagocytosis, we optimized the OPA assay using pooled immune serum produced by live-attenuated *S.* Typhimurium CVD 1931 (*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*) and measured the killing of *S.* Typhimurium D65. We determined the effect of combining HL-60 cells and baby rabbit complement (BRC) and immune sera (I) or nonimmune sera (NI) in various combinations. OPA titer was determined as the reciprocal of the highest serum dilution that produced >50% killing in comparison to bacteria incubated with BRC and HL-60 cells. The dashed line represents the lowest dilution tested. Results shown are the titers obtained from a single experiment. Titers obtained for the conditions containing immune serum were verified by repeating the experiment using a different pool of immune serum from the same mice. Similar OPA titers were obtained (I + HL-60 + BRC, 1:204,800; I + HL60, 1:100; and I + BRC, 1:100).

tested for OPA with HL-60 cells and BRC in different combinations against an invasive clinical isolate of *S.* Typhimurium D65 from Mali, as shown in Fig. 1. In the absence of HL-60 cells, there was no detectable bacterial killing. Thus, at a concentration of 12.5%, BRC was unable to mediate killing, as shown previously (16). When HL-60 cells were incubated with bacteria and serum without BRC, limited OPA titers were observed for immune mice (1:400) but not for nonimmune mice. However, when BRC was included in the assay, the OPA titer for immune sera increased >100-fold. It was therefore determined that a sublethal concentration of 12.5% BRC is required for optimal OPA in this assay.

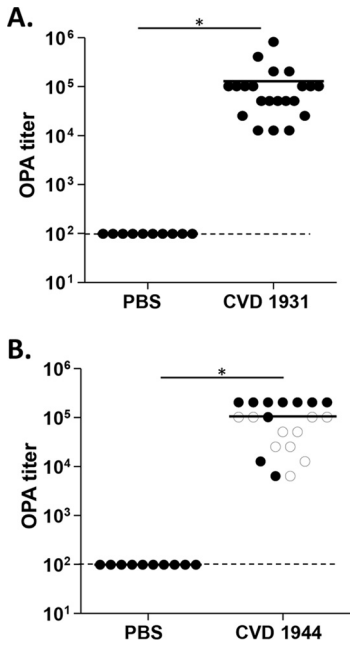

FIG 2 OPA titers for individual mice immunized with *S.* Typhimurium CVD 1931 and *S.* Enteritidis CVD 1944. (A) OPA titers against wild-type *S.* Typhimurium D65 in serum samples from individual mice immunized with CVD 1931 (*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*) or PBS. (B) OPA titers against wild-type *S.* Enteritidis SO1 in serum samples from individual mice immunized with CVD 1944 (*S.* Enteritidis R11 Δ*guaBA* Δ*clpX*) or PBS. OPA titer was determined as the reciprocal of the highest serum dilution that produced >50% killing in comparison to bacteria incubated with BRC and HL-60 cells. Open circles, serum samples that were retested; *, $P < 0.05$ (Student's *t* test). Dashed line, the lowest dilution tested.

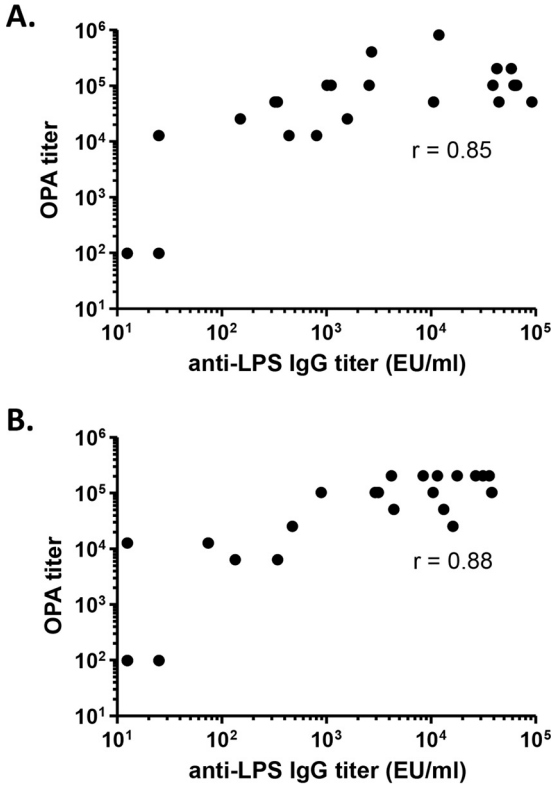

FIG 3 Correlation between anti-LPS serum IgG and OPA titers. Titers elicited by *S.* Typhimurium CVD 1931 (*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*) (A) and *S.* Enteritidis CVD 1944 (*S.* Enteritidis R11 Δ*guaBA* Δ*clpX*) (B). Data were analyzed using Spearman's correlation coefficient.

Using our optimized OPA assay, we determined OPA titers of serum samples from individual mice immunized with live-attenuated NTS vaccines. We tested samples from groups that received the live-attenuated *S.* Typhimurium vaccine strain CVD 1931 (*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*) against the target strain *S.* Typhimurium D65 from which the vaccine was derived. As expected, significantly higher OPA titers were measured in postvaccination sera than in samples from PBS-treated controls (Fig. 2A). Similarly, when we tested serum samples from mice immunized with the live-attenuated *S.* Enteritidis vaccine strain CVD 1944 (*S.* Enteritidis R11 Δ*guaBA* Δ*clpX*) against the virulent target strain *S.* Enteritidis S01, also isolated from the blood of a Malian child, we observed significantly higher OPA titers than for samples from mice treated with PBS (Fig. 2B). To account for possible lot-to-lot variation in BRC from the supplier, we picked 10 different serum samples from mice immunized with the live-attenuated *S.* Enteritidis vaccine strain CVD 1944 (*S.* Enteritidis R11 Δ*guaBA* Δ*clpX*) that were previously tested (Fig. 2B, open circles) for OPA titers with BRC lot 31325 and retested them in an OPA assay with BRC

TABLE 1 Seroconversion and anti-LPS serum IgG and OPA geometric mean titers (GMT)

Vaccine	Anti-LPS serum IgG titer			OPA titer		
	Preimmune GMT (95% CI)	Immune GMT (95% CI)	Seroconversion[a]	Preimmune GMT (95% CI)	Immune GMT (95% CI)	Seroconversion[a]
S. Typhimurium CVD 1931	12.5 (12.5–12.5)	4,337 (1,423–13,218)	20/21	100 (100–100)	71,223 (43,412–116,849)	21/21
S. Enteritidis CVD 1944	12.5 (12.5–12.5)	3,210 (1,104–9,329)	19/20	100 (100–100)	65,258 (36,925–115,331)	20/20

[a] No. of mice with ≥4-fold rise in titer.

lots 31325, 34636, and 19837. In our hands, we observed the OPA titers of the individual serum samples to be exactly the same for all three BRC lots. Furthermore, no differences in OPA titers were observed when the 10 immune serum samples were tested a second time on a different day with the same lot of BRC (lot 31325). A significant correlation was seen between OPA titers and antilipopolysaccharide (anti-LPS) serum IgG titers for *S.* Typhimurium and *S.* Enteritidis (Spearman's correlation coefficients of 0.85 and 0.88, respectively; $P < 0.0001$ for both) (Fig. 3). Using seroconversion as the parameter, all of the *S.* Typhimurium- and *S.* Enteritidis-vaccinated mice showed a >4-fold increase in OPA titer compared to preimmune serum, while 20 of 21 *S.* Typhimurium-vaccinated mice and 19 of 20 *S.* Enteritidis-vaccinated mice seroconverted serum IgG anti-LPS titer (Table 1). The fact that the *S.* Typhimurium- and *S.* Enteritidis-vaccinated mice were protected (86% and 76% vaccine efficacies, respectively) against a lethal challenge (7) suggests that the OPA assay not only provides a measure of biological function but appears to be highly sensitive in detecting seroconversion following vaccination. The specificity of the OPA antibodies remains to be determined.

Robust, reproducible assays to measure immune responses elicited by candidate vaccines are required by regulatory agencies for eventual licensure. Here, we have adapted a well-characterized pneumococcus OPA assay accepted by regulatory agencies to evaluate pneumococcal conjugate vaccine formulations for measurement of OPA antibodies against *Salmonella*. Although the contribution of opsonophagocytic antibody activity in controlling *Salmonella* infections is unknown, the assay described herein may be used to characterize NTS vaccine immune responses, allowing quantification of functional antibody titers. The robustness of this assay, a measure of its reproducibility, was determined by using different lots of BRC and testing the assay on different days where the sera were subjected to repeated freeze-thaw cycles. We found the assay to be reproducible, with no differences observed in the OPA titers of the immune sera. We previously observed a similar robustness for SBA titers using immune mouse serum samples. Although SBA assays for NTS have been described, the need for an OPA assay is still compelling, as immune sera may lack SBA activity but still possess OPA that represents a correlate of protection for vaccines. Together with the SBA assay that we described previously, these assays may be useful in determining NTS correlates of protection, thereby allowing vaccine developers to predict vaccine performance.

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