Comparison of the Antibodies in Lymphocyte Supernatant and Antibody-Secreting Cell Assays for Measuring Intestinal Mucosal Immune Response to a Novel Oral Typhoid Vaccine (M01ZH09)

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Antibody-secreting cell (ASC) and antibodies in lymphocyte supernatant (ALS) assays are used to assess intestinal mucosal responses to enteric infections and vaccines. The ALS assay, performed on cell supernatants, may represent a convenient alternative to the more established ASC assay. The two methods, measuring immunoglobulin A to *Salmonella enterica* serovar Typhi lipopolysaccharide, were compared in volunteers vaccinated with a live-attenuated typhoid vaccine M01ZH09. The specificity of the ALS assay compared to the ASC assay was excellent (100%), as was sensitivity (82%). The ALS assay was less sensitive than the ASC assay at ≤42 spots/10^6 peripheral blood lymphocytes.

After vaccination or infection, antigen-specific antibody (immunoglobulin [Ig])-secreting cells briefly circulate systemically before homing to mucosal effector sites, such as the intestine (7, 8, 12). Both the solid-phase antibody-secreting cell (ASC; or enzyme-linked immunospot [ELISPOT]) assay and the human lymphocyte supernatant (antibodies in lymphocyte supernatant [ALS]) assay are used semiquantitatively to assay mucosal immune responses (2–4, 6, 7, 11). The ASC assay enumerates "spots" formed by Ig-producing cells bound to a nitrocellulose plate after incubation of peripheral blood lymphocytes (PBL) with specific antigen, whereas the ALS assay measures, by enzyme-linked immunosorbent assay, antibody in the supernatant of incubated PBL (2, 6, 7). The ALS assay has greater flexibility than the ASC assay, since antibody measurements can be performed later on frozen lymphocyte supernatants (2–4, 11). Comparison of the ALS and ASC assays has been extensively reviewed for tuberculosis and cholera, but the assays have not been compared for a systemic enteric illness such as a typhoid vaccine model (11, 13, 14). We compared the sensitivity and specificity of ALS and ASC assays in 31 adult volunteers vaccinated with a candidate oral typhoid vaccine, M01ZH09 (*Salmonella enterica* serovar Typhi [Ty2 S. Typhi $ssaV^- aroC^-$]) (5, 9, 10).

An open-label, randomized trial was performed at the University of Vermont, approved by the institutional review board, and carried out under a Food and Drug Administration investigational new drug application. Thirty-one healthy adult volunteers aged 18 to 50 years old with no history of typhoid vaccination received 5×10^9 CFU of M01ZH09 after fasting for ≥2 h, as described previously (10). Whole blood for PBL was collected in heparinized cell preparation tubes (Vacutainer CPT; Becton Dickinson, San Diego, CA) on the day of vaccination (day 0) and day 7 postvaccination.

ASC assay. The IgA ASC assay to *S. enterica* serovar Typhi lipopolysaccharide (LPS) was performed as described previously (1, 6, 7). PBL were plated at 2.5×10^6/ml, 5.0×10^6/ml, and 1×10^7/ml on nitrocellulose microtiter plates (Millipore, Billerica, MA). Wells were coated with *S. enterica* serovar Typhi LPS (Sigma, St. Louis, MO) in 1× Reggiardo's buffer or were uncoated. PBL from volunteers, positive and negative controls, were incubated overnight at 37°C in 5% CO_2. After washing with phosphate-buffered saline (PBS)-Tween, an anti-human IgA–alkaline phosphatase conjugate was added and the mixture was incubated for 1 h. 5-Bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium substrate was added to each well for 30 min in the dark, and the reaction was stopped by washing the microtiter plates. ASC "spots" were manually counted by two observers, photographed, and expressed as antibody-secreting cells/10^6 PBL. A result of ≥4 ASC per 10^6 PBL was defined as a positive response. Previous testing in clinical trials has validated the use of this positive cutoff as over >3 standard deviations from the mean of negative control ASC counts (0 to 2/10^6) (10).

ALS assay. PBL were diluted to 1×10^7 cells/ml in plating medium (RPMI, 10% fetal calf serum, and 1% pencillin–streptomycin). An aliquot of the diluted PBL was centrifuged, and supernatant was frozen at −20°C as the "time zero" sample. The remainder of the PBL were plated on a 96-well plate at 1×10^6 cells/ml and 1×10^7 cells/ml, with 6 wells/volunteer/concentration and incubated at 37°C in 5% CO_2 for 24, 72, or 96 h. At each time point, cell supernatants were frozen as described above (2, 11).

To determine supernatant levels of *S. enterica* serovar Typhi LPS-specific IgA, 96-well microtiter plates were coated over-

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night at 4°C with *S. enterica* serovar Typhi LPS (0.5 μg/ml) (Sigma, St. Louis, MO) in Reggiardo's buffer. Plates were washed and blocked with 3% bovine serum albumin in PBS for 2 h at 37°C. A standard curve, linear at 5 to 70 U/ml, of *S. enterica* serovar Typhi LPS IgA-positive sera in plating medium was used. IgA-negative serum was used as a negative control, and IgA in the standard serum was set arbitrarily at 40,000 U/ml. Three sets of quality control (QC) samples were used (high QC, 48 U/ml; mid QC, 27 U/ml; and low QC, 12 U/ml) throughout the plate. Frozen cell supernatants were added in duplicate, after thawing at room temperature and dilution to 1:10, 1:50, and 1:100 in plating medium. Samples under 5 U/ml and all day 0 samples were rediluted at 1:5. No sample needed dilution to >1:100. Plates were incubated for 1 h at 37°C. After washing, 1° antibody (goat anti-human IgA-biotin conjugate at 1:2,000) and streptavidin-horseradish peroxidase were added sequentially, each followed by incubation for 1 h at 37°C and washing. Tetramethyl benzidine substrate was added in the dark, the mixture was incubated at room temperature, and then the reaction was stopped with H_2SO_4. Plates were read on a Biotek enzyme-linked immunosorbent assay reader (Colchester, VT) at 450 nm.

The optical density of standards, minus blanks, was plotted against concentration and described by a four-parameter logistic fit. Optical density was interpreted against the standard curve, and concentrations of anti-LPS IgA were determined. All plates met acceptance criteria (R^2 of the standard curve fit > 0. 950; accuracy for the QC control samples within 25.0% of the expected concentration; and replicate coefficients of variation (based on absorbance) of <15.0%.

Both the ASC and ALS assays demonstrated responses that were 100% specific to vaccine exposure. All day 0 ASC assays were negative for spot-forming cells. Day 7 ASC assays demonstrated a range of ASC (Table 1). Three of 31 (9.7%) plates demonstrated 0 to 3 spots and were considered "negative." Twenty-eight of 31 (90.3%) plates were "positive" for ≥4 ASC. Positive plate spot counts ranged from 5 to 1,040/10^6 PBL.

Similarly, all day 0 ALS assays were negative at all time points of incubation (0, 24, 72, and 96 h). At 72 and 96 h, ALS responses peaked in most subjects. In most samples, positive ALS assay IgA results trended closely to the ASC result (Fig. 1). Divergence occurred in two samples with very-high-positive ASC plates (no. 03 and 06). The ALS assay data demonstrated concordance to ASC results in 26 of 31 (84%) of the volunteers (Table 1) and a sensitivity of 82% (23/28). Five subjects who demonstrated an ASC response of 7 to 34 spots/10^6 cells had no response by ALS, even upon triplicate testing of both cell concentrations used in the ALS assay (10^6 and 10^7/ml) and using decreasing dilutions. However, concordance was exact when ASC counts were >42 spots/10^6 PBL. All positive ALS assays demonstrated a positive ASC result (100% positive predictive value of ASC response).

The ASC assay is used in early-phase vaccine trials as a measure of oral and enteric vaccine intestinal immunogenicity (1, 6, 7). ASC, a solid-phase assay usually performed on fresh PBL, measures the ability of vaccination to induce antigen-specific IgA antibody-secreting cells, which contribute to specific mucosal immunity. The IgA, secreted into supernatant, is measured by ALS (2–4, 11, 13, 14). ALS offers the convenience of freezing and "banking" lymphocyte supernatant for later

TABLE 1. Antigen-specific mucosal antibody responses in subjects vaccinated with M01ZH09 by ASC and ALS assays[a]

Subject	No. of spots/10^6 cells by assay:						
	ASC		ALS				
	Day 0	Day 7	Day 0 (all times)[b]	Day 7			
				0 h	24 h	72 h	96 h
003	0	1,040	0	0	116	267	154
004	0	624	0	0	453	426	397
006	0	560	0	0	1,145	2,145	3,200
005	0	400	0	0	255	481	486
028	0	396	0	0	235	497	347
022	0	362	0	0	369	616	351
001	0	209	0	0	105	244	225
015	0	199	0	0	494	893	1,101
021	0	198	0	0	93	156	63
008	0	172	0	0	199	391	636
019	0	172	0	0	64	133	68
025	0	154	0	0	62	173	100
007	0	149	0	0	49	71	102
017	0	148	0	0	237	289	283
009	0	140	0	0	130	186	323
014	0	88	0	0	56	132	101
024	0	59	0	0	69	77	123
020	0	58	0	0	10	17	7
002	0	55	0	0	25	68	ND[c]
011	0	42	0	0	32	0	0
031	0	42	0	0	16	70	0
018	0	34	0	0	0	0	0
029	0	25	0	0	0	0	36
027	0	19	0	0	31	22	32
023	0	16	0	0	0	0	0
030	0	12	0	0	0	0	0
012	0	7	0	0	0	0	0
026	0	5	0	0	0	0	0
032	0	3	0	0	0	0	0
016	0	1	0	0	0	0	0
013	0	0	0	0	0	0	0

[a] Results represent responses in 31 adult subjects vaccinated with M01ZH09 as measured by ASC and ALS assays performed at days 0 and 7 postvaccination. Note that plates for three subjects (032, 016, and 013) demonstrated 0 to 3 spots and were considered "negative."

[b] All incubation times for day 0 (0, 24, 72, and 96 h).

[c] ND, not determined.

measurement of antibody or cytokine levels (2–4). In *S. enterica* serovar Typhi, a positive ASC response has been associated with vaccine efficacy in field trials (6). ALS has not independently been correlated with efficacy but has been demonstrated to be more sensitive than specific serum or intestinal IgA (3, 4).

Our data demonstrate that the ALS and ASC results show an excellent concordance. Both assays are highly specific to vaccine exposure (100%), as demonstrated by the negative results prevaccination and also by examining the ALS supernatant preincubation, 7 days postvaccination (day 7, time zero). When compared to the ASC assay, the ALS assay is also 100% specific. The concordance and sensitivity of ALS versus ASC assay are also high (84% and 82%, respectively), as is the positive predictive value (100%). Interestingly, the ALS assay was negative for several ($n = 5$) samples that demonstrated a modest to moderate ASC response (4 to 34/10^6 PBL), but matched ASC results for ≥42 spots/10^6 cells. Although the ALS assay appears less sensitive than the ASC assay for IgA to *S. enterica* serovar Typhi LPS, the >42 spots/10^6 cells may
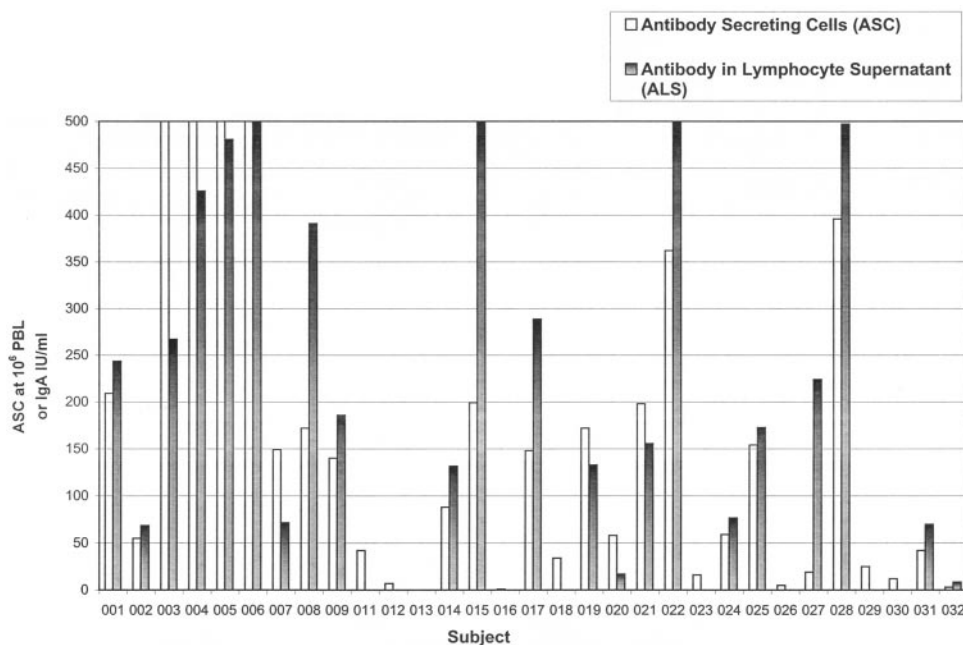
FIG. 1. The *S. enterica* serovar Typhi LPS IgA ASC and ALS assays demonstrate concordance in volunteers vaccinated with oral typhoid vaccine M01ZH09.

correlate with clinical efficacy. In addition, the ALS and ASC assays appear to diverge at very high counts (>400). However, when ASC counts are extremely high, ASC counts may be imprecise, since extensive sample dilution may not be possible with limited PBL.

Our data suggest that the ASC and ALS assays demonstrate excellent specificity and sensitivity in an oral typhoid vaccine model, particularly at ASC counts of ≥ 42 spots/10^6 cells. Future work will determine whether similar ASC levels correlate with vaccine efficacy or immunogenicity. The ALS assay is a flexible and robust alternative to the ASC assay and may be easier to validate as a model of vaccine immunogenicity used to support vaccine licensure.

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