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/10⁶ 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 or other enteric infections were enrolled. Thirty-one healthy adult volunteers aged 18 to 50 years old with no history of typhoid or other enteric infections were enrolled. The study protocol was reviewed by the Advisory Committee on Human Vaccine Trials at the University of Vermont College of Medicine, Unit of Infectious Diseases, 110 Stafford Building, 95 Carrigan Lane, Burlington, VT 05405. Phone: (802) 656-5822. Fax: (802) 656-0881. E-mail: beth.kirkpatrick@uvm.edu.

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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 H2SO4. 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 (based on absorbance) of the expected concentration; and replicate coefficients of variation were arbitrary at 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 were negative for spot-forming cells. Day 7 ASC assays demonstrated responses that were 100% specific to vaccine exposure (100%), as demonstrated by the negative results of several (n = 5) samples that demonstrated a modest to moderate response (4 to 34/10^6 PBL), matched ASC results for ≥42 spots/10^6 PBL. Although the ALS assay appears less sensitive than the ASC assay for IgA to *S. enterica* serovar Typhi LPS, the >42 spots/10^6 PBL may

### Table 1. Antigen-specific mucosal antibody responses in subjects vaccinated with M01ZH09 by ASC and ALS assays

| Subject | ASC | ALS |
|---------|-----|-----|
| Day 0   | Day 7 | Day 0 (all times) | Day 7 |
| No. of spots/10^6 cells by assay: | 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 | 255 | 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 |
| 031     | 0    | 42 | 0 | 0 | 32 | 0 | 0 |
| 018     | 0    | 34 | 0 | 0 | 0 | 0 | 0 |
| 029     | 0    | 25 | 0 | 0 | 0 | 0 | 0 |
| 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 |

* 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.”

a All incubation times for day 0 (0, 24, 72, and 96 h).

b ND, not determined.
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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