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# Point-of-Use Mixing of Influenza H5N1 Vaccine and MF59 Adjuvant for Pandemic Vaccination Preparedness: Antibody Responses and Safety. A Phase 1 Clinical Trial

Mark J. Mulligan,<sup>1</sup> David I. Bernstein,<sup>2</sup> Sharon Frey,<sup>3</sup> Patricia Winokur,<sup>4</sup> Nadine Rouphael,<sup>1</sup> Michelle Dickey,<sup>2</sup> Srilatha Edupuganti,<sup>1</sup> Paul Spearman,<sup>5</sup> Edwin Anderson,<sup>3</sup> Irene Graham,<sup>3</sup> Diana L. Noah,<sup>6</sup> Brian Mangal,<sup>7</sup> Sonnie Kim,<sup>8</sup> and Heather Hill<sup>7</sup> for the National Institute of Allergy and Infection Diseases Vaccine and Treatment Evaluation Units

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**Background.** Avian influenza A/H5N1 has threatened human health for nearly 2 decades. Avian influenza A vaccine without adjuvant is poorly immunogenic. A flexible rapid tactic for mass vaccination will be needed if a pandemic occurs.

**Methods.** A multicenter, randomized, blinded phase 1 clinical trial evaluated safety and antibody responses after point-of-use mixing of influenza A/Indonesia/05/2005 (H5N1) vaccine with MF59 adjuvant. Field-site pharmacies mixed 3.75, 7.5, or 15 mcg of antigen with or without MF59 adjuvant just prior to intramuscular administration on days 0 and 21 of healthy adults aged 18–49 years.

**Results.** Two hundred and seventy subjects were enrolled. After vaccination, titers of hemagglutination inhibition antibody  $\geq 1:40$  were achieved in 80% of subjects receiving 3.75 mcg + MF59 vs only 14% receiving 15 mcg without adjuvant ( $P < .0001$ ). Peak hemagglutination inhibition antibody geometric mean titers for vaccine + MF59 were  $\sim 65$  regardless of antigen dose, and neutralizing titers were 2- to 3-fold higher. Vaccine + MF59 produced cross-reactive antibody responses against 4 heterologous H5N1 viruses. Excellent safety and tolerability were demonstrated.

**Conclusions.** Point-of-use mixing of H5N1 antigen and MF59 adjuvant achieved target antibody titers in a high percentage of subjects and was safe. The feasibility of the point-of-use mixing should be studied further.

**Keywords.** adjuvant; antibody; avian influenza; H5N1; MF59; pandemic preparedness; point-of-use mixing; vaccine.

The enduring pandemic threat of Avian influenza A/H5N1 was underscored in January 2014 when the

first North American H5N1 death was documented in a Canadian who returned from China [1, 2]. For almost 2 decades preceding this fatality, the pandemic potential of avian H5N1 influenza has been of concern [3, 4]. More than 30 sporadic cases of H5N1 infection occurred in 2013, for a total of 648 laboratory-confirmed illnesses, and  $\sim 60\%$  mortality was reported to the World Health Organization by 16 countries from 2003 to 2013 [5]. The repeated outbreaks of novel and variant avian and swine influenza viruses in humans have led to efforts to produce vaccines against these pandemic or potential pandemic viruses.

Clinical trials of candidate H5N1 vaccines revealed that these hemagglutinins (HAs) were poor vaccine antigens

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[6–8], but antibody responses could be improved substantially with adjuvants [9]. Because vaccine supplies may be limited, it is also important to lower the antigen dose required to induce protection in order to make limited vaccine supplies available for protection of more people. For stockpiled vaccine antigens that require an adjuvant to achieve protective antibody titers, 1 rapid response approach would be to match the relevant antigen to a stockpiled adjuvant at pandemic onset and mix the reagents at designated field sites—the mix-and-match approach tested here.

The squalene-containing oil-in-water emulsion adjuvant, MF59, improved the antibody response, permitted dose sparing, and enhanced efficacy [10] when paired with inactivated influenza vaccines [11–14]. MF59-adjuvanted seasonal influenza vaccines were first licensed for the elderly [15] by the European Union in 1997 and—along with MF59-adjuvanted 2009 H1N1 pandemic vaccines—have subsequently been licensed in more than 30 countries with an excellent safety profile and over 65 million doses delivered [16].

MF59-adjuvanted vaccines have not been licensed in the United States. However, the US Department of Health and Human Services (HHS) Biomedical Advanced Research and Development Authority (BARDA) has procured in the National Pre-pandemic Influenza Vaccine Stockpile both H5N1 vaccines and the oil-in-water adjuvants MF59 and AS03. Here, we studied a mix-and-match tactic that paired a stockpiled H5N1 antigen from one manufacturer with stockpiled MF59 adjuvant from another manufacturer, and we demonstrated the potential of point-of-use mixing as a flexible logistical tactic for rapid deployment of stockpiled vaccine reagents should an H5N1 pandemic occur.

## METHODS

### Regulatory Reviews and Subject Consent

The Division of Microbiology and Infectious Diseases (DMID) 10-0016 protocol and informed consent forms were reviewed, approved, and monitored for subjects' safety and sites' Good Clinical Practices by DMID, the Food and Drug Administration, and the institutional review boards (IRBs) at Emory University, University of Iowa, Saint Louis University, and Cincinnati Children's Hospital Medical Center. Each subject provided written consent before performance of study procedures. The study was registered at ClinicalTrials.gov with identifier NCT01317745.

### Study Design

The trial was a randomized, double-blind, active-controlled, multicenter, phase 1 clinical trial in 270 healthy male and female subjects aged 18–49 years. Full inclusion and exclusion criteria are available at Clinicaltrials.gov identifier NCT01317745. After 25 subjects were excluded from vaccine dose 2, mostly for clinically insignificant safety laboratory abnormalities, the study

was amended in August 2011 to loosen grading scales for international normalized ratio (INR), partial thromboplastin time (PTT), and alanine aminotransferase (ALT).

The objectives were to assess the safety, reactogenicity, and immunogenicity of point-of-use mixing of H5N1 vaccine from one manufacturer and MF59 adjuvant from another manufacturer. Consenting eligible subjects were randomized 2:1 to vaccine + MF59 vs unadjuvanted vaccine arms, and they received 3.75, 7.5, or 15 mcg of vaccine based on HA content administered intramuscularly (IM) on days 0 and 21.

Clinic visits occurred on days 0, 8, 21, 29, 42, 201, and 386, and telephone calls occurred on days 1, 23, 81, and 141. Safety and tolerability were evaluated by collection of the following: serious adverse events (SAE) or new chronic medical conditions throughout the 13-month study period; adverse events (AEs) through day 42; clinical safety laboratories before and 8 days after each vaccination; and using a memory aid and local and systemic reactogenicity for 8 days after each vaccination. Additional monitoring for AEs of special interest (AESI) was conducted. The AESI were a predefined group of neuroinflammatory disorders; musculoskeletal, gastrointestinal, and skin disorders; metabolic diseases; and autoimmune conditions.

### Randomization and Masking

After a subject signed the IRB-approved consent form and met eligibility criteria, randomization took place just before vaccination. This process was done via the Internet using the enrollment module in the study database created by EMMES Corporation, the study Statistical and Data Coordinating Center. The enrollment module randomized the subject, and a Sequence Number and coded Treatment Number (to correspond to the treatment assignment) were displayed for study staff. Vaccine preparation was performed by the site pharmacist, and vaccination was performed by an unblinded vaccine administrator who was a study clinician licensed to administer medications and vaccines. This individual was not involved in study-related assessments or had no subject contact for data collection after vaccine administration. All study-related assessments were performed by blinded study personnel. Study subjects and laboratory personnel were also blinded to group assignment.

### Vaccine, Adjuvant, and the Mix-and-Match Procedure

The subvirion inactivated monovalent vaccine from influenza strain A/Indonesia/05/2005 (H5N1) was manufactured by Sanofi Pasteur, Inc. (Swiftwater, PA). The squalene oil-in-water adjuvant MF59 was manufactured by Novartis Vaccines and Diagnostics, Inc. (Marburg, Germany) and administered as 9.75 mg of squalene per dose. The US Department of Health and Human Services/BARDA provided the vaccine and adjuvant from the US National Pre-pandemic Influenza Vaccine Stockpile. Phosphate-buffered saline (PBS) diluent was obtained from Sanofi Pasteur, Inc. Vaccine, adjuvant, and PBS were stored

in the 4 Vaccine and Treatment Evaluation Unit (VTEU) pharmacies at 4°C until use. On the day of vaccination, a pharmacist performed mixing under a laminar flow hood using aseptic technique according to United States Pharmacopeia (USP) 797 guidelines. Once mixed, the preparations were stored in vials at room temperature and used within 8 hours. A final volume of 0.5 mL was administered IM in the deltoid by needle and syringe within 15 minutes of drawing into the syringe.

### Antibody Assays

Venous blood was collected for hemagglutination inhibition (HAI) antibody and neutralizing antibody (NAb) assays on days 0, 8, 21, 29, 42, 201, and 386. Sera were stored at -20°C or colder before shipping to a central repository on dry ice. Hemagglutination inhibition and NAb responses were measured as previously described at a single central laboratory (Southern Research, Birmingham, AL) [17, 18]. The NAb assay is also designated in the literature as the microneutralization assay [19]. Sera were tested against the homologous H5N1 clade 2.1.3 A/Indonesia/05/2005 reassortant virus (obtained from the Centers for Disease Control and Prevention [CDC]) as well as 4 heterologous H5N1 strains: A/Vietnam/1203/2004 (clade 1; CDC), A/Anhui/1/2005 (clade 2.3.4; CDC), A/turkey/Turkey/01/2005 (clade 2.2.1; obtained from National Institute for Biological Standards and Control (NIBSC), and A/Hubei/1/2010 (clade 2.3.2.1; CDC). Sera were tested in duplicate, and the initial dilution of the series was defined as 1:10 per the US Food and Drug Administration (FDA) recommendations. Sera without assay activity were scored as 1:5.

### Statistics

Demographics data were summarized by treatment group using descriptive statistics. Safety and reactogenicity data were summarized by treatment group using frequency counts, percentages, and exact 95% confidence intervals (CIs). The possibility of a dose-response or adjuvant-response relationship was explored by using logistic regression (none vs mild, moderate or severe, for local and systemic symptoms separately), adjusted by age and gender. Analyses were conducted separately for each vaccination. The homologous or heterologous antibody titers from the HAI and NAb assays were summarized by treatment group based on geometric mean titers (GMTs) and distribution of titers (emphasizing the proportion of subjects achieving HAI and NAb titers of  $\geq 1:40$ ) at study days 8, 21, 29, 42, 201, and 386. Geometric mean titers were analyzed on a logarithmic scale, with 95% CIs calculated using the normal approximation, then converted back to the original scale. Distributions of titers were compared between groups using Fisher's exact test. The correlation between HAI and NAb titers was explored using Pearson's correlation coefficient. General linear models with adjuvant as a fixed effect, dose as a continuous variable, and the interaction of the 2 were fit to the log-transformed titer data

to examine the dose response for both HAI and NAb titers. Models were fit separately for day 21 and day 42.

### Analysis Populations

The safety and tolerability population included any subject who received  $\geq 1$  vaccination. The intent-to-treat (ITT) population included all eligible subjects who received  $\geq 1$  vaccination and contributed both prevaccination and at least 1 postvaccination blood sample for which valid results were reported. The modified ITT (mITT) population included all subjects who received both vaccinations regardless of window. Vaccine storage temperature excursions and the receipt of a disallowed nonstudy vaccine excluded some subjects from this population. The per protocol (PP) population was defined as all subjects from the mITT population who received both vaccinations within the specified time windows.

### Role of the Funding Source

The US government's National Institute of Allergy and Infection Diseases (NIAID) contracted with the 4 VTEUs, the central laboratory, and the statistical and data coordinating center for the performance of the study. The National Institute of Allergy and Infection Diseases participated in the conception, design, regulatory approvals, monitoring, and reporting of the study. The corresponding author (M. J. M.) had full access to all of the data and had final responsibility for the decision to submit for publication.

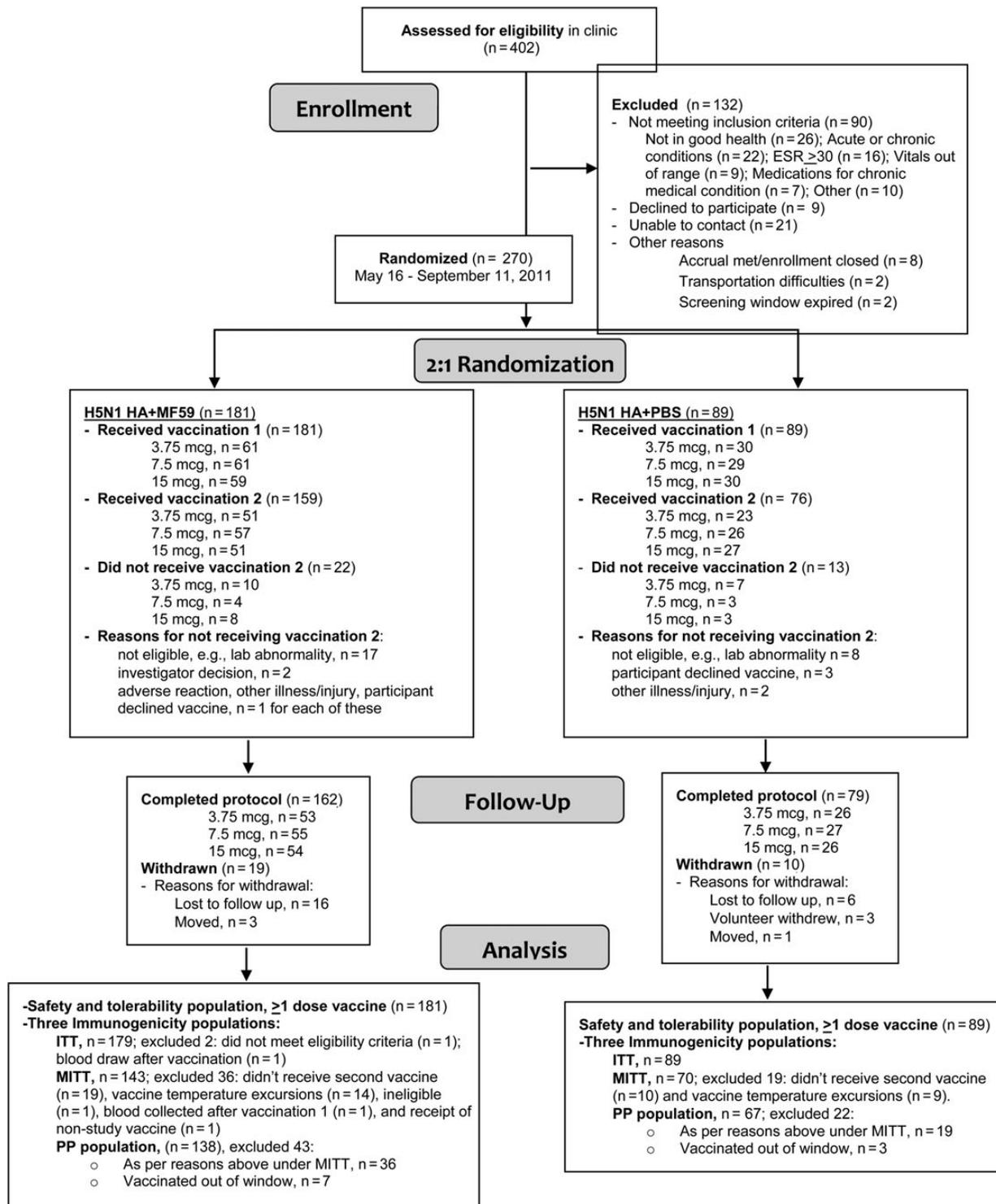
## RESULTS

### Enrollment and Demographics

Between May and September 2011, 402 interested volunteers were assessed for eligibility, and 270 were eligible, randomized, and vaccinated with dose 1 (Figure 1). Overall, the subjects were as follows: slightly more male (56%) than female; younger adults with median age 27.7 years; non-Hispanic (99%); and racially diverse with 71% Caucasian, 20% black/African-American, 6% Asian, and 3% multiracial (Supplementary Table 1). The demographic profiles did not vary significantly across the 6 study groups. The original safety laboratory grading scales for INR, PTT, and ALT (SGPT) were too stringent and caused 25 subjects to be excluded from vaccine 2 for insignificant elevations. The study was amended to correct this problem.

### Antibody Responses Against Vaccine Strain H5N1 Virus

Antibody data for the PP population are presented because the results for the ITT and mITT populations were not substantially different. For the vaccine + MF59 groups, the priming vaccination induced only low HAI GMTs of  $\sim 10$  on day 21, but the boost produced significantly higher GMTs of  $\sim 65$  that peaked on day 8 after the boost (Figure 2A). By 6 or 12 months post-boost, the HAI titers had fallen to near or below the level of detection (Figure 2A; Table 1A). For the unadjuvanted vaccine

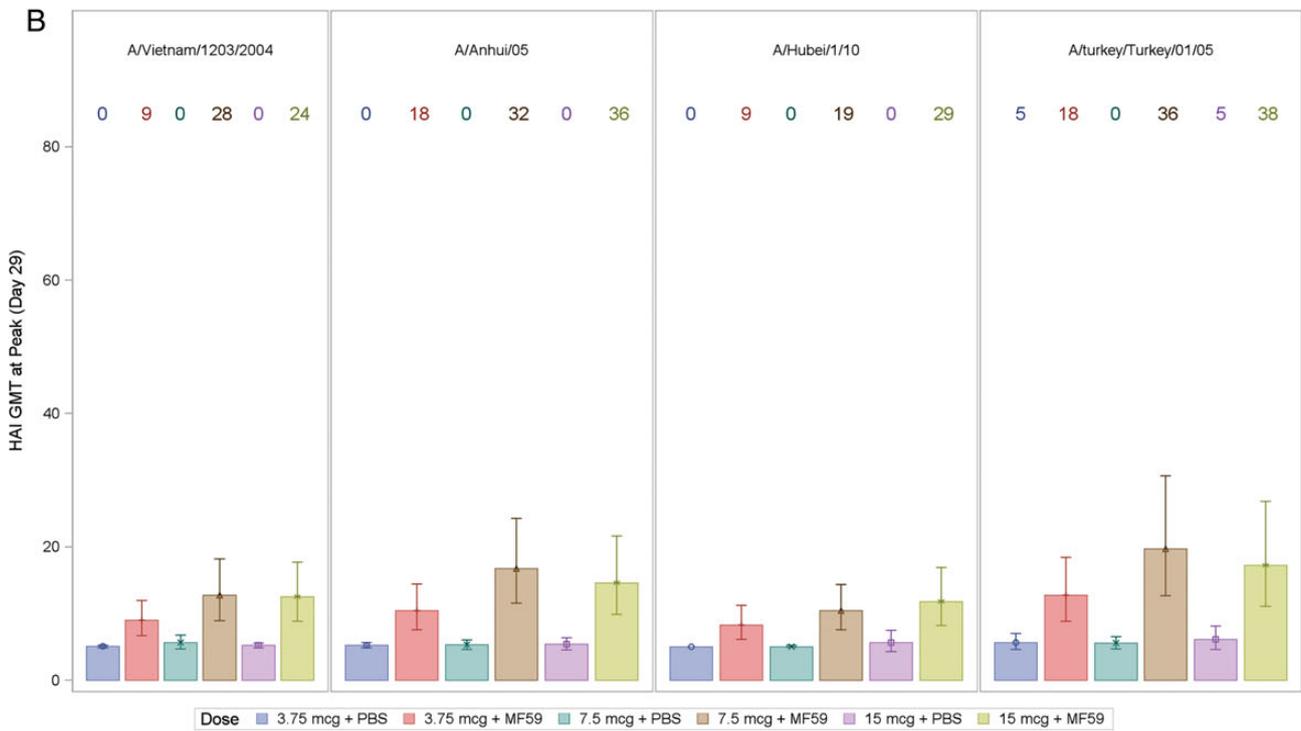
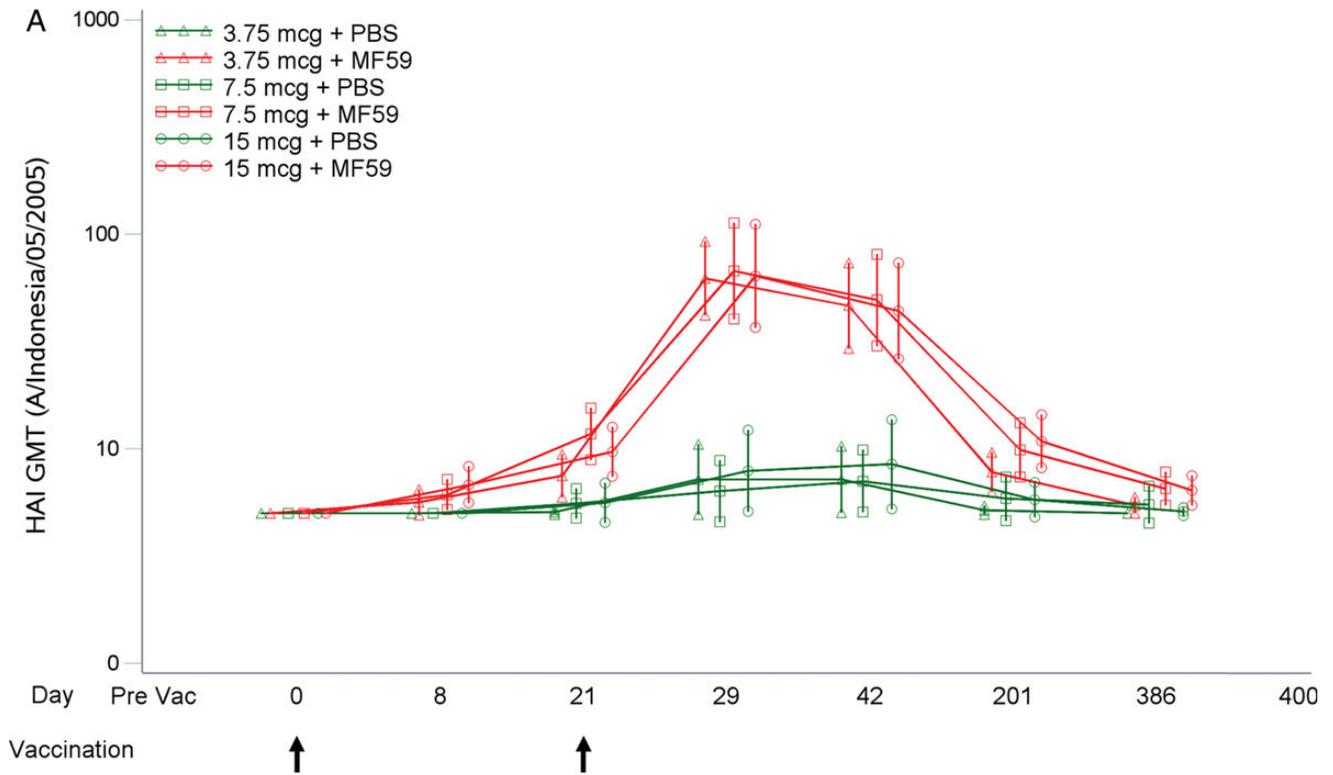


**Figure 1.** CONSORT flow diagram.

groups, neither the prime nor the boost produced an HAI GMT  $\geq$ 10 against the vaccine strain regardless of antigen dose. Neutralizing antibody GMTs also rose significantly after the vaccine + MF59 boosts (Figure 3A) and were 2- to 3-fold higher than HAI GMTs (Table 1B). Both HAI and NAb GMTs were

significantly higher for the adjuvanted vaccine groups than the unadjuvanted vaccine groups on study days 21, 29, 42, 201, and 386 ( $P < .0001$ ).

For vaccine + MF59, the percentages of subjects that achieved HAI titers of  $\geq$ 1:40 at any time for the 3.75, 7.5, and 15 mcg



**Figure 2.** Hemagglutination inhibition (HAI) antibody responses vs vaccine strain or 4 heterologous H5N1 viruses. (A) Hemagglutination inhibition antibody geometric mean titers (GMT) vs the homologous A/Indonesia/05/2005 virus (vaccine strain) by study day and group; 95% confidence interval (CI; vertical bars) and days of vaccinations (arrows) are shown. No subject had a detectable ( $\geq 1:10$ ) prevaccination HAI titer; undetectable titers were assigned a value of 5. (B) Hemagglutination inhibition antibody GMT at day 8 after boost and percentage of subjects achieving titer of  $\geq 1:40$  vs 4 heterologous H5N1 viruses. X-axis, 3 dosage levels for vaccine + MF59 or unadjuvanted vaccine. Y-axis, GMT with 95% CI. H5N1 strains are identified at the top of the bar plots. Numbers above the bars are the percentages of subjects achieving an HAI titer  $\geq 1:40$ .

**Table 1. HAI and NAb GMT and Proportions of Subjects With Titer  $\geq 1:40$  Against the Vaccine Strain, by Day for Each Study Group.**

Study Group	Day 0		Day 8		Day 21		Day 8 Post Dose 2		Day 21 Post Dose 2		Day 180 Post Dose 2		Day 365 Post Dose 2																																																																																																																														
	N	GMT (95% CI)	N	GMT (95% CI)	N	GMT (95% CI)	N	GMT (95% CI)	N	GMT (95% CI)	N	GMT (95% CI)	N	GMT (95% CI)																																																																																																																													
<b>A. HAI GMT to Homologous A/Indonesia/05/2005</b>																																																																																																																																											
3.75 mcg + MF59	55	5.0	55	5.6 (4.9–6.5)	55	7.5 (6.0–9.4)	44	62.2 (41.9–92.4)	44	46.5 (29.4–73.4)	43	7.8 (6.3–9.6)	39	5.5 (5.0–5.9)																																																																																																																													
3.75 mcg + PBS	27	5.0	27	5.0	27	5.1 (4.9–5.2)	21	7.2 (4.9–10.5)	21	7.2 (5.0–10.3)	21	5.2 (4.9–5.4)	20	5.0																																																																																																																													
7.5 mcg + MF59	55	5.0	55	6.1 (5.2–7.2)	54	11.7 (8.9–15.5)	47	67.5 (40.4–112.8)	46	49.4 (30.2–80.8)	47	9.9 (7.4–13.2)	43	6.5 (5.4–7.8)																																																																																																																													
7.5 mcg + PBS	27	5.0	27	5.0	26	5.6 (4.8–6.5)	22	6.3 (4.6–8.8)	23	7.1 (5.1–9.9)	22	5.9 (4.6–7.4)	22	5.5 (4.5–6.7)																																																																																																																													
15 mcg + MF59	54	5.0	54	6.8 (5.6–8.3)	53	9.7 (7.4–12.6)	42	64.0 (36.8–111.5)	41	43.9 (26.2–73.5)	40	10.8 (8.1–14.4)	37	6.4 (5.4–7.5)																																																																																																																													
15 mcg + PBS	27	5.0	27	5.0	27	5.6 (4.5–6.9)	22	7.9 (5.1–12.2)	21	8.5 (5.3–13.7)	19	5.8 (4.8–7.0)	17	5.1 (4.9–5.3)																																																																																																																													
<b>B. NAb GMT to Homologous A/Indonesia/05/2005</b>																																																																																																																																											
3.75 mcg + MF59	55	5.3 (5.1–5.6)	55	6.4 (5.6–7.4)	55	12.5 (9.7–16.0)	44	116.8 (81.0–168.3)	44	123.4 (84.8–179.5)	43	34.9 (26.9–45.2)	39	18.8 (14.5–24.3)																																																																																																																													
3.75 mcg + PBS	27	5.1 (4.9–5.2)	27	6.1 (4.5–8.3)	27	6.0 (4.8–7.5)	21	14.9 (9.0–24.6)	21	14.6 (8.9–24.1)	21	10.3 (7.7–13.9)	20	6.5 (5.4–7.8)																																																																																																																													
7.5 mcg + MF59	55	5.1 (5.0–5.3)	55	7.0 (6.0–8.1)	54	16.0 (12.1–21.0)	47	149.7 (98.0–228.7)	46	135.6 (91.4–201.1)	47	40.1 (30.3–53.1)	43	20.3 (15.1–27.4)																																																																																																																													
7.5 mcg + PBS	27	5.0	27	5.4 (5.1–5.7)	26	5.9 (5.0–7.1)	22	9.8 (6.2–15.7)	23	11.5 (7.3–18.0)	22	11.0 (7.2–16.9)	22	7.4 (5.2–10.6)																																																																																																																													
15 mcg + MF59	54	5.3 (5.0–5.5)	54	8.7 (7.3–10.5)	53	15.0 (11.7–19.2)	42	165.4 (106.5–256.7)	41	140.9 (95.2–208.7)	40	43.1 (34.0–54.6)	37	20.6 (16.0–26.5)																																																																																																																													
15 mcg + PBS	27	5.1 (4.9–5.4)	27	6.2 (5.4–7.2)	27	7.7 (5.7–10.6)	22	15.1 (8.8–25.8)	21	15.9 (9.4–26.8)	19	15.7 (10.3–23.9)	17	8.3 (5.8–12.0)																																																																																																																													
Study Group																																																																																																																																											
<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th rowspan="2">Visit</th> <th colspan="2">3.75 mcg</th> <th colspan="2">7.5 mcg</th> <th colspan="2">15 mcg</th> </tr> <tr> <th>MF59</th> <th>PBS</th> <th>MF59</th> <th>PBS</th> <th>MF59</th> <th>PBS</th> </tr> </thead> <tbody> <tr> <td colspan="7"><b>C. Proportion of Subjects with an HAI titer <math>\geq 1:40</math> for the Homologous A/Indonesia/05/2005 Virus.</b></td> </tr> <tr> <td colspan="7"><b>Day 0</b></td> </tr> <tr> <td>N Response/N Tested</td> <td colspan="2">0/55</td> <td colspan="2">0/27</td> <td colspan="2">0/54</td> </tr> <tr> <td>Proportion Response (95% CI)</td> <td colspan="2">0 (0–0.06)</td> <td colspan="2">0 (0–0.13)</td> <td colspan="2">0 (0–0.06)</td> </tr> <tr> <td colspan="7"><b>Day 8</b></td> </tr> <tr> <td>N Response/N Tested</td> <td colspan="2">1/55</td> <td colspan="2">0/27</td> <td colspan="2">3/55</td> </tr> <tr> <td>Proportion Response (95% CI)</td> <td colspan="2">0.02 (0–0.10)</td> <td colspan="2">0 (0–0.13)</td> <td colspan="2">0.05 (0.01–0.15)</td> </tr> <tr> <td colspan="7"><b>Day 21</b></td> </tr> <tr> <td>N Response/N Tested</td> <td colspan="2">5/55</td> <td colspan="2">0/27</td> <td colspan="2">10/54</td> </tr> <tr> <td>Proportion Response (95% CI)</td> <td colspan="2">0.09 (0.03–0.20)</td> <td colspan="2">0 (0–0.13)</td> <td colspan="2">0.19 (0.09–0.31)</td> </tr> <tr> <td colspan="7"><b>Day 8 - Post Dose 2</b></td> </tr> <tr> <td>N Response/N Tested</td> <td colspan="2">35/44</td> <td colspan="2">3/21</td> <td colspan="2">30/47</td> </tr> <tr> <td>Proportion Response (95% CI)</td> <td colspan="2">0.80 (0.65–0.90)</td> <td colspan="2">0.14 (0.03–0.36)</td> <td colspan="2">0.64 (0.49–0.77)</td> </tr> <tr> <td colspan="7"><b>Day 21 - Post Dose 2</b></td> </tr> <tr> <td>N Response/N Tested</td> <td colspan="2">27/44</td> <td colspan="2">2/21</td> <td colspan="2">29/46</td> </tr> <tr> <td>Proportion Response (95% CI)</td> <td colspan="2">0.61 (0.45–0.76)</td> <td colspan="2">0.10 (0.01–0.30)</td> <td colspan="2">0.63 (0.48–0.77)</td> </tr> </tbody> </table>															Visit	3.75 mcg		7.5 mcg		15 mcg		MF59	PBS	MF59	PBS	MF59	PBS	<b>C. Proportion of Subjects with an HAI titer <math>\geq 1:40</math> for the Homologous A/Indonesia/05/2005 Virus.</b>							<b>Day 0</b>							N Response/N Tested	0/55		0/27		0/54		Proportion Response (95% CI)	0 (0–0.06)		0 (0–0.13)		0 (0–0.06)		<b>Day 8</b>							N Response/N Tested	1/55		0/27		3/55		Proportion Response (95% CI)	0.02 (0–0.10)		0 (0–0.13)		0.05 (0.01–0.15)		<b>Day 21</b>							N Response/N Tested	5/55		0/27		10/54		Proportion Response (95% CI)	0.09 (0.03–0.20)		0 (0–0.13)		0.19 (0.09–0.31)		<b>Day 8 - Post Dose 2</b>							N Response/N Tested	35/44		3/21		30/47		Proportion Response (95% CI)	0.80 (0.65–0.90)		0.14 (0.03–0.36)		0.64 (0.49–0.77)		<b>Day 21 - Post Dose 2</b>							N Response/N Tested	27/44		2/21		29/46		Proportion Response (95% CI)	0.61 (0.45–0.76)		0.10 (0.01–0.30)		0.63 (0.48–0.77)	
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<b>C. Proportion of Subjects with an HAI titer <math>\geq 1:40</math> for the Homologous A/Indonesia/05/2005 Virus.</b>																																																																																																																																											
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N Response/N Tested	0/55		0/27		0/54																																																																																																																																						
Proportion Response (95% CI)	0 (0–0.06)		0 (0–0.13)		0 (0–0.06)																																																																																																																																						
<b>Day 8</b>																																																																																																																																											
N Response/N Tested	1/55		0/27		3/55																																																																																																																																						
Proportion Response (95% CI)	0.02 (0–0.10)		0 (0–0.13)		0.05 (0.01–0.15)																																																																																																																																						
<b>Day 21</b>																																																																																																																																											
N Response/N Tested	5/55		0/27		10/54																																																																																																																																						
Proportion Response (95% CI)	0.09 (0.03–0.20)		0 (0–0.13)		0.19 (0.09–0.31)																																																																																																																																						
<b>Day 8 - Post Dose 2</b>																																																																																																																																											
N Response/N Tested	35/44		3/21		30/47																																																																																																																																						
Proportion Response (95% CI)	0.80 (0.65–0.90)		0.14 (0.03–0.36)		0.64 (0.49–0.77)																																																																																																																																						
<b>Day 21 - Post Dose 2</b>																																																																																																																																											
N Response/N Tested	27/44		2/21		29/46																																																																																																																																						
Proportion Response (95% CI)	0.61 (0.45–0.76)		0.10 (0.01–0.30)		0.63 (0.48–0.77)																																																																																																																																						



dose levels were 80%, 64%, and 67%; and for Nab, percentages were 93%, 83%, and 90% (Table 1C and D). Because no participant had a prevaccination HAI or Nab titer >10, HAI seroconversion (minimum 4-fold rise in titer to  $\geq 1:40$ ) rates equaled the  $\geq 1:40$  rates and are not shown separately. Dose-response modeling of the HAI and Nab GMTs did not reveal any statistically significant antigen dose effect for either adjuvanted or unadjuvanted vaccines, although a trend for adjuvanted vaccine was apparent for Nab GMT (Figure 3B, left panel). Gender did not influence HAI or Nab responses.

Hemagglutination inhibition and Nab titers in individual sera generally correlated well with a Pearson's coefficient of 0.9 across all time points. However, a number of sera were noted to be discordant; ie, they had undetectable HAI titers but detectable Nab titers that ranged from 1:10 to 1:640. We then looked for the presence of these discordant HAI- Nab+ sera at each individual study visit and found they were more common at early (day 8 and 21) or late (day 201 or 386) visits when Pearson's coefficients were lower at 0.5–0.7. At the peak postvaccination time points (days 29 or 42), the HAI- Nab+ sera were less common and Pearson's correlations were higher at 0.9.

#### **Cross-reactive Antibody Responses Against Heterologous H5N1 Clades and Strains**

The clade 2.1 A/Indonesia/05/2005 vaccine (7.5 mg + adjuvant group) produced day 29 (peak) HAI titers  $\geq 1:40$  against clade 1 A/Vietnam/1203/2004, clade 2.3 A/Anhui/1/2005, clade 2.3 A/Hubei/1/2010, or clade 2.2 A/turkey/Turkey/01/2005 viruses in 28%, 32%, 19%, and 36% of subjects, respectively (Figure 2B); Nab titers  $\geq 1:40$  were achieved in 2%, 51%, 26%, and 64% (Figure 3B). We analyzed the relationships of the heterologous virus HAI or Nab GMTs at days 21 and 42 with the amino acid homology of their HA1 (variable globular head) or HA2 (conserved stem) proteins relative to the vaccine strain. There was no correlation.

#### **Safety**

No deaths occurred. Three SAEs reported for 2 subjects were not related to vaccine. Among 270 enrolled subjects, 183 unsolicited non-SAEs occurred in 110 subjects (41%) through day 21 after second vaccination, of which 49 events (27%) were related to vaccination (Supplementary Figure 1). The distributions of the related events among MedDRA System Organ Classes were similar across the 6 treatment groups. Two severe unsolicited nonserious AEs were judged by the investigators as unrelated to vaccination: vomiting in a 7.5 mcg + MF59 subject and abdominal pain and reflux in a 15 mcg + MF59 subject. A subject in the 15 mcg unadjuvanted group developed alopecia areata 12 days after second vaccination, which was judged as related by the blinded study investigator. Clinical hematology and chemistry laboratory results did not differ across the 6 study groups (data not shown). No AESIs were reported.

#### **Tolerability**

Solicited local symptoms or signs were reported by a higher percentage of subjects for vaccine + MF59 vs unadjuvanted vaccine (~75% vs ~40%) ( $P < 0.001$ ) and were as follows: mostly mild pain and tenderness; present mainly on days 1–3; not associated with antigen dose; and not worse with second vaccination (Supplemental Figure S2). Systemic reactogenicity was not significantly different for adjuvanted vs unadjuvanted vaccines.

## **DISCUSSION**

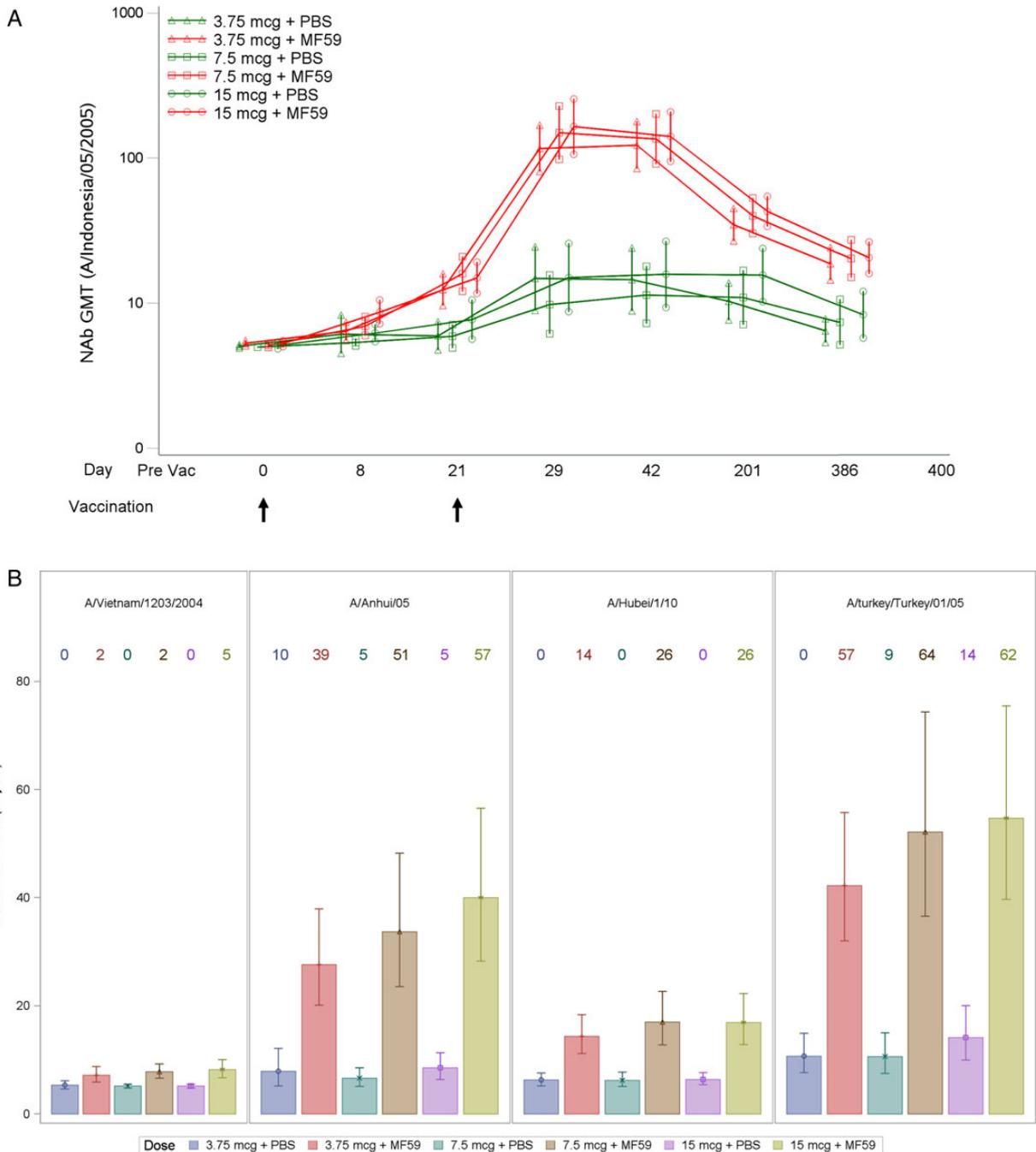
We conclude that H5N1 antigen and MF59 adjuvant from different manufacturers can be safely combined at the point-of-care and used to produce protective levels of postvaccination antibodies in most subjects. Additional logistical analyses would be needed to judge the following: (1) whether this tactic could be feasible for mass vaccination in a pandemic; (2) which sites could perform field mixing; and (3) what are the manpower requirements. Our experience at 4 university research clinics indicates that the mixing can be readily performed in laminar flow hoods by experienced pharmacists.

The on-site mixing of MF59 enhanced postvaccination responses so that a high percentage of subjects at any antigen dose developed HAI and Nab titers of  $\geq 1:40$ . The tolerability, safety, and antibody responses observed with point-of-use mixing of H5N1 antigen + MF59 from 2 manufacturers are similar to the experience when H5N1 antigen + MF59 were produced by a single manufacturer [9, 11, 13, 20].

The first dose of vaccine + MF59 clearly primed B and T cellular responses so that the second (booster) dose induced a rapid anamnestic antibody response with peak titers occurring less than 1 month after first vaccine. This quick response is desirable for rapid protection in a future pandemic. However, the duration of antibody was limited; by 6 months after boosting, most subjects had HAI titers that neared or fell below the threshold of detection. Neutralizing antibody had better durability, likely relating to the 2- to 3-fold higher peak titers achieved. Because a future pandemic would likely have an at-risk period extending several months, improved durability of antibody is an important area of future research.

The MF59 adjuvant is reported to induce a local proinflammatory environment that enhanced immune responses [21]. Preclinical data demonstrate that MF59 achieves this enhancement by targeting dendritic cells, monocytes/macrophages, and granulocytes, with a range of effects including increased antigen uptake, the release of chemoattractants, and induction of cell differentiation [22, 23]. Detailed transcriptome and systems analyses of the oil-in-water adjuvants are needed and are underway [24, 25].

Figure 4 demonstrates the dynamic shifting in postvaccination circulating antibody quality, particularly with MF59 use.

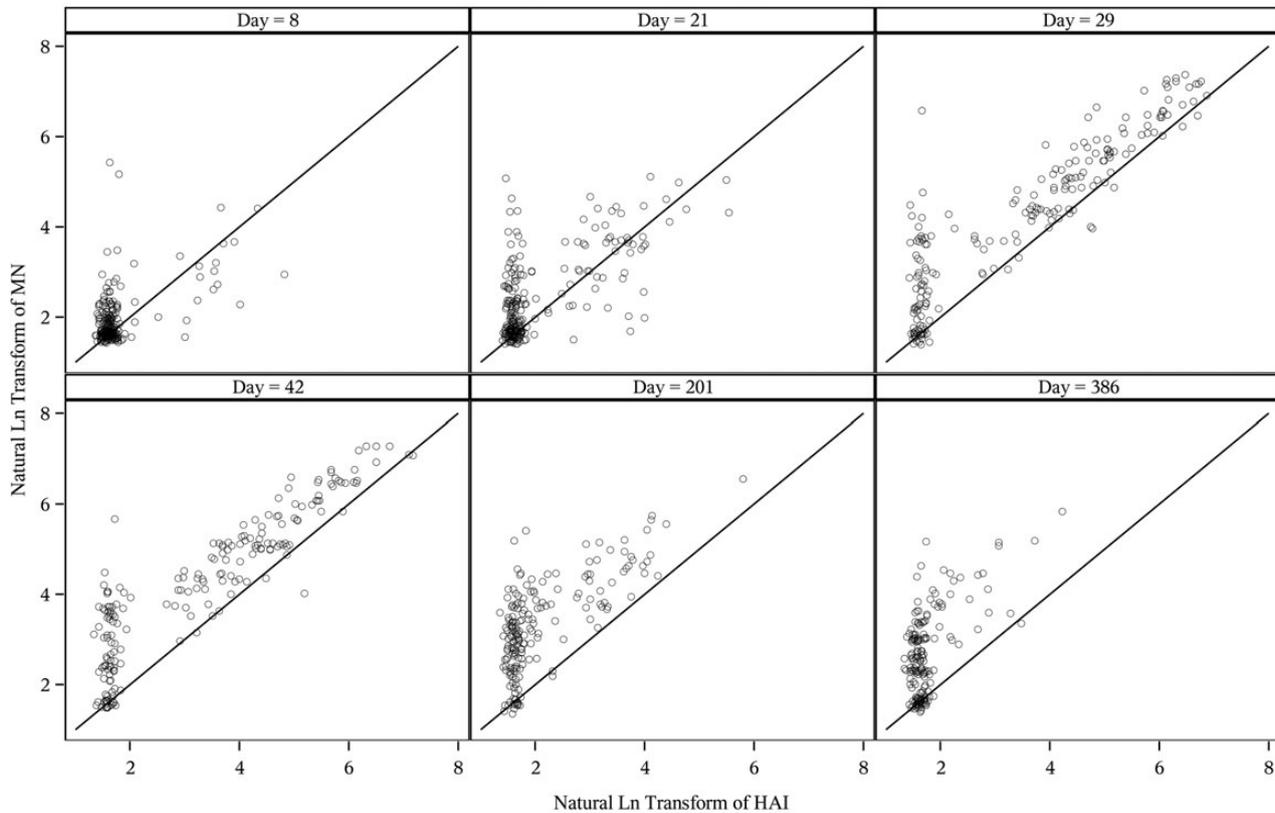


**Figure 3.** Neutralizing antibody (NAb) responses vs vaccine strain or 4 heterologous H5N1 viruses. (A) Neutralizing antibody geometric mean titers (GMT) vs the A/Indonesia/05/2005 virus (vaccine strain) by study day and group; 95% confidence interval (vertical bars) and days of vaccinations (arrows) are shown. (B) Neutralizing antibody GMT at day 8 after boost, and percentage of subjects achieving titer  $\geq 1:40$  vs 4 heterologous H5N1 viruses. Description otherwise as in Figure 2 legend.

Previous work reported an ability of the MF59 adjuvant to produce postvaccination temporal changes in H5-specific antibody quality through broadening of the serum antibody repertoire from conserved HA2 stem epitopes to the HA1 head including its receptor-binding domain [26]. We believe the early or late discordant sera (HA– NAb+) reflect antibodies directed primarily to conserved HA2 stem epitopes.

A dose-sparing effect of MF59 adjuvant was clearly demonstrated. The (lowest) 3.75 mcg antigen dose produced HAI titers  $\geq 1:40$  in 80% of subjects. By comparison, the FDA-licensed dosage for unadjuvanted H5N1 A/Vietnam/1203/2004 vaccine was 90 mcg given twice [27], a 24-fold higher dosage (without adjuvant) that achieved HAI titers  $\geq 1:40$  in just 58% of subjects [7]. It is important to note that our study did not define the

Scatter Plot of MN vs HAI for A/Indonesia/05/2005 By Visit  
(Per-Protocol Population)



Pearson Correlation Coefficients:  
Day 8 = 0.5318, Day 21 = 0.6700, Day 28 = 0.9070, Day 42 = 0.9033, Day 201 = 0.6946, Day 386 = 0.5958

**Figure 4.** Correlation of geometric mean titers (GMTs) from hemagglutination inhibition (HAI) and neutralizing antibody (designated as MN) assays against vaccine strain A/Indonesia/05/2005. Each circle is an individual participant; filled circles, participants received unadjuvanted vaccine; open circles, participants received adjuvanted vaccine. All participants were analyzed at each postvaccination time point for this analysis. At day 0 (data not shown), all HAI titers were undetectable and 18 subjects (7%) had NAb titers of 10 in both ( $n = 7$ ) or 1 ( $n = 11$ ) assay replicate.

minimal adjuvanted antigen dose that achieved the maximal antibody level, an area for further study.

Both safety and tolerability of vaccination after point-of-use mixing of H5N1 antigen with MF59 adjuvant were demonstrated. Because of theoretical or real [28, 29] concerns about provoking autoimmune diseases through molecular mimicry when strong adjuvants are added to vaccine antigens, the trial protocol designated AESIs. None were detected in this relatively small study. The safety profile of MF59 is well supported by published clinical trial experience with 23 000 subjects receiving influenza vaccines [16, 30].

As the segmented RNA genomes of circulating H5N1 viruses continue to drift in their avian hosts, the static stockpiled H5N1 vaccines become ever more dissimilar to contemporary strains. The antibody cross-reactivity results in this study indicated that an optimal public health response to a future H5N1 pandemic would require a clade- or strain-specific vaccine, as opposed to stockpiled vaccines matched to earlier H5N1 clades. With

widely available technologies and production timelines, this rapid strain-specific approach is not currently achievable, but recent advances in rapid technologies have been reported [31, 32].

The cross-reactive antibody responses produced by adjuvanted vaccine did not correlate with conservation of HA primary structure (AA sequence). The discrepancies in cross-reactive antibody responses to different H5N1 clades and strains likely resulted from mutations in conformational (rather than linear) antibody epitopes in evolved viral HA1 and HA2 target proteins.

Fortunately, if an individual vaccinated with stockpiled vaccine subsequently was exposed to a drifted H5N1 pandemic virus, cross-reactive circulating antibody and boosting of anamnestic antibody responses would be expected to provide some protection. Ideally, a pandemic clade- and/or strain-specific H5N1 vaccine boost would be given as soon as available. A number of data support the value of the heterologous prime-boost approach [8, 20, 33]. We previously showed that a delayed

heterologous boost produces higher levels of antibodies to both the prime and the boost strains [8].

In conclusion, our data indicate that a point-of-use mix-and-match tactic to deliver 2 doses of the most relevant stockpiled H5N1 antigen + MF59 adjuvant would offer several advantages in an H5N1 pandemic: safety, rapidity, flexibility, immunogenicity, cross-reactivity, and dose sparing.

Although this report was under review, a complementary study using AS03 adjuvant mixed onsite with H5N1 antigen was published [19]. That study differed primarily in use of AS03 adjuvant rather than MF59, it was performed in a different population (at 4 additional VTEU clinics), and it reported HAI titers  $\geq 40$  in 86%–100% of study participants.

## Supplementary Material

Supplementary material is available online at *Open Forum Infectious Diseases* (<http://OpenForumInfectiousDiseases.oxfordjournals.org/>).

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