19 to 23) with an H5N1 whole-virus formulation containing 3.75 μg, 7.5 μg, 15 μg, or 30 μg of hemagglutinin antigen with a 0.2% alum adjuvant or 7.5 μg or 15 μg of hemagglutinin antigen without adjuvant. There was no placebo group. Subjects and investigators were unaware of the dose of vaccine administered within the subgroups (Fig. 1, and the Supplementary Appendix). Blood samples were taken for serologic testing before the first dose of vaccine and on day 21 after the first and second doses.

Using a diary provided by the investigators, subjects were asked to record daily oral body temperature (using study-issued digital thermometers), local reactions, and systemic adverse events for 7 days after each vaccination. On days 7 and 21 after each vaccination, subjects were asked to return for a review of the diary and assessment for any adverse events.

ASSAYS

We evaluated all immunogenicity outcomes against the influenza-virus strain used in the vaccine (A/Vietnam/1203/2004) according to hemagglutination-inhibition and virus-neutralization assays. To assess cross-reactivity of antibodies, all assays were also conducted with known related influenza strains — for example, an original prototype clade 3 strain (A/Hong Kong/156/1997) and a clade 2 strain (A/Indonesia/05/2005).

Using a hemagglutination-inhibition or virus-neutralization assay, we investigated secondary
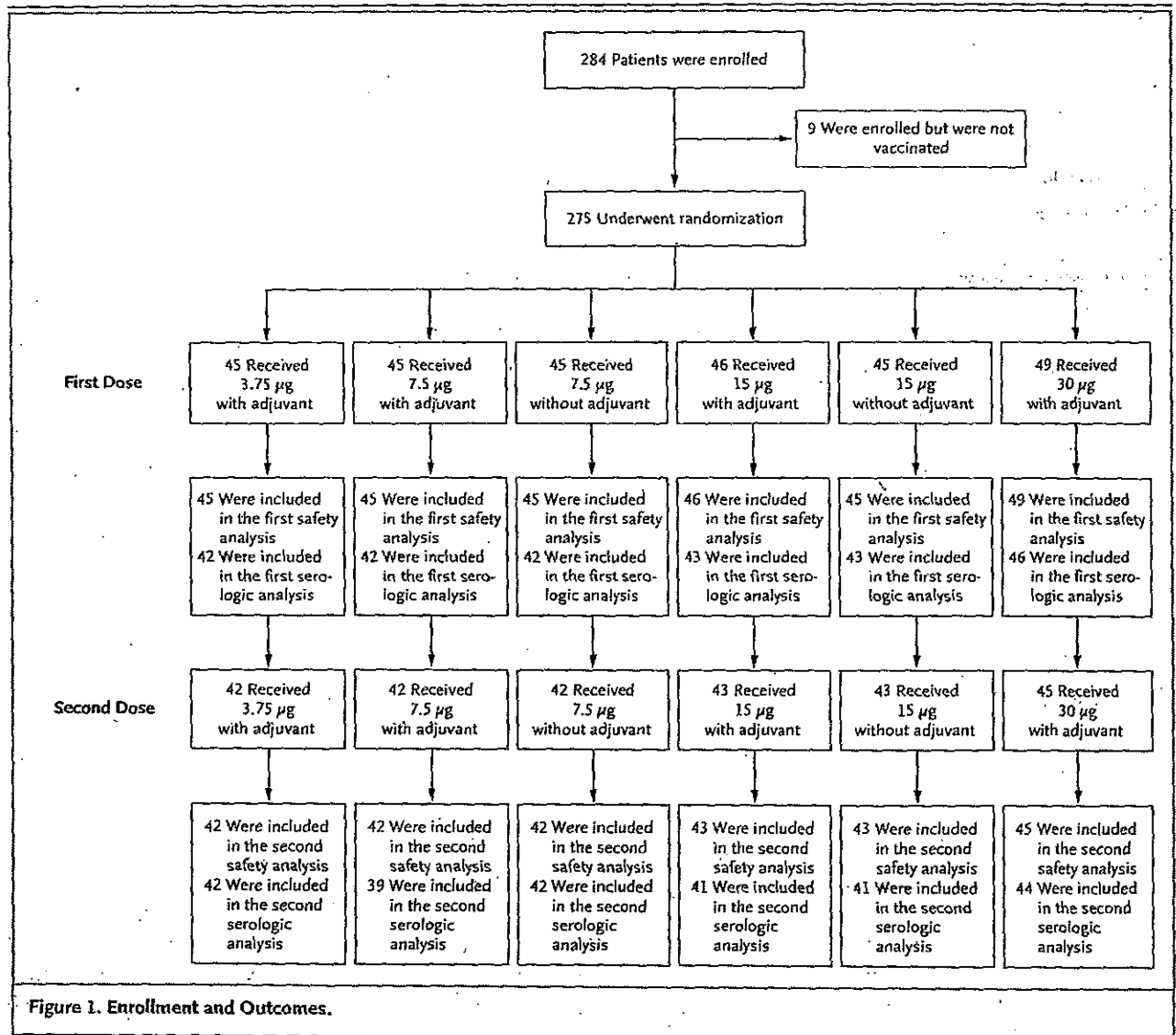

Figure 1. Enrollment and Outcomes.

191

Table 1. Proportion of Subjects with Injection-Site and Systemic Reactions within 7 Days after the First and Second Doses of Vaccine.

Variable	3.75 µg with Adjuvant	7.5 µg with Adjuvant	7.5 µg without Adjuvant	15 µg with Adjuvant	15 µg without Adjuvant	30 µg with Adjuvant
First dose						
No. of subjects	45	45	45	46	45	49
			percent (95% confidence interval)			
Injection-site reaction						
Any	29 (16–44)	22 (11–37)	11 (4–24)	28 (16–43)	20 (10–35)	24 (13–39)
Pain	27 (15–42)	20 (10–35)	9 (2–21)	26 (14–41)	18 (8–32)	24 (13–39)
Erythema*	0 (0–8)	2 (0–12)	2 (0–12)	4 (1–15)	0 (0–8)	0 (0–7)
Swelling*	0 (0–8)	0 (0–8)	0 (0–8)	2 (0–12)	0 (0–8)	2 (0–11)
Induration*	0 (0–8)	2 (0–12)	0 (0–8)	0 (0–8)	4 (1–15)	2 (0–11)
Ecchymosis*	0 (0–8)	0 (0–8)	0 (0–8)	0 (0–8)	2 (0–12)	2 (0–11)
Systemic reaction						
Any	51 (36–66)	31 (18–47)	38 (24–53)	30 (18–46)	47 (32–62)	18 (9–32)
Fever†	2 (0–12)	4 (1–15)	0 (0–8)	4 (1–15)	2 (0–12)	2 (0–11)
Headache	31 (18–47)	18 (8–32)	20 (10–35)	13 (5–26)	24 (13–40)	6 (1–17)
Malaise	13 (5–27)	11 (4–24)	4 (1–15)	13 (5–26)	9 (2–21)	6 (1–17)
Myalgia	9 (2–21)	16 (6–29)	4 (1–15)	9 (2–21)	9 (2–21)	2 (0–11)
Shivering	0 (0–8)	9 (2–21)	7 (1–18)	9 (2–21)	2 (0–12)	0 (0–7)
Second dose						
No. of subjects	42	42	42	43	43	45
			percent (95% confidence interval)			
Injection-site reaction						
Any	17 (7–31)	12 (4–26)	14 (5–29)	19 (8–33)	16 (7–31)	13 (5–27)
Pain	14 (5–29)	10 (3–23)	12 (4–26)	19 (8–33)	16 (7–31)	11 (4–24)
Erythema*	0 (0–8)	2 (0–13)	2 (0–13)	0 (0–8)	0 (0–8)	0 (0–8)
Swelling*	0 (0–8)	2 (0–13)	0 (0–8)	2 (0–12)	0 (0–8)	0 (0–8)
Induration*	5 (1–16)	0 (0–8)	0 (0–8)	2 (0–12)	0 (0–8)	0 (0–8)
Ecchymosis*	0 (0–8)	2 (0–13)	0 (0–8)	0 (0–8)	2 (0–12)	2 (0–12)
Systemic reaction						
Any	31 (18–47)	24 (12–39)	26 (14–42)	28 (15–44)	44 (29–60)	18 (8–32)
Fever†	0 (0–8)	2 (0–13)	5 (1–16)	0 (0–8)	7 (1–19)	2 (0–12)
Headache	19 (9–34)	10 (3–23)	5 (1–16)	9 (3–22)	12 (4–25)	13 (5–27)
Malaise	5 (1–16)	7 (1–19)	5 (1–16)	2 (0–12)	12 (4–25)	9 (2–21)
Myalgia	12 (4–26)	2 (0–13)	2 (0–13)	2 (0–12)	7 (1–19)	0 (0–8)
Shivering	0 (0–8)	2 (0–13)	5 (1–16)	2 (0–12)	7 (1–19)	0 (0–8)

* Listed are injection-site reactions with a diameter of more than 1 cm.
† Fever was defined as an oral temperature of 38°C (100.4°F) or more.

immunogenicity outcomes by analyzing the antibody response 21 days after the first and second doses of vaccine; the increase in the antibody response 21 days after the first and second doses, as compared with baseline; and the number of subjects with seroconversion (which we defined as a minimum increase by a factor of 4 in the titer) 21 days after the first and second doses, as compared with baseline.

The hemagglutination-inhibition assay is the standard test for detection of antibodies against influenza after infection or vaccination. However,

192

Table 2. Proportion of Subjects with a Virus-Neutralization Antibody Titer of 1:20 or More.

Virus Strain and Day	3.75 µg with Adjuvant	7.5 µg with Adjuvant	7.5 µg without Adjuvant	15 µg with Adjuvant	15 µg without Adjuvant	30 µg with Adjuvant
A/Vietnam/1203/2004 (clade 1)						
Day 0						
No./total no. (%)	0/42	3/42 (7.1)	0/42	1/43 (2.3)	0/43	0/46
95% CI	0.0–8.4	1.5–19.5	0.0–8.4	0.1–12.3	0.0–8.2	0.0–7.7
Day 21						
No./total no. (%)	9/42 (21.4)	11/42 (26.2)	17/42 (40.5)	7/43 (16.3)	17/43 (39.5)	5/46 (10.9)
95% CI	10.3–36.8	13.9–42.0	25.6–56.7	6.8–30.7	25.0–55.6	3.6–23.6
Day 42						
No./total no. (%)	29/42 (69.0)	25/39 (64.1)	32/42 (76.2)	25/41 (61.0)	29/41 (70.7)	29/44 (65.9)
95% CI	52.9–82.4	47.2–78.8	60.5–87.9	44.5–75.8	54.5–83.9	50.1–79.5
A/Indonesia/05/2005 (clade 2)						
Day 0						
No./total no. (%)	1/42 (2.4)	1/42 (2.4)	0/42	1/43 (2.3)	0/43	0/46
95% CI	0.1–12.6	0.1–12.6	0.0–8.4	0.1–12.3	0.0–8.2	0.0–7.7
Day 21						
No./total no. (%)	5/42 (11.9)	5/42 (11.9)	10/42 (23.8)	1/43 (2.3)	7/43 (16.3)	3/46 (6.5)
95% CI	4.0–25.6	4.0–25.6	12.1–39.5	0.1–12.3	6.8–30.7	1.4–17.9
Day 42						
No./total no. (%)	12/42 (28.6)	14/39 (35.9)	19/42 (45.2)	3/41 (7.3)	15/41 (36.6)	13/44 (29.5)
95% CI	15.7–44.6	21.2–52.8	29.8–61.3	1.5–19.9	22.1–53.1	16.8–45.2
A/Hong Kong/156/1997 (clade 3)						
Day 0						
No./total no. (%)	0/42	4/42 (9.5)	2/42 (4.8)	2/43 (4.7)	1/43 (2.3)	1/46 (2.2)
95% CI	0.0–8.4	2.7–22.6	0.6–16.2	0.6–15.8	0.1–12.3	0.1–11.5
Day 21						
No./total no. (%)	9/42 (21.4)	13/42 (31.0)	20/42 (47.6)	9/43 (20.9)	18/43 (41.9)	7/46 (15.2)
95% CI	10.3–36.8	17.6–47.1	32.0–63.6	10.0–36.0	27.0–57.9	6.3–28.9
Day 42						
No./total no. (%)	28/42 (66.7)	25/39 (64.1)	32/42 (76.2)	26/41 (63.4)	32/41 (78.0)	34/44 (77.3)
95% CI	50.5–80.4	47.2–78.8	60.5–87.9	46.9–77.9	62.4–89.4	62.2–88.5

this assay may be insensitive for the detection of anti-H5 antibodies.[10,11] For this reason, immunogenicity analyses focused on a determination of functional neutralizing-antibody responses. Since most licensing authorities typically request data regarding hemagglutination-inhibition assays or single radial hemolysis, these determinations are also reported but only for the vaccine virus strain A/Vietnam/1203/2004. (For details on hemagglutination-inhibition and virus-neutralization assays and single radial hemolysis,[12-14] see the Supplementary Appendix.)

STATISTICAL ANALYSIS

The protocol called for the recruitment of 45 subjects per study group. With this number of subjects, the 95% confidence interval for the percentage of subjects with an antibody response that was associated with protection did not extend more than 15% from the observed rate, assuming a seroprotection rate of approximately 80%.

We used the likelihood-ratio chi-square test to compare the number of subjects with local or systemic reactions within 7 days after vaccination among the various vaccine formulations. For bi-

Table 3. Geometric Mean of the Increase from Baseline (GMI) and Proportion of Subjects with Seroconversion.*

Virus Strain and Day	3.75 μg with Adjuvant		7.5 μg with Adjuvant		7.5 μg without Adjuvant	
	GMI	Seroconversion	GMI	Seroconversion	GMI	Seroconversion
	value (95% CI)	% (95% CI)	value (95% CI)	% (95% CI)	value (95% CI)	% (95% CI)
A/Vietnam/1203/2004 (clade 1)						
Day 21	2.0 (1.6–2.4)	11.9 (4.0–25.6)	2.0 (1.6–2.5)	9.5 (2.7–22.6)	3.2 (2.4–4.2)	35.7 (21.6–52.0)
Day 42	4.4 (3.5–5.6)	54.8 (38.7–70.2)	4.0 (3.1–5.2)	51.3 (34.8–67.6)	5.3 (4.1–6.9)	69.0 (52.9–82.4)
A/Indonesia/05/2005 (clade 2)						
Day 21	1.7 (1.4–1.9)	4.8 (0.6–16.2)	1.6 (1.3–1.9)	7.1 (1.5–19.5)	2.2 (1.8–2.8)	19.0 (8.6–34.1)
Day 42	2.8 (2.3–3.4)	19.0 (8.6–34.1)	2.7 (2.1–3.4)	28.2 (15.0–44.9)	3.2 (2.5–4.0)	31.0 (17.6–47.1)
A/Hong Kong/156/1997 (clade 3)						
Day 21	2.3 (1.8–2.9)	16.7 (7.0–31.4)	2.3 (1.8–2.8)	14.3 (5.4–28.5)	3.4 (2.5–4.7)	38.1 (23.6–54.4)
Day 42	5.8 (4.4–7.7)	69.0 (52.9–82.4)	5.2 (3.8–7.1)	51.3 (34.8–67.6)	5.9 (4.3–8.1)	66.7 (50.5–80.4)

* Seroconversion was defined as an increase in the virus-neutralization titer by a factor of 4 or more.

nary variables (i.e., seroprotection and seroconversion), response rates and 95% confidence intervals were computed for each strain and time point. The confidence intervals were interpreted in a descriptive manner, and no adjustment for multiplicity was made.[15]

In addition, for the log-transformed values of virus-neutralization titers and single radial hemolysis, a longitudinal analysis was performed within a repeated mixed-model framework of analysis of covariance. Changes from baseline were analyzed, accounting for the fixed effects of vaccine formulation, day, sex, age, baseline titer, interaction between the vaccine formulation and day, and random effects for subjects. Vaccine formulations without adjuvant were compared with formulations with adjuvant within this model. Comparisons were also made between groups receiving 7.5 μg and 15 μg of hemagglutinin antigen without adjuvant. We calculated the proportion of subjects with a virus-neutralization titer of 1:20 or more and that of subjects with results of 25 mm² or more on single radial hemolysis, using a generalized linear model with repeated measurements and the general-estimating-equations method (see the Supplementary Appendix).

RESULTS

STUDY POPULATION

A total of 275 subjects between the ages of 18 and 45 years received the first dose of vaccine, and 257 received the second dose. All vaccinated subjects were included in the safety analysis. Two subjects who initially gave their consent withdrew from the study because of nonserious adverse events, including four events in one subject (chills, fatigue, malaise, and insomnia) and one event in the second subject (papular rash); the majority of these symptoms abated within 24 hours. Immunogenicity data were available for 258 subjects for the first dose of vaccine and for 249 subjects for the second dose of vaccine.

SAFETY

The rates of occurrence of injection-site and systemic reactions during the first 7 days after each dose of vaccine are presented in Table 1. No serious, vaccine-related adverse events were recorded. There were two serious adverse events recorded in two subjects: hospitalization due to a contusion of the left foot and hospitalization for an elective abortion.

The most commonly occurring injection-site reaction after vaccination was pain, which occurred in 9 to 27% of subjects; the most frequently reported systemic reaction was headache, which occurred in 6 to 31% of subjects.

There were no significant differences between the vaccine formulations with respect to local reactions after the first dose and the second dose of vaccine (P=0.32 and P=0.97, respectively, for all comparisons). With respect to systemic reactions, a slight difference was observed between the vaccine formulations after the first dose of vaccine (P=0.01), a finding that was largely due

194

15 μg with Adjuvant		15 μg without Adjuvant		30 μg with Adjuvant	
GMI value (95% CI)	Seroconversion % (95% CI)	GMI value (95% CI)	Seroconversion % (95% CI)	GMI value (95% CI)	Seroconversion % (95% CI)
1.9 (1.5–2.4)	11.6 (3.9–25.1)	3.1 (2.5–4.0)	34.9 (21.0–50.9)	2.1 (1.8–2.5)	13.0 (4.9–26.3)
3.9 (3.0–5.0)	46.3 (30.7–62.6)	5.7 (4.3–7.5)	68.3 (51.9–81.9)	4.6 (4.0–5.4)	61.4 (45.5–75.6)
1.4 (1.2–1.7)	2.3 (0.1–12.3)	2.3 (1.8–2.9)	16.3 (6.8–30.7)	1.7 (1.5–2.0)	2.2 (0.1–11.5)
2.5 (2.1–2.9)	9.8 (2.7–23.1)	3.6 (2.9–4.5)	43.9 (28.5–60.3)	2.9 (2.5–3.5)	29.5 (16.8–45.2)
2.0 (1.5–2.7)	11.6 (3.9–25.1)	3.3 (2.5–4.3)	30.2 (17.2–46.1)	1.9 (1.6–2.3)	15.2 (6.3–28.9)
4.9 (3.7–6.5)	53.7 (37.4–69.3)	7.8 (5.7–10.6)	75.6 (59.7–87.6)	5.7 (4.6–7.0)	63.6 (47.8–77.6)

to an unexpectedly low rate of headache observed in the group receiving the 30-μg formulation with adjuvant. No difference was shown regarding systemic reactions after the second dose of vaccine (P=0.15).

IMMUNE RESPONSE

At 21 days after the first and second doses, functional neutralizing antibodies against strain A/Vietnam/1203/2004 were detected in patients receiving any of the six formulations. Table 2 shows the rates of response in subjects with a virus-neutralization titer of 1:20 or more, and Table 3 shows the geometric mean increase (GMI) of the titer from baseline and the percentage of seroconversion. Numerically, the formulations without adjuvant induced the highest rates of a virus-neutralization titer of 1:20 or more after the first dose (40.5% and 39.5% for 7.5 μg and 15 μg without adjuvant, respectively) and the second dose (76.2% and 70.7% for 7.5 μg and 15 μg without adjuvant, respectively) (Table 2). Similar results were obtained with respect to GMI (Table 3), since the highest GMIs were obtained for the formulations without adjuvant (5.3 and 5.7 for 7.5 μg and 15 μg without adjuvant, respectively) (Table 3). Among subjects with seroconversion (an increase in the titer by a factor of at least 4 after immunization), the highest rates of response were again seen in subjects who received a 7.5-μg or 15-μg formulation without adjuvant (69.0% and 68.3%, respectively) (Table 3).

Statistical analysis with the use of a mixed-model on log-transformed virus-neutralization values confirmed that the formulations without adjuvant induced significantly higher immune responses than did the formulations with adjuvant (P<0.001). There were no significant differences between the two formulations without adjuvant or among the four formulations with adjuvant. All vaccine formulations showed a similar ratio of increase in antibody titer between day 21 and day 42, as shown by the nonsignificant interaction between vaccine formulation and day (Table 4, and Table 4 in the Supplementary Appendix).

Table 5 compares the presumed rates of seroprotection, as measured by hemagglutination-inhibition assay (i.e., the proportion of subjects with a titer ≥40) and single radial hemolysis (i.e., the proportion of subjects with an area of ≥25 m² on single radial hemolysis). Numerically, the formulations without adjuvant again were more immunogenic than those with adjuvant. On single radial hemolysis, the percentage of seroprotection 21 days after the second dose of vaccine without adjuvant was 78.6% for the 7.5-μg dose and 61.0% for the 15-μg dose. Single radial hemolysis for H5N1 antibodies appeared to be more sensitive than hemagglutination-inhibition assay, since the equivalent values for hemagglutination-inhibition assay were 47.6% and 26.8%, respectively.

We also analyzed changes from baseline in results on single radial hemolysis using a mixed-model analysis of covariance for the log-transformed values, and the results were similar to those obtained for the virus-neutralization titers. Again, we observed a significant effect of the

Table 4. Mixed-Model Analysis of Log-Transformed Values of Virus-Neutralization Titer.

Effects and Comparison	A/Vietnam/ 1203/2004 (Clade 1)	A/Indonesia/ 05/2005 (Clade 2)	A/Hong Kong/ 156/1997 (Clade 3)
		P Value	
Effect			
Vaccine formulation	0.004	0.001	0.01
Day 21 vs. day 42	<0.001	<0.001	<0.001
Baseline	<0.001	<0.001	<0.001
Sex	0.009	0.08	0.01
Age	0.41	0.18	0.03
Vaccine formulation–day interaction	0.06	0.36	0.01
Comparison			
With adjuvant vs. without adjuvant	<0.001	<0.001	<0.001
Without adjuvant, 7.5 μg vs. 15 μg	0.80	0.97	0.70

vaccine formulations, with formulations without adjuvant showing higher response rates than those with adjuvant. There was no significant difference between the two formulations without adjuvant or among the formulations with adjuvant (Table 4, and Table 5 in the Supplementary Appendix).

CROSS-NEUTRALIZATION

The 7.5-μg and 15-μg formulations without adjuvant showed high levels of cross-reactivity against the A/Hong Kong strain (76.2% and 78.0%, respectively, with a neutralizing titer of ≥1:20) (Table 2). The responses against the clade 2 strain were somewhat lower (with rates of a virus-neutralization titer of ≥1:20 of 45.2% and 36.6% for the 7.5-μg and 15-μg formulations without adjuvant, respectively) (Table 2).

We also analyzed the virus-neutralization response to the heterologous strains using the mixed model. Results were similar to those for the homologous strain. Formulations without adjuvant elicited significantly higher immune responses than those with adjuvant. Antibody titers increased significantly from baseline, independently of the vaccine dose (Table 4, and Tables 3 and 4 in the Supplementary Appendix).

The reverse cumulative distribution curves for antibody titers after the first and second doses of vaccine against all three strains support the finding of higher immunogenicity from the formulations without adjuvant (Fig. 2). Analysis of rates of seroprotection with homologous and heterologous immune responses showed results that were consistent with those obtained by direct analysis of values of virus-neutralization titers and single radial hemolysis (Tables 6 and 7 in the Supplementary Appendix).

DISCUSSION

It has been reported that whole-virus trivalent influenza vaccines are more immunogenic than subvirion vaccines but are also more prone to cause adverse reactions.[5] In our study, a monovalent whole-virus H5N1 vaccine had a side-effect profile similar to that of subvirion H5N1 formulations described previously.[2,3,16] Most important, the low rate of fever among subjects in our study (2 to 7%) compares favorably with that reported both for subvirion H5N1 vaccines and for an egg-derived whole-virus H5N1 vaccine with adjuvant.[2,3,6,16] However, it should be noted that reporting systems and characteristics of the subjects differ among the various studies.

With respect to immunogenicity, the highest neutralizing-antibody response after the second dose of vaccine (76.2%) was obtained with the 7.5-μg formulation without adjuvant, which was equivalent to a rate of seroconversion of 69.0% and represented an increase by a factor of 4 or more in the neutralization titer after two doses of vaccine (Tables 2 and 3). These data are also similar to the levels of immunogenicity reported in a study of an egg-derived whole-virus H5N1 vaccine, in which 96% of subjects who received

196

Dose with or without Adjuvant	Assay	Seroprotection			Seroconversion		GMI	
		Day 0	Day 21	Day 42	Day 21	Day 42	Day 21	Day 42
		percent (95% CI)					value (95% CI)	
3.75 μg with adjuvant	HI	2.4 (0.1–12.6)	33.3 (19.6–49.5)	40.5 (25.6–56.7)	33.3 (19.6–49.5)	38.1 (23.6–54.4)	2.7 (1.7–4.4)	4.5 (2.4–8.4)
	SRH	4.8 (0.6–16.2)	26.2 (13.9–42.0)	50.0 (34.2–65.8)	21.4 (10.3–36.8)	47.6 (32.0–63.6)	1.7 (1.2–2.3)	2.9 (2.0–4.2)
7.5 μg with adjuvant	HI	4.8 (0.6–16.2)	35.7 (21.6–52.0)	38.5 (23.4–55.4)	35.7 (21.6–52.0)	35.9 (21.2–52.8)	3.2 (1.9–5.4)	3.6 (1.9–6.8)
	SRH	4.8 (0.6–16.2)	26.2 (13.9–42.0)	35.9 (21.2–52.8)	21.4 (10.3–36.8)	33.3 (19.1–50.2)	1.7 (1.2–2.3)	2.3 (1.5–3.4)
7.5 μg without adjuvant	HI	0.0 (0.0–8.4)	47.6 (32.0–63.6)	47.6 (32.0–63.6)	47.6 (32.0–63.6)	47.6 (32.0–63.6)	4.5 (2.7–7.6)	5.3 (3.0–9.5)
	SRH	7.1 (1.5–19.5)	69.0 (52.9–82.4)	78.6 (63.2–89.7)	61.9 (45.6–76.4)	73.8 (58.0–86.1)	4.8 (3.2–7.2)	6.3 (4.3–9.1)
15 μg with adjuvant	HI	0 (0.0–8.2)	14.0 (5.3–27.9)	14.6 (5.6–29.2)	14.0 (5.3–27.9)	14.6 (5.6–29.2)	1.5 (1.1–2.2)	1.7 (1.1–2.7)
	SRH	4.7 (0.6–15.8)	16.3 (6.8–30.7)	39.0 (24.2–55.5)	11.6 (3.9–25.1)	36.6 (22.1–53.1)	1.4 (1.1–1.8)	2.2 (1.6–3.2)
15 μg without adjuvant	HI	0 (0.0–8.2)	25.6 (13.5–41.2)	26.8 (14.2–42.9)	25.6 (13.5–41.2)	26.8 (14.2–42.9)	2.8 (1.6–4.9)	3.2 (1.7–6.0)
	SRH	2.3 (0.1–12.3)	41.9 (27.0–57.9)	61.0 (44.5–75.8)	39.5 (25.0–55.6)	58.5 (42.1–73.3)	2.8 (1.9–4.2)	4.7 (3.1–7.1)
30 μg with adjuvant	HI	0 (0.0–7.7)	34.8 (21.4–50.2)	36.4 (22.4–52.2)	34.8 (21.4–50.2)	36.4 (22.4–52.2)	3.4 (2.0–5.7)	4.5 (2.4–8.6)
	SRH	2.2 (0.1–11.5)	21.7 (10.9–36.4)	58.1 (42.1–73.0)	19.6 (9.4–33.9)	58.1 (42.1–73.0)	1.5 (1.2–2.0)	3.6 (2.5–5.2)

Table 5. Antibody Response to the Homologous Virus Strain after the First and Second Doses of Vaccine.*

* GMI denotes geometric mean of the increase, HI hemagglutination-inhibition assay, and SRH single radial hemolysis.

two doses of 5-μg or 10-μg formulations had a neutralization titer of 1:20 or more,[6] although differences in assay systems must be taken into account in making such direct comparisons.

Lower rates of seroprotection and seroconversion (as defined in the guidelines of the Committee for Proprietary Medicinal Products[17]) were obtained with the hemagglutination-inhibition assay than with the virus-neutralization assay, which supports the finding that the hemagglutination-inhibition assay is less sensitive for detection of anti-H5 antibodies, as reported previously.[10,11] In our study, single radial hemolysis, which is considered to have a sensitivity equivalent to that of the hemagglutination-inhibition assay for seasonal influenza strains,[18] was shown to be more sensitive than the hemagglutination-inhibition assay for H5N1.

The lack of enhancement of vaccine immunogenicity by the use of alum adjuvant at the doses studied here was consistent with data from a previous study, which showed that no effect of alum adjuvant was seen with a 15-μg dose of subvirion vaccine, and a 7.5-μg formulation without alum was more immunogenic than the formulation with adjuvant.[3] In the previous study, an enhanced immune response with the use of alum was seen only with the 30-μg formulation. We did not investigate this dose without alum in our study.

However, other studies have described substantial positive effects of other adjuvants on H5N1 immunogenicity. The use of an oil-in-water–based emulsion in a 3.8-μg dose of split-virus vaccine resulted in 82% seroconversion, as compared with 4% seroconversion without adjuvant.[16] The addition of another oil-in-water–based adjuvant (MF-59) to an H5N3 vaccine was also associated with a substantial increase in antibody response.[19]
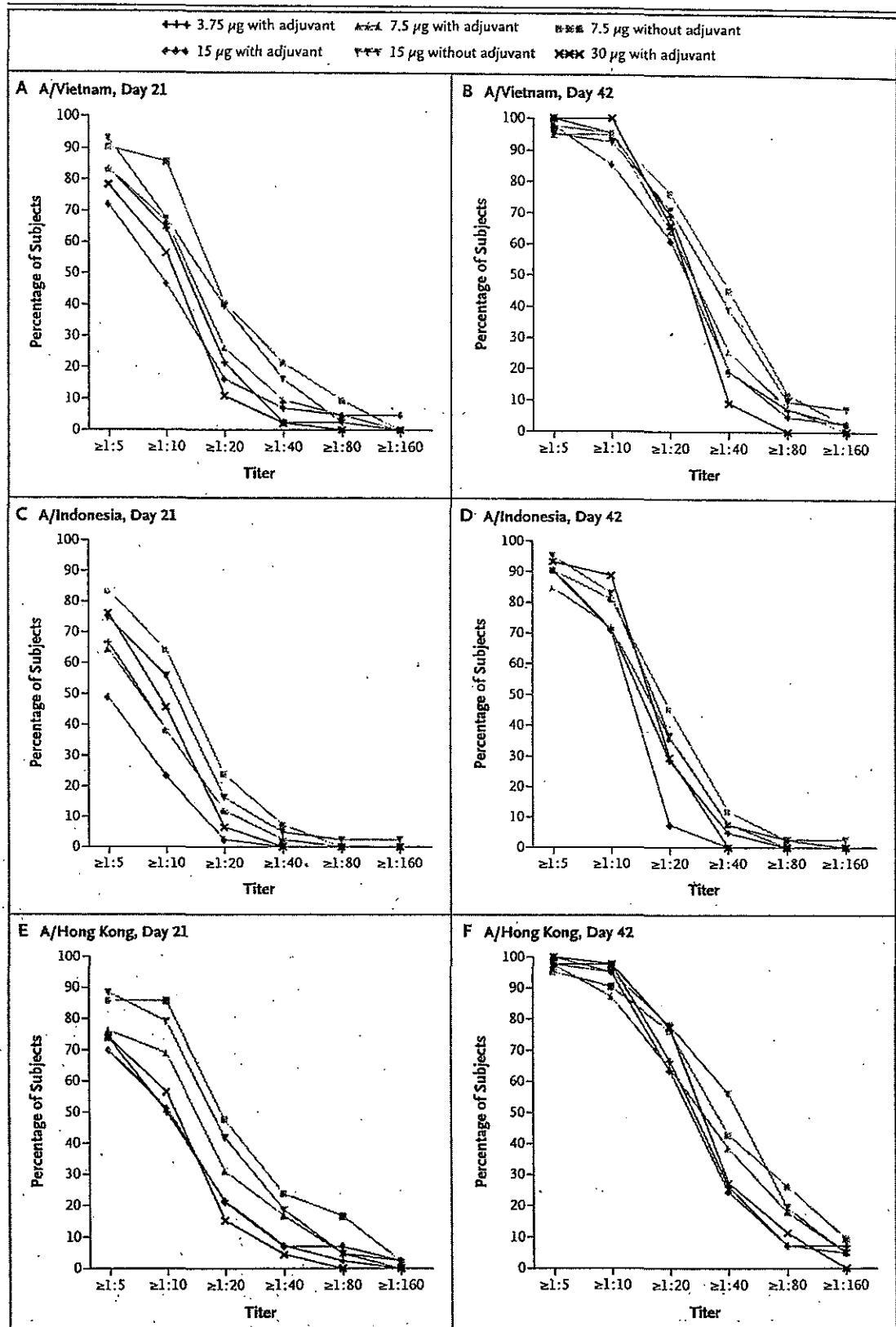
197

198

Our data also showed that the whole-virus clade 1–based vaccine can induce a substantial cross-neutralizing response against clade 2 and clade 3 strains. The results described in Table 2 are encouraging: after two doses of 7.5-µg of the formulation without adjuvant, the proportions of subjects with neutralizing titers of 1:20 or more were 45% of those immunized against the clade 2 Indonesia strain and 76% of those immunized against the clade 3 Hong Kong strain. However, there is no available evidence to indicate which neutralizing titer is sufficient to confer protection. Most studies of H5N1 split-virus and whole-virus vaccines have not described attempts to determine the cross-reactivity of antibodies to other H5N1 virus strains. However, a recent study of a novel split-virus vaccine with adjuvant also showed high levels of cross-neutralization against a clade 2 strain.[16] In addition, in a study involving 15 subjects, two doses of an H5N3 vaccine with MF-59 as adjuvant induced intermediate levels of cross-reactivity to antigenically distinct H5N1 strains, and three doses induced high levels of cross-reactivity.[20]

The apparent absence of a dose–response relationship in our study may be surprising. However, it is in agreement with a number of studies of vaccine for pandemic influenza. Leroux-Roels et al. reported no relationship between the dose of antigen and the neutralizing-antibody response for H5N1 formulations with adjuvant,[16] and there appeared to be an inverse dose–response relationship with respect to responses to the clade 2 strain. A number of other studies involving other pandemic-strain vaccines — H9N2,[21] H5N3,[19] and H2N2[22] — have shown no dose–response relationship or even a reduced response at higher

doses. The reasons for these findings are unclear, but at least with respect to vaccines with adjuvant, it has been speculated that the ratio of adjuvant to antigen may be critical in determining the immune-enhancing effect rather than the antigen concentration alone.[19] For other viral vaccines, particularly those with soluble proteins, it has been reported that there are distinct dose–response relationships for induction of various cytokines. In many studies, responses similar to those mediated by type 2 helper T cells have been elicited at low doses of vaccine, and responses similar to those mediated by type 1 helper T cells have been elicited at higher doses.[23] Further studies focusing on T-cell responses will be required to investigate this phenomenon. In addition, these studies will be extended by the use of antigen doses lower than 3.75 µg to confirm and extend the results obtained in our study.

Our study provides initial safety and immunogenicity data for a whole-virus H5N1 vaccine produced on Vero cell culture. It also shows that a broadly reactive immune response to clade 2 and clade 3 of H5N1 virus can be obtained with the use of a low-dose clade 1 vaccine without adjuvant. Since we observed no significant dose–response relationship, the 7.5-µg formulation without adjuvant has been chosen for further development.

199