ORIGINAL ARTICLE

Trial of 2009 Influenza A (H1N1) Monovalent MF59-Adjuvanted Vaccine

Tristan W. Clark, M.R.C.P., Manish Pareek, M.R.C.P., Katja Hoschler, Ph.D., Helen Dillon, M.R.C.P., Karl G. Nicholson, M.D., F.R.C.P., Nicola Groth, M.D., and Iain Stephenson, M.D., F.R.C.P.

ABSTRACT

BACKGROUND

The 2009 pandemic influenza A (H1N1) virus has emerged to cause the first pandemic of the 21st century. Development of effective vaccines is a public health priority.

METHODS

We conducted a single-center study, involving 176 adults, 18 to 50 years of age, to test the monovalent influenza A/California/2009 (H1N1) surface-antigen vaccine, in both MF59-adjuvanted and nonadjuvanted forms. Subjects were randomly assigned to receive two intramuscular injections of vaccine containing 7.5 μg of hemagglutinin on day 0 in each arm or one injection on day 0 and the other on day 7, 14, or 21; or two 3.75-μg doses of MF59-adjuvanted vaccine, or 7.5 or 15 μg of nonadjuvanted vaccine, administered 21 days apart. Antibody responses were measured by means of hemagglutination-inhibition assay and a microneutralization assay on days 0, 14, 21, and 42 after injection of the first dose.

RESULTS

The most frequent local and systemic reactions were pain at the injection site and muscle aches, noted in 70% and 42% of subjects, respectively; reactions were more common with the MF59-adjuvanted vaccine than with nonadjuvanted vaccine. Three subjects reported fever, with a temperature of 38°C or higher, after either dose. Antibody titers, expressed as geometric means, were higher at day 21 among subjects who had received one dose of MF59-adjuvanted vaccine than among those who had received one dose of nonadjuvanted vaccine ($P<0.001$ by the microneutralization assay). By day 21, hemagglutination-inhibition and microneutralization antibody titers of 1:40 or more were seen in 77 to 96% and 92 to 100% of subjects receiving MF59-adjuvanted vaccine, respectively, and in 63 to 72% and 67 to 76% of those receiving nonadjuvanted vaccine, respectively. By day 42, after two doses of vaccine, hemagglutination-inhibition and microneutralization antibody titers of 1:40 or more were seen in 92 to 100% and 100% of recipients of MF59-adjuvanted vaccine, respectively, and in 74 to 79% and 78 to 83% of recipients of nonadjuvanted vaccine, respectively.

CONCLUSIONS

Monovalent 2009 influenza A (H1N1) MF59-adjuvanted vaccine generates antibody responses likely to be associated with protection after a single dose is administered. (ClinicalTrials.gov number, NCT00943358.)

From the Infectious Diseases Unit, University Hospitals Leicester and Department of Inflammation, Infection and Immunity, University of Leicester, Leicester, United Kingdom (T.W.C., M.P., H.D., K.G.N., I.S.); the Respiratory Virus Laboratory, Health Protection Agency, London (K.H.); and Novartis Vaccines and Diagnostics, Siena, Italy (N.G.). Address reprint requests to Dr. Stephenson at the Infectious Diseases Unit, Level 6, Windsor Bldg., Leicester Royal Infirmary, Leicester, United Kingdom, or at iain.stephenson@uhl-tr.nhs.uk.

Drs. Clark and Pareek contributed equally to this article.

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The emergence of the 2009 pandemic influenza A (H1N1) virus demonstrates the unpredictable nature of influenza.[1] The virus has the potential to cause disease, death, and socioeconomic disruption,[2,3] and modeling suggests that the effect of the virus can be reduced by immunization.[4] The development of effective vaccines is a public health priority.

Traditional seasonal influenza vaccines are produced from reassortant vaccine strains grown in hens' eggs. However, demand for vaccine against the 2009 H1N1 virus will most likely exceed the supply if this method of manufacturing is solely used. Cell culture provides an additional platform for the manufacture of vaccines that may be more easily scaled up during periods of heightened demand.[5-7]

Serologic analysis suggests that after seasonal vaccination in children and young adults, there is little evidence of cross-reactive antibodies against the 2009 H1N1 virus,[8] with no evidence of protection from the seasonal vaccine.[9] The efficacy of conventional vaccines prepared from avian influenza strains is disappointingly low, even after two doses.[10-14] The addition of oil-in-water–emulsion adjuvant enhances immunogenicity and induces cross-reactive antibodies against antigenically drifted variants.[12-16] The use of such adjuvants in 2009 influenza A (H1N1) vaccines has been suggested by the World Health Organization.[17]

Vaccination programs for 2009 influenza A (H1N1) are under way, but the optimal formulation is unknown. The need for high-yield vaccine strains, limitations of the supply and production capacity of egg-based vaccines, and the possible requirement of two doses in some groups may delay an effective immunization program.

We present the clinical and immunogenicity profiles of the 7.5-μg dose of the monovalent influenza A/California/2009 (H1N1) MF59-adjuvanted surface-antigen vaccine, derived from cell culture, administered to adults 18 to 50 years of age. Two doses of 7.5 μg of MF59-adjuvanted vaccine were given concurrently on day 0 or were given 7, 14, or 21 days apart; or two doses of 3.75 μg of MF59-adjuvanted vaccine or 7.5 or 15 μg of nonadjuvanted vaccine were given 21 days apart. Our earlier report of the preliminary results is available at NEJM.org.

Methods

The study was designed by one academic author and one industry author; the academic author was responsible for managing the data and drafting the manuscript. The data were fully accessible and interpreted by all the authors, who vouch for the accuracy and completeness of the data and analyses. The U.K. Medicines and Healthcare Products Regulatory Agency and the Leicestershire, Rutland, and Northamptonshire Ethics Committee approved the study. University Hospitals Leicester was the main sponsor; the vaccine was manufactured by Novartis, who provided funding but had no role in the conduct of the study or in preparation of the manuscript.

Vaccine

The 2009 H1N1 vaccine virus (New York Medical College [NYMC] X-179A) was generated from the influenza A/California/7/2009 strain with the use of classical reassortant methods. The gene segments encoding the hemagglutinin, neuraminidase, and the polymerase PB1 were derived from the influenza A/California/7/2009 strain, with the remaining genes taken from the influenza A/PR8/8/34 virus used as a backbone for influenza vaccines. The strain was supplied by the Centers for Disease Control and Prevention and is a pandemic vaccine strain recommended for use in vaccine development. The seed virus was grown in Madin–Darby Canine Kidney (MDCK) cell culture by means of standard processes similar to those used for the development of Optaflu vaccines against interpandemic influenza. The vaccine was formulated and produced by Novartis (Marburg, Germany) as an inactivated surface-antigen H1N1 vaccine, with or without MF59 adjuvant, and was supplied in 0.5-ml prefilled single-dose syringes. Each MF59-adjuvanted vaccine contained 7.5 μg of H1 hemagglutinin, 9.75 mg of the squalene MF59, 1.175 mg of polysorbate 80, and 1.175 mg of sorbitan trioleate in buffer. Each nonadjuvanted vaccine contained 15 μg of H1 hemagglutinin in buffer. Hemagglutinin content in the final vaccine was initially determined by means of reverse-phase high-performance liquid chromatography, because single-radial diffusion reagents were unavailable. Subsequently, hemagglutinin content

of the final product, determined by means of single-radial diffusion reagents, was approximately 20% lower than the estimated content. Vaccine was stored at 2 to 8°C until use.

STUDY DESIGN

We conducted a single-center, phase 1, randomized study from July through September 2009 at Leicester Royal Infirmary (Leicester, United Kingdom). Subjects were screened for eligibility and provided written informed consent. (For eligibility criteria, see the Supplementary Appendix, available with the full text of this article at NEJM.org.)

The first 75 subjects enrolled were randomly assigned, in a 1:1:1 ratio, to receive two doses of 7.5 μg of MF59-adjuvanted vaccine, either concurrently administered on day 0 (i.e., one injection of the vaccine containing twice the antigen and adjuvant content of a single vaccine) or administered in two doses, one at day 0 and the other at day 7, 14, or 21. Serum samples for antibody measurements were collected on days 0, 14, 21, and 28.

The next 101 subjects enrolled were randomly assigned, in a 1:1:1:1 ratio, to receive two doses of 7.5 or 3.75 μg of MF59-adjuvanted vaccine (for the latter dose, by administering half the contents of the adjuvanted-vaccine syringe for each), two 15-μg doses of nonadjuvanted vaccine, or two 7.5-μg doses of nonadjuvanted vaccine (by administering half the contents of the nonadjuvanted-vaccine syringe for each) — with one injection at day 0 and the other at day 21. Serum samples were collected on days 0, 14, 21, and 42.

The vaccine was administered by intramuscular injection into the deltoid muscle of the nondominant arm, or in both arms if both doses were given on day 0. Subjects were observed for 30 minutes after each injection, and for the next 7 days they recorded, in self-completed diaries, the severity of unsolicited and solicited local symptoms (pain, bruising, erythema, and swelling) and systemic symptoms (chills, malaise, muscle aches, nausea, and headache), oral temperature, and use of analgesics. Symptoms were graded as follows: none; mild, if they did not interfere with normal activities; moderate, if they resulted in interference with normal activities; and severe, if they prevented engagement in daily activities and necessitated medical attention. Adverse reactions were defined as any reaction that persisted beyond 7 days after vaccination. Serious adverse reactions were defined as any reaction that necessitated medical attention or hospitalization during the study period.

LABORATORY ASSAYS

Antibody responses were detected by means of microneutralization and hemagglutination-inhibition assays, according to standard methods,[18,19] at the Centre for Infections, Health Protection Agency (London), and with the use of cell-culture X-179A H1N1 vaccine virus (see the Vaccine section above) and egg-grown NIBRG-121 virus — a reverse-genetic virus containing hemagglutinin and neuraminidase from the influenza A/California/7/2009 strain (see the Supplementary Appendix for details). Serum samples obtained from subjects were tested with the use of 1:2 serial dilutions. For hemagglutination-inhibition assays, serum samples were tested at an initial dilution of 1:8, and those that were negative for the antibody were assigned a titer of 1:4. Serum specimens were analyzed to determine absolute end-point titers. For microneutralization assays, serum samples were tested at an initial dilution of 1:10,[20] and those that were negative were assigned a titer of 1:5. The final dilution was 1:320, and samples for which the end-point titers were greater were assigned a value of 1:640. Specimens were tested in duplicate, and the geometric mean values were used in analyses.

STATISTICAL ANALYSIS

The group sizes used are usual for phase 1 studies and were not based on power calculations. Data analysis was undertaken with the use of Stata software (version 9.2, StataCorp).

For solicited and unsolicited adverse reactions, the percentages of subjects (point estimates and 95% confidence intervals) with postvaccination reactions were based on the frequency and severity of the reported responses after vaccination. Exact (Clopper–Pearson) confidence intervals are reported for all proportional end points. We used a two-sided Fisher's exact test to compare proportions between vaccine groups. All reported P values are two-sided, with no adjustment for multiple testing; values of 0.05 or less were considered to indicate statistical significance.

For immunogenicity analyses, the geometric mean antibody titers at each time point were used. Geometric mean titers and 95% confidence intervals were computed by taking the exponent ($\log_{10}$) of the mean and of the lower and upper

limits of the 95% confidence intervals of the $\log_{10}$-transformed titers. Geometric mean titers were compared between each pair of vaccine groups by means of one-way analysis of variance on the $\log_{10}$-transformed titers with Bonferroni correction for multiple pairwise comparisons, if appropriate. The proportions of subjects in whom seroconversion (a prevaccination hemagglutination-inhibition antibody titer ≤1:10 and a postvaccination titer ≥1:40 or a prevaccination titer ≥1:10 and an increase in the titer by a factor of four or more) or a hemagglutination-inhibition antibody titer of 1:40 or more was achieved were compared between each group with the use of a two-sided Fisher's exact test. Separate analyses were performed for the hemagglutination-inhibition and microneutralization assays. Because there are no established immune correlates for microneutralization, in that analysis we assessed the proportion of subjects who had seroconversion (an increase in the antibody titer by a factor of four or more) and a microneutralization titer of 1:40 or more.

RESULTS

We enrolled 176 subjects. A total of 101 subjects received two 7.5-μg doses of MF59-adjuvanted vaccine (with the two doses administered concurrently at day 0 [in 25 subjects] or one dose at day 0 and one at day 7 [in 25 subjects], at day 14 [in 25 subjects], or at day 21 [in 26 subjects]). The other 75 subjects each received two doses of 3.75 μg of MF59-adjuvanted vaccine (25 subjects), or 7.5 or 15 μg of nonadjuvanted vaccine (25 subjects each), separated by 21 days (i.e., doses at day 0 and at day 21).

Both doses of vaccine were given in 175 of the 176 subjects (99%); 1 subject in the group receiving 15 μg of nonadjuvanted vaccine did not attend the second vaccination visit. In all, 322 of the 325 issued diary cards (99%) were returned. Serum samples were obtained from 175 of the 176 subjects (99%) and 97 of the 101 subjects (96%) from whom samples were required at days 21 and day 42, respectively, according to protocol. In addition, serum samples were obtained on day 14 from 166 of the 176 subjects (94%). Data from all 176 subjects were included in safety and immunogenicity analyses (see the Supplementary Appendix).

The median age was 33 years (range, 18 to 50); 65% of the subjects were women, 82% were white, and 37% had previously received seasonal influenza vaccine (Table 1). The baseline characteristics were similar among the seven groups.

Table 1. Baseline Characteristics of the Study Subjects, According to Vaccine Group.

	MF59-Adjuvanted Vaccine					Nonadjuvanted Vaccine	
	3.75 μg, Days 0 and 21 (N=25)	7.5 μg, Days 0 and 21 (N=26)	7.5 μg, Days 0 and 14 (N=25)	7.5 μg, Days 0 and 7 (N=25)	7.5 μg, Both Doses on Day 0 (N=25)	7.5 μg, Days 0 and 21 (N=25)	15 μg, Days 0 and 21 (N=25)
Race or ethnic group — no. (%)*							
White	17 (68)	21 (81)	19 (76)	23 (92)	21 (84)	19 (76)	25 (100)
South Asian	4 (16)	4 (15)	3 (12)	1 (4)	4 (16)	5 (20)	0
Black	3 (12)	1 (4)	3 (12)	0	0	1 (4)	0
Chinese	1 (4)	0	0	1 (4)	0	0	0
Sex — no. (%)							
Female	13 (52)	17 (65)	15 (60)	19 (76)	14 (56)	20 (80)	16 (64)
Male	12 (48)	9 (35)	10 (40)	6 (24)	11 (44)	5 (20)	9 (36)
Previous receipt of seasonal influenza vaccine — no. (%)	7 (28)	9 (35)	11 (44)	11 (44)	9 (36)	7 (28)	11 (44)
Age — yr							
Median	31	35	29	32	34	34	30
Range	20–48	20–49	23–49	18–50	19–49	23–49	24–49

* Race or ethnic group was self-reported.

Table 2. Solicited Local and Systemic Adverse Effects within 7 Days after Receipt of Either Dose of MF59-Adjuvanted or Nonadjuvanted Vaccine, According to Vaccine Group.*

Effect	MF59-Adjuvanted Vaccine					Nonadjuvanted Vaccine	
	3.75 μg, Days 0 and 21 (N=25)	7.5 μg, Days 0 and 21 (N=26)	7.5 μg, Days 0 and 14 (N=25)	7.5 μg, Days 0 and 7 (N=25)	7.5 μg, Both Doses on Day 0 (N=25)†	7.5 μg, Days 0 and 21 (N=25)	15 μg, Days 0 and 21 (N=25)
	percent (95% confidence interval)						
Local reaction							
Pain‡							
None	44 (24–65)	27 (12–48)	24 (9–45)	44 (24–65)	16 (5–36)	64 (43–82)	58 (37–78)
Mild	40 (21–61)	58 (37–78)	48 (28–69)	48 (28–69)	76 (55–91)	36 (18–58)	38 (19–59)
Moderate	16 (5–36)	15 (4–35)	28 (12–49)	8 (1–26)	8 (1–26)	0 (0–14)	4 (0–21)
Severe	0 (0–14)	0 (0–13)	0 (0–14)	0 (0–14)	0 (0–14)	0 (0–14)	0 (0–14)
Redness diameter							
0 mm	96 (80–100)	96 (80–100)	84 (64–96)	88 (69–98)	88 (69–98)	80 (59–93)	79 (58–93)
1–4 mm	0 (0–14)	0 (0–13)	16 (5–36)	12 (3–31)	12 (3–31)	16 (5–36)	13 (3–32)
≥5 mm	4 (0–20)	4 (0–20)	0 (0–14)	0 (0–14)	0 (0–14)	4 (0–20)	8 (1–27)
Swelling diameter							
0 mm	92 (74–99)	96 (80–100)	80 (59–93)	96 (80–100)	84 (64–96)	92 (74–99)	92 (73–99)
1–4 mm	4 (0–20)	4 (0–20)	16 (5–36)	0 (0–14)	12 (3–31)	8 (1–26)	4 (0–21)
≥5 mm	4 (0–20)	0 (0–13)	4 (0–20)	4 (0–20)	4 (0–20)	0 (0–14)	4 (0–21)
Bruising diameter							
0 mm	96 (80–100)	92 (75–99)	84 (64–96)	92 (74–99)	92 (74–99)	96 (80–100)	92 (73–99)
1–4 mm	0 (0–14)	4 (0–20)	8 (1–26)	4 (0–20)	4 (0–20)	4 (0–20)	8 (1–27)
≥5 mm	4 (0–20)	4 (0–20)	8 (1–26)	4 (0–20)	4 (0–20)	0 (0–14)	0 (0–14)
Systemic reaction							
Muscle aches§							
None	72 (51–88)	58 (37–78)	60 (39–79)	64 (43–82)	36 (18–58)	72 (51–88)	58 (37–78)
Mild	16 (5–36)	31 (14–52)	20 (7–41)	32 (15–54)	52 (31–72)	28 (12–49)	38 (19–59)
Moderate	12 (3–31)	11 (2–30)	20 (7–41)	4 (0–20)	12 (3–31)	0 (0–14)	4 (0–21)
Severe	0 (0–14)	0 (0–13)	0 (0–14)	0 (0–14)	0 (0–14)	0 (0–14)	0 (0–14)
Chills							
None	92 (74–99)	88 (70–98)	88 (69–98)	100 (86–100)	84 (64–96)	92 (74–99)	92 (73–99)
Mild	4 (0–20)	8 (1–25)	4 (0–20)	0 (0–14)	8 (1–26)	0 (0–14)	4 (0–21)
Moderate	4 (0–20)	4 (0–20)	8 (1–26)	0 (0–14)	8 (1–26)	8 (1–26)	4 (0–21)
Severe	0 (0–14)	0 (0–13)	0 (0–14)	0 (0–14)	0 (0–14)	0 (0–14)	0 (0–14)
Malaise							
None	84 (64–96)	69 (48–86)	88 (86–100)	96 (80–100)	76 (55–91)	96 (80–100)	92 (73–99)
Mild	12 (3–31)	27 (12–48)	8 (1–26)	4 (0–20)	24 (9–45)	4 (0–20	4 (0–21)
Moderate	4 (0–20)	4 (0–20)	4 (0–20)	0 (0–14)	0 (0–14)	0 (0–14)	4 (0–21)
Severe	0 (0–14)	0 (0–13)	0 (0–14)	0 (0–14)	0 (0–14)	0 (0–14)	0 (0–14)
Headache							
None	68 (47–85)	61 (41–80)	56 (35–76)	80 (59–93)	60 (39–79)	56 (35–76)	71 (49–87)
Mild	24 (9–45)	35 (17–56)	28 (12–49)	12 (3–31)	36 (18–58)	36 (18–58)	21 (7–42)
Moderate	8 (1–26)	4 (0–20)	16 (5–36)	8 (1–26)	4 (0–20)	8 (1–26)	8 (1–27)
Severe	0 (0–14)	0 (0–13)	0 (0–14)	0 (0–14)	0 (0–14)	0 (0–14)	0 (0–14)

Table 2. (Continued)

Effect	MF59-Adjuvanted Vaccine					Nonadjuvanted Vaccine	
	3.75 μg, Days 0 and 21 (N=25)	7.5 μg, Days 0 and 21 (N=26)	7.5 μg, Days 0 and 14 (N=25)	7.5 μg, Days 0 and 7 (N=25)	7.5 μg, Both Doses on Day 0 (N=25)†	7.5 μg, Days 0 and 21 (N=25)	15 μg, Days 0 and 21 (N=25)
	percent (95% confidence interval)						
Nausea							
None	96 (80–100)	81 (61–93)	84 (64–96)	92 (74–99)	88 (69–98)	88 (69–98)	79 (58–93)
Mild	4 (0–20)	11 (2–30)	12 (3–31)	4 (0–20)	12 (3–31)	8 (1–26)	17 (5–37)
Moderate	0 (0–14)	8 (1–25)	4 (0–20)	4 (0–20)	0 (0–14)	4 (0–20)	4 (0–21)
Severe	0 (0–14)	0 (0–13)	0 (0–14)	0 (0–14)	0 (0–14)	0 (0–14)	0 (0–14)
Fever, temp. >38°C	0 (0–14)	0 (0–13)	4 (0–20)	0 (0–14)	4 (0–20)	4 (0–20)	0 (0–14)
Analgesic use	24 (9–45)	8 (1–25)	16 (5–36)	0 (0–14)	20 (7–41)	16 (5–36)	8 (1–27)

* Subjects used a subjective scale to grade adverse events. Symptoms were considered mild if they did not interfere with daily activities, moderate if they caused some impairment, and severe if they affected daily activities and necessitated medical attention.
† For the group who had received both doses concurrently on day 0, any local reaction in either arm is reported.
‡ Pain at injection site was reported more frequently in groups receiving MF59-adjuvanted vaccine than in those receiving nonadjuvanted vaccine (P=0.003 by Fisher's exact test).
§ Muscle aches were reported more frequently in the group receiving both doses of MF59-adjuvanted vaccine on day 0 than in those who received one dose of MF59-adjuvanted vaccine (P=0.02 by Fisher's exact test).

SAFETY ANALYSIS

Solicited local and systemic reactions during the first 7 days following any vaccine dose are shown in Table 2. Overall, 80% of subjects reported adverse reactions after either vaccine dose (73% after the first and 60% after the second). The frequency or severity of reactions did not increase after the second dose was received (see the Supplementary Appendix). All self-reported reactions were graded as mild or moderate and were generally self-limited, resolving within a 72-hour period. No dose–response relationship was observed for either vaccine type for any reaction.

The most frequent local reaction after any vaccine was pain at the injection site, reported by 61% of subjects. Overall, pain was more frequent after injection of the MF59-adjuvanted vaccine than with nonadjuvanted vaccine (65% vs. 39%, P=0.003). In general, pain was not accompanied by redness, swelling, or bruising, although bruising was reported to be severe in one subject — affecting an area 20 mm in diameter — after the first dose, with resolution within 72 hours. No severe local reactions were reported.

The most frequent systemic reaction was muscle ache, reported by 40% of subjects. There was no significant difference in frequency or severity of systemic reactions after receipt of MF59-adjuvanted vaccine and those after receipt of nonadjuvanted vaccine, except in subjects who received two doses of MF59-adjuvanted vaccine on day 0, who reported a greater frequency of muscle ache than did subjects who received one dose of MF59-adjuvanted vaccine on day 0 (P=0.02). A total of 13% of subjects reported use of analgesics. Three subjects reported fever, defined as a temperature of 38°C or more (after the first dose in two subjects and after the second dose in the third), but none required antipyretic medication. No severe systemic reactions were reported.

Fifteen unsolicited adverse events were reported (see the Supplementary Appendix). After receiving MF59-adjuvanted vaccine, three subjects reported self-limiting diarrhea (that resolved within the 48-hour period after the first dose); one subject took over-the-counter loperamide. Three subjects reported coryza that resolved within 72 hours. One subject reported toothache that resolved after 5 days. One subject reported a transient itchy rash on the right forearm that resolved within 48 hours. After receiving nonadjuvanted vaccine, two subjects reported musculoskeletal pain that resolved after 48 hours. Two subjects reported coryza that resolved within 72 hours. One subject each reported diarrhea, itching, or sore throat that resolved within 48 hours.

A probable vaccine-related adverse reaction, reported after receipt of the 7.5 μg of MF59-adju-

vanted vaccine, is described in the Supplementary Appendix. Briefly, this subject (who received two doses of the MF59-adjuvanted vaccine on day 0) reported a purpuric rash on the lower limbs on day 17, with resolution within 72 hours. Further questioning revealed that she had consulted with her family practitioner in May 2009 for an intermittent leg rash within the 12-month period before the study. Investigations including complete blood count and biochemical profile showed normal values, but an autoimmune profile was positive for antinuclear and extractable nuclear-antigen antibodies. She had not received medication, and this medical history was not known at enrollment. Follow-up for the rash included a normal complete blood count and biochemical profile. Results of autoimmune testing were unchanged from those in May 2009.

IMMUNOGENICITY AGAINST THE 2009 H1N1 VIRUS

Antibody responses against the vaccine strain were detected by the hemagglutination-inhibition assay (titer >1:8) and the microneutralization assay (titer >1:10) before vaccination in 16% and 31% of subjects, respectively; this frequency was unrelated to age (P=0.72 by hemagglutination-inhibition assay and P=0.32 by microneutralization assay) or previous receipt of seasonal vaccine (P=0.14 and P=0.18, respectively).

There was no significant dose–response relationship regarding the geometric mean titers, at any postvaccination visit, for MF59-adjuvanted vaccine (P=0.71 by hemagglutination-inhibition assay and P=0.43 by microneutralization assay on day 14; P=0.63 and P=0.42, respectively, on day 21; and P=0.86 and P=0.75, respectively, on day 42) or for nonadjuvanted vaccine (P=0.74 and P=0.49, respectively, on day 14; P=0.99 and P=0.62, respectively, on day 21; and P=0.27 and P=0.88, respectively, on day 42).

On day 14, geometric mean titers, as measured with the use of hemagglutination-inhibition assay (Table 3) and microneutralization assay (Table 4), were higher in subjects who received two 7.5-μg doses of MF59-adjuvanted vaccine by that time, as compared with those who had received one dose only (P=0.03 by hemagglutination-inhibition assay and P<0.001 by microneutralization assay). After the administration of one dose, the microneutralization antibody titers were greater in subjects who had received the MF59-adjuvanted vaccine than in those who had received nonadjuvanted vaccine (P<0.001).

On day 21, microneutralization antibody titers were higher among subjects who had received two 7.5-μg doses of MF59-adjuvanted vaccine than among those who had received one dose only by that time (P=0.03). After one dose of either vaccine, the MF59-adjuvanted vaccine induced greater titers than nonadjuvanted vaccine (P<0.001 by microneutralization assay).

On day 42, after two doses of either vaccine, geometric mean titers were higher in groups receiving the MF59-adjuvanted vaccine than in those receiving the nonadjuvanted vaccine (P=0.007 by hemagglutination-inhibition assay and P<0.001 by microneutralization assay).

Table 3 shows the ratio of the antibody titer measured at each postvaccination visit and the titer measured at the prevaccination visit, and the percentages of subjects with seroconversion and with an antibody titer of 1:40 or more, as measured with the hemagglutination-inhibition assay.

There was no significant dose–response relationship regarding the rates of seroconversion with MF59-adjuvanted vaccine (P=0.78 by the hemagglutination-inhibition assay and P=1.00 by the microneutralization assay on day 14; P=0.29 and P=1.00, respectively, on day 21; and P=1.00 and P=1.00, respectively, on day 42) or with nonadjuvanted vaccine (P=0.23 and P=1.00, respectively, on day 14; P=0.23 and P=1.00, respectively, on day 21; and P=0.74 and P=1.00, respectively, on day 42). There was no significant difference in the rates of seroconversion between subjects who had a detectable prevaccination antibody titer and those who did not (day 14, P=0.46; day 21, P=0.61; and day 42, P=1.00). On day 14, the percentages of subjects with seroconversion and with an antibody titer of 1:40 or more were higher (P=0.007 and P=0.002, respectively) among subjects who had received two doses of the MF59-adjuvanted vaccine than among those who had received only one. On day 21, as compared with subjects who had received one dose of MF59-adjuvanted vaccine, those who had received two doses did not differ significantly in the percentage of subjects with seroconversion (P=0.06) but did have a greater percentage with an antibody titer of 1:40 or more (P=0.03). Although MF59-adjuvanted vaccine

Table 3. Antibody Responses as Measured with the Hemagglutination-Inhibition Assay, According to Vaccine Group.*

Value	MF59-Adjuvanted Vaccine					Nonadjuvanted Vaccine	
	3.75 μg, Days 0 and 21 (N=25)	7.5 μg, Days 0 and 21 (N=26)	7.5 μg, Days 0 and 14 (N=25)	7.5 μg, Days 0 and 7 (N=25)	7.5 μg, Both Doses on Day 0 (N=25)	7.5 μg, Days 0 and 21 (N=25)	15 μg, Days 0 and 21 (N=25)
Day 0							
Geometric mean titer (95% CI)	6.7 (4.4–10.3)	6.1 (4.0–9.1)	6.6 (4.2–10.4)	4.8 (3.7–6.3)	6.0 (3.8–9.5)	5.1 (3.9–6.5)	7.1 (4.6–10.9)
Antibody titer ≥1:40 — % (95% CI)	4 (0–20)	12 (2–30)	12 (3–31)	4 (0–20)	8 (1–26)	4 (0–20)	12 (3–31)
Day 14†							
Geometric mean titer (95% CI)	197.2 (100.0–388.5)	174.9 (79.4–385.6)	155.8 (62.7–387.2)	416.5 (260.9–664.9)	294.8 (165.9–523.5)	105.4 (47.8–232.9)	87.2 (36.9–206.2)
Geometric mean ratio (95% CI)	29.4 (13.9–62.1)	28.9 (12.5–66.5)	23.7 (8.9–62.2)	86.7 (52.3–143.8)	49.2 (24.6–98.2)	20.7 (9.5–44.8)	12.3 (5.2–29.2)
Seroconversion — % (95% CI)	78 (56–93)	76 (55–91)	68 (47–85)	96 (79–100)	91 (72–99)	71 (49–87)	52 (30–74)
Antibody titer ≥1:40 — % (95% CI)	87 (66–97)	80 (59–93)	72 (51–88)	100 (86–100)	96 (78–100)	71 (49–87)	57 (34–78)
Day 21‡							
Geometric mean titer (95% CI)	199.1 (106.2–373.1)	157.4 (73.8–335.7)	288.7 (150.6–553.7)	282.9 (160.2–499.7)	256.1 (158.0–415.2)	96.1 (44.8–206.2)	95.6 (41.2–221.7)
Geometric mean ratio (95% CI)	29.7 (14.4–61.2)	25.9 (11.5–58.9)	43.8 (20.6–93.2)	58.9 (32.4–107.0)	42.7 (22.7–80.5)	18.9 (8.8–40.4)	13.5 (5.6–32.7)
Seroconversion — % (95% CI)	88 (69–98)	73 (52–88)	88 (69–98)	92 (74–99)	88 (69–98)	72 (51–88)	52 (31–73)
Antibody titer ≥1:40 — % (95% CI)	92 (74–99)	77 (56–91)	92 (74–99)	96 (80–100)	92 (74–99)	72 (51–88)	63 (41–81)
Day 42§							
Geometric mean titer (95% CI)	305.4 (213.4–437.2)	321.3 (200.4–515.1)	ND	ND	ND	116.6 (65.3–208.0)	194.3 (90.5–417.0)
Geometric mean ratio (95% CI)	45.6 (26.9–77.5)	53.0 (29.5–95.5)	ND	ND	ND	22.9 (12.7–41.2)	27.4 (12.2–61.9)
Seroconversion — % (95% CI)	92 (74–99)	92 (73–99)	ND	ND	ND	79 (58–93)	74 (52–90)
Antibody titer ≥1:40 — % (95% CI)	100 (86–100)	92 (73–99)	ND	ND	ND	79 (58–93)	74 (52–90)

* Geometric mean titers are the ratios of the antibody level at the day of interest and at day 0. Percentages of subjects are based on the total number of subjects tested. Seroconversion was defined as a prevaccination titer of 1:10 or less and a postvaccination titer of 1:40 or more or a prevaccination titer of greater than 1:10 and an increase in the titer by a factor of four or more. "Antibody titer ≥1:40" denotes a titer of 1:40 or greater at each postvaccination visit. ND denotes not done.

† At day 14, the geometric mean ratio, the rate of seroconversion, and the percentage of subjects with an antibody titer of 1:40 or more were all significantly greater (P=0.03, P=0.007, and P=0.002, respectively) in the two groups that had received two 7.5-μg doses of MF59-adjuvanted vaccine by that time (the first on day 0 and the second on day 0 or day 7) than in the two groups that had received one dose only.

‡ At day 21, the percentage of subjects with an antibody titer of 1:40 or more was greater (P=0.03) in the groups that had received two 7.5-μg doses of MF59-adjuvanted vaccine by that time (the first on day 0 and the second on day 0, day 7, or day 14) than in the group that had received one dose only.

§ At day 42, the geometric mean ratio, the rate of seroconversion, and the percentage of subjects with an antibody titer of 1:40 or more were all significantly greater (P=0.007, P=0.05, and P=0.007, respectively) in the groups that had received MF59-adjuvanted vaccine than in those that had received nonadjuvanted vaccine.

Table 4. Antibody Responses as Measured with the Microneutralization Assay, According to Vaccine Group.*

Value	MF59-Adjuvanted Vaccine					Nonadjuvanted Vaccine	
	3.75 μg, Days 0 and 21 (N=25)	7.5 μg, Days 0 and 21 (N=26)	7.5 μg, Days 0 and 14 (N=25)	7.5 μg, Days 0 and 7 (N=25)	7.5 μg, Both Doses on Day 0 (N=25)	7.5 μg, Days 0 and 21 (N=25)	15 μg, Days 0 and 21 (N=25)
Day 0							
Geometric mean titer (95% CI)	8.1 (5.4–12.2)	12.9 (8.0–20.7)	13.1 (7.8–22.1)	9.8 (6.6–14.5)	10.4 (5.9–18.1)	7.4 (5.2–10.6)	6.9 (5.1–9.4)
Antibody titer ≥40 — % (95% CI)	8 (1–26)	19 (7–39)	16 (5–36)	16 (5–36)	12 (3–31)	12 (3–31)	4 (0–20)
Day 14†							
Geometric mean titer (95% CI)	251.8 (139.9–453.0)	338.7 (246.4–465.6)	285.4 (185.1–439.9)	502.2 (406.7–620.0)	606.5 (557.8–659.5)	131.2 (73.3–234.7)	95.0 (42.9–210.7)
Seroconversion — % (95% CI)	87 (66–97)	84 (64–96)	84 (64–96)	96 (79–100)	91 (72–99)	67 (45–84)	67 (43–85)
Antibody titer ≥40	87 (66–97)	100 (86–100)	92 (74–99)	100 (86–100)	100 (85–100)	75 (53–90)	67 (43–85)
Day 21‡							
Geometric mean titer (95% CI)	266.6 (164.9–430.9)	335.4 (239.5–469.9)	407.2 (301.4–550.3)	448.9 (335.1–601.5)	582.8 (518.3–655.3)	110.6 (63.5–192.5)	88.2 (41.5–187.4)
Seroconversion — % (95% CI)	92 (74–99)	92 (75–99)	96 (80–100)	96 (80–100)	92 (74–99)	68 (47–85)	67 (45–84)
Antibody titer ≥40 — % (95% CI)	92 (74–99)	100 (87–100)	100 (86–100)	100 (86–100)	100 (86–100)	76 (55–91)	67 (45–84)
Day 42§							
Geometric mean titer (95% CI)	433.8 (320.2–587.7)	406.9 (308.6–536.6)	ND	ND	ND	156.6 (89.5–273.9)	166.4 (91.3–303.5)
Seroconversion — % (95% CI)	92 (73.9–99.0)	96 (78.9–99.9)	ND	ND	ND	80 (59–93)	78 (56–93)
Antibody titer ≥40 — % (95% CI)	100 (86–100)	100 (86–100)	ND	ND	ND	83 (63–95)	78 (56–93)

* Geometric mean titers are the ratios of the antibody level at the day of interest and at day 0. Percentages of subjects are based on the total number of subjects tested. Seroconversion was defined as an increase in the antibody titer by a factor of four or more. Because no threshold titer for seroprotection has been established for the microneutralization assay, the percentage of subjects with titers of 1:40 or greater are reported. "Antibody titer ≥40" denotes a titer of 1:40 or greater at each postvaccination visit. ND denotes not done.

† At day 14, the geometric mean titer, the rate of seroconversion, and the percentage of subjects with an antibody titer of 1:40 or more were all significantly greater ($P<0.001$, $P=0.02$, and $P=0.003$, respectively) in the groups that had received a single dose of MF59-adjuvanted vaccine than in those that had received nonadjuvanted vaccine. The geometric mean titer was greater ($P<0.001$) in the groups that had received two 7.5-μg doses of MF59-adjuvanted vaccine by that time (the first on day 0 and the second on day 0 or day 7) than in the two groups that had received one dose only.

‡ At day 21, the geometric mean titer, the rate of seroconversion, and the percentage of subjects with an antibody titer of 1:40 or more were all significantly greater ($P<0.001$, $P=0.002$, and $P=0.001$, respectively) in the groups that had received one dose of MF59-adjuvanted vaccine by that time (the first on day 0 and the second on day 0, day 7, or day 14) than in those that had received nonadjuvanted vaccine. The geometric mean titer was greater ($P=0.03$) in the groups that had received two 7.5-μg doses of MF59-adjuvanted vaccine by that time than in the group that had received one dose only.

§ At day 42, the geometric mean titer, the rate of seroconversion, and the percentage of subjects with an antibody titer of 1:40 or more were all significantly greater ($P<0.001$, $P=0.04$, and $P=0.001$, respectively) in the groups that had received MF59-adjuvanted vaccine than in those that had received nonadjuvanted vaccine.

induced more seroconversions and antibody titers of 1:40 or more than nonadjuvanted vaccine at each postvaccination visit, the difference was not significant (day 14, $P=0.22$ and $P=0.09$, respectively; and day 21, $P=0.07$ and $P=0.06$, respectively). On day 42, after two vaccine doses, the percentages of subjects with seroconversion and an antibody titer of 1:40 or more were higher among the MF59-adjuvanted vaccine groups ($P=0.05$ and $P=0.007$, respectively) than among the nonadjuvanted vaccine groups.

Table 4 shows the ratio of the antibody titer measured at each postvaccination visit and the titer measured at the prevaccination visit, and the

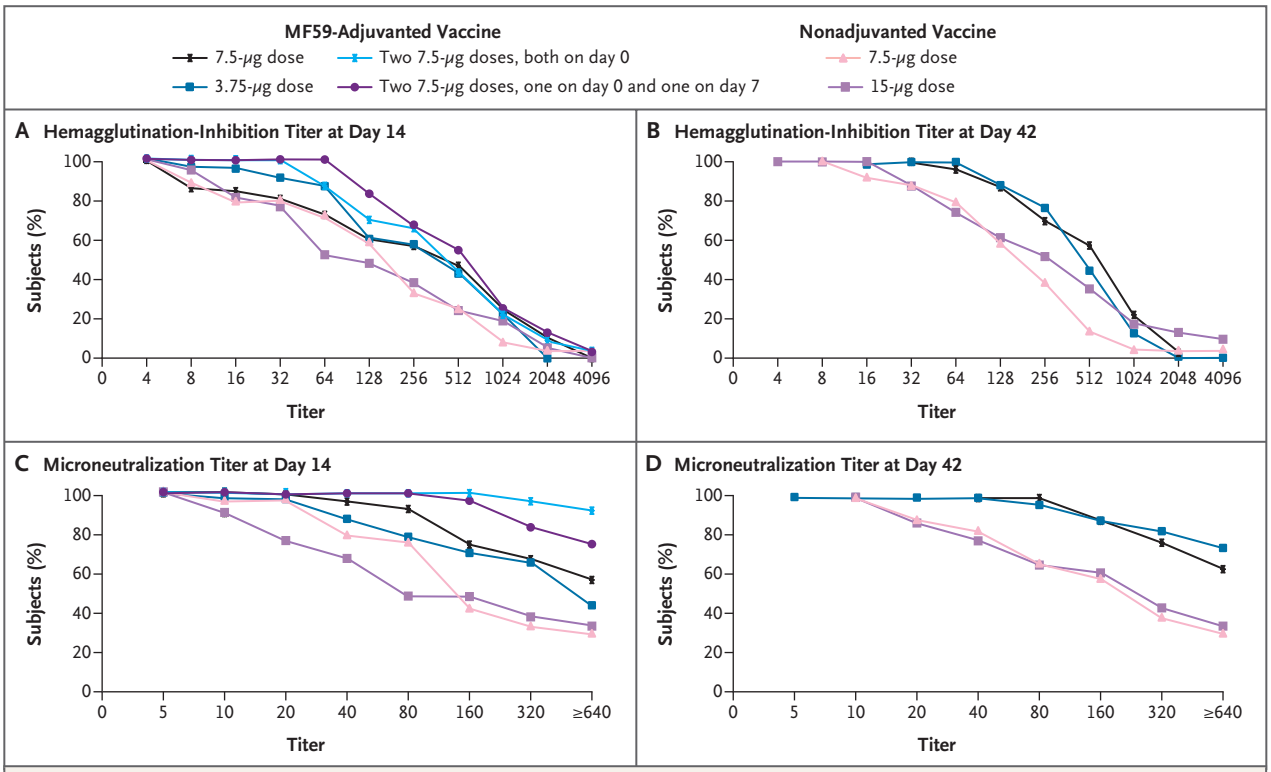

Figure 1. Reverse Cumulative-Distribution Curves of Antibody Titers in Serum Samples Obtained on Days 14 and 42, According to Number and Timing of Vaccine Doses.

The percentages of subjects are based on the total number of subjects tested in each of the vaccine groups. All vaccine groups received the first of two doses on day 0. One group received the second dose of 7.5 μg of MF59-adjuvanted vaccine on day 0, one group on day 7, and one group on day 14; the three other groups received the second vaccine dose on day 21. Hemagglutination-inhibition titers (expressed as the reciprocal of the dilution) are shown at day 14 (Panel A) and day 42 (Panel B). Microneutralization titers are shown at day 14 (Panel C) and day 42 (Panel D). Titers are expressed as reciprocal of the dilution and are given on a $\log_2$ scale.

percentages of subjects with seroconversion and antibody titers of 1:40 or more, as measured with the microneutralization assay. On days 14 and 21, there were no significant differences between subjects who had received two doses of MF59-adjuvanted vaccine and those who had received one dose in the rate of seroconversion (day 14, P=0.20; and day 21, P=0.64) or in the percentage with titers of 1:40 or more (day 14, P=0.50; and day 21, P=1.00). On each postvaccination visit, the rate of seroconversion and the percentage of subjects with titers of 1:40 or more were greater after receipt of MF59-adjuvanted vaccine than after receipt of nonadjuvanted vaccine (day 14, P=0.02 and P=0.003, respectively; day 21, P=0.002 and P=0.001, respectively; and day 42, P=0.04 and P=0.001, respectively).

Figure 1 shows the distribution of antibody titers at day 14 and day 42, according to vaccine group. At day 14, hemagglutination-inhibition titers of 1:32 or more were achieved in 84% of subjects receiving MF59-adjuvanted vaccine and in 77% of subjects receiving nonadjuvanted vaccine. Microneutralization titers of 1:40 or more were achieved in 94% subjects receiving MF59-adjuvanted vaccine and in 73% of subjects receiving nonadjuvanted vaccine. After two doses, at day 42, hemagglutination-inhibition titers of 1:32 or more were achieved in 100% of subjects receiving MF59-adjuvanted vaccine and in 87% of subjects receiving nonadjuvanted vaccine. Microneutralization titers of 1:40 or more were achieved in 100% subjects receiving MF59-adjuvanted vaccine and in 80% of subjects receiving nonadjuvanted vaccine.

Responses against the NIBRG-121 virus were similar to those against the 2009 X-179A H1N1 vaccine virus, as measured by means of the hemagglutination-inhibition assay (see the Supplementary Appendix).

DISCUSSION

Data from studies of other inactivated influenza vaccines suggest that hemagglutination-inhibition antibody titers of 1:40 or more provide partial protection, and this titer was achieved by most of the subjects given one dose, with or without adjuvant, in this clinical trial. Effective vaccination should reduce illness and virus transmission,[4] although this may be challenging, as global demand for vaccine will probably exceed manufacturing capacity and will be met only by implementing a range of production approaches. Large-scale vaccine production with newly characterized viruses can be challenging if low egg growth limits the supply of antigen. Our vaccine was produced from a classical egg-derived seed virus propagated in a MDCK cell line.[7,21] Cell-culture systems may provide a faster response and greater scale-up than egg production. Cell-culture seasonal influenza seed viruses also show better antigenic matching to clinical isolates than egg-passaged strains.[5-8,21]

Clinical experience with avian and human influenza A/H1N1–subunit vaccines in subjects who did not have detectable levels of preexisting antibody suggests that two doses are required to induce a hemagglutination-inhibition antibody titer of 1:40 or more.[10-15,22,23] Traditionally, dosing intervals of 21 to 28 days are used, often delaying effective immunization. We evaluated rapid immunization schedules involving two doses of MF59-adjuvanted vaccines, since flexible dosing would be useful for authorities organizing immunizations. However, our data suggest that a single immunization against the 2009 pandemic influenza A (H1N1) virus would be sufficient to induce a hemagglutination-inhibition antibody titer of 1:40 or more.

Our findings add to observations that oil-in-water–emulsion adjuvants are well tolerated, with systemic reactogenicity similar to that of nonadjuvanted inactivated seasonal vaccines, but are associated with increased local pain at the site of administration.[24] For avian subvirion vaccines, oil-in-water–emulsion adjuvants are important to induce long-lasting cross-reactive immunity.[10,12-15] Although 2009 pandemic influenza A (H1N1) isolates are antigenically homogenous, induction of broadly cross-reactive antibodies would be a desirable characteristic of the first vaccines against the 2009 H1N1 virus, and serum samples obtained after the administration of candidate vaccines, either adjuvanted or nonadjuvanted, should be assessed against emerging antigenic variant strains. The addition of MF59 adjuvant to 2009 influenza A (H1N1) monovalent vaccine increases the speed and magnitude of the antibody response; however, nonadjuvanted vaccine also induced satisfactory immune responses, which is consistent with early reports of other 2009 H1N1 subunit vaccines.[25] The 2009 H1N1 virus is antigenically distinct from recently circulating seasonal H1N1 strains, so the response to a single dose of vaccine suggests there may a greater degree of preexisting immunity in the population than expected. Sixteen percent of subjects had detectable prevaccination levels of hemagglutination-inhibition antibody, a finding that is consistent with results of seroepidemiologic studies.[11] Although we excluded subjects with previous respiratory illnesses, asymptomatic infection with influenza A (H1N1) viruses cannot be ruled out, since local activity was present during the study.

Interpretation of immunogenicity data for the vaccine against the 2009 pandemic influenza A (H1N1) virus is complicated by a lack of recognized immune correlates. The insensitivity of hemagglutination-inhibition assays to some avian hemagglutinin has required that microneutralization assays, hemagglutination-inhibition assays involving horse erythrocytes, or single radial hemolysis be used.[10,18,26] Because there is significant laboratory variation in testing,[20] efforts to develop biologic standards for serologic assays of influenza A (H1N1) viruses are under way.

The safety and immunogenicity of these and alternative candidate vaccines against the 2009 H1N1 virus, including egg-derived, whole-virion, recombinant, and live-attenuated vaccines, must be assessed in high-risk populations, including children, the elderly, and other persons whose immunologic profiles may differ from those of young adults.[8] In addition, the duration of antibody responses and their ability to be boosted after revaccination should be established to predict protection against future pandemic waves.

Finally, although seasonal influenza vaccines have an established safety profile, there are occasional case reports of unusual reactions, including vasculitis.[24,27] MF59, a proprietary oil-in-water–emulsion adjuvant, was first licensed for use in seasonal influenza vaccines in 1997. Over 40 million doses have been delivered in Europe, and

over 16,000 doses administered in clinical trials, with no excess reports of autoimmune conditions.[28] It is important to ensure post-marketing surveillance during any mass use of a pandemic-virus vaccine, with or without adjuvant. These results may be useful for planning of immunization schedules, and comparison with other vaccine options as they become available.

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