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Immunogenicity, Boostability, and Sustainability of the Immune Response after Vaccination against Influenza A Virus (H1N1) 2009 in a Healthy Population▽

Elisabeth Huijskens,[1]* John Rossen,[1] Paul Mulder,[2] Ruud van Beek,[3] Hennie van Vugt,[1] Johannes Verbakel,[1] Guus Rimmelzwaan,[3] Marion Koopmans,[3,4] and Marcel Peeters[1]†

Laboratory of Medical Microbiology and Immunology, St. Elisabeth Hospital, Tilburg, Netherlands[1]; Laboratory for Microbiology and Infection Control, Amphia Hospital, Breda, Netherlands[2]; Department of Medical Microbiology, Erasmus Medical Center, Rotterdam, Netherlands[3]; and National Institute of Public Health and the Environment, RIVM, Bilthoven, Netherlands[4]

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The emergence of a new influenza A virus (H1N1) variant in 2009 led to a worldwide vaccination program, which was prepared in a relatively short period of time. This study investigated the humoral immunity against this virus before and after vaccination with a 2009 influenza A virus (H1N1) monovalent MF59-adjuvanted vaccine, as well as the persistence of vaccine-induced antibodies. Our prospective longitudinal study included 498 health care workers (mean age, 43 years; median age, 44 years). Most (89%) had never or only occasionally received a seasonal influenza virus vaccine, and 11% were vaccinated annually (on average, for >10 years). Antibody titers were determined by a hemagglutination inhibition (HI) assay at baseline, 3 weeks after the first vaccination, and 5 weeks and 7 months after the second vaccination. Four hundred thirty-five persons received two doses of the 2009 vaccine. After the first dose, 79.5% developed a HI titer of ≥40. This percentage increased to 83.3% after the second dose. Persistent antibodies were found in 71.9% of the group that had not received annual vaccinations and in 43.8% of the group that had received annual vaccinations. The latter group tended to have lower HI titers ($P = 0.09$). With increasing age, HI titers decreased significantly, by 2.4% per year. A single dose of the 2009 vaccine was immunogenic in almost 80% of the study population, whereas an additional dose resulted in significantly increased titers only in persons over 50. Finally, a reduced HI antibody response against the 2009 vaccine was found in adults who had previously received seasonal influenza virus vaccination. More studies on the effect of yearly seasonal influenza virus vaccination on the immune response are warranted.

On 11 June 2009, the World Health Organization declared the first influenza pandemic in 41 years (32). It was caused by a triple-reassortant influenza A (H1N1) virus that contained genes from avian, human, and swine influenza viruses. The hemagglutinin (HA) of this virus was derived from viruses that had been circulating in swine since their initial introduction during the influenza A virus (H1N1) pandemic in 1918 (8). Due to this separation in host species, each with its own dynamics of influenza virus evolution, the viral HA was antigenically very distinct from that of the circulating seasonal influenza virus H1N1 strains (7). Up to 6 January 2011, a total of 2,193 patients had been hospitalized and 63 patients had died in the Netherlands, with a greater risk of complications found in the age group of 5 to 14 years (27). The age distribution showed the highest impact in relatively young persons, correlating with the presence of cross-reactive antibodies with the H1N1 pandemic strain in older people that were induced originally after infection against H1N1 viruses that circulated before 1957 (15, 20, 33).

The emergence of the new influenza A virus (H1N1) variant led to a worldwide vaccination program, which was prepared in a relatively short period of time. Vaccination is the most important preventive measure for reducing morbidity and mortality and may also influence virus transmission. In the Netherlands, clinics used the 2009 influenza A virus (H1N1) monovalent MF59-adjuvanted vaccine (Focetria; Novartis), an inactivated influenza virus vaccine containing the influenza virus A/California/7/2009 (H1N1)v-like strain (X-179A) and the adjuvant MF59. MF59, an oil-in-water emulsion, is an adjuvant developed to improve the performance of vaccines in general (2) and has been shown to improve immune response to seasonal influenza virus vaccines in adults as well as in children (24, 30).

The aim of this study was to measure the humoral immunity before and after vaccination with the 2009 influenza A virus (H1N1) monovalent MF59-adjuvanted vaccine and to examine the duration of the immune response.

MATERIALS AND METHODS

Study design. From November 2009 through June 2010, we conducted a prospective, longitudinal study at St. Elisabeth Hospital and TweeSteden Hospital, Tilburg, Netherlands. Health care workers of both hospitals (≥18 years; if pregnant, only after 13 weeks of pregnancy) were eligible for inclusion. We collected demographic characteristics, including age, sex, comorbidity (scored as asthma, chronic obstructive pulmonary disease [COPD], diabetes, hypo- and hyperthyroidism, rheumatoid arthritis, psoriasis, inflammatory bowel diseases, celiac disease, autoimmune sclerosis, anemia, sarcoidosis, prolactinoma, arthro-

* Corresponding author. Mailing address: Laboratory of Medical Microbiology and Immunology, St. Elisabeth Hospital, P.O. 747, 5000 AS Tilburg, Netherlands. Phone: 31 13 5392650. Fax: 31 13 5441264. E-mail: i.huijskens@gmail.com.

† Marcel Peeters passed away on 6 June 2011.

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sis, gout, nephropathy, and carcinoma), and influenza virus vaccination status, by means of a short questionnaire.

Blood samples were collected prior to the first vaccination against influenza A virus (H1N1) 2009, before the second vaccination (3 weeks later), before the vaccination with trivalent seasonal influenza virus vaccine (5 weeks after the second vaccination), and 7 months after the second vaccination against influenza A virus (H1N1) 2009. The trivalent seasonal influenza virus vaccine consisted of influenza A virus H1N1 (2007), influenza A virus H3N2 (2007), and influenza B (2008) virus.

A comparison was made between the group of participants who had never or occasionally received seasonal influenza virus vaccination and those who had been vaccinated with seasonal influenza virus vaccine each year. Occasionally, vaccinated was defined as having received seasonal influenza virus vaccination sporadically but not yearly in the past. The study was approved by the local medical ethics committee. Written informed consent was obtained from all participants in the study.

Serology. Virus-specific antibodies were measured by a hemagglutination inhibition (HI) assay, using egg-grown A/California/7/2009 A (H1N1) pandemic virus and fresh red blood cells (RBCs) of turkeys in Alsever's solution (Biotrading, Netherlands). Virus had been purified by centrifugation through a sucrose gradient and concentrated to create an influenza virus zonal pool preparation. Four hemagglutinating units were yielded with a virus antigen dilution of 1:16. Serum samples were heat inactivated at 56°C and treated overnight at 37°C with receptor-destroying enzyme (RDE, cholera filtrate; CosmosBiomedical Ltd., Derbyshire, United Kingdom). The serum-antigen mixture was incubated for 45 min at room temperature, and subsequently RBCs (1%) were added to each well of the microtiter plate. A negative and a positive control were used, as were serum controls for each specimen to detect nonspecific hemagglutination. Finally, plates were read promptly when the RBC control had completely settled.

The HI titer was the reciprocal of the highest dilution of serum that inhibited virus-induced hemagglutination. Titers below the detection limit of 10 were assigned a value of 5, and 1,280 was the endpoint titration and also the highest dilution tested.

Comparison of serological data from different laboratories is complicated by a lack of standardization. This is caused by the use of various influenza virus strains and different receptor-destroying enzymes (homemade or commercially bought) and also by differences in quality and nature of red blood cells (1). Therefore, there is a poor reproducibility of the HI assay between laboratories. To overcome this problem, red blood cells and receptor-destroying enzyme were bought commercially, and we used a candidate international standard for antibody titers to pandemic H1N1 virus to correct our data (22).

Statistical analysis. For immunogenicity analyses, the geometric mean antibody titers (GMT) at each time point were used. GMT were computed by taking the antilog of the mean of the log-transformed titers. Linear mixed-model analyses for repeated measurements were performed with the log-transformed titer as the dependent variable measured at 3 weeks (before the second vaccination), 5 weeks after the second vaccination (before the vaccination with seasonal influenza virus vaccine), and 7 months (after the second vaccination).

The following independent variables were initially entered simultaneously in the model, as were the interaction terms between these variables: time (with the three levels just described), age at baseline (years), history of seasonal influenza virus vaccination (yes/no), and comorbidity (yes/no). Along with these variables, the time-dependent explanatory indicator variable "seasonal vaccination in 2010" was entered into the model. By definition, this variable was 0 in all persons attending the first- and second-measurement wave and took the value 1 in 32 out of 137 persons attending the last-measurement wave, while remaining 0 in the other 105 persons attending this wave. Moreover, gender and its interaction term with time postvaccination were included in the initially entered set of explanatory variables because of sex differences in immune antibody responses to vaccines described in the literature (16, 17).

A final model was obtained after stepwise backwards elimination. At each step, the term with the highest P value above 0.10 was deleted from the model, with higher-order terms having precedence over lower-order terms in the elimination procedure. In the final model that remained, all terms had a P value below 0.10. Estimated effects and their confidence limits were back-transformed by taking the antilog and expressed as percent changes. Differences between the group who were never/occasionally vaccinated and the group who were regularly vaccinated were tested using Fisher's exact test for dichotomous variables or the two-sample t test for titers after logarithmic transformation. All analyses were performed using PASW Statistics 18 (IBM Company, Chicago, IL).

TABLE 1. Demographic characteristics of the persons

Characteristic	Value for:		
	All persons[a] (n = 498)	Persons not annually vaccinated with seasonal influenza virus vaccine (n = 443)	Persons annually vaccinated with seasonal influenza virus vaccine (n = 54)
Age (yr)			
Mean	43	43	48
Median	44	44	49
Range	19–66	19–63	19–66
No. (%) vaccinated:			
Before 2000			32 (59.2)
From 2001–2004			13 (24.1)
After 2005			9 (16.7)
No. (%) of gender			
Male	155 (31.1)	131 (29.6)	24 (44.4)
Female	343 (68.9)	312 (70.4)	30 (55.6)
No. (%) with comorbidity			
None	457 (91.8)	416 (93.9)	40 (74.1)
COPD/asthma	9 (1.8)	4 (0.9)	5 (9.2)
Diabetes mellitus	3 (0.6)	0 (0)	3 (5.6)
Thyroidism	6 (1.2)	6 (1.4)	0 (0)
Rheumatoid arthritis	4 (0.8)	3 (0.7)	1 (1.9)
Other	19 (3.8)	14 (3.1)	5 (9.2)
Total with comorbidity	41 (8.2)	27 (6.1)	14 (25.9)

[a] For one of the patients, the status of the seasonal influenza virus vaccination was missing.

RESULTS

Initially, 498 persons were included, a serum blood sample was taken, and the first vaccination was given. Three weeks later, 435 persons also received the second vaccination. In addition, a third serum sample was taken from 341 persons 5 weeks after the second vaccination. At 7 months after the second vaccination, a fourth serum sample was collected from 137 persons. Of these, 32 persons (28 persons who had not received annual vaccinations and 4 persons who had received annual seasonal influenza virus vaccinations) received a trivalent seasonal influenza virus vaccination in January 2010. One person was excluded due to nonspecific reactions in the HI assay. The median age of the persons was 44 years (range, 19 to 66 years), 69% of them were female, and 11% had a history of annual vaccinations against seasonal influenza virus (60% for more than 10 years) (Table 1).

Immunogenicity. At baseline, 22 of the 498 persons (4.4%) had HI titers of ≥40 (Table 2). These titers were more frequently observed in persons receiving annual seasonal influenza virus vaccination (6 of 54 [11.1%]) than in persons without a history of seasonal influenza virus vaccination (16 of 443 [3.6%]) (P = 0.023). After the first vaccination, 346 of the 435 persons who returned for a second vaccination (79.5%) produced an immune response. After the second vaccination, this increased to 83.3% (284 of the 341 persons who had a third serum sample taken).

Seven months after the second vaccination, 94 (68.6%) of

TABLE 2. Immunogenicity after the first and second doses of the monovalent MF59-adjuvanted influenza virus H1N1 (2009) vaccine, as measured by a hemagglutination inhibition assay

Immunogenicity endpoint	Value for:		Total	*P* value
	Persons never or occasionally vaccinated with seasonal influenza virus vaccine	Persons always vaccinated with seasonal influenza virus vaccine		
Baseline				
No. of persons	443	54	498[b]	
Geometric mean titer	5.8	7.2	5.9	0.019
No. (%) of persons with HI titer of ≥1:40	16 (3.6)	6 (11.1)	22 (4.4)	0.023
3 wk after first dose				
No. of persons	383	51	435[b]	
Geometric mean titer (IQR)[a]	70.9 (40–160)	47.0 (20–160)	67.1 (40–160)	0.025
No. (%) of persons with HI titer of ≥1:40	310 (80.9)	36 (70.6)	346 (79.5)	0.095
5 wk after second dose				
No. of persons	297	44	341	
Geometric mean titer (IQR)	74.2 (40–160)	53.1 (40–135)	71.1 (40–160)	0.049
No. (%) of persons with HI titer of ≥1:40	249 (83.8)	35 (79.5)	284 (83.3)	0.52
7 mo after second dose				
No. of persons	121	16	137	
Geometric mean titer (IQR)	55.1 (20–80)	30.8 (20–40)	51.5 (20–80)	0.081
No. (%) of persons with HI titer of ≥1:40	87 (71.9)	7 (43.8)	94 (68.6)	0.041

[a] IQR, interquartile range.
[b] For one of the patients, the status of the seasonal influenza virus vaccination was missing.

the 137 persons who gave a fourth serum sample had HI titers of ≥40, but the difference between the groups had increased remarkably: in the group without a history of annual vaccination, 87 persons (71.9%) had a titer of ≥40, whereas in the group who had received annual vaccinations, only 7 of the 16 persons (43.8%) had a titer of ≥40 ($P = 0.041$). The trivalent seasonal influenza virus vaccine, given to 32 of the 137 persons, had no influence on the reduction in HI titer of the pandemic H1N1 virus.

The estimated final model using linear mixed modeling showed a significant interaction between age and time post-vaccination ($P = 0.004$) and a nearly significant effect of history of previous vaccination ($P = 0.098$), which were therefore both included in the final model. No significant effects were found for the other interaction terms or for comorbidity, gender, and seasonal influenza virus vaccination in 2010; therefore, they were all eliminated. A history of previous vaccination with seasonal influenza virus vaccine resulted in a nearly significant 30.6% lower titer (95% confidence interval [95% CI], 55.0% lower to 7.1% higher).

The significant age-by-time interaction caused the effect of age at baseline on titer to be modified by time after vaccination as follows. Per year of age increase at baseline, the corresponding estimated decrease in titer was 2.4% (95% CI, 3.4 to 1.4; $P < 0.00005$) at 3 weeks (after the first vaccination), 1.4% (95% CI, 2.3 to 0.4; $P = 0.0041$) at 5 weeks (after the second vaccination), and 2.6% (95% CI, 4.3 to 1.0; $P = 0.0020$) at 7 months (after the second vaccination).

Alternatively, one may explain the age-by-time interaction as the effect of time on titer being modified by age. The estimated percent changes in titer between the first and second vaccinations and between the first vaccination and 7 months after the second vaccination are presented in Table 3 at 30, 40, 50, and 60 years of age at baseline. For example, between the first and second vaccinations there was a significant increase in titer of 15.1% (95% CI, 6.4 to 24.5; $P < 0.0005$) at 50 years of age, whereas at 60 years of age this increase in titer was 28.0% (95% CI, 13.2 to 44.8; $P < 0.0005$). Between the first vaccination and 7 months after the second vaccination, the age-by-time interaction was less pronounced, as a significant decrease in titer of about 25% was seen at 40, 50, and 60 years of age.

DISCUSSION

This study demonstrates that a single dose of a monovalent MF59-adjuvanted influenza virus vaccine with influenza A virus (H1N1) 2009 produced an antibody response in 346 of 435 persons (79.5%). In addition, it was shown that a second vaccination had little or no additional effect on the antibody titers in persons under 50 years of age. However, significant in-

TABLE 3. Estimated percent change in titers compared with titers found after the first vaccination, adjusted for seasonal influenza virus vaccination by using linear mixed modeling

Time point tested	% change in titer	*P* value	95 % CI	
			Lower	Upper
After second dose at age (yr):				
30	−7.0	0.24	−17.7	5.0
40	3.4	0.40	−4.3	11.8
50	15.1	<0.0005	6.4	24.5
60	28.0	<0.0005	13.2	44.8
After 7 mo at age (yr):				
30	−22.6	0.11	−43.5	6.1
40	−24.4	0.006	−38.0	−7.7
50	−26.1	0.001	−38.5	−11.3
60	−27.9	0.025	−45.8	−4.0

creases in the proportion of persons with protective level HI titers were observed in older persons following booster vaccination. Finally, a statistically significant correlation was observed between increasing age and more rapid decline in HI titer over time.

The response to the first dose of the pandemic influenza virus vaccine was sufficient to fulfill the European licensure criteria for immunogenicity of influenza virus vaccines, in line with results of previous studies (7). The advice to provide an additional second vaccine dose was a matter of debate in our country and elsewhere but was recommended based on the concern that risk groups might have a less favorable response to a single vaccine dose, as had been described for seasonal influenza virus vaccines (14, 18). Indeed, we found a clear age-specific effect in response to vaccination, despite the presence of a strong adjuvant.

We found that age at time of vaccination itself had a negative effect on the HI titer. In the literature, conflicting results of the effect of age on the immune response to influenza virus vaccine have been described (3, 12, 26). In a quantitative review, Goodwin et al. described that the antibody response in the elderly (>65 years) is considerably lower than that in younger adults (12). In contrast, Remarque et al. described that lower HI titers were not caused by age or aging of the immune system but probably by differences in priming histories and concomitant diseases (26).

Our data indicate that this may be the case, as responses were lower and persisted for shorter times in persons with a history of seasonal influenza virus vaccination prior to pandemic influenza virus vaccination. This effect has repeatedly been observed in clinical and animal studies on trivalent seasonal influenza virus vaccines (5, 21, 28, 29). The mechanism behind it remains to be discovered, but it is tempting to speculate about the observation of Bodewes et al., who found a T cell-mediated protective effect of natural influenza virus infection to subsequent infection with heterologous strains in ferrets (4). This heterosubtypic immunity supposedly is suppressed by regular vaccination, thus influencing the impact of a subsequent infection with an antigenically distinct influenza virus strain (5).

Studies during the pandemic remained inconclusive, but some suggested increased severity of pandemic influenza in persons with prior seasonal influenza virus exposures. Skowronski et al. reported an increased risk of medically attended influenza virus H1N1 (2009) illness after prior vaccination with trivalent seasonal influenza virus vaccine 2008–2009 (29), but these findings have never been corroborated by others. Obviously, an alternative explanation may be that such observations are confounded by the fact that vaccination is often limited to older persons and persons with comorbidities, who therefore possibly have impaired immune functions (6, 12).

In any case, in our study, the clear increase in proportion of persons over 50 years with an adequate response to vaccination following a booster vaccination showed that the decision to provide a 2-dose schedule was advantageous for this age group. Moreover, our data suggest that immune responses after vaccination need to be evaluated more carefully, taking vaccination history into account.

A limitation of our study was that vaccination of the health care workers in our hospitals commenced in week 46 of 2009, which coincided with the pandemic influenza peak in the Netherlands. However, in this study, we found baseline protective antibodies (HI titers of ≥40) in only 4.4% of the study population, compared with 4 to 31% found in other studies, which suggests that the majority of study participants had not encountered pandemic influenza before vaccination (13, 19, 25). In addition, if people were naturally infected during the study period, we expected those effects to be randomly spread over the groups.

Previous studies reported the presence of some level of cross-reactive antibodies in persons born before 1957. Such cross-reactive antibodies may be present in these persons because the H1N1 subtype viruses before 1957 were more similar to the H1N1 (2009) than the H1N1 viruses that reemerged in the 1970s. We detected no difference in preexisting baseline titers to the 2009 H1N1 strain between individuals born before or after 1957, and no stronger immunologic response in HI titer was seen in this group (15, 20). This might be due to the small number of persons born before 1957 in this study.

A larger proportion of persons who were vaccinated annually than those who were unvaccinated had preexisting antibodies to the 2009 H1N1 strain. This finding resembles those of other studies, and the most likely explanation is that seasonal influenza virus vaccination induced antibodies that cross-react with 2009 H1N1 virus to a certain extent (15, 34).

Contrary to other studies, we did not find a difference in antibody response between men and women. Data from clinical trials of seasonal influenza virus vaccines reveal pronounced differences between antibody responses in men and women. HI titers are consistently higher in women than men, suggesting that women may be better protected against influenza disease (16, 17). Although the mechanisms underlying sex-specific differences in immune development are poorly understood, women have higher absolute $CD4^+$ lymphocyte count and production of TH1 cytokines after immunization, as well as more sustained responses to antigenic challenge (9–11, 31). The absence of this difference in antibody response between men and women may be due to the use of MF59 as an adjuvant in the vaccine, which is known to enhance the immune response (2, 23, 24, 30).

Conclusions. In conclusion, in our population the first vaccination with a monovalent MF59-adjuvanted influenza virus H1N1 (2009) vaccine resulted in a titer of ≥40 in 79.5% of the participants, and 71.9% of the participants had persisting antibodies for 6 months. Overall, a second dose of the vaccine conferred little additional benefit, but in some age groups (50 to 60 years) a significant increase of HI titer was seen. Increasing age had an unfavorable effect on the postvaccination HI antibody titers of the participants. For each additional year of age, a 2.4% reduction in GMT was found.

There was a reduced HI antibody response, although not significant, in adults who annually received seasonal influenza virus vaccination compared with that in adults who never or occasionally received seasonal influenza virus vaccination, suggesting a possible unfavorable effect on immunogenicity for adults who annually received seasonal influenza virus vaccination. The mechanism underlying this negative effect of previous vaccination is unclear and remains to be elucidated.

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