A(H1N1)pdm09 Vaccination of Health Care Workers: Improved Immune Responses in Low Responders Following Revaccination

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Background. We conducted a clinical trial in October 2009 to evaluate the immunogenicity of the AS03-adjuvanted influenza vaccine (pH1N1 vaccine) in health care workers (HCWs). By 2 weeks after vaccination, 97% had protective hemagglutinin inhibition (HI) titers (≥40) however, 16% were low responders (LR) and failed to maintain a protective response 90 days after vaccination.

Methods. We analyzed the humoral responses (HI, antibody-secreting cell [ASC], and serum immunoglobulin G [IgG]) in 15 LRs and 25 control HCWs. Twelve LRs were revaccinated with the pH1N1 vaccine, and 7 were subsequently vaccinated with the 2010 seasonal trivalent influenza vaccine. We conducted a long-term analysis of the humoral and CD4+ T-helper (Th) 1 responses.

Results. The LRs had a slower HI antibody response than the control HCWs, with protective antibody titers not reached until 2 weeks after vaccination in the majority of the participants. The LRs also had significantly lower IgG ASCs at day 7 and HA1-specific serum IgG responses at day 21, compared with the control HCWs. Revaccination with the pH1N1 vaccine elicited rapid HI antibody, ASC, memory B cell, and multifunctional CD4+ Th1 cell responses.

Conclusion. This study shows that revaccination of low-responding HCWs with the pH1N1 vaccine is required for maintaining long-term protection.

Clinical Trials Registration. NCT01003288.
In the current study, we conducted a kinetic analysis of serum antibody and B cell responses of low responders (LRs) who had HI titers (≤40) at 3 months after a single dose of the pH1N1 vaccine. Furthermore, we revaccinated 12 LRs with a second dose of the pH1N1 vaccine and, subsequently, the TIV and report the long-term humoral and CD4⁺ Th1 responses.

**MATERIALS AND METHODS**

**Participants and Study Design**

In October 2009, we vaccinated 207 frontline HCWs at the Haukeland University Hospital (HUH, Bergen, Norway) with a single dose of the pH1N1 vaccine [5]. In the current study, 40 HCWs were retrospectively selected on the basis of seroconversion and HI antibody response at 3 months after vaccination. On this basis, a cohort of 15 LRs who failed to seroconvert or maintain a protective HI antibody response (titer, ≥40) at day 90 after vaccination was selected. Further criteria for inclusion of LRs in this study were informed consent for revaccination and/or availability of peripheral blood mononuclear cells (PBMCs) on day 7, which were run in the enzyme-linked immunospot assay (ELISPOT) assay. The control group consisted of 25 HCWs who had seroconverted and had a protective HI titer at 3 months after vaccination and had donated PBMCs for the ELISPOT assay at 7 days after vaccination and serum samples on all sampling days. Table 1 shows the demographic characteristics, influenza vaccination history, and underlying risk factors. One participant from the control group and 2 from the LR group reported having hypertension, and another 3 participants from the LR group had either type-1 diabetes, arthritis, or asthma.

The LRs were offered revaccination, and 12 participants decided to be revaccinated 5 months after primary vaccination; 3 HCWs refused revaccination. The revaccinated group consisted of 4 men and 8 women, with a mean age (± standard deviation) of 40.3 ± 12.7 years (Table 1). Four participants in the revaccinated group had either hypertension, type-1 diabetes, arthritis, or asthma (Table 1).

As part of the annual TIV campaign for HCWs at HUH, 7 of 12 participants in the revaccinated group and 9 of 25 control HCWs were given a single dose of the 2010 TIV at approximately 210 days after revaccination with the pH1N1 vaccine.

**Ethics and Regulatory Approval**

All participants provided written informed consent before inclusion in the study, which was approved by the regional ethics committee and the Norwegian Medicines Agency (www.clinicaltrials.gov, NCT01003288). The inclusion and exclusion criteria for this study are described elsewhere [5].

**Vaccines Used in the Study**

Pandemrix (pH1N1 vaccine; GlaxoSmithKline) is a monovalent split virus (X179A) vaccine adjuvanted with oil-in-water adjuvant AS03. The seasonal TIV (Influvac; Solvay) was composed of 15 μg HA of influenza A(H1N1)pdm09–like, A/Perth/16/2009 (H3N2)–like, and B/Brisbane/60/2008–like strains.

**Vaccination and Blood Sample Collection**

All volunteers received a single dose (3.75 μg HA) of the pH1N1 vaccine intramuscularly in the deltoid muscle. The revaccinated LRs received a second pH1N1 vaccine dose (3.75 μg HA) at approximately 5 months (± 2 weeks) after primary vaccination. All participants provided clotted blood samples (10 mL) at 0, 21, and 90 days after revaccination. Some participants donated blood at 7 and 14 days after primary vaccination and 7, 14, 21, and 210 days after revaccination. After TIV vaccination, clotted blood samples were collected before vaccination (day 0) and at days 21, 90, and 180 after vaccination. All serum samples were aliquoted and coded with a unique identification number and stored at −80°C before use in the serological assays. An additional 40 mL of blood was collected from some individuals at each time point, and the PBMCs were isolated as published elsewhere [12]. Fresh PBMCs were used in all relevant assays. An overview of the vaccination schedule, including the number of participants at each time point, is presented in Figure 1.
Antibody Assays
Pre- and post-vaccination serum samples from each individual were tested at the same time in the HI assay using the X179A virus and 0.7% turkey red blood cells, as described elsewhere [5].

The influenza A(H1N1)pdm09 HA1 (eEnzyme) and X179A whole virus–specific serum IgG response after primary vaccination was quantified by enzyme-linked immunosorbent assay (ELISA) [13].

B Cell Assays
The virus (X179A)–specific IgG, IgA, and IgM antibody-secreting cell (ASC) response after primary vaccination and revaccination was determined by ELISPOT using fresh PBMCs according to Cox et al (1994) [14]. The numbers of IgG, IgA, and IgM ASCs were evaluated at 7 days after vaccination, because this has previously been shown to be the peak response after influenza vaccination [14].

The X179A-specific IgG memory B cell (MBC) response after revaccination was quantified by ELISPOT as described elsewhere [15]. Results are presented as virus-specific IgG MBCs per 1 × 10⁶ PBMCs.

Intracellular Cytokine Staining of CD4+ T cells
PBMCs from revaccinated participants were stimulated overnight with the X179A split virus and stained intracellularly for Th1 cytokines (interferon [IFN]–γ), interleukin 2 (IL-2), and tumor necrosis factor[TNF]–α), and the percentage of CD4+ Th1 cells secreting either single or multiple cytokines was determined by multiparametric flow cytometry, as described elsewhere [16].

Statistics
No sample size calculation was made for this exploratory trial. Statistical analyses were performed by the Kruskal–Wallis and Mann–Whitney U tests, the Friedman test with Dunns postest, or Spearman correlation (GraphPad Prism, version 5.00, for Mac; GraphPad Software). Differences in T cell responses were
RESULTS

The Hemagglutination Inhibition Antibody Response After Primary Vaccination

The kinetics of the HI responses to influenza A(H1N1)pdm09 after primary vaccination were retrospectively analyzed and compared between the HCWs identified as low responders and the control HCWs. Before vaccination, all of the LRs and 21 of 25 control HCWs had HI titers under the protective level of 40 (Figure 2A). By 7 days after vaccination, 21 of 25 control HCWs had protective HI titers (geometric mean titer [GMT], 176); however, only 3 of 7 LRs had HI titers ≥ 40 (GMT, 21). By 14 days after vaccination, 6 of 7 LRs and all control HCWs had HI titers ≥ 40, and no statistically significant increase in the HI response was observed in either group at day 21. The HI titers continued to be substantially lower in the LRs (GMT, 117 and 81), compared with the control HCWs (GMT, 606 and 462) at 14 and 21 days after vaccination, respectively. At day 90, the HI titers were generally < 40 in the LRs (49-fold lower than in the control HCWs).

The Serum IgG Response After Primary Vaccination

The serum IgG antibody response to influenza A(H1N1)pdm09 was analyzed by ELISA using whole virus as the immobilized antigen. Significantly higher post-vaccination concentrations of influenza A(H1N1)pdm09–specific IgG were detected at day 21 in the LR group and at days 21 and 90 in the control group, compared with prevaccination. However, no statistically significant differences were observed between the LR and controls at any matched time point (results not shown). Therefore we proceeded to analyze the HA1-specific...
serum response (Figure 2B). Very low levels of anti-HA1 IgG were detected prior to vaccination in LR and controls, which increased in both groups at day 7 and peaked at day 14, with no significant difference observed between the 2 groups. At day 21, the HA1-specific IgG response was significantly ($P < .01$) lower in the LRs than in the control HCWs. The responses continued to decrease at day 90, when a tendency toward lower IgG responses in the LRs than in the control HCWs was observed; however, this was not statistically significant ($P = .07$).

The Antibody Secreting Cell Response After Primary Vaccination

This analysis revealed that both LRs and control HCWs had a dominant IgG ASC response with mean virus-specific IgG ASCs per $1 \times 10^5$ PBMC of 65 and 120, respectively (Figure 2C). Comparison of class-matched virus-specific ASC response showed that the control HCWs had significantly ($P < .05$) higher numbers of IgG and IgA ASCs, compared with LRs, but the IgM response was similar in the 2 groups. In LRs, the IgG ASC response was significantly higher than the IgM response ($P < .01$), but not compared with the IgA response, whereas in control HCWs, the IgG ASC response was significantly higher than the IgM ($P < .001$) and IgA ($P < .01$) response (data not shown). Of note, there was no significant correlation between HI titers at any time point and ASC responses for either group (data not shown).

The HI Antibody Response After Revaccination

Before revaccination, 11 of 12 LRs had antibody titers below the protective HI titer and 5 had no detectable antibody (HI titer, <10) to the influenza A(H1N1)pdm09 virus (Figure 3A). By 7 days after vaccination, 11 of 12 LRs had a protective antibody titer, and no further significant increase in the HI titer was detected from days 7 through 21. HI responses after pH1N1 revaccination met all 3 of European Medicinal Agency’s Committee for Medicinal Products for Human Use (CHMP) immunogenicity criteria by 7 days after revaccination...
The long-term HI antibody response was measured 210 days after pH1N1 revaccination; 4 of 12 participants had HI titers ≥40 to the influenza A(H1N1)pdm09 virus, 4 had low HI titers, and 4 had no detectable HI antibody response.

As part of the annual influenza vaccination program at the hospital, participants were vaccinated with the 2010 TIV 210 days after revaccination with the pH1N1 vaccine. As shown in Figure 3A, 6 of 7 participants elicited a protective HI antibody response against the influenza A(H1N1)pdm09 virus 21 days after TIV vaccination (GMT, 129). The HI antibody response decreased 90–180 days after TIV vaccination with GMT of 51 and 29, respectively. The CHMP immunogenicity criteria were met 21 days after TIV vaccination, which was the time chosen for serum sample collection.

The ASC Response After Revaccination With the pH1N1 Vaccine

To further characterize the humoral response of the 12 revaccinated individuals, we evaluated the ASC response 7 days after revaccination (Figure 3B). Revaccination induced a significantly higher influenza A(H1N1)pdm09 virus–specific IgG ASC response, compared with the IgM (P < .001) and IgA (P < .05) responses, similarly to the response in the control group after primary vaccination. Furthermore, we compared the IgG ASC response of the revaccinated group (Figure 3B) with that of the LRs at 7 days after primary vaccination (Figure 2C) and found no significant differences between the 2 groups. Of note, only 4 of the 7 participants who were evaluated for ASC response after primary vaccination were present in the revaccination group. However, the day 7 IgG ASC response of the revaccinated group (Figure 3B) was significantly lower (P < .001) than that of the control HCWs at 7 days after the primary vaccination (Figure 2C).

The Memory B Cell Response After Revaccination

Five months after primary vaccination and immediately before revaccination, we detected influenza A(H1N1)pdm09 virus–reactive MBCs in all participants (day 0) (Figure 3C). Although the MBC frequencies were low, they were not significantly different than the MBC responses of the control
HCWs at the same time point (mean of 797, compared with 1092 cells per 1 × 10⁶ PBMC, respectively). The antigen-specific IgG MBC frequencies increased at day 7 after revaccination and peaked at day 21 (Figure 3C). The MBC frequencies at day 21 were significantly higher (P < .05) than those at day 0 and increased 2–8 fold. At day 210, the frequencies of MBCs had decreased to levels similar to those on day 0.

When analyzing the individual MBC responses to pH1N1 revaccination (Figure 4), we found that the responses between individuals were heterogeneous. Nevertheless, the individual responses demonstrated a dominant peak in the IgG MBC response at day 21 after revaccination. Furthermore, we found a significant correlation between MBC frequencies at days 7, 21, and 210 after revaccination and HI titers at days 7, 14, and 21 after revaccination (Table 2).

TIV vaccination did not significantly increase the MBC response (Figure 3C), although 4 of the 7 analyzed individuals had an increase in MBC frequencies at day 21.

Polyfunctional CD4⁺ T Cell Response After Revaccination

Figure 5A shows that the frequency of Th1 CD4⁺ T cells that express a single cytokine (IFN-γ, IL-2, or TNF-α) was significantly (P < .05) higher 7, 14, and 21 days after revaccination, compared with the pre-revaccination levels. Similarly, the percentage of CD4⁺ T cells simultaneously expressing either 2 (IFN-γ/IL-2, IFN-γ/TNF-α, or IL-2/TNF-α) or 3 (IFN-γ/IL-2/TNF-α) cytokines was significantly higher 7, 14, and 21 days after revaccination, compared with prevaccination levels (Figure 5B). A peak response was observed at day 21 after revaccinations for both single and multiple cytokine-expressing CD4⁺ T cells, regardless of the cytokine measured. Furthermore, a significant positive correlation was observed from the day 7 CD4⁺ T cell response to the HI titers at days 21 and 210 after revaccination (Table 2).

**DISCUSSION**

Studies of avian candidate pandemic vaccines have shown that 2 doses of adjuvanted vaccines are required to elicit protective antibody responses in naive persons. However, one dose of pH1N1 vaccine resulted in protective HI titers by 6–7 days after vaccination [5]. We observed that protective HI titers were not maintained in 16% of HCWs at 3 months after vaccination, and these HCWs were thus offered a second dose of vaccine (revaccination). In this study, we retrospectively analyzed B cell responses in 15 LRs after the first dose of pH1N1 vaccine and prospectively analyzed B and T cell responses in 12 HCWs after revaccination. One-third of the LRs reported having underlying medical conditions, but whether this may have directly contributed to their low serum antibody response is unclear.

In addition to having low HI titers, the LRs also had substantially lower ASC and HA1-specific serum antibody responses, compared with control HCWs after primary vaccination. The LRs were also found to have a slower HI antibody response, compared with the control HCWs, with protective antibody titers not reached until 2 weeks after vaccination in the majority of the participants. Influenza A (H1N1)pdm09 vaccine immunogenicity has been associated with persons with high-avidity IgG antibodies toward the HA1 subunit [18]; however, we did not see any significant differences in the avidity to HA1 between the LRs and control HCWs after primary pH1N1 vaccination (Supplementary

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**Table 2. Spearman Correlation Coefficients Between HI Titers and anti-A(H1N1)pdm09 IgG Memory B Cell Frequencies and Subsets of Cytokine Producing CD4⁺ Th1 Cells at Different Time Points After Revaccination**

<table>
<thead>
<tr>
<th>Memory B Cell Frequencies</th>
<th>CD4⁺Th1 Cells - Day 7</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
<td>Day 21</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.921***</td>
<td>0.795**</td>
</tr>
<tr>
<td>Day 14</td>
<td>ns</td>
<td>0.681*</td>
</tr>
<tr>
<td>Day 21</td>
<td>ns</td>
<td>0.727*</td>
</tr>
<tr>
<td>Day 210</td>
<td>ns</td>
<td>ns</td>
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Abbreviation: ns, not significant.

* The significant values are shown in the table. All non-significant values are either labeled as not significant or not shown.

**a** I = IFN-γ, 2 = IL-2, T = TNF-α.

**b** Total number of influenza-specific CD4⁺ Th1 cells secreting indicated cytokine.

**c** Total influenza-specific CD4⁺ Th1 cells secreting any Th1 cytokine.

*P < .05.

**P < .01.

***P < .001.
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The CD4+ T cell cytokine (Th1) response before and 7, 14, and 21 days after pH1N1 revaccination. The cells were stained for intracellular cytokines and the percentage of CD4+ T cells secreting either single (Figure 5A) or multiple (Figure 5B) Th1 cytokines was measured by multiparametric flow cytometry. *p* group significantly different by Student t test (*P* < .05) from the day 0 (pre-revaccination) group.

**Figure 5.** The CD4+ T cell cytokine (Th1) response before and 7, 14, and 21 days after pH1N1 revaccination. The cells were stained for intracellular cytokines and the percentage of CD4+ T cells secreting either single (Figure 5A) or multiple (Figure 5B) Th1 cytokines was measured by multiparametric flow cytometry. *p* group significantly different by Student t test (*P* < .05) from the day 0 (pre-revaccination) group.

The humoral immune response was further characterized by determining the ASC frequencies 7 days after pH1N1 vaccination, which has previously been shown to correspond to the peak plasmablast (CD19−CD20−CD27highCD38high) response [14].

In the current study, the numbers of IgG and IgA ASCs observed in the control HCWs were higher than those in the ASC responses previously reported for seasonal influenza vaccines [13, 14]. This along with serological results suggests that the AS03-adjuvanted pH1N1 vaccine generates stronger B cell responses than nonadjuvanted seasonal influenza vaccines. The control HCWs had significantly higher IgG frequencies and IgG/IgM ratios 7 days after vaccination, compared with the LRs. The ASC response and rapid HI response at day 7 in the control HCWs are consistent with a recall response to vaccination [14] originating from activation of cross-reactive MBCs generated by previous influenza infection and/or vaccination [19–21]. Furthermore, immunoglobulin sequence data have shown that the majority of influenza-specific IgG ASCs isolated 1 week after influenza A[H1N1]pdm09 vaccination have gone through extensive affinity maturation, thus indicating a dominant recall response [21]. In contrast, the lower ASC and HI responses in the LRs may have resulted from a smaller repertoire of cross-reactive MBCs or, as recently suggested, may be attributable to genetic factors [22]. Revaccination did not significantly increase the ASC frequencies of LRs, consistent with previous observations in participants receiving 2 doses of other influenza vaccines [12, 23]. The IgG ASC response after revaccination did not correlate with HI titers and remained significantly lower, compared with the control HCWs after primary vaccination.

The humoral response was further investigated by evaluating the influenza-specific IgG MBCs (CD3−CD19−CD20−CD27* [15]). MBCs can rapidly differentiate into plasmablasts secreting IgG antibodies after antigen re-encounter and may possess antigen-reactive plasticity through broad cross-reactivity and the ability to go through secondary affinity maturation to altered antigenic epitopes (eg, drifted HA) [24–26]. We detected an IgG MBC response in LRs before revaccination, which suggests that, even if influenza-specific serum immunoglobulin titers decrease to below protective levels because of a reduction of plasma cells, the LRs may still have some protection through long-lived MBCs [27, 28]. After revaccination, the antigen-specific IgG MBC frequencies increased at day 7 and peaked at day 21, similar to the kinetics observed previously for nonadjuvanted TIV and in participants primed with recombinant HA of influenza A/Hong Kong/156/97 (H5N1) vaccine and boosted with influenza A/Vietnam/1203/04 (H5N1) vaccine [19, 29]. Of interest, the MBC response 21 and 210 days after revaccination significantly correlated with the day 7, 14, and 21 HI antibody responses (Table 2), suggesting that the early HI antibody response is an indicator of the long-term protective response elicited by pH1N1 vaccination with adjuvanted vaccine. TIV vaccination failed to elicit a significant increase in the MBC response, therefore suggesting
that the adjuvanted vaccine may be a better inducer of B cell memory than seasonal vaccines.

CD4+ T cells play a key role in anti-influenza immunity [30–33], and recent reports suggest that multifunctional CD4+ T cells that simultaneously express IFN-γ, TNF-α, and IL-2 are functionally superior at inducing anti-influenza immunity than are single cytokine producers [34, 35]. Of importance, studies have shown that the CD4+ Th1 response after primary influenza vaccination with H5N1-adjuvanted vaccine accurately predicts the protective serological responses after booster immunization and, thus, could have implications for early identification of LRs or nonresponders when evaluating future pandemic adjuvanted influenza vaccines [36, 37]. A similar correlation was observed in the present study in which, after revaccination, the day 7 influenza-specific CD4+ Th1 cell response had a significant positive correlation with day 21 and long-term HI titers (Table 2). Therefore, our data suggest that the early CD4+ Th1 response can predict the long-term serological responses and their persistence in LRs after pH1N1 vaccination, and this is in line with our previous observation in humans with a candidate pandemic H5N1 vaccine [37]. Furthermore, the day 7 influenza-specific single IFN-γ, single TNF-α, and double IFN-γ/TNF-α cytokine-producing CD4+ Th1 cell frequencies were positively correlated with the long-term IgG MBC response (Supplementary Table 1) at day 210 after revaccination, suggesting a role for this T cell subset in mediating the long-lived MBC response.

We conclude that HCWs who responded well to one dose of pH1N1 vaccine had a rapid serum antibody response, probably because of a recall response to pH1N1 vaccine primed through prior influenza infection and/or vaccination. In contrast, the LRs had a slow HI antibody response and lower numbers of ASCs and reduced serum antibodies specific to HA1. Revaccination of LRs boosted the serum HI titers, ASC, CD4+ Th1 cell, and MBC responses, which may allow for long-term protection. Frontline HCWs are extensively exposed to pandemic influenza infection; therefore, vaccination is an important step for their protection and will prevent nosocomial transfer to patients. This study shows the need to monitor the immune response after pandemic influenza vaccination to allow identification of HCWs who require revaccination to maintain a long-lasting protective immune response.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.