BRIEF REPORT

Cross-Reactivity to Highly Pathogenic Avian Influenza H5N1 Viruses after Vaccination with Nonadjuvanted and MF59-Adjuvanted Influenza A/Duck/Singapore/97 (H5N3) Vaccine: A Potential Priming Strategy

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(See the editorial commentary by Schwartz and Gellin and the article by Lipatov, on pages 1207–9 and 1216–20, respectively.)

Antigenically well-matched vaccines against highly pathogenic avian influenza H5N1 viruses are urgently required. Human serum samples after immunization with MF59 or nonadjuvanted A/duck/Singapore/97 (H5N3) vaccine were tested for antibody to 1997–2004 human H5N1 viruses. Antibody responses to 3 doses of nonadjuvanted vaccine were poor and were higher after MF59-adjuvanted vaccine, with seroconversion rates to A/HongKong/156/97, A/HongKong/213/03, A/Thailand/16/04, and A/Vietnam/1203/04 of 100% ($P < .0001$), 100% ($P < .0001$), 71% ($P = .0004$), and 43% ($P = .0128$) in 14 subjects, respectively, compared with 27%, 27%, 0%, and 0% in 11 who received nonadjuvanted vaccine. These findings have implications for the rational design of pandemic vaccines against influenza H5.

Influenza A viruses unpredictably undergo antigenic shift, resulting in occasional human pandemics. Newly shifted influenza strains typically emerge in Asia, where the close proximity of humans, birds, and swine facilitates virus reassortment [1]. Human-avian influenza virus reassortants were responsible for the previous 2 influenza pandemics, during 1957 and 1968.

The first association of respiratory disease with highly pathogenic avian influenza (HPAI) H5N1 was in Hong Kong in 1997, when 18 cases occurred in humans during outbreaks of HPAI H5N1 among poultry markets [2]. In 2003, antigenically distinct HPAI H5N1 viruses reemerged in a family visiting southern China [3, 4]. During 2004–2005, 44 deaths (76%) of 55 cases in humans in Vietnam, Thailand, and Cambodia were associated with extensive HPAI H5N1 outbreaks in poultry across Asia [5–7]. These recent H5N1 strains are again antigenically and phylogenetically distinct from the human 1997 and 2003 H5N1 viruses [6, 7]. Reassortment of an H5N1 virus with a cocirculating human virus could potentially generate a strain capable of pandemic spread.

Our ability to combat pandemic influenza depends on the availability of effective vaccine produced from egg-grown virus. However, conventional manufacture of vaccines derived from HPAI viruses is not possible because of technical issues [8]. Although reverse genetics enables the generation of attenuated reference vaccine strains that contain desired surface glycoproteins, regulatory and legal issues have delayed the clin-

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ical evaluation of these vaccine candidates. One strategy, adopted after 1997, was the use of conventionally prepared vaccine from an apathogenic but antigenically related virus to induce antibody to 1997 H5N1 viruses. Nonadjuvanted or MF59-adjuvanted A/duck/Singapore/97 (Dk/Sing/97; H5N3) surface-antigen vaccine was assessed in adults [9]. Although 2 doses of 30 μg of nonadjuvanted vaccine were poorly immunogenic, the addition of MF59 adjuvant boosted antibody responses to levels associated with protection. Revaccination 16 months later boosted antibody responses to levels that were significantly higher than those after 2 doses [10].

Traditional inactivated H1N1 and H3N2 influenza vaccines induce relatively strain-specific serum antibody and are ineffective against drifted viruses and unrelated strains [11]. It is unknown whether vaccine prepared from a more antigenically distant H5 strain could induce antibody that would be capable of neutralizing newly emerged human H5 strains until a well-matched vaccine becomes approved for clinical use. To address this, we used serum samples from subjects before and after the administration of 2 and 3 doses of nonadjuvanted or MF59-adjuvanted Dk/Sing/97 vaccine [9, 10] to establish the breadth of antibody responses to HPAI H5N1 viruses isolated from humans during 1997–2004.

SUBJECTS, MATERIALS, AND METHODS

Subjects, materials, and methods. A clinical study, described elsewhere [9, 10], was conducted in 2 parts during 1999–2000 at the Leicester Royal Infirmary (Leicester, UK). Participants were healthy volunteers 18–45 years old who provided signed, informed consent.

Chiron Vaccines prepared monovalent surface antigen and MF59-adjuvanted vaccine from Dk/Sing/97 (H5N3), under biosafety level ⩾2 containment and by use of conventional procedures, that contained one-half (7.5 μg), normal (15 μg), or double (30 μg) the antigen content of hemagglutinin contained in interpandemic vaccines. Briefly, 65 volunteers were assigned 2 doses, with administration separated by 21 days, that contained 7.5, 15, or 30 μg of H5 hemagglutinin in conventional or MF59-adjuvanted vaccine, by intramuscular injection [9]. Sixteen months later, all original subjects were invited to participate in an additional study; 26 subjects received the same vaccine formulation and dose that they had before [10]. Serum samples were collected before and 21 days after each vaccination and were stored at −30°C at the Health Protection Agency (Colindale, UK).

Serum samples from before vaccination and 21 days after each vaccination were sent on dry ice to the Centers for Disease Control and Prevention (CDC; Atlanta, GA) for testing by virus microneutralization (MN) against HPAI H5N1 viruses isolated from humans during 1997–2004. Previous tests, by MN, of serum samples drawn before the third vaccine dose were negative to Dk/Sing/97 [10]. If sufficient volume was available, IgG responses were detected by H5-specific ELISA.

The MN assay was based on a methodology described elsewhere [12]. Pre- and postvaccination serum samples were tested together in triplicate. End-point titers represented the highest serum dilution that resulted in 50% neutralization of 100 $TCID_{50}$ of virus. On that basis of criteria established elsewhere [12], seroconversion was defined as a titer of ⩾1:80 and demonstration of ⩾4-fold increases in titers from prevaccination titers. Sheep antiserum to A/Hong Kong/213/2003 (HK/213/03) was used as positive control in assays. Tests with HPAI H5N1 viruses were conducted under biosafety level ⩾3 containment. HPAI H5N1 viruses isolated from humans—A/Hong Kong/156/97 (HK/156/97), HK/213/03, A/Vietnam/1203/2004 (VN/1203/04), and A/Thailand/16/2004 (Thai/16/04)—were grown in 10-day-old embryonated hens' eggs; allantoic fluid was harvested 26 h after inoculation and was stored at −80°C until use.

The ELISA assay was based on a methodology described elsewhere [12] by use of rHA H5 HK/156/97 and VN/1203/04 (Protein Sciences). Pre- and postvaccination samples were tested together with age-matched negative control human serum samples. The ELISA titer for test samples was calculated as the reciprocal of the highest dilution of test serum that gave an absorbance at 490 nm (A_{490}) value greater than the mean A_{490} ± 3 SDs of the negative controls at an equivalent dilution of serum. On the basis of previous experience, seroconversion was defined as a titer of ⩾1:1600 and demonstration of ⩾4-fold increases in titer from prevaccination titers.

Statistical analyses. Geometric mean titers (GMTs) and ratios of neutralizing antibody and H5-specific ELISA with 95% confidence intervals (CIs) were calculated by taking the exponential (base 10) of the least-squares means and of the lower and upper limits of associated 95% CI of $\log_{10}$-transformed titers. Least-squares means, 95% CIs, and P values were calculated by general linear model with factors for dose and vaccine type. Titers below the detection limit were assigned a level of one-half that limit. The proportions of subjects who achieved seroconversion after 2 and 3 doses of vaccine were compared by χ^2 test.

RESULTS

Population. Twenty-six subjects received 3 doses of vaccine. Fifteen subjects received MF59-adjuvanted vaccine: 6, 3, and 6 received doses that contained 7.5, 15, and 30 μg of hemagglutinin, respectively. Eleven subjects received nonadjuvanted vaccine: 3, 6, and 2 received doses that contained 7.5, 15, and 30 μg of hemagglutinin, respectively. All groups were similar with respect to age, sex, and ethnicity [9, 10].

Antibody responses. There was no significant dose-response relationship between GMT of neutralizing antibody or H5-specific IgG following doses of 7.5, 15, or 30 μg H5 hemagglutinin against any antigen tested (table 1). Therefore, for further analysis, seroconversions and GMT antibody responses before and after vaccination were calculated after grouping all doses of MF59-adjuvanted vaccine and nonadjuvanted vaccine together.

Neutralizing antibody responses. Table 1 shows GMTs of neutralizing antibody, number of seroconversions, and ratios of post- to prevaccination GMTs for each vaccine group before and after each vaccination. After 2 vaccine doses, more subjects seroconverted to Dk/Sing/97 ($P = .0013$) and HK/156/97 ($P < .0001$) after they received MF59-adjuvanted vaccine than did those who received nonadjuvanted vaccine, but the response to 2003 and 2004 isolates was equally poor (7%–14% vs. 0%–9%). After 3 vaccine doses, the seroconversion rates with MF59-adjuvanted vaccine were significantly higher than with nonadjuvanted vaccine to all antigens tested.

Antibody responses after 2 doses of MF59-adjuvanted vaccine were higher than those after 2 doses of nonadjuvanted vaccine for Dk/Sing/97 ($P = .0002$), HK/156/97 ($P = .0006$), and HK/213/03 ($P = .0487$) but not for VN/1203/04 or Thai/16/04. After the third vaccine dose, antibody responses with MF59-adjuvanted vaccine were significantly higher than those with nonadjuvanted vaccine for each antigen tested (all $P < .0001$). Also, the GMTs of antibody after 3 doses of MF59-adjuvanted vaccine were higher than the GMTs of antibody after 2 doses of vaccine for each antigen tested (all $P < .0001$). In contrast, this boosting effect of the third vaccine dose was weaker with nonadjuvanted vaccine and was present only for Dk/Sing/97 ($P = .0246$), HK/156/97 ($P = .0244$), and HK/213/03 ($P = .0116$) and not for VN/1203/04 or Thai/16/04. Two doses of MF59-adjuvanted vaccine induced higher increases in GMT than did nonadjuvanted vaccine to Dk/Sing/97 and HK/156/97 ($P = .0002$) and to all antigens tested after 3 doses ($P < .0001$).

H5-specific ELISA. Table 2 shows seroconversion rates and GMTs of IgG to rHA HK/156/97 and VN/1203/04 before and after vaccination. After 2 or 3 doses of vaccine, more subjects seroconverted to HK/156/97 and VN/1203/04 after they received MF59-adjuvanted vaccine than did those who received nonadjuvanted vaccine. After 3 doses of vaccine, antibody responses with MF59-adjuvanted vaccine were higher than those with nonadjuvanted vaccine for both antigens ($P < .0001$). The GMTs of antibody after 3 doses of MF59-adjuvanted vaccine were higher than those after 2 doses to HK/156/97 ($P = .0413$) and VN/1203/04 ($P = .0007$). This boosting effect of the third dose was not seen with nonadjuvanted vaccine.

DISCUSSION

The reemergence of HPAI H5N1 influenza in humans in 2003 and 2004 highlights a continuing public health threat. Patients infected with HPAI H5N1 rapidly develop acute respiratory distress, with case-fatality rates of 33%–76% [2, 5, 6]. An emerging H5N1 pandemic would place significant burdens on global health care systems, and planning for the response should include an emergency vaccination strategy. We have demonstrated that an MF59-adjuvanted H5N3 vaccine, but not a nonadjuvanted vaccine, based on an earlier antigenically distinct H5 virus can induce cross-reactive neutralizing antibody responses to recent HPAI H5N1 variants isolated from humans. These results have implications for pandemic vaccination strategies.

In keeping with previous findings, 3 doses of conventional Dk/Sing/97 surface-antigen vaccine was poorly immunogenic, even against HK/156/97-like strains to which it is antigenically closely related [9, 10]. The addition of MF59 as an adjuvant significantly increased antibody titers. Revaccination >1 year later further boosted antibody responses; this may be important, because further waves of infection may occur after the first outbreak of a pandemic. More important, 3 doses of MF59-adjuvanted vaccine induced broadly cross-reactive antibody capable of neutralizing antigenically distinct HPAI H5N1 viruses isolated from humans during 1997–2004. Although correlates of immunity remain undetermined, it is possible that preexisting broad anti–H5 antibody may confer at least partial protection against infection with 2004 HPAI H5 viruses, and this should be explored in animal models.

The ability of an H5 vaccine to induce broad cross-reactive immune responses could be crucially important in the early response to an emerging pandemic, when global demand for vaccine would exceed production capability. The mechanisms by which adjuvants enhance cross-reactivity of immune responses to influenza are not well understood. In mice, heterosubtypic cross-protection against challenge with influenza viruses, including HPAI H5N1, can be induced by the use of influenza vaccines by use of mucosal adjuvants or immunostimulating complexes [13, 14].

The measurement of antibody responses to this H5N3 vaccine was limited by serum sample volumes. The detection of antibody to influenza by the HI test, which is routinely used for the licensure of interpandemic vaccines, is insensitive for the detection of antibody to avian hemagglutinin and cannot be used to adequately assess immunogenicity of H5 vaccine candidates [9, 10, 12]. Virus neutralization tests are more sensitive for the detection of H5-specific antibody and may be more strain specific for the detection of antibody to human influenza strains [15], which is why we used them. However, no standardized protocol for the detection of neutralizing antibody exists, and, because correlates of immune protection have yet to be determined, licensure of vaccines by neutralizing antibody is not feasible. This should be addressed as part of the strategy to develop vaccines against avian influenza, and an international comparative study of influenza virus neutraliza-

Table 1. Geometric mean titers (GMTs) of neutralizing antibody and seroconversions to H5N1 viruses isolated from humans during 1997–2004 before and after vaccination with 2 and 3 doses of nonadjuvanted or MF59-adjuvanted influenza A/duck/Singapore/97 (H5N3) vaccine

	Geometric mean antibody titer (95% CI)				GMR[a]		Frequency of seroconversions, no. (%)[b]		
Test antigen, doses of vaccine received	MF59 ($n=15$)	Plain ($n=11$)	*P*, dose[c]	*P*, vaccine type	MF59/plain	*P*, vaccine type	MF59 ($n=15$)[d]	Plain ($n=11$)	*P*, vaccine type
A/duck/Singapore/97 (H5N3)									
Prevaccination	22 (20–24)	25 (22–27)	.405	.1525					
2	107 (82–140)	43 (31–59)	.2649	.0002	2.7 (1.7–4.3)	.0002	9 (64)	0 (0)	.0013
3	377 (276–514)	72 (51–102)	.9133	<.0001	5.8 (3.4–9.9)	<.0001	14 (100)	2 (18)	<.0001
A/Hong Kong/156/97 (H5N1)									
Prevaccination	22 (19–25)	28 (24–32)	.7029	.0245					
2	151 (112–203)	62 (43–88)	.651	.0006	3.1 (1.8–5.3)	.0002	13 (93)	1 (9)	<.0001
3	894 (620–288)	102 (68–154)	.5383	<.0001	11.0 (5.8–20.9	<.0001	14 (100)	3 (27)	<.0001
A/Hong Kong/213/03 (H5N1)									
Prevaccination	16 (13–19)	14 (11–18)	0.2218	.3990					
2	47 (34–65)	28 (19–41)	0.8694	.0487	1.6 (0.9–2.9)	.1426	2 (14)	1 (9)	.7543
3	573 (373–881)	54 (33–88)	0.963	<.0001	9.5 (4.7–19.1)	<.0001	14 (100)	3 (27)	.0001
A/Vietnam/1203/04 (H5N1)									
Prevaccination	16 (13–19)	19 (15–23)	0.6631	.2449					
2	23 (19–30)	23 (18–31)	0.5922	.9803	1.2 (0.8–1.8)	.3896	1 (7)	0 (0)	.388
3	72 (56–92)	23 (18–31)	0.3464	<.0001	3.7 (2.1–6.5)	.0001	6 (43)	0 (0)	.0128
A/Thailand/16/04 (H5N1)									
Prevaccination	16 (12–21)	25 (18–34)	0.0584	.0379					
2	44 (32–61)	40 (28–58)	0.7054	.6638	1.7 (0.9–3.3)	.094	2 (14)	0 (0)	.2119
3	134 (100–180)	37 (26–51)	0.6531	<.0001	5.5 (2.7–11.5)	<.0001	10 (71)	0 (0)	.0004

NOTE. CI, confidence interval.

[a] Geometric mean ratio (GMR) (postvaccination GMT:prevaccination GMTs) achieved for all combined doses of MF59-adjuvanted vaccine to GMR for all combined doses of nonadjuvanted vaccine after 2 or 3 doses of vaccine.

[b] Defined as at least a 4-fold increase and achieving MN titer $\geq$1/80 [12], with *P* values generated by χ^2 of combined MF59-adjuvanted vaccine vs. combined nonadjuvanted vaccine.

[c] Generated by a general linear model with vaccine as a factor.

[d] Fourteen of 15 serum samples were available after 2 and 3 doses of MF59-adjuvanted vaccine.

Table 2. Geometric mean titers (GMTs) of antibody and frequency of seroconversions as detected by H5-specific ELISA to recombinant H5 hemagglutinin (rHA) before and after 2 or 3 doses of MF59-adjuvanted or nonadjuvanted A/duck/Singapore/97 (H5N3) vaccine.

	Geometric mean antibody titers (95% CI)				GMR[a]		Frequency of seroconversion, no. (%)[b]		
rHA, doses of vaccine received	MF59[c]	Plain[d]	*P*, dose	*P*, vaccine type	MF59/plain	*P*, vaccine type	MF59[c]	Plain[d]	*P*, vaccine type
A/Hong Kong/156/97 (H5)									
Prevaccination	170 (95–304)	166 (88–311)	.8445	.9667					
2	6547 (2790–15,361)	696 (310–1564)	.4025	.0036	10.3 (3–36)	.0011	9 (100)	3 (10)	.0016
3	27,535 (11,200–67,699)	453 (177–1161)	.8557	<.0001	56.4 (11.7–267.9	<.0001	12 (100)	3 (27)	.0003
A/Vietnam/1203/04 (H5)									
Prevaccination	100 (100–100)	100 (100–100)	1.000	1.000					
2	1008 (244–4165)	100 (26–384)	.4414	.0903	10.1 (1.4–71.3)	.0233	4 (44)	0 (0)	.0177
3	23,718 (7816–71971)	129 (40–410)	.1354	<.0001	184.3 (37.0–917.5)	<.0001	12 (100)	1 (9)	<.0001

NOTE. CI, confidence interval.

[a] Geometric mean ratio (GMR) (postvaccination GMT:prevaccination GMT) achieved for all combined doses of MF59-adjuvanted vaccine to GMR for all combined doses of nonadjuvanted vaccine after 2 or 3 doses of vaccine.

[b] Defined as at least a 4-fold increase in titer from prevaccination and achieving a titer of $\geqslant$1:1600 [12].

[c] Thirteen, 9, and 12 serum samples were available for before vaccination and after 2 and 3 doses of MF59-adjuvanted vaccine, respectively.

[d] Eleven, 10, and 11 serum samples from prevaccination and after 2 and 3 doses of plain vaccine, respectively.

tion tests is planned. The serum samples that we used have been tested against Dk/Sing/97 by neutralization tests performed by the CDC and the UK Health Protection Agency, and titers correlated well.

HPAI H5 viruses cannot be grown efficiently in large quantities for vaccine production, because they are lethal to eggs that support their growth. Heightened biocontainment further constrains vaccine manufacture derived from HPAI viruses. Attenuated H5 virus vaccine candidates for HK/156/97, HK/213/03, and VN/1194/04 have been generated by reverse genetics. However, their clinical evaluation has not yet been undertaken, in part because of issues surrounding use of genetically modified organisms, intellectual property rights, and, for earlier viruses, the requirement of mammalian cell lines to be of human vaccine quality [8].

Although attempts to generate a high-growth reassortant with Dk/Sing/97 suitable for large-scale production in eggs have proved unsuccessful, the findings of the present study have implications for pandemic planning. The immunopotentiating ability of adjuvants, such as MF59, could optimize the use of limited antigen in an emerging pandemic but may also enhance broader cross-reactive immune responses. Clearly, a pandemic vaccination strategy based on a 3-dose schedule would not be practical. However, it may be possible to prime individuals at high risk (e.g., health care workers) during the early stages of an emerging H5 pandemic with an adjuvanted vaccine produced from a previously prepared H5 strain, even if it is antigenically distinct, while waiting for an optimally matched and regulatory-approved vaccine. Our findings support the development of vaccine virus libraries that represent the most likely pandemic virus subtypes.

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