CONCISE COMMUNICATION

Correlates of Immune Protection Induced by Live, Attenuated, Cold-Adapted, Trivalent, Intranasal Influenza Virus Vaccine

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The authors conducted a 2-year, multicenter, double-blind, placebo-controlled efficacy field trial of live, attenuated, cold-adapted, trivalent influenza vaccine administered by nasal spray to children 15–71 months old. Overall, vaccine was 92% efficacious at preventing culture-confirmed infection by influenza A/H3N2 and influenza B. Because influenza A/H1N1 did not cause disease during the years in which this study was conducted, the authors sought to determine vaccine efficacy and correlates of immune protection against experimental challenge with 10^7 $TCID_{50}$ of attenuated H1N1 (vaccine strain) by intranasal spray. Prechallenge assessments included serum hemaglutination-inhibiting (HAI) antibody and nasal wash IgA antibody to H1N1. Vaccine was 83% efficacious (95% confidence interval, 60%–93%) at preventing shedding of H1N1 virus after challenge. Any serum HAI antibody or any nasal wash IgA antibody was correlated with significant protection from H1N1 infection as indicated by vaccine-virus shedding, and high efficacy against H1N1 challenge was demonstrated.

The development of live, attenuated, cold-adapted, trivalent influenza vaccine that is given intranasally may improve vaccine usage and provide a simple and convenient approach for the prevention of influenza in children as well as adults [1, 2]. Techniques to update the antigenic composition of live, attenuated vaccine annually have shown that new antigens can be reliably conferred in the trivalent live vaccine and that the live vaccine has predictable levels of attenuation, immunogenicity, and high efficacy [2–4]. Previously, we had shown that vaccine given to children in an efficacy field trial was 95% efficacious at preventing influenza A/H3N2 that was antigenically matched to the strain in the vaccine and 86% efficacious against a significantly drifted influenza A/H3N2 [2, 3]. Furthermore, vaccine was 91% efficacious against influenza B during the earlier field trial [2]. H1N1 did not circulate during either of the 2 years of the pivotal efficacy field trial. Therefore, to develop surrogate data on vaccine efficacy against viral shedding, we challenged children with high-dose monovalent H1N1 vaccine 6 months after vaccination. We took this opportunity to evaluate serum and secretory antibody as correlates of immune protection induced by the vaccine.

Materials and Methods

Vaccine and placebo. Children had previously been vaccinated intranasally with trivalent live, attenuated, cold-adapted influenza vaccine containing, in year 1, $10^{6.7}$ $TCID_{50}$ of A/Texas/36/91–like (H1N1), A/Wuhan/359/95–like (H3N2), and B/Harbin/7/94–like virus. In year 2, children were revaccinated with $10^{7.0}$ intranasal vaccine containing A/Shenzhen/227/95–like (H1N1), A/Wuhan/359/95–like (H3N2), and B/Harbin/7/94–like virus. The challenge virus for this study was monovalent cold-adapted influenza A/H1N1 vaccine (Aviron, Mountain View, CA) that was frozen in single-dose intranasal applicators. Each challenge dose contained $10^{7.0}$ $TCID_{50}$ of influenza A/Shenzhen 227/95–like H1N1 virus in egg allantoic fluid with sucrose-phosphate-glutamate. The vaccine was

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stored frozen at −20°C; thawed vaccine could be stored as long as 8 h at refrigerator temperature (2°–8°C) prior to use.

Subjects. Healthy children were 34–91 months old at the time of recruitment into the vaccine challenge study, which was ~20 months after the start of the field trial. Only children who had previously participated in the 2-year field trial of live, attenuated influenza vaccine were eligible [2, 3]. Subjects had received vaccine or placebo in the fall of 1996 and were revaccinated with either vaccine or placebo from September through November 1997. Antigens contained in the vaccine matched those recommended for inclusion in the trivalent inactivated vaccine. The H1N1 antigens were A/Texas in year 1 and A/Shenzhen 227/95–like in year 2. Informed consent was obtained from a parent or guardian. Vaccine virus challenge was done in April through June 1998.

Study design. The primary efficacy endpoint of the study was shedding of vaccine virus in respiratory secretions on days 1–4 after vaccine virus challenge. Between 20 and 40 subjects were recruited into the study from each of the 10 clinical centers. Subjects and staff remained blinded as to their original assignment to vaccine or placebo throughout the study. The data-coordinating center examined the allocation distribution after approximately half the subjects for each site had been recruited, to ensure that the original 2 : 1 randomization was maintained across all sites. Serum and nasal wash samples were obtained prior to challenge.

Laboratory studies and case definitions. Study sites attempted to collect viral culture specimens from all subjects daily for 4 days after vaccine virus challenge. Rhesus monkey kidney tissue–culture cells were inoculated with fresh respiratory secretions within 4 h of collection, or as soon as possible thereafter, to cultivate influenza viruses. The case definition of vaccine virus shedding was any shedding detected on days 1–4 after challenge.

Sera were assayed for the presence of hemagglutination-inhibiting (HAI) antibodies to H1N1 virus (A/Shenzhen) at Aviron as described elsewhere [5]. HAI antibody titers ≤1 : 4 were considered as representing seronegative children. Nasal washes were assayed at Vanderbilt University for the presence of IgA to H1N1 by means of a kinetic ELISA as described elsewhere [6]. Microneutralization antibody titers were determined at Aviron as follows: receptor-destroying enzyme (RDE)–treated serum samples were stored at 4°C and used within 48 h in the microneutralization assay. Eleven 2-fold serial dilutions, starting at 1 : 10 and ending at 1 : 10 240, were made in culture medium, and 0.1 mL of each dilution was mixed with 50 $TCID_{50}$ of vaccine H1N1 virus in 0.1 mL of medium. This concentration of virus consistently gave 100% cytopathic effect for control wells. After incubation at 33°C for 60 min, the resulting serum antibody–virus antigen mixture was transferred to a 96-well culture plate with Madin Darky canine kidney (MDCK) cell monolayer and incubated at 33°C for 6 days in 5% CO_2 incubator. The serum-neutralizing antibody titer of a given sample was determined as the reciprocal of the last serum dilution with no detectable cytopathic effect.

Data collection and statistical analyses. Data were monitored on site and entered by use of the double-data-entry method. Statistical analyses used SAS 6.12 (SAS, Inc., Cary, NC) and StatXact 2.0 (Cytel Software, Cambridge, MA). Efficacy-point estimates were computed as $100 \times (1 - \text{relative risk}) = 100 \times (1 - P_V/P_P)$, where P_V and P_P indicate observed proportion of virus shedding in vaccine- and placebo-group children, respectively. Efficacy confidence intervals used Koopman's method for the ratio of binomials. A log-rank test was used to compare the time at which virus shedding first terminated for each group.

Results

Two hundred twenty-two children were enrolled and challenged with monovalent H1N1 vaccine virus; 144 had previously been vaccinated with intranasal live, attenuated trivalent influenza vaccine, and 78 had previously received placebo. There were no statistically significant differences between the age, sex, race, day care enrollment, or household compositions of vaccine and placebo groups enrolled into this challenge study. Of the 222 children, 219 had serum and 199 had nasal washes done that produced sufficient volume for laboratory studies; 222, 222, 219, and 221 children were tested for vaccine virus shedding on days 1, 2, 3, and 4 after challenge, respectively.

Safety and efficacy against virus shedding. No serious adverse events were associated with vaccine virus challenge, and no significant differences were noted in the occurrence of runny nose or nasal congestion in children (13% of previously vaccinated children vs. 9% of children previously receiving placebo; P = NS) or fever (2 of 144 previously vaccinated children vs. 0 of 78 children previously receiving placebo; P = NS) on day 2 after vaccination, the day of observed differences after dose 1 during year 1 in the efficacy trial [2].

Six of 144 vaccinees and 19 of 78 children previously receiving placebo shed vaccine virus on 1 or more days after challenge (table 1); vaccine efficacy was 83% (95% confidence interval [CI], 60%–93%) against H1N1 shedding after challenge. Days 2 and 3 were the most frequent days of shedding. Previously vaccinated children terminated viral shedding significantly sooner than did previous placebo recipients (P = .0001, log-rank test).

Correlates of immune protection. One hundred twenty-six vaccinated children and 66 placebo-receiving children had both serum antibody and nasal wash antibody measured before H1N1 challenge. There were significant differences in serum HAI antibody and nasal wash IgA antibody levels between the vaccinated children and placebo-receiving subjects prior to challenge (figure 1). Previously vaccinated subjects had significantly higher nasal wash and serum antibody titers. The presence of any serum antibody or nasal wash IgA significantly correlated with protection from viral shedding (table 1).

Serum from 219 of the 222 subjects was assayed for HAI antibody. Overall, the presence of any serum antibody provided significant protection from viral shedding. Both seropositive vaccinated subjects and seropositive placebo-receiving subjects were protected from challenge virus, as indicated by 2% and 0% shedding after challenge. Among seronegative placebo-receiving subjects, 19 (37%) of 51 shed challenge virus; however, among seronegative vaccinated subjects, only 4 (9%) of 46 shed (χ^2 = 10.9, P = .001). HAI antibody correlated with protection,

Table 1. Effects of intranasal challenge with influenza strain H1N1 on virus shedding in children according to previous vaccine group, serum antibody status, nasal IgA status, and microneutralizing antibody status.

Group	No. challenged	No. shedding (%)[b]	No. shedding/no. tested, according to prechallenge HAI antibody status[a]		No. shedding/no. tested, according to prechallenge nasal IgA antibody status		No. shedding/no. without either serum HAI or nasal IgA (no. with serum microneutralizing antibody)[f]
			Seronegative (HAI≤1 : 4)[c]	Seropositive (HAI≥1 : 8)	IgA negative[d]	IgA positive[e]	
Vaccine	144	6 (4)	4/46	2/97	5/41	1/90	4/16 (12)
Placebo	78	19 (24)	19/51	0/25	16/45	3/23	16/35 (1)
All	222	25	23/97	2/122	21/86	4/113	

[a] HAI, hemagglutination-inhibiting.

[b] Vaccine efficacy against H1N1 shedding, 83% (CI, 60%–93%); duration of shedding was significantly shorter among previously vaccinated subjects who did shed virus (log-rank test, $P = .0001$). Among previously vaccinated children, 2 shed on day 1, 5 on day 2, 1 on day 3, and 0 on day 4; among placebo recipients, 2 shed on day 1, 10 on day 2, 9 on day 3, and 6 on day 4.

[c] Seronegative children who were previously vaccinated were significantly protected from challenge virus shedding when compared with seronegative children who were not previously vaccinated, 4 of 46 vs. 19 of 51 ($\chi^2 = 10.9$, $P = .001$). Serum HAI antibody significantly predicted reduced shedding of challenge virus (logistic regression against viral shedding, $P = .0001$).

[d] Previously vaccinated children were less likely to shed challenge virus, even if no IgA was detected, when compared with IgA negative placebo-receiving subjects: 5 of 41 versus 16 of 45, ($\chi^2 = 6.3$, $P < .01$). Serum HAI antibody significantly predicted reduced shedding of challenge virus; (logistic regression against viral shedding, $P = .0001$).

[e] Nasal IgA significantly predicted protection from challenge virus shedding (logistic regression against viral shedding, $P = .001$).

[f] Significantly more children with a history of vaccine but with no HAI or nasal IgA had serum microneutralizing antibody than did placebo-receiving subjects, 12 of 16 vs. 1 of 35 ($\chi^2 = 30$, $P < .001$); vaccine efficacy, 45% (95 % CI, 22%–79%).

but, in addition to serum HAI antibody, some factor was contributing to protection of vaccinated subjects who were seronegative. One hundred ninety-nine of the 222 children had nasal wash specimens assayed for IgA antibodies. Overall, the presence of IgA antibody in prechallenge nasal wash specimens was significantly correlated with protection from vaccine virus challenge. Among the children with prechallenge nasal wash IgA, only 1 (1%) of 90 previously vaccinated children and 3 (13%) of 23 placebo-receiving children shed challenge virus. Among IgA-negative children, 5 (12%) of 41 of previously vaccinated children and 16 (36%) of 45 of placebo-receiving children shed challenge virus ($\chi^2 = 6.3$, $P < .01$).

Sixteen (11%) of 144 vaccinees and 35 (44%) of 78 of placebo-receiving subjects had neither serum HAI antibody nor nasal wash IgA at the time of challenge. Despite the lack of serum and nasal wash IgA antibody, the vaccinated children had fewer challenge virus infections. Although the 95% CI included 0, a history of vaccine in the absence of antibody had efficacy of 45% (95% CI, 22%–79%). Many of those subjects (12 of 16) had low levels of antibody, as indicated by microneutralization antibody assay. This low level of immunity was reflected in reduced viral shedding among challenged, vaccinated subjects.

Discussion

In the absence of natural infection with influenza A/H1N1 during a multiyear field trial, we challenged a randomly chosen subset of 222 children with 10^7 $TCID_{50}$ of H1N1 vaccine virus, to obtain surrogate data on vaccine efficacy against this virus. Despite an interval of 6–8 months between vaccination and challenge, the vaccine provided high efficacy (83%; 95% CI, 60%–93%) against shedding of challenge virus. We believe similarly high efficacy would be found during natural exposure to H1N1 epidemic strains. The evidence for this is as follows: (1) prevention of shedding during challenge is believed to reflect reduced or absent viral replication in the upper airways; (2) despite 6–8 months of waning immunity, both serum and nasal wash antibody correlated with protection, and vaccination temporally closer to the epidemic (1–3 months) should provide higher levels of nasal IgA and serum HAI antibody; (3) during natural challenge, we observed similar high efficacy against H3N2 (86%–95% efficacy, depending on the strain) and influenza B (91% efficacy); and (4) a large efficacy field trial with bivalent H1N1 plus H3N2 live, attenuated vaccine showed high efficacy of H1N1 vaccines in both adults and children [7].

The presence of either serum antibody or nasal wash IgA correlated with protection from infection by H1N1 as indicated by prevention of viral shedding. Serum antibody alone was the best predictor of protection among the placebo recipients. All placebo-receiving subjects who were infected by the challenge virus had undetectable HAI antibody prior to challenge. However, it is expected that natural infection would have occurred in this group at least 3 years previously, whereas H1N1 has not circulated in the United States in this interval, and the children had not been previously vaccinated for influenza. Thus, many children in this group had serum antibody from distant natural infection, and many have lost nasal wash antibody (10 of 24 had serum HAI but low or undetectable nasal wash IgA). In contrast, intranasally vaccinated children more often had nasal wash IgA, and many had very high levels of IgA (figure 1). In this vaccinated group, the presence of either serum antibody or nasal wash IgA was highly correlated with protection from H1N1 experimental challenge; however, nasal wash IgA was the stronger correlate.

Some vaccinated children had neither serum HAI antibody nor nasal wash IgA. Most of these children (75%) had low

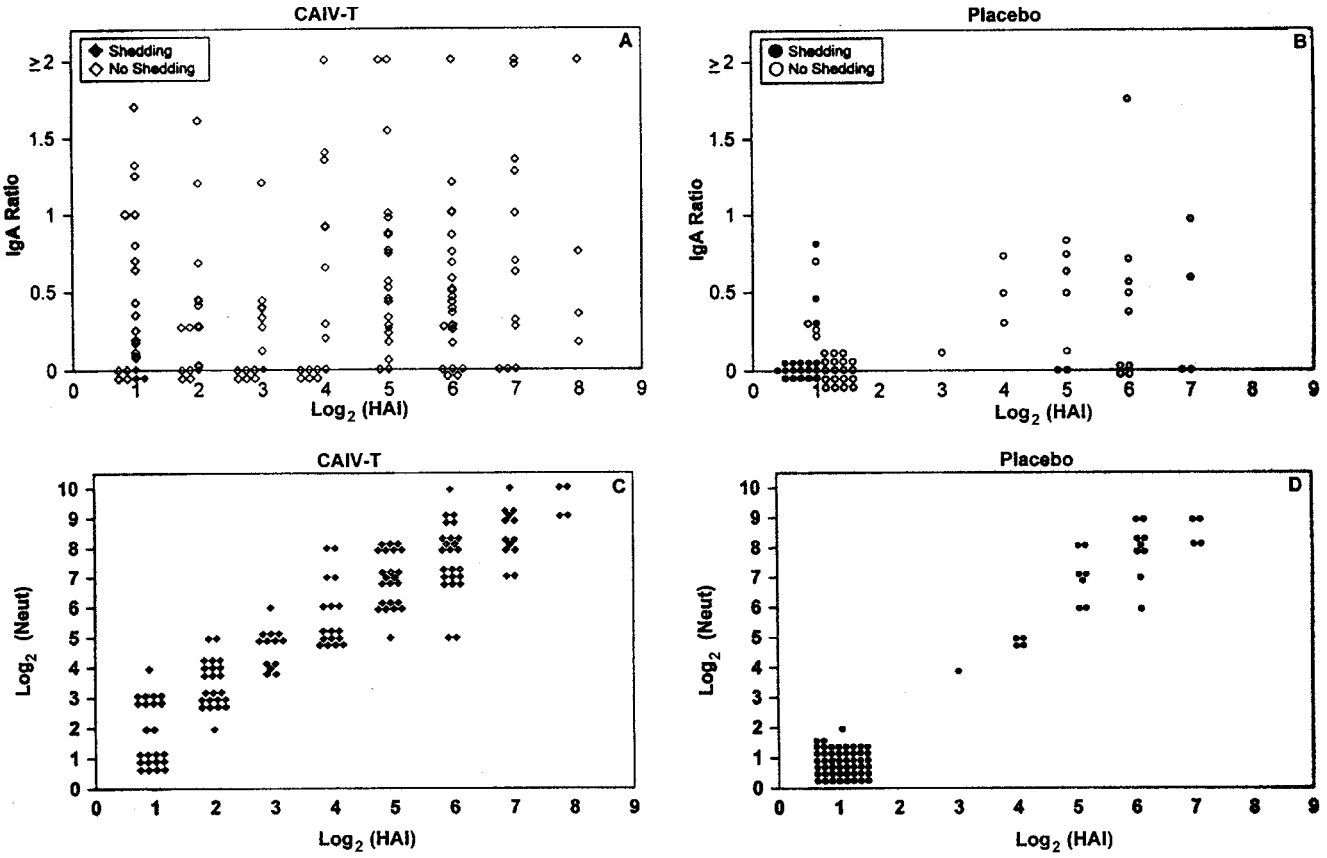

Figure 1. Serum hemaglutination-inhibiting (HAI) antibody titer, nasal wash IgA ratio (the ratio of influenza-specific IgA index to total IgA), and viral shedding (*solid points*) or no viral shedding (*open points*) of H1N1 challenge virus among children who received vaccine (*upper left*) or placebo (*upper right*) 6–8 months earlier. Serum HAI antibody titer correlated with serum microneutralization antibody titer for previously vaccinated subjects (*lower left;* $r = .78$, $P = .0001$) and for placebo-receiving subjects (*lower right;* $r = .87$, $P = .0001$). CAIV-T, trivalent cold-adapted influenza virus; HAI, serum HAI antibody titer; Neut, serum microneutralization antibody titer.

microneutralizing antibody titers (1 : 10 or 1 : 20), in contrast to HAI-negative placebo-receiving subjects, among whom 97% had negative microneutralizing antibody titers (<1 : 10). The low levels of immunity that were not detected by the HAI or IgA assays may have contributed to efficacy. Alternatively, the presence of memory B cells or of cellular immunity may have contributed to protection by the live vaccine. Memory B cells are known to be present in the respiratory tract of animals, and these can be mobilized in as little as 24 h. Perhaps the vaccinated children retained this type of activity; it may have protected them from detectable viral replication after challenge [8]. These possibilities require further investigation.

Most studies of correlates of immune protection against influenza have focused on serum HAI antibody [9–14]. The results of these studies generally agree that serum HAI is correlated with protection, but protective levels of antibody have varied with the prevalent virus subtype. An HAI antibody titer of 1 : 32 is commonly said to be protective [15]. Most of the studies were conducted when H3N2 virus strains were present, and protective levels of antibody have varied from 1 : 20 to 1 : 80, with higher levels of antibody being more protective against H3N2 [9–14]. The few studies on the correlates of immune protection against influenza A/H1N1 or influenza B have found that low levels of serum HAI antibody are correlated with protection against these viruses. In adults, 1 : 10 HAI antibody correlated with protection against influenza B in 1 study [10], and 1 : 20 correlated with such protection in another [12]; however, the 1 : 20 HAI antibody did not protect children [12]. Another study found that 1 : 64 HAI against influenza B was protective in adults the following year [11]. Protection against H1N1 virus infection was given by 1 : 20 HAI antibody in children, and any antibody protected adults [12]. The multiple studies of serum HAI antibody provide clear evidence that HAI antibody correlates with protection; however, the studies leave significant room for discussion regarding the absolute amount needed to confer protection. Furthermore, the data mentioned earlier suggest that the antibody levels needed to protect against H3N2 may be higher than those needed to pro-

tect against H1N1 or B. Also, the presence of IgA with or without serum HAI antibody confounds analysis of the correlates of protection. Clements et al. [13] compared the correlates of immune protection induced by live, attenuated intranasal vaccine with inactivated parenteral vaccine after experimental challenge with wild-type influenza virus. Serum HAI antibody correlated with protection after inactivated vaccine but not after live vaccine; in contrast, live vaccine induced nasal wash antibody that correlated with protection. In the present study, either serum or nasal wash antibody correlated with immune protection against H1N1.

The safety and the high efficacy against illness caused by influenza A/H3N2 (including an H3N2 strain variant not included in the vaccine), influenza B, and now influenza A/H1N1 suggest that this vaccine is suitable for general use to prevent influenza. The understanding that serum antibody and nasal wash IgA are independent correlates of immunity in children is a significant step in explaining how this vaccine exerts efficacy in children.

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References

1. Nichol KL, Mendelman PM, Mallon KP, et al. for the Live Attenuated Influenza Virus Vaccine in Healthy Adults Trial Group. Effectiveness of live, attenuated intranasal influenza virus vaccine in healthy, working adults: a randomized controlled trial. JAMA **1999**; 282:137–45.
2. Belshe RB, Mendelman PM, Treanor J, et al. The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine in children. N Engl J Med **1998**; 338:1405–12.
3. Belshe RB, Gruber WC, Mendelman PM, et al. Efficacy of annual vaccination with live attenuated, cold-adapted, trivalent intranasal influenza virus vaccine against a variant (A/Sydney) not contained in the vaccine. J Pediatr **2000**; 136:168–75.
4. Clancy RL. Mucosal vaccines for the prevention of influenza. Drugs **1995**; 50:587–94.
5. WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza. The 1995–96 WHO Influenza Reagent Kit for the Identification of Influenza Isolates, Appendix A. Atlanta, GA: Centers for Disease Control and Prevention, **1995**:15-9.
6. Boyce TG, Gruber WC, Coleman-Dockery SD, et al. Mucosal immune responses to trivalent live attenuated intranasal influenza vaccine in children. Vaccine **1999**; 18:82–8.
7. Edwards KM, Dupont WD, Westrich MK, Plummer WD Jr, Palmer PS, Wright PF. A randomized controlled trial of cold-adapted and inactivated vaccines for the prevention of influenza A disease. J Infect Dis **1994**; 169: 68–76.
8. Yarchoan R, Strober W, Murphy BR, Nelson DL. In vitro production of IgA antibody by human lymphocytes. In: Strober W, Lamm ME, McGhee JR, James S, eds. Mucosal Immunity and Infections at Mucosal Surfaces. New York: Oxford University Press, **1988**:94–102.
9. Edmondson WP Jr, Rothenberg R, White PW, Gwaltney JM Jr. A comparison of subcutaneous, nasal, and combined influenza vaccination. II. Protection against natural challenge. Am J Epidemiol **1971**; 93:480–6.
10. Evans AS. Serologic studies of acute respiratory infections in military personnel. Yale J Biol Med **1975**; 48:201–9.
11. Hjordis MF, Cooney MK, McMahan R, Bor E, Grayston JT. Single-dose monovalent A_2/Hong Kong influenza vaccine efficacy 14 months after immunization. JAMA **1971**; 217:1067–71.
12. Fox JP, Cooney MK, Hall CE, Hjordis MF. Influenza virus infections in Seattle families, 1975–1979. Am J Epidemiol **1982**; 116:228–42.
13. Clements ML, Betts RF, Tierney EL, Murphy BR. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. J Clin Microbiol **1986**; 24:157–60.
14. Potter CW. Determinants of immunity to influenza infection in man. Br Med Bull **1979**; 35:69–75.
15. National Institutes of Health. Specific immunity in influenza—summary of Influenza Workshop III. J Infect Dis **1973**; 127:220–36.