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Seasonal H1N1 2007 influenza virus infection is associated with elevated pre-exposure antibody titers to the 2009 pandemic influenza A (H1N1) virus

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Abstract

The new influenza strain detected in humans in April 2009 has caused the first influenza pandemic of the 21st century. A cross-reactive antibody response, in which antibodies against seasonal H1N1 viruses neutralized the 2009 pandemic influenza A (H1N1) virus (2009 pH1N1), was detected among individuals aged >60 years. However, factors other than age associated with such a cross-reactive antibody response are poorly documented. Our objective was to examine factors potentially associated with elevated pre-exposure viro-neutralization and hemagglutination-inhibition antibody titers against the 2009 pH1N1. We also studied factors associated with antibody titers against the 2007 seasonal H1N1 virus. One hundred subjects participating in an influenza cohort were selected. Sera collected in 2008 were analysed using hemagglutination inhibition and viro-neutralization assays for the 2009 pH1N1 virus and the 2007 seasonal H1N1 virus. Viro-neutralization results were explored using a linear mixed-effect model and hemagglutination-inhibition results using linear-regression models for interval-censored data. Elevated antibody titers against 2009 pH1N1 were associated with seasonal 2007 H1N1 infection (viro-neutralization, p 0.006; hemagglutination-inhibition, p 0.018). Elevated antibody titers were also associated with age in the viro-neutralization assay (p <0.0001). Seasonal 2007 H1N1 infection is an independent predictor of elevated pre-exposure antibody titers against 2009 pH1N1 and may have contributed to lowering the burden of the 2009 pH1N1 pandemic.

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Introduction

In April 2009 a new influenza virus strain, the 2009 pandemic influenza A (H1N1) virus (2009 pH1N1), with genes derived from avian, human and classical swine lineages, emerged in humans [1]. The virus spread rapidly worldwide, leading the World Health Organization (WHO) to declare the first influenza pandemic of the 21st century, in June 2009. Descriptions of the clinical and epidemiological characteristics of cases in Mexico, the US and the UK, where sustained human-to-human transmission occurred, showed that 75% of confirmed cases involved persons aged between 10 and 50 years [2], as during seasonal H1N1 epidemics [3]. A cross-reactive antibody response, in which antibodies against seasonal H1N1 viruses neutralized the 2009 pH1N1 virus, was detected in 7% of individuals aged <30 years and in 30% of those aged >60 years [4–6], suggesting that individuals born before 1957, when H1N1 was replaced by the H2N2 subtype, might be better protected against this new viral subtype [7]. This was further supported by reports of memory type T-cell immune responses against 2009 pH1N1 in some adults, suggesting cross-reactivity with influenza H1N1 strains circulating prior to 1957 [8].

However, factors other than age may be associated with such a cross-reactive antibody response. In particular, the

potential protective effect of recent seasonal H1N1 virus infection against 2009 pH1N1 infection is poorly documented.

We therefore conducted a cross-sectional study of selected subjects from a large cohort originally designed to study recurrent seasonal influenza infection, in order to identify factors associated with pre-exposure neutralizing and hemagglutination-inhibition antibody titers against the 2009 pH1N1 virus. To determine the specificity of any predictive factors thus identified for 2009 pH1N1, we also studied factors associated with neutralizing and hemagglutination-inhibition antibody titers against the 2007 seasonal H1N1 virus.

Methods

Study design

The study was nested within a cohort of adult volunteers followed-up over 3 years. The main research objective of the cohort was to determine the risk of influenza virus infection according to the viral strain, host immune status and environmental factors.

Participants in the cohort were recruited during the 2007–2008 seasonal influenza epidemic (2007–2008) by 36 general practitioners (GPs) located throughout France, during a medical visit for influenza-like illness (ILI) or another acute disorder unrelated to ILI. A stratified sampling protocol was used. The strata were defined by age (10-year age groups from 20 to 79 years), and by the reasons for seeking medical advice (ILI or another acute disorder). ILI was defined by abrupt onset of fever above 38°C, stiffness and cough. Each GP was asked to include one or two subjects within each of the 12 strata. Follow-up medical visits took place at 1-year intervals, with additional medical visits for any episode of ILI. The cohort study is planned to end in September 2011.

The study was approved by the Ile de France V ethics committee (#07715) and the French Data Protection Authority (#1261460). Each participant gave his or her written informed consent.

Three hundred and seventy-nine subjects (165 with a diagnosis of ILI, 214 with another diagnosis) were recruited during the 2007–2008 season (from 7 January to 16 March 2008), of whom 100 were selected for this study. The sample of 100 participants selected from the cohort was constructed to include an equal number of subjects within each stratum. In keeping with our objectives, participants with a diagnosis of seasonal H1N1 virus infection were over-represented in the ILI strata.

Data collection

Data were collected by the GPs during the visits, using a dedicated electronic case report form.

At inclusion, the reason for the medical visit, chronic illnesses (any condition requiring permanent treatment) and 2007 influenza vaccination status were recorded by the GP. The 2007 influenza vaccine contained the following strains: A/Solomon Islands/3/2006 (H1N1), A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004. Nasopharyngeal swabs (VIROCULT®, KITVIA) were collected from participants with ILI at inclusion, for virological confirmation of influenza. The sera used in this study were collected during the follow-up medical visit after the 2007–2008 influenza season, between 22 May and 10 October 2008.

Laboratory studies

RT-PCR. Nasopharyngeal swabs collected during medical visits for ILI were placed in transport medium and kept at 2–4°C. All samples were centralized in the Virology Laboratory of Necker Hospital (Paris). One-step RT-PCR was used for separate amplification of influenza A and B viruses (see Supporting Information).

The influenza viruses that circulated during the 2007–2008 season were A/Brisbane/59/2007 (H1N1) (61%), B/Florida/4/2006 (36%) and A/Brisbane/10/2007 (H3N2) (3%) [9].

Participants who were PCR-positive for seasonal 2007 H1N1 virus infection were classified as 2007 H1N1-positive, and others as 2007 H1N1-negative. Participants who were PCR-positive for seasonal 2007 type B virus infection were classified as 2007 B-positive, and others as 2007 B-negative.

Viro-neutralization (VN) and hemagglutination inhibition (HI) assays. All sera were tested in VN and HI assays (see Supporting Information).

The blood samples were tested for antibodies against 2009 pH1N1 by using the A/New Mexico/04/2009 strain in the VN assay and the OPYFLU-1 strain in the HI assay.

Antibodies against the 2007 seasonal H1N1 virus were detected by using the A/Brisbane/59/2007 strain in the VN assay and the Marseille 2007 strain in the HI assay.

Serial two-fold dilutions of 1/10-diluted sera were prepared, from 1/20 to 1/5120.

The VN assay used virus-like particles (EPIXIS SA, Lyon, France) [10]. Sera were heat-inactivated for 30 min at 56°C and tested in triplicate. The percentage of viro-neutralization (PN) was calculated for each triplicate. All values (n = 3) recorded for each VN assay dilution (n = 8) were taken into account.

For the HI assay, the titration endpoint was the highest dilution that exhibited complete inhibition of hemagglutination in two independent readings [11].

Statistical methods

The main outcome measure was the percentage of viro-neutralization at each dilution. Based on *a posteriori* analysis and estimates of response variability (effect size of 0.6), the sample size had more than 90% power to identify factors associated with PN [12].

Multivariate models were developed to identify factors associated with PN and/or HI antibody titers. The following variables were included in the multivariate models: age, sex, 2007 influenza vaccination, 2007 H1N1 status, 2007 B status, any respiratory illness that occurred between January and July 2008 after the inclusion episode, and the serum dilution (VN analysis only). As in previous studies [5], age was categorized into two groups: 20–49 years and 50–79 years. An interaction between the age group and 2007 H1N1 status was tested for in the models.

To identify factors associated with the PN, we used linear mixed-effect models that took into account the hierarchical structure of PN data (see Supporting Information). The models included three random effects, taking into account correlations among triplicates at a given dilution, among different dilutions for a given participant (nested effect) and a residual variation [13].

We used linear regression models for interval-censored data to identify factors associated with the HI antibody titer [14,15].

Geometric mean titers (GMTs) were calculated for HI assays as described in [16]. The non-parametric Wilcoxon rank sum test was used to compare GMTs between age groups and between 2007 H1N1-positive and negative subjects. Spearman's correlation was used to test the association between pandemic and seasonal PN or HI antibody titers. Statistical tests were two tailed, with a type I error of 5%.

SAS software version 9.1 (SAS Institute, Inc., Cary, NC, USA) was used for all analyses.

Results

The mean age was 51 years (SD = 16), 47 (47%) of the participants were under 50, and 53 participants (53%) were women. Fifty participants (50%) had chronic illnesses; of these, 11 were receiving statins, nine were receiving betablockers and four were receiving steroids. Fifty-one participants (51%) had ILI at inclusion; of these, 35 were positive for 2007 H1N1, seven were positive for 2007 B, and nine were negative for both 2007 H1N1 and B. Of the 35 participants who were positive for the 2007 H1N1, 25 (71%) were under 50.

Subsequent respiratory illness, between January and June 2008, was reported in 10 participants (10%). Forty participants (40%) had received the 2007 influenza vaccine.

The PN against 2009 pH1N1 was positively associated with age and 2007 H1N1 positivity (Table 1). The interaction between these two covariates was not significant (p 0.88). The PN was highest in 50- to 79-year-old 2007 H1N1-positive subjects and lowest in 20- to 49-year-old 2007 H1N1-negative subjects, with an average difference of 20.6% between these two groups (p <0.0001) (Fig. 1).

The PN differences between the four groups of participants (20- to 49-year-old 2007 H1N1-positive, 20- to 49-year-old 2007 H1N1-negative, 50- to 79-year-old 2007 H1N1-positive, 50- to 79-year-old 2007 H1N1-negative) were statistically significant for each serum dilution (p <0.0001) (Fig. 1).

TABLE 1. Factors associated with the percentage of viro-neutralization and with hemagglutination antibody titers against the 2009 pandemic influenza A (H1N1) virus

	Viro-neutralization assay[a]			Hemagglutination assay[b]		
	Coefficient	Standard error	p-value	Coefficient	Standard error	p-value
Sex (male vs. female)	−0.052	0.19	0.79	0.093	0.096	0.33
Age group (50–79 vs. 20–49)	0.82	0.22	0.0003	0.013	0.11	0.91
Influenza vaccination 2007 (yes vs. no)	0.21	0.23	0.37	0.16	0.12	0.16
2007 H1N1 status (positive vs. negative)	0.63	0.23	0.0080	0.26	0.11	0.019
2007 B status (positive vs. negative)	0.34	0.38	0.37	−0.038	0.18	0.98
Respiratory illness, winter 2007–2008 (yes vs. no)	0.11	0.32	0.74	−0.071	0.15	0.64

[a]Estimates from a multivariate linear mixed-effects model (see manuscript and Supporting Information for details) A positive (or negative) significant coefficient indicates a positive (or negative) association between the tested factors and the percentage of neutralization, adjusted for all other factors presented in the table.
[b]Estimates from a multivariate regression model for interval-censored data. A positive (or negative) significant coefficient indicates a positive (or negative) association between the tested factors and the HI titer adjusted for all other factors presented in the table.

Elevated HI antibody titers against the 2009 pHINI virus were associated with 2007 HINI positivity but not with age (Table 1).

The GMT of HI antibodies against the 2009 pHINI virus did not differ according to age (p 0.93) but was higher in participants who were 2007 HINI-positive (GMT, 42; 95% CI, 36–49) than in participants who were 2007 HINI-negative (GMT, 37; 95% CI, 32–43), p 0.043.

PN and HI titers against the 2007 seasonal HINI virus were positively associated with 2007 HINI positivity and with 2007 influenza vaccination but did not differ according to the age group (PN, p 0.063; HI, p 0.31) (Table 2).

The GMT of HI antibodies against the 2007 seasonal HINI virus was higher in participants who were positive for 2007 HINI (162; 95% CI, 127–206) than in participants who were 2007 HINI-negative (75; 95% CI, 63–90) (p <0.0001).

We found a negative association between PN against the 2007 seasonal HINI virus and B infection (p 0.037), which was due to a single participant with a low level of PN: when the analysis was repeated without this participant, the regression coefficient was no longer significant (p 0.096).

We did not found any association between PN and HI titers against the 2009 pA/HINI virus or 2007 seasonal HINI virus and treatment for chronic illness (p >0.20).

PN and HI antibody titers correlated positively with each other (2009 pHINI virus, p 0.0017; 2007 seasonal HINI virus, p 0.0015).

Antibody titers against the 2009 pHINI virus also correlated with titers against the 2007 seasonal HINI virus (PN, r = 0.70, p <0.0001; HI, r = 0.27, p 0.0056).

Four of the 100 participants selected for this study reported influenza-like illness during the 2009 pandemic season; only one was positive for the 2009 pHINI. This participant, 25 years of age, was negative for the 2007 HINI and had not received the 2007–2008 seasonal vaccination. PN on his sera in the present study (collected in June 2008) was very low (<30%) against the seasonal 2007 HINI and 2009 pHINI. Compared with the three negative patients, this patient presented the highest number of symptoms and the longest sick leave from work.

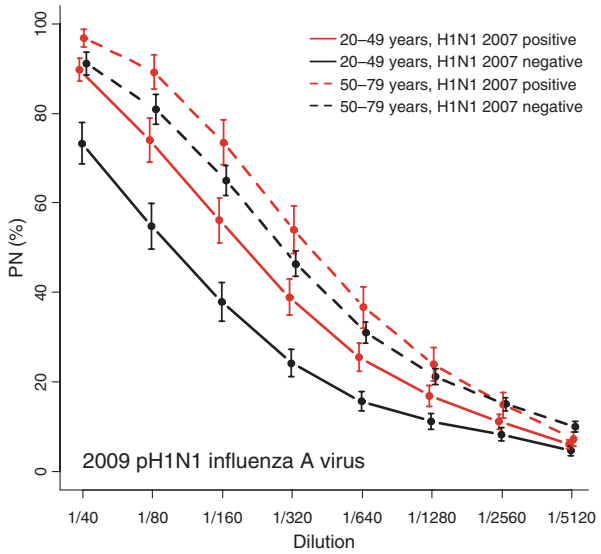

FIG. 1. Percentage of viro-neutralization (PN) against the 2009 pandemic influenza A (HINI) virus according to age and 2007 HINI status. The PN differed across the curves at each dilution (p <0.0001).

Discussion

In sera collected between May and October 2008, we found that the PN against the 2009 pHINI virus increased with age. This relationship was expected, and suggests that exposure to HINI viruses that circulated before 1957 may have conferred substantial cross-protective immunity to the 2009 pHINI virus [4–7]. In particular, because the immune

TABLE 2. Factors associated with the percentage of viro-neutralization and with hemagglutination antibody titers against the 2007 seasonal HINI virus

	Viro-neutralization assay[a]			Hemagglutination assay[b]		
	Coefficient	Standard error	p-value	Coefficient	Standard error	p-value
Sex (male vs. female)	0.15	0.28	0.60	0.26	0.14	0.064
Age group (50–79 vs. 20–49)	0.61	0.31	0.054	−0.16	0.17	0.34
Influenza vaccination 2007 (yes vs. no)	0.72	0.33	0.035	0.46	0.17	0.0075
2007 HINI status (positive vs. negative)	0.74	0.33	0.028	1.04	0.17	<0.0001
2007 B status (positive vs. negative)	−1.14	0.54	0.037	0.17	0.25	0.50
Respiratory illness, winter 2007–2008 (yes vs. no)	−0.76	0.46	0.10	0.32	0.23	0.16

[a]Estimates from a multivariate linear mixed-effects model (see manuscript and Supporting Information for details) A positive (or negative) significant coefficient indicates a positive (or negative) association between the tested factors and the percentage of neutralization, adjusted for all other factors presented in the table.
[b]Estimates from a multivariate regression model for interval-censored data. A positive (or negative) significant coefficient indicates a positive (or negative) association between the tested factors and the HI titer adjusted for all other factors presented in the table.

response is greatest to antigens to which exposure occurred first in childhood (the so called 'original antigenic sin' [17,18]), old people may differ from young people regarding immune status against the 2009 pHINI virus if influenza strains with antigenic properties closely related to the 2009 pHINI have been circulating in the past.

However, elevated PN and HI antibody titers against the 2009 pHINI virus were also positively associated with seasonal 2007 HINI influenza infection. This relationship has not previously been reported in humans but is consistent with results obtained in a guinea pig model, in which prior exposure to HINI and H3N2 seasonal influenza A strains provided partial immunity against 2009 pHINI infection [19]. This finding is supported by evidence that memory cytotoxic T lymphocytes established by seasonal influenza A viruses had cross-reactivity against 2009 pHINI [20].

During the 2009 pHINI pandemic, one study participant, 25 years of age and negative for the 2007 HINI, was positive for the 2009 pHINI. PN on his sera in the present study (collected in June 2008) was very low against the seasonal 2007 HINI and the 2009 pHINI. However, this single case was not sufficient to draw conclusions on a trend regarding the rate of infection or clinical severity of the 2009 pHINI pandemic in relation to the pre-exposure antibody titers against the 2009 pHINI.

Seasonal 2007–2008 influenza vaccination was not associated with elevated PN or HI antibody titers against the 2009 pHINI virus but, as expected, was associated with elevated PN and HI antibody titers against the 2007 seasonal HINI virus. This finding is consistent with previous reports [5,21] and suggests that immunity to HA protein elicited by vaccination does not cross-protect against viruses bearing variant HA molecules [22]. Alternatively, the breadth and intensity of the antibody response to natural infection may be greater than that elicited by inactivated viral particles.

PN and the HI antibody titer against the 2007 seasonal HINI virus were both positively associated, as expected, with 2007 HINI infection. It is noteworthy that they were also associated with 2007–2008 seasonal influenza vaccination. We also found that the HI antibody titer against the 2007 seasonal HINI virus was higher in 2007 HINI-positive subjects but did not increase with age. The lack of association between age and the PN or HI antibody titer against the 2007 seasonal HINI virus was not surprising, as the participants were stratified for ILI during winter 2007: as ILI was mostly caused by the 2007 seasonal HINI virus and because infection was logically associated with high antibody response to this strain, the stratification led to similar numbers of participants with high or low titers in each age group.

We chose to explore factors related to PN and HI antibody titers by using regression models rather than by studying factors associated with a seroprotection rate, for two reasons. First, no seroprotection rate has been defined for neutralization assays, and second, the statistical power of our study would have been undermined if the participants had been categorized around an arbitrary seroprotection threshold.

In conclusion, age and recent exposure to seasonal HINI viruses may have independently contributed to the unexpectedly low morbidity and mortality of the 2009 pHINI pandemic.

Author Contributions

All the authors contributed to writing the paper. Study concept and design: M. Lemaitre and F. Carrat. Analysis and interpretation of data: M. Lemaitre, X. De Lamballerie, N. Salez, M. Leruez-Ville, A.-C. Fluckiger, P. Garrone and F. Carrat. Preparation of the manuscript: M. Lemaitre, X. De Lamballerie, M. Leruez-Ville, A.-C. Fluckiger, P. Garrone and F. Carrat.

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Transparency Declaration

F. Carrat has received consulting fees from Roche, Aventis and Chiron-Novartis and attended sponsor-funded meetings. D. Klatzmann is a co-founder of and holds shares in Epixis.

P. Garrone and A.-C. Fluckiger are employees of Epixis. M. Leruez-Ville, X. De Lamballerie and M. Lemaitre declare they have no potential conflicts of interest. The cohort study was supported by a grant from Agence Nationale de la Recherche and Region Ile-de-France and was sponsored by Université Paris 6 Pierre et Marie Curie. This work was supported by a grant from the programee de recherches H1N1—Aviesan Institut de Microbiologie and Maladies Infectieuses and a grant from Institut de Veille Sanitaire. The funding sources and the sponsor had no role in the design, analysis or reporting of the study or in the decision to submit the manuscript for publication.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data Source/Measurement.

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