Relationship of Preexisting Influenza Hemagglutination Inhibition, Complement-Dependent Lytic, and Antibody-Dependent Cellular Cytotoxicity Antibodies to the Development of Clinical Illness in a Prospective Study of A(H1N1)pdm09 Influenza in Children

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Abstract

The hemagglutination inhibition (HAI) antibody titer is considered the primary immune correlate of protection for influenza. However, recent studies have highlighted the limitations on the use of the HAI titer as a correlate in at-risk populations such as children and older adults. In addition to the neutralization of cell-free virus by antibodies to hemagglutinin and interference of virus release from infected cells by antibodies to neuraminidase, influenza virus-specific antibodies specifically can bind to infected cells and lyse virus-infected cells through the activation of complement or natural killer (NK) cells, via antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent lysis (CDL). We evaluated preexisting HAI, CDL, and ADCC antibodies in young children enrolled in a prospective cohort study of dengue during the epidemic with influenza A(H1N1)pdm09 virus to determine associations between preexisting antibodies and the occurrence of clinical or subclinical influenza virus infection. Though both preexisting HAI and CDL antibodies were associated with protection against clinical influenza, our data suggested that CDL was not a better correlate than HAI. We found that ADCC antibodies behaved differently from HAI and CDL antibodies. Unlike HAI and CDL antibodies, preexisting ADCC antibodies did not correlate with protection against clinical influenza. In fact, ADCC antibodies were detected more frequently in the clinical influenza group than the subclinical group. In addition, in contrast to HAI and CDL antibodies, HAI and the ADCC antibodies titers did not correlate. We also found that ADCC, but not CDL or HAI antibodies, positively correlated with the ages of the children.

Introduction

THE HEMAGGLUTINATION INHIBITION (HAI) antibody titer is considered the primary immune correlate of protection for influenza. An HAI titer of 1:40 is associated with protection of 50–70% against clinical influenza (10,19). Though a recent paper by Ng *et al.* estimated that HAI titers of 1:40 against A(H1N1)pdm09 and B(Victoria lineage) were associated with 48% [95% confidence interval (CI) 30–62%] and 55% [95% CI 32–70%] protection against polymerase chain reaction (PCR)-confirmed infection in children (17), other studies suggest that HAI titers should not be used alone to assess vaccine efficacy, especially in

high-risk groups such as children and older adults. Black *et al.* found that in children, who have less prior experience with influenza virus infection or vaccination, a higher titer of 1:110 was associated with the conventional 50% protective rate and that the 1:40 titer was associated with only 22% protection (1). In a study by Ohmit *et al.*, H3N2 influenza cases had lower pre- and post-vaccination mean HAI titers than noncases, but a small number of cases had “protective” HAI antibodies ranging from 64 to 3,028. These studies highlight the limitations of the use of the HAI titer as a correlate in at-risk populations, and support the search for additional determinants of protection when evaluating influenza vaccines.

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In addition to the neutralization of cell-free virus by antibodies to hemagglutinin and interference of virus release from infected cells by antibodies to neuraminidase, influenza virus-specific antibodies specifically can bind to antigens on the surface of infected cells, leading to the activation of natural killer (NK) cells (through cross-linking of Fc γ receptors) and complement, resulting in the lysis of virus-infected cells via antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent lysis (CDL). While the HAI assay detects antibodies directed against the globular head of the HA, CDL and ADCC antibodies may be directed against other influenza proteins expressed on the surface such as the neuraminidase (NA), nucleoprotein (NP), and M1 and M2 matrix proteins (12,16,30).

The role of CDL has been investigated in such viral systems as HIV, hepatitis B virus, and herpes virus (9,11,20). In a previous study, we reported that 3 of 10 young adults who received A/USSR/77 vaccine, naïve to this H1N1 subtype, had preexisting CDL but no HAI antibodies against this novel H1N1 virus (21). More recently, using human monoclonal antibodies cloned from plasmablasts obtained from patients infected with the 2009 pandemic H1N1 strain and from healthy adults vaccinated with seasonal influenza vaccine (28,29), we found that HA stalk-specific monoclonal antibodies that do not inhibit hemagglutination could mediate CDL and were more cross-reactive to temporally distant H1N1 strains than hemagglutination-inhibiting and neutralizing MABs (26).

Recently, there has been renewed interest in the role that ADCC antibodies may play in influenza infection. In an early study by Hashimoto *et al.*, ADCC antibodies were detected at higher levels (1 to 2 log₁₀) than HAI antibodies after experimental live attenuated intranasal influenza vaccination in young children; these antibodies were detected earlier and were broadly cross-reactive within a subtype (7). Similarly, Vella *et al.* reported that adult subjects who received the A/Victoria/3/75(H3N2) inactivated influenza vaccine showed a significant rise in ADCC specific immune lysis (SIL) and developed ADCC antibody titers similar to naturally infected subjects (27). Grandea *et al.* found that monoclonal antibodies derived from healthy subjects, which recognized a highly conserved epitope within the ectodomain of the influenza matrix 2 protein, protected mice from lethal challenge with H5N1 and A(H1N1)pdm09 influenza virus strains, with *in vitro* evidence suggesting that ADCC and/or CDL antibodies mediated this protection (6). A recent paper by Dilillo *et al.* demonstrated that Fc–Fc γ R interactions are required for stalk-specific, but not globular head-specific, monoclonal antibody-mediated protection during *in vivo* challenge in mice, suggesting a role for ADCC in protection against influenza via stalk-specific antibodies (2).

Traditionally, ADCC antibodies have been measured by lysis of target cells using chromium release assays. Recently, an ADCC flow cytometry assay was reported, which focuses on the activation of NK cells rather than lysis of target cells, measuring the percentage of NK cells that express the degranulation marker CD107a or the antiviral cytokine IFN γ (13). Using this method and serum samples from adults older and younger than 45 years of age obtained before the 2009 A(H1N1)pdm09 influenza pandemic, Jegaskanda *et al.* showed that a higher percentage of the

adults >45 years of age had cross-reactive ADCC antibodies to the pandemic virus, which may have contributed to protection from influenza (14).

ADCC antibody responses in other acute self-limited viral infections such as measles and herpes simplex viruses (HSV) suggest that ADCC may play an important role in protection (5,15). In adults with acute measles, titer changes (between specimens obtained) in ADCC titers but not CDL or neutralizing titers correlated with reduction in viremia (5). In neonatal HSV infection, high levels of maternal or neonatal anti-HSV ADCC antibodies and neonatal neutralizing antibodies were shown to correlate independently with an absence of disseminated infection (15).

In this study, we evaluated HAI, CDL, and ADCC antibodies in young children (9 months–11 years) enrolled in a prospective cohort study of dengue and influenza infection during the epidemic with influenza A(H1N1)pdm09 virus in order to determine associations between preexisting antibody profiles and the subsequent occurrence of subclinical and symptomatic PCR + influenza infection.

Materials and Methods

Study subjects and blood samples

Male and female Thai subjects ($n=1886$), aged from 9 months to 11 years, were enrolled in a 2010–2011 prospective cohort study of dengue and influenza infection in Kamphaeng Phet province in north-central Thailand. Informed consent from parent(s) or guardian(s), as well as informed assent from all children ≥ 7 years old, was obtained. The study protocol was approved by the Institutional Review Boards of the Thai Ministry of Public Health and the Walter Reed Army Institute of Research. Serum samples were collected at enrollment from May to September 2010 prior to the presumed peak dengue virus transmission season (termed “pre-surveillance”) and in January to March 2011 after the end of the peak dengue season (termed “post-surveillance”). Though influenza activity is detected year round in Thailand, peak influenza activity is seen primarily from June to November (23). Enhanced passive surveillance for acute febrile illness was conducted during the intervening period. Acute febrile illnesses were detected when the subject called study staff or sought medical attention at the public health office. Subjects/parents were reminded every month by study staff to report any febrile illnesses (“enhanced” surveillance). There were a total of 186 subjects with 173 acute febrile illnesses during the surveillance period. In addition to being tested for dengue virus infection, acute and convalescent sera from children with acute febrile illnesses underwent influenza HAI testing, and acute nasal and throat swabs underwent influenza PCR (Fig. 1).

Influenza vaccine coverage information is incomplete in this cohort, with no data collected at enrollment. However, at the post-surveillance visit, four subjects in the cohort ($n=1,886$) reported receiving an influenza vaccination in 2010.

For the ADCC assay, peripheral blood mononuclear cell samples were obtained from healthy subjects >18 years old at the University of Massachusetts Medical School (UMMS) as a source of NK cells. Informed consent was obtained from each subject, and the study was approved by the UMMS Institutional Review Board.

FIG. 1. Study design, surveillance, and sample collection overview. PCR, polymerase chain reaction.

Influenza viruses

The A/California/7/09(H1N1) strain used in the CDL and ADCC antibody assays was a gift from Dr. Robert Ryall of Sanofi Pasteur. The A/California/08/2009 (H1N1) strain was used by the Armed Forces Research Institute of Medical Sciences (AFRIMS) in the HAI assays.

HAI assay

HAI testing was done on the pre- and post-surveillance sera at AFRIMS in Bangkok, Thailand.

CDL assay

We modified a previously developed assay in order to measure levels of CDL antibodies in these sera (8,21). A549 cells (human lung epithelial cell line, ATCC® CCL-185™) were seeded at 0.7×10^6 cells/well in a 6-well plate in 4 mL of F-12K and 10% fetal bovine serum (FBS) for 6 h. Cells were washed $2 \times$ with PBS and then infected at an MOI of 5–10 with the A/California/7/09(H1N1) virus diluted in Dulbecco's PBS with 0.1% bovine serum albumin (BSA) for 1 h (target cells). After washing the cells, F-12K and 2% FBS medium was added to the wells, and the plate was incubated overnight (15–17 h) in a tissue culture incubator. TrypLE Express (Life Technologies) was added to each well (0.5–0.7 mL) and incubated at 37°C until A549 cells detached (about 10 min). Medium (1 mL of RPMI 5%) was added to each well, and the cells were centrifuged and then labeled with $^{51}\text{Chromium}$ for 60 min. After washing $3 \times$ with RPMI with 5% FBS, these target cells were added at 2,000 cells/well in U-bottom 96-well plates. Two-fold serial dilutions (1:20–1:2560) of heat inactivated donor serum were added to appropriate wells in replicates of three. The plates were then incubated at 37°C for 1 h. Low-Tox® Guinea Pig Complement at a final concentration of 1:20 (Cedarlane Laboratories) was added to the plates. RENEX detergent was added to maximum release wells, and medium was added to minimum release wells. Plates were spun at $250 \times g$ for 5 min. The plates were incubated in the tissue culture incubator for 2 h. Supernatants were harvested from wells using Skatron Supernatant Collection System and counted in a gamma counter. Calculation of % SIL at each serum dilution = (average count per minute (CPM) of experimental release – average CPM of complement minimum release) /

(average CPM of maximum release – average CPM of complement minimum release) $\times 100$. The highest serum dilution showing >15% SIL was defined as the CDL end-point titer. For calculation of mean endpoint titers, an undetectable CDL titer was assigned a value of 10.

ADCC assay

A549 cells were seeded at 0.7×10^6 cells/well in a 6-well plate in F-12K and 10% FBS. The A549 cells were then infected at an MOI of 5–10 with the A/California/7/09 (H1N1) virus diluted in Dulbecco's PBS and 0.1% BSA for 1 h (target cells). After washing cells twice with Dulbecco's PBS, F-12K and 2% FBS were added to the wells, and the plate was incubated overnight (15–17 h) in a tissue culture incubator. After washing the cells, they were trypsinized using TrypLE Express/well (0.7 mL; Life Technologies) and incubated at 37°C until the A549 cells detached. Cells were washed, centrifuged, and then labeled with $^{51}\text{Chromium}$ for 60 min. Target cells were then washed three times with RPMI and 5% FBS, and added at 2,000 cells/well in U-bottom 96-well plates. Fourfold serial dilutions (1:20–1:327,680) of heat-inactivated donor serum were added to appropriate wells in replicates of three. The plates were then incubated at 37°C for 1 h. NK cells were purified (RosetteSep™ Human NK Cell Enrichment Cocktail; Stem Cell Technologies) by negative selection from whole blood obtained from healthy subjects (in a preliminary experiment, purified cells were 99.9% CD3-negative and 83.9% double positive for CD56 and CD16). Enriched NK cells were added at an E:T ratio of 5:1/well. RENEX detergent was added to maximum release wells, and medium was added to minimum release wells. Plates were spun at $250 \times g$ for 5 min. Plates were incubated at 37°C in a tissue culture incubator for 2 h. Supernatants were then harvested from wells using the Skatron Supernatant Collection System and counted in a gamma counter. Calculation of % SIL at each serum dilution = % lysis of sample – % lysis of NK cells alone. Calculation of % lysis at each serum dilution = (average CPM of experimental release – average CPM of minimum release) / (average CPM of maximum release – average CPM of minimum release) $\times 100$. Calculation of % lysis of NK cells = (average CPM of NK cell release – average CPM of minimum release) / (average CPM of maximum release – average CPM of minimum release) $\times 100$.

The highest serum dilution showing >15% SIL was defined as the ADCC endpoint titer. In a previous paper, Hashimoto *et al.* used 10% as a cutoff (7). For calculation of mean endpoint titers, an undetectable ADCC titer was assigned a value of 5.

Statistical analysis

All statistical analyses were performed using GraphPad Prism v6.00 for Windows (GraphPad Software). Mean titers were calculated by transforming data to log scale for all computations and comparisons. One-way between different subjects analysis of variance (ANOVA) was conducted to compare the differences of HAI, CDL, or ADCC antibodies between the PCR+ influenza group, the seroconversion group, and the nonseroconversion group. *Post hoc* comparisons using the Tukey Multiple Comparisons Test were performed when the ANOVA result was significant ($p < 0.05$). Pearson correlation coefficient r was computed to assess the relationship between HAI, CDL, and ADCC titers and the relationship between age and HAI, CDL, and ADCC titers. A p -value of < 0.05 was considered statistically significant. Chi square analysis was performed using K.J. Preacher's "Calculation for the chi square test: An interactive calculation tool for chi square tests of goodness of fit and independence" (April 2001; <http://quantpsy.org>).

Results

Study groups

In this study, we compared pre-surveillance CDL, ADCC, and HAI antibodies in three groups: (1) PCR+ confirmed cases; (2) seroconversion group (subclinical), which included subjects who did not have PCR+ illness and who were identified by a fourfold increase in HAI titers from the pre- to the post-surveillance period; and (3) non-seroconversion group, which included subjects with no history of PCR+ illness or HAI seroconversion from the pre- to post-surveillance period. Out of 1,886 subjects en-

rolled in the prospective study, there were 34 cases of febrile PCR-confirmed A(H1N1)pdm09 influenza (infections by other influenza A subtypes or influenza B viruses were excluded from the analyses). There were a total of 314 subjects who seroconverted to the A(H1N1)pdm09 influenza virus. Sera from all of the PCR+ confirmed cases ($n = 34$) and from a similar number of randomly selected subjects from the seroconversion group ($n = 38$) and from the nonseroconversion group ($n = 35$) were tested in CDL and ADCC assays. Subjects in the PCR+ group were older ($M_{\text{age}} = 7.6 \pm 2.8$ years) compared to the seroconversion group ($M_{\text{age}} = 3.1 \pm 2.2$ years) and the nonseroconversion group ($M_{\text{age}} = 2.4 \pm 1.2$ years; Tukey's comparison test $p < 0.0001$).

Pre-surveillance HAI antibodies and development of clinical illness

HAI antibody titers were analyzed, given their importance as the primary immune correlate of protection against influenza (22). The mean log₁₀ titer HAI titer for the PCR+ group (0.76 ± 0.19) was significantly lower compared to the seroconversion (1.21 ± 0.35) and the nonseroconversion groups (1.24 ± 0.49 ; $p < 0.0001$; Fig. 2). We also compared the number of subjects with detectable pre-surveillance HAI, CDL, and ADCC antibody titers in each of these groups by chi square analysis because of the large number of subjects with undetectable titers (Table 1). We found a significantly smaller number of subjects in the PCR+ group with pre-surveillance HAI titers $\geq 1:20$ or $\geq 1:40$ (defined by some as a protective level of HAI antibodies) compared to the seroconversion or the nonseroconversion groups ($p = 0.0001$ for $\geq 1:20$ and $p = 0.015$ for $\geq 1:40$; Table 2).

Pre-surveillance CDL antibodies and development of clinical illness

Very few children had any detectable CDL antibodies in the pre-surveillance sera (Table 1). Similar to the HAI antibodies, the mean log₁₀ CDL titer for the PCR+

FIG. 2. Log₁₀ mean A(H1N1)pdm09 antibody titers in the three study groups as determined by the (A) hemagglutination inhibition (HAI) assay, (B) complement-dependent lytic (CDL) assay, and (C) antibody-dependent cellular cytotoxicity assay (ADCC). Error bars represent one standard deviation. Asterisk represents statistically significant difference in antibody titers between the two groups ($p < .05$, Tukey's Multiple Comparisons Test). Numbers in parenthesis represent the number of subjects in each group with undetectable antibody titers. Undetectable CDL and ADCC titers were defined as less than 20; undetectable HAI titer was defined as < 10 . For calculation of mean titers, we assigned 1:5 for undetectable HAI, 1:10 for undetectable CDL, and 1:5 for undetectable ADCC.

TABLE 1. AGE AND PRE-SURVEILLANCE HAI, CDL, AND ADCC TITERS TO THE A(H1N1)PDM09 VIRUS FOR EACH SUBJECT BY GROUP

	ADCC	HAI	CDL	Age (years)
<i>PCR+</i>				
KPSIII-0387	ND*	ND	ND	3.5
KPSIII-0787	ND	ND	ND	5.5
KPSIII-0652	ND	ND	ND	6.1
KPSIII-0658	ND	ND	ND	6.2
KPSIII-0794	ND	ND	ND	6.6
KPSIII-0916	ND	ND	ND	7.2
KPSIII-1241	ND	ND	ND	7.2
KPSIII-1088	ND	ND	ND	7.5
KPSIII-1492	ND	ND	ND	8.3
KPSIII-1217	ND	ND	ND	9.4
KPSIII-1296	ND	ND	ND	11.4
KPSIII-0116	ND	10	ND	2.3
KPSIII-0354	ND	10	ND	4.1
KPSIII-0377	ND	40	ND	3.2
KPSIII-1181	20	ND	ND	10.8
KPSIII-0961	20	ND	ND	7.8
KPSIII-1389	80	ND	ND	7.8
KPSIII-0714	80	ND	ND	6.2
KPSIII-0021	80	ND	ND	2.8
KPSIII-0619	80	ND	ND	5.9
KPSIII-1001	80	ND	ND	8.1
KPSIII-1410	80	ND	ND	8.8
KPSIII-1091	80	ND	80	10.6
KPSIII-1375	80	ND	ND	11.2
KPSIII-1160	80	ND	ND	11.8
KPSIII-0069	80	20	ND	2.2
KPSIII-0710	320	ND	ND	6.1
KPSIII-1359	320	ND	ND	8.8
KPSIII-1582	320	ND	ND	9.0
KPSIII-1059	320	ND	ND	10.3
KPSIII-1386	320	ND	ND	11.4
KPSIII-1348	1280	ND	ND	8.3
KPSIII-1525	1280	ND	ND	10.2
KPSIII-1581	1280	ND	40	10.5
<i>Seroconversion</i>				
KPSIII-0114	ND	ND	ND	0.8
KPSIII-0110	ND	ND	ND	1.1
KPSIII-0141	ND	ND	ND	1.3
KPSIII-0056	ND	10	ND	0.9
KPSIII-0077	ND	10	ND	1.3
KPSIII-0138	ND	10	ND	1.4
KPSIII-0172	ND	10	ND	1.4
KPSIII-0156	ND	10	ND	1.5
KPSIII-0033	ND	10	ND	1.8
KPSIII-0037	ND	10	ND	2.8
KPSIII-0433	ND	10	ND	3.7
KPSIII-0452	ND	10	ND	3.7
KPSIII-0484	ND	10	ND	3.8
KPSIII-0417	ND	10	ND	3.8
KPSIII-0389	ND	10	ND	4.0
KPSIII-0002	ND	20	ND	0.8
KPSIII-0031	ND	20	ND	1.9
KPSIII-0155	ND	20	ND	2.5
KPSIII-0106	ND	20	ND	2.6
KPSIII-0027	ND	20	ND	2.7
KPSIII-0059	ND	20	ND	2.7
KPSIII-0100	ND	20	ND	2.9
KPSIII-0458	ND	20	ND	3.4
KPSIII-0090	ND	40	ND	2.4

TABLE 1. (CONTINUED)

	ADCC	HAI	CDL	Age (years)
KPSIII-0179	ND	40	ND	2.7
KPSIII-0167	ND	40	ND	2.8
KPSIII-0096	ND	160	320	1.9
KPSIII-0372	20	10	ND	3.3
KPSIII-0188	20	20	ND	2.7
KPSIII-1207	80	ND	ND	10.8
KPSIII-0101	80	20	ND	2.6
KPSIII-0125	80	80	160	1.9
KPSIII-1332	320	ND	ND	7.6
KPSIII-0413	1280	20	160	3.0
KPSIII-1557	1280	40	160	7.3
KPSIII-0463	1280	40	320	3.6
KPSIII-1094	5120	40	160	7.0
KPSIII-1949	5120	40	640	7.0
<i>Nonseroconversion</i>				
KPSIII-0030	ND	ND	ND	1.0
KPSIII-0061	ND	ND	ND	1.0
KPSIII-0185	ND	ND	ND	0.9
KPSIII-0032	ND	ND	ND	2.8
KPSIII-0065	ND	ND	ND	2.8
KPSIII-0040	ND	ND	ND	2.8
KPSIII-0089	ND	ND	ND	2.8
KPSIII-0062	ND	10	ND	0.8
KPSIII-0079	ND	10	ND	0.9
KPSIII-0129	ND	10	ND	1.0
KPSIII-0017	ND	10	ND	1.1
KPSIII-0020	ND	10	ND	2.8
KPSIII-0451	ND	10	ND	3.7
KPSIII-0494	ND	10	ND	3.7
KPSIII-0333	ND	10	ND	3.8
KPSIII-0088	ND	20	ND	0.8
KPSIII-0176	ND	20	ND	0.8
KPSIII-0003	ND	20	ND	2.8
KPSIII-0492	ND	20	ND	3.6
KPSIII-0472	ND	20	ND	3.7
KPSIII-0334	ND	20	ND	3.8
KPSIII-0119	ND	80	ND	2.7
KPSIII-0148	ND	80	ND	2.7
KPSIII-0164	20	ND	ND	1.0
KPSIII-0145	20	ND	ND	2.8
KPSIII-0083	20	10	ND	1.0
KPSIII-0323	20	10	ND	3.8
KPSIII-0075	80	80	320	0.8
KPSIII-0328	80	80	320	3.7
KPSIII-0344	80	160	320	3.8
KPSIII-0024	320	40	320	2.8
KPSIII-0373	320	80	160	3.7
KPSIII-0453	320	80	320	3.7
KPSIII-0487	1280	160	640	3.6
KPSIII-0126	5120	80	640	2.8

HAI, hemagglutination inhibition; CDL, complement-dependent lysis; ADCC, antibody-dependent cellular cytotoxicity; PCR, polymerase chain reaction; ND, antibodies below detectable levels. Undetectable CDL and ADCC titers were defined as <20; undetectable HAI titer was defined as <10.

group (1.04 ± 0.18) was significantly lower than in the nonseroconversion group (1.35 ± 0.66) ($p < 0.05$; Fig. 2). In addition, fewer subjects in the PCR+ group had pre-surveillance CDL titers $\geq 1:20$ or $\geq 1:40$ compared to the seroconversion or the nonseroconversion groups, but the differences did not reach statistical significance (Table 2).

(continued)

TABLE 2. ANTIBODY AND STUDY GROUP CROSS-TABULATION FOR CHI SQUARE ANALYSIS

	Number of subjects in study group with antibody titer			p-Value
	PCR+	Sero conversion	Non-sero conversion	
HAI antibody titer:				
≥20	2	20	16	0.0001
<20	32	18	19	
HAI antibody titer:				
≥40	1	9	10	0.0148
<40	33	29	25	
CDL antibody titer:				
≥20	2	7	8	0.135
<20	32	31	27	
CDL antibody titer:				
≥40	2	7	8	0.135
<40	32	31	27	
ADCC antibody titer:				
≥20	20	11	12	0.0245
<20	14	27	23	
ADCC antibody titer:				
≥40	18	9	8	0.001
<40	16	29	27	

Pre-surveillance ADCC titers and development of clinical illness

In contrast to HAI and CDL antibodies, a higher number of subjects in the PCR+ group compared to the seroconversion and the nonseroconversion group had ADCC titers $\geq 1:20$ or $\geq 1:40$ ($p=0.025 \geq 1:20$ and $p=0.001 \geq 1:40$; Table 2). Although not statistically significant, the mean log₁₀ ADCC titer was higher in the PCR+ group (1.57 ± 0.84) compared to the seroconversion group (1.22 ± 0.95) and the nonseroconversion group (1.18 ± 0.81 ; Fig. 2).

Comparisons between HAI, CDL, and ADCC titers

In most subjects, CDL titers were equal to or slightly higher than HAI titers, while ADCC titers in many instances were higher than CDL or HAI titers (Table 1). A Pearson product correlation coefficient was computed to assess the relationship between the pre-surveillance HAI, CDL, and ADCC titers. There was a strong positive correlation between HAI and CDL pre-season titers ($r=0.69$, $p<0.0001$) and between CDL and ADCC titers ($r=0.63$, $p<0.0001$) but

only a weak correlation between HAI and ADCC titers ($r=0.20$, $p=0.04$; Fig. 3).

Relationship between age and HAI, CDL, and ADCC titers

As discussed above, we found a significant age difference between the PCR+ and the seroconversion and non-seroconversion groups. We analyzed the relationship between age and the antibodies measured in this study. A statistically significant positive correlation was seen between age and ADCC titers ($r=0.45$, $p<0.0001$), and a statistically significant inverse correlation was seen between age and HAI titers ($r=-0.39$, $p<0.0001$; Fig. 4).

Discussion

This is the first study to examine the relationship between preexisting ADCC and CDL antibodies in comparison to HAI antibodies and the subsequent development of clinical influenza, represented by the PCR+ group, in young children. We found that preexisting HAI and CDL titers correlated with protection against influenza illness (Fig. 2). However, chi square analysis suggested that CDL titers may not be a better correlate than HAI (Table 2). In both the PCR+ and the seroconversion groups, there was no case in which CDL titers were observed without HAI titers. We also found, in the seroconversion group, that the presence of preexisting “protective” HAI titers (>40) did not prevent infection.

Notably, we found that ADCC antibodies behaved differently from HAI and CDL antibodies. ADCC antibodies were detected more frequently in the PCR+ group than in the seroconversion and nonseroconversion group ($p=0.0245$). Mean ADCC titers in the PCR+ group were also higher compared to either the seroconversion or non-seroconversion groups, though this trend did not reach statistical significance. Correlation with age was also different between ADCC antibodies and HAI or CDL antibodies (Fig. 4). Higher ADCC titers in older children likely reflect prior exposure to influenza antigens. However, our data suggest that these levels of ADCC titers were not sufficient for protection. Epstein *et al.* analyzed the impact of prior H1N1 influenza infection and susceptibility to H2N2 infection during the H2N2 pandemic of 1957. They showed that the impact of heterosubtypic immunity induced by H1N1 infection in previous years prior to H2N2 infection was observed only in adults not in children (4), which may be consistent with our results. Using a murine model, Di-Lillo *et al.* demonstrated that anti-stalk monoclonal antibodies required Fc-FcγR interactions in order to neutralize

FIG. 3. Correlation of antibody titers in pre-surveillance serum scatter plot displaying the relationship between different pre-surveillance antibody titers ($n=107$). Parentheses contain the number of subjects with the same antibody titer if $n>3$.

FIG. 4. Correlation of age and antibody titers scatter plot displaying the relationship between age and either CDL (*left* panel), ADCC (*middle* panel), and HAI (*right* panel) log₁₀ titers. Pearson correlation coefficients (*r*) and *p*-values are shown. In displaying antibody titers, 1:5 was assigned for undetectable HAI, 1:10 for undetectable CDL, and 1:5 for undetectable ADCC.

influenza virus *in vivo* through ADCC (2). In our study, in these children, stalk-specific antibodies may have represented only a small portion of the ADCC antibodies measured and may not have been in high enough concentration to have a biologically protective effect. Repeated exposures to influenza virus may be required to develop sufficient levels of these stalk-specific ADCC antibodies to provide protection. Another reason for the lack of a protective impact by ADCC antibodies may be that ADCC antibodies impact viral clearance better in acute systemic infections such as in measles (5) and in neonatal HSV infections (15) than in the prevention of localized respiratory tract infections such as influenza. Also, ADCC antibodies may exert their primary effect by enhancing cross-reactive T-cell responses and increasing immunopathology by a mechanism called antibody-dependent enhancement, as we have assessed *in vitro* (24, 25), or acting through immunomodulation rather than through lysis of infected cells (18). The statistically significant age difference between the groups, especially the clinical PCR+ and the subclinical seroconversion groups, with both groups potentially exposed to the virus through either infection or vaccination, was an unexpected finding, since younger children are usually at higher risk. One possible explanation is that older children may have been exposed more to influenza virus in the school setting compared to younger children who stayed home. Although the experimental design was not ideal, these analyses revealed interesting relationships between age and these three functionally different antibodies.

In summary, this is the first study to examine the relationship between preexisting HAI, CDL, and ADCC antibodies and the subsequent development of clinical influenza in young children. Preexisting HAI and CDL antibodies were associated with protection against PCR+ influenza, but ADCC antibodies were not. The strength of this study was in the prospective cohort design which included pre- and post-surveillance blood sampling, coupled with enhanced passive surveillance for febrile illness that allowed for the identification of symptomatic influenza cases by PCR and subclinical influenza cases by pre- and post-surveillance HAI seroconversion. Although each of the groups studied contained a relatively small number of subjects, we were able to show significant differences in preexisting HAI and CDL antibodies between the PCR+ and seroconversion groups. The major limitation of this study was the statisti-

cally significant difference in the average age of the study groups. Children who developed PCR+ influenza illness were significantly older than children in either the seroconversion or nonseroconversion group. Confirmation of these results in a larger prospective study, including age-matched children and adult subjects, is needed. Understanding additional correlates of immunity to influenza including potential cross-reactive components of immune protection such as ADCC may prove to be helpful for the development of improved influenza vaccines (3,22).

Acknowledgments

This work was supported by the National Institutes of Health (U19 AI57319 and 5P01 AI034533), the Military Infectious Disease Program, and by the Global Emerging Infections Surveillance and Response System, a Division of the Armed Forces Health Surveillance Center. We would like to acknowledge Dr. Alan Rothman for assistance with the logistics for this study. We thank Dr. Robert Ryall from Sanofi Pasteur for his gift of the A/California/7/09 strain used in the CDL and ADCC assays. We would also like to acknowledge Dr. Louise Maranda for her advice on the statistical analysis of the data, and Dr. Jin-Sheng Wen for establishing the ADCC assay. The views expressed in this article are those of the authors and do not represent the official policy or position of the U.S. Department of the Army, Department of Defense, or U.S. Government or the Royal Thai Army.

Author Disclosure Statement

No competing financial interests exist.

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