High Antibody-Dependent Cellular Cytotoxicity Antibody Titers to H5N1 and H7N9 Avian Influenza A Viruses in Healthy US Adults and Older Children

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Human influenza is a highly contagious acute respiratory illness that is responsible for significant morbidity and excess mortality worldwide [1]. In addition to neutralizing antibodies, there are antibodies that bind to influenza virus–infected cells and mediate lysis of the infected cells by natural killer (NK) cells (antibody-dependent cellular cytotoxicity [ADCC]) or complement (complement-dependent lysis [CDL]). We analyzed sera obtained from 16 healthy adults (18–63 years of age), 52 children (2–17 years of age), and 10 infants (0.75–1 year of age) in the United States, who were unlikely to have been exposed to the avian H7N9 subtype of influenza A virus, by ADCC and CDL assays. As expected, none of these sera had detectable levels of hemagglutination-inhibiting antibodies against the H7N9 virus, but we unexpectedly found high titers of ADCC antibodies to the H7N9 subtype virus in all sera from adults and children aged ≥8 years.

Keywords. avian influenza viruses; H7N9 subtype; H5N1 subtype; antibody-dependent cellular cytotoxicity; antibody-dependent cell-mediated cytotoxicity; ADCC, complement-dependent lysis; hemagglutination-inhibition; non-neutralizing antibody.

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In humans, ADCC antibodies against seasonal influenza viruses were detected at higher levels (1 to 2 logs) than HAI antibodies in children and adults [9, 20], but we found that CDL antibody titers were in a similar range to HAI antibody titers [21]. Previously, we reported that 3 of 10 young adults who were naive to influenza virus A/USSR/77(H1N1) had preexisting CDL antibodies but no HAI antibodies against this subtype [22], and more recently Jegaskanda et al reported that healthy adults who were unlikely to have been exposed to A(H5N1) or A(H1N1)pdm09 had cross-reactive ADCC antibodies to HAs of A(H5N1) and A(H1N1)pdm09 [23]. This suggests that ADCC and CDL antibodies have greater subtype cross-reactivity than conventional neutralizing antibodies, which may have implications for the development of a universal influenza vaccine [24]. In our recent study of young Thai children, we found that ADCC antibody titers against A(H1N1)pdm09 increased with age, whereas CDL or HAI antibodies titers did not [25], suggesting that sets of antibodies contributing to ADCC and CDL activities overlap but are not exactly the same.

ADCC and/or CDL antibodies are nonneutralizing antibodies and are thought to help control viral infection [26, 27]. In influenza virus infection, these nonneutralizing antibodies may be important against novel influenza viruses arising from reassortment with animal influenza A viruses [28], against which the majority of human population have no or very low levels of neutralizing antibodies. The seropositivity for A(H5N1) is considered to be 1%–2% [29]. One seroprevalence study of new avian influenza A(H7N9) viruses in China reported no detectable levels of neutralizing antibodies in 1544 sera collected in 2012 from poultry workers prior to the outbreaks in the area [30]. Another study conducted during the 2013 A(H7N9) outbreak reported that seropositivity for A(H7N9) was 0.8% in the general population but 13.9% among poultry workers [31].

Recently Jegaskanda et al analyzed sera from 62 healthy adult Australians and found 1 serum specimen with ADCC antibodies against seasonal influenza virus A(H7N9) [32]. In the present study, we first analyzed serum samples obtained from infants and adults in the United States using our CDL and ADCC assays and found that all adults and 2 of 10 infants had detectable ADCC antibodies to A(H7N9). We then analyzed sera collected from children aged 2–17 years and observed that titers of ADCC antibodies to A(H7N9) increased with age, which may have been induced by exposure to seasonal influenza viruses.

**METHODS**

**Samples**

Samples tested in this study were prevaccination sera obtained from 16 healthy adults (age, 18–63 years) who were about to receive licensed influenza vaccines under an approved University of Massachusetts Medical School (UMMS) Institutional Review Board protocol and random sera obtained from 10 infants ≤1 year old and 52 children 2–17 years old, purchased from a commercial company (Bioreclamation, Westbury, New York).

A whole-blood sample obtained under a UMMS IRB–approved protocol from 1 healthy adult subject was used as a source of NK cells.

**Viruses Used**

Influenza viruses A/Anhui/1/2013(H7N9), A/Hong Kong/156/97(H5N1), A/Brisbane/59/2007(H1N1), and B/Florida/4/2006 were obtained from the Centers for Disease Control and Prevention, and influenza virus A/Victoria/361/2011(H3N2) was obtained from Biodefense and Emerging Infections Research Resources. All experiments using A/Anhui/1/2013(H7N9) and A/Hong Kong/156/97(H5N1) were performed in the UMMS enhanced biosafety level 3 laboratory.

**HAI Assay**

HAI assays were performed with 2-fold serial dilutions of serum of 1:5–1:5120, using a standard protocol with some modifications as previously described [33, 34]. For statistical analysis, an undetectable HAI antibody titer was assigned a value of 2.5.

**CDL Assay**

The CDL assay method was previously described [25]. We used a chromium-release assay to calculate the percentage specific immune lysis (SIL) of infected A549 cells (human lung epithelial cell line; ATCC CCL-185), using 2-fold serial dilutions (1:10–1:1280) of heat-inactivated serum in the presence of Low-Tox Guinea Pig Complement at a final concentration of 1:20 (Cedarlane Laboratories, Burlington, North Carolina). A549 cells were infected at a multiplicity of infection (MOI) of 5–10 with A/Anhui/1/2013(H7N9) or A/Hong Kong/156/97(H5N1). Experiments were performed in replicates of 3. A549 cells infected with A(H7N9) or A(H5N1) were not lysed by complement alone in our CDL assays. The highest serum dilution showing ≥15% SIL was defined as the CDL antibody end point titer. For statistical analysis, an undetectable CDL antibody titer was assigned a value of 5. Serum from an adult subject with CDL antibodies against seasonal A(H1N1) was included as a positive control in all assays.

**ADCC Assay**

The ADCC assay method was previously described [25]. We used a chromium-release assay to calculate the percentage SIL of infected A549 cells, using 4-fold serial dilutions (1:20–1:327 680) of heat-inactivated donor serum in the presence of enriched NK cells (RosetteSep Human NK Cell Enrichment Cocktail; Stem Cell Technologies, Canada) isolated from a fresh whole-blood specimen obtained from a healthy subject at an effector:target ratio of 5:1/well. A549 cells were infected at a MOI of 5–10 with A/Anhui/1/2013(H7N9) or A/Hong Kong/156/97(H5N1). Lysis of infected target cells by NK cells alone without
sera added was 13.2% and 13.7% against A(H7N9)-infected cells and 11.2% against A(H5N1)-infected cells. When calculating SIL values, data on lysis of NK cells alone were subtracted. The highest serum dilution showing ≥15% SIL was defined as the ADCC antibody end point titer. For statistical analysis, an undetectable ADCC titer was assigned a value of 5, and for end point titers above the range of serum dilutions tested, a titer of 327 680 was assigned. A serum specimen from an adult with ADCC antibodies against seasonal A(H1N1) was included as a positive control in all assays.

**Statistical Analysis**
Statistical analyses were performed using Microsoft Office Excel 2007 and an online utility [35]. All antibody titers were transformed to log10 scale for all computations and comparisons. A P value of <.05 was considered to indicate statistical significance.

**RESULTS**

**Comparison of Titers of HAI, CDL, and ADCC Antibodies to A/Anhui/1/2013 (H7N9) in US Infants and Adults**

We analyzed HAI, CDL, and ADCC antibodies against A/Anhui/1/2013(H7N9) in serum samples obtained from 10 infants (age, ≤1 year) and 16 adults (age, 18–63 years). We intended to use infant sera as negative controls containing no or few residual maternal antibodies. As expected, there were no subjects with detectable HAI antibodies to the A/Anhui/1/2013(H7N9) (Supplementary Table 1). Sera from 6 of 16 adults had detectable CDL antibodies to this virus. None of the infants had sera with detectable CDL antibodies (Supplementary Table 1 and Figure 1). In contrast, sera from 2 of 10 infants and all adults had detectable ADCC antibodies to the virus (Supplementary Table 1 and Figure 1). Immunoglobulin G (IgG) depletion of an adult serum specimen used as a positive control eliminated ADCC activity.
against A(H7N9)-infected target cells, and purified IgG from the same serum specimen retained ADCC activity (data not shown).

**Comparison of Titers of CDL, ADCC, and HAI Antibodies to A/Hong Kong/156/97(H5N1)**

We were interested in whether these findings would be observed for another avian influenza virus, A/Hong Kong/156/97 (H5N1). No detectable HAI antibodies to A/Hong Kong/156/97 (H5N1) were observed in either adult or infant sera (Supplementary Table 1). Similar to A(H7N9), CDL antibodies to A(H5N1) were not detected in sera from any infants; however, in 7 of 16 adult sera, CDL antibodies were detected (Supplementary Table 1 and Figure 1). Detectable ADCC antibody titers were seen in sera from 2 of 10 infants and all adults (Supplementary Table 1 and Figure 1).

**Correlation of Titers of ADCC and CDL Antibodies to A/Anhui/1/2013(H7N9) and A/Hong Kong/156/97(H5N1)**

We observed a highly positive (r = 0.93) and significant correlation between A/Anhui/1/2013(H7N9) and A/Hong Kong/156/97(H5N1) ADCC antibody titers in all subjects (P = .0000000000078; Figure 2). Of note, the only 2 infant sera (BRH688264 and BRH688272) that had detectable ADCC antibodies to A/Hong Kong/156/97(H5N1) were the same 2 that had detectable ADCC antibodies to A/Anhui/1/2013(H7N9) (Supplementary Table 1). All adult sera had high titers of ADCC antibodies to both avian influenza viruses.

Six of the 16 adult serum samples tested had detectable CDL antibodies against A/Anhui/1/2013(H7N9), 7 had detectable CDL antibodies against A/Hong Kong/156/97(H5N1), but only 3 had CDL antibodies to both viruses (Supplementary Table 1 and Figure 2). No infant serum specimen had CDL antibodies against these viruses. Using the χ² test, we found no correlation between detection of CDL antibodies to A/Anhui/1/2013(H7N9) and detection of those to A/Hong Kong/156/97(H5N1) (data not shown).

**Age Distribution of Titers of ADCC Antibodies to A/Anhui/1/2013 (H7N9) in US Children**

We analyzed 52 sera from US children aged 2–17 years to determine titers of HAI and ADCC antibodies against A/Anhui/1/2013(H7N9). As expected, no subjects had detectable HAI antibodies to A/Anhui/1/2013(H7N9) (Supplementary Table 2). Thirty-four children had detectable ADCC antibodies against the virus. All samples obtained from children ≥8 years old had detectable ADCC antibodies, and the ADCC antibody titers increased with age (r = 0.49; P = .00069; Supplementary Table 2 and Figure 3). Analysis of samples obtained from children ≤7 years old, however, revealed no significant correlation between ADCC antibody titer and age (r = 0.21; P = .19).

**Figure 2.** Correlation between complement-dependent lysis (CDL) and antibody-dependent cellular cytotoxicity (ADCC) antibody titers to A/Anhui/1/2013 (H7N9) and A/Hong Kong/156/97(H5N1) in US infants and adults. Samples under the detection limit were plotted at 0.70 in the log₁₀ scale (equivalent to a 1:5 dilution) for both assays. Parentheses denote the number of subjects (≥2) with the same titer combination.

**Figure 3.** Age distribution of antibody-dependent cellular cytotoxicity (ADCC) antibody titers to A/Anhui/1/2013(H7N9) in US children. Samples under detection limit were assigned as a dilution of 1:5 (0.70 on the log₁₀ scale). The experiment was performed in triplicate. All samples were assayed in the same experiment. y = 0.12x + 1.1, r = 0.46, P = .00069.
Exposure to Seasonal Influenza Viruses and Generation of ADCC Antibodies Against A/Anhui/1/2013(H7N9)

We hypothesized that children may generate subtype–cross-reactive ADCC antibodies through exposure to seasonal influenza viruses. We analyzed the same 52 serum samples to determine HAI titers against seasonal influenza A(H3N2), A(H1N1), and influenza B, although influenza B is unlikely to induce high-titer, subtype–cross-reactive antibodies to influenza A [36]. Thirty-nine children had detectable HAI antibodies against A/Victoria/361/2011(H3N2). Similar to findings for ADCC antibodies against A(H7N9), all but 1 child aged ≥8 years had detectable HAI antibodies against A(H3N2) (Supplementary Table 2 and Figure 4). HAI antibody titers against A(H3N2), however, did not correlate with age (ages 2–17 years, r = 0.046 and P = .75; ages 2–7 years, r = 0.21 and P = .19). Half of the children had detectable HAI antibodies against A/Brisbane/59/2007(H1N1). All but 1 child aged ≥8 years had detectable HAI antibodies against A(H1N1) (Supplementary Table 2 and Figure 4). HAI antibody titers against A(H1N1) increased with age (ages 2–17 years, r = 0.57 and P = .0000098; ages 2–7 years, r = 0.62 and P = .000015). The smaller number of children who had HAI antibodies against A/Brisbane/59/2007(H1N1), compared with the number who had HAI antibodies against A/Victoria/361/2011(H3N2), may be explained partly by the replacement of seasonal A(H1N1) strains with the A(H1N1)pdm09 strain in recent years. Thirty-two children had detectable HAI antibodies against B/Florida/4/2006. All children aged ≥8 years had detectable HAI antibodies against influenza B (Supplementary Table 2). HAI antibody titers against influenza B also increased with age (ages 2–17 years, r = 0.53 and P = .000062; ages 2–7 years, r = 0.43 and P = .0050; Supplementary Figure 1).

Titers of HAI antibodies against A(H3N2) and A(H1N1) correlated with titers of ADCC antibodies against A(H7N9) in the group aged 2–17 years (A(H3N2), r = 0.34 and P = .013; A(H1N1), r = 0.39 and P = .0039) and the group aged 2–7 years (A(H3N2), r = 0.47 and P = .0019; A(H1N1), r = 0.38 and P = .014; Figure 5). As expected, titers of HAI antibodies against influenza B did not correlate with titers of ADCC antibodies against A(H7N9) (Supplementary Figure 2). We performed a χ² test to see whether there were correlations between detection of HAI antibodies against seasonal influenza viruses and the detection of ADCC antibodies against A(H7N9) (Table 1 and Supplementary Table 3). Detection of HAI antibodies against A(H1N1) correlated with detection of ADCC antibodies against A(H7N9) (ages 2–17 years, P = .0036; ages 2–7 years, P = .051; Figure 5). The correlation between detection of HAI antibodies against A(H3N2) and the detection of the ADCC antibodies against A(H7N9) did not reach statistical significance (ages 2–17 years, P = .092; ages 2–7 years, P = .23; Figure 5). As expected, there was no correlation between the detection of HAI antibodies against influenza B and detection of ADCC antibodies against A(H7N9).

Twenty-three children had HAI antibodies against both A(H3N2) and A(H1N1) (Table 2 and Supplementary Table 2). Among these children, 87% had ADCC antibodies against A(H7N9). In contrast, 53% of the children who had HAI antibodies against either A(H3N2) or A(H1N1) and 40% of those who had no HAI antibodies against influenza A had ADCC antibodies against A(H7N9) (Supplementary Table 2). When HAI antibody titers and the detection of the ADCC antibodies were compared, the higher the A(H3N2) or A(H1N1) HAI antibody titer a subject had, the greater the likelihood that the subject had A(H7N9) ADCC antibodies (Supplementary Table 4).

**DISCUSSION**

Here, we report the presence of high titers of ADCC antibodies to the avian H7N9 subtype of influenza A virus in sera from healthy US adults and older children who are unlikely to have been exposed to avian influenza viruses, and the absence of these antibodies in sera from the majority of infants. Since the adult sera had similarly high titers of ADCC antibodies to avian A(H5N1), to which they are also unlikely to have been...
exposed, the observed subtype cross-reactivity is not a phenomenon unique to a peculiar virus strain but a more general characteristic of human ADCC antibody responses to avian influenza A viruses. All children aged ≥ 8 years also had ADCC antibodies to avian A(H7N9).

These results are in contrast with those from the study by Jegaskanda et al, who found that serum specimen from only 1 of 62 healthy adult Australians had ADCC antibodies to A(H7N9) [32]. The readouts of our ADCC assay and their ADCC assay are different. They quantitated activation of NK cells (interferon-γ or CD107a expression detected by fluorescence-activated cell sorting) in the presence of serum and purified recombinant H7 HA protein. ADCC antibodies detected by their assay are, therefore, specific to HA. We quantitated lysis of A(H7N9)-infected cells by NK cells in the presence of serum. ADCC antibodies measured in this assay can include antibodies to all influenza A proteins expressed on infected cell surface. Different results produced by these 2 assays suggest that antibodies binding to conserved epitopes on NA, NP, and M2 contribute to the ADCC activity against A(H7N9)-infected cells detected by our ADCC assay. In addition, the different results could be related to the location of the subjects whose sera were analyzed.

Although 2 ADCC antibody epitopes have been identified on the globular head of the H1 HA [13], they are not subtype cross-reactive and are unlikely to be involved in subtype-cross-reactive ADCC antibody responses to A(H7N9) or A(H5N1). Jegaskanda et al detected anti-NA (N1) ADCC antibodies in some of the intravenous immunoglobulin preparations tested [32]. Subtype-cross-reactive antigenic sites have been identified on NA [37]. Anti-NP ADCC antibodies were detected in macaques either infected with A(H1N1)pdm09 or vaccinated with trivalent inactivated influenza vaccine [38]. It is important to determine whether anti-NP antibodies are also involved in subtype-cross-reactive ADCC antibody response in humans. Although levels of anti-M2 antibodies were found to be low to nonexistent in humans [39, 40], a recent study that used transfected cell lines expressing M2 on their surface reported that anti-M2 antibodies were detected in a higher percentage of individuals than previously thought, especially in adults aged ≥ 40 years (38%–50%) [41].

Figure 5. Correlation between hemagglutination inhibition (HAI) antibody titers against seasonal influenza A and antibody-dependent cellular cytotoxicity (ADCC) antibody titers against A(H7N9) in children aged 2–7 years and those aged 2–17 years. The top panels show correlation between titers of HAI antibodies against A/Victoria/361/2011(H3N2) and ADCC antibodies against A/Anhui/1/2013(H7N9) in children aged 2–7 years (y = 0.47x + 0.78, r = 0.47, P = .0019; left graph) and children aged 2–17 years (y = 0.39x + 1.1, r = 0.34, P = .013; right graph). The bottom panels show correlations between titer of HAI antibodies against A/Brisbane/59/2007(H1N1) and ADCC antibodies to A/Anhui/1/2013(H7N9) in children aged 2–7 years (y = 0.64x + 1.1, r = 0.38, P = .014; left graph) and children aged 2–17 years (y = 0.70x + 1.2, r = 0.39, P = .0039; right graph). Parentheses denote the number of subjects (≥ 2) with the same titer combination. Samples under the detection limit were assigned a dilution of 1:5 for ADCC antibody titers (0.70 on the log10 scale) and 1:2.5 for HAI antibody titers (0.40 on the log10 scale).
We measured HAI antibodies to seasonal influenza A(H3N2), A(H1N1), and influenza B to determine whether influenza virus exposure induced subtype–cross-reactive ADCC antibodies to A(H7N9), and we found some correlations between HAI antibody responses to seasonal influenza A, but not influenza B, and ADCC antibodies to A(H7N9). Four children had HAI antibodies to seasonal influenza A that were under the detection limit but ADCC antibodies to A(H7N9). It is possible that this ADCC assay is more sensitive than the HAI assay, which is supported by the relatively higher titers of ADCC antibodies than HAI antibodies to influenza A in human sera [9, 20]. Alternatively, their HAI titers may have waned to below the detectable limit.

Concerning the contribution of ADCC and other nonneutralizing antibodies to protection, a reanalysis of the archival records from the Cleveland Family Study suggested the presence of heterosubtypic immunity in adults, not in children. That study was conducted before and during the 1957 A(H2N2) pandemic and found that A(H1N1) infection in previous years had a protective effect against A(H2N2) infection in 1957 only in adults [42]. Both antibodies and T cells may also be involved in this heterosubtypic immunity. Recently, we observed in Thai children in a 2010–2011 study that titers of ADCC antibodies against A(H1N1)pdm09 increased with age, while titers of CDL and HAI antibodies did not [25]. This age distribution of ADCC antibodies parallels that of heterosubtypic immunity. Of note, in the Thai study we measured ADCC antibodies against circulating A(H1N1)pdm09, not subtype–cross-reactive ADCC antibodies.

We also observed moderate titers of subtype–cross-reactive CDL antibodies against A(H7N9) and A(H5N1) in sera from 38%–44% of adults. But, contrary to ADCC antibody titers, there was no correlation between titers of CDL antibodies against A(H7N9) and those against A(H5N1), suggesting that a different set of antibodies may be involved in the subtype–cross-reactive ADCC and CDL antibody responses.

### Table 1. Correlation Between the Presence of Detectable Hemagglutination Inhibition (HAI) Antibodies Against Seasonal Influenza A Viruses and of Antibody-Dependent Cellular Cytotoxicity (ADCC) Antibodies Against A(H7N9) in Children Aged 2–7 Years and Those Aged 2–17 Years

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χ² statistic = 1.4
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χ² statistic = 3.8
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χ² statistic = 9.3
P = .026

### Table 2. Correlation Between the Presence of Detectable Hemagglutination Inhibition (HAI) Antibodies Against Influenza A(H3N2) and A(H1N1) and of Antibody-Dependent Cellular Cytotoxicity (ADCC) Antibodies Against A(H7N9) in Children

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χ² statistic = 9.3
P = .026

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χ² statistic = 9.3
P = .026

### Notes

- a Antibodies were detected (≥1:5).
- b Antibodies were under detection limit (<1:5).
- c Antibodies were detected (≥1:20).
- d Antibodies were under detection limit (<1:20).
There is always a question of whether antiviral immune responses other than virus neutralization, such as the ADCC and CDL antibodies, may be immunopathological. A study in mice suggests that NK cells may contribute to pathology [43]. Another study reported that, with a lower infectious dose, this was not seen [44], suggesting a fine balance between protection and immunopathology. Recently, DiLillo et al demonstrated that Fc-Fe receptor γ interactions are required for stalk-specific, but not globular head-specific, monoclonal antibody–mediated protection against influenza virus challenge in mice at lower antibody concentrations but are not needed at higher concentrations [14]. Another earlier report, however, demonstrated that NK cells were not necessary for antibody-mediated influenza virus clearance [45]. As for roles of complement, although A (H5N1) infection of mice suggested that increased complement activation was associated with enhanced disease, studies using knockout mice demonstrated that C3 was required for protection [46]. We should point out that results from mouse models of influenza virus infection should be interpreted cautiously because the immunological history of humans is very different from that of laboratory mice.

Recently, some perspectives have been offered concerning the relative contributions of the immune system to pathology and protection. La Gruta et al concluded that the same immunological factors mediating tissue damage during the anti-influenza virus immune response are also critical for efficient elimination of influenza virus [47]. More generally, a new framework was proposed to understand microbial pathogenesis. It has been suggested that both too weak and too strong host responses to microorganisms lead to host damage [48]. The contribution of ADCC and CDL antibodies to protection and/or to immunopathology may also be relative and context dependent and deserves to be investigated in prospective controlled clinical studies.

Supplementary Data

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

Notes

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Potential conflicts of interest. The authors received research funding from GlaxoSmithKline for an influenza virus vaccine study, which is not related to the study described in this article.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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