Virus-Specific Antibody Secreting Cell, Memory B-cell, and Sero-Antibody Responses in the Human Influenza Challenge Model

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**Background.** Antibodies play a major role in the protection against influenza virus in human. However, the antibody level is usually short-lived and the cellular mechanisms underlying influenza virus-specific antibody response to acute infection remain unclear.

**Methods.** We studied the kinetics and magnitude of influenza virus-specific B-cell and serum antibody responses in relation to virus replication during the course of influenza infection in healthy adult volunteers who were previously seronegative and experimentally infected with seasonal influenza H1N1 A/Brisbane/59/07 virus.

**Results.** Our data demonstrated a robust expansion of the virus-specific antibody-secreting cells (ASCs) and memory B cells in the peripheral blood, which correlated with both the throat viral load and the duration of viral shedding. The ASC response was obviously detected on day 7 post-infection when the virus was completely cleared from nasal samples, and serum hemagglutination-inhibition antibodies were still undetectable. On day 28 postinfection, influenza virus-specific B cells were further identified from the circulating compartment of isotype-switched B cells.

**Conclusions.** Virus-specific ASCs could be the earliest marker of B-cell response to a new flu virus infection, such as H7N9 in humans.

**Keywords.** B cells; human challenge model; influenza A virus.

Antibody-secreting cells (ASCs) and memory B cells (MBCs) constitute the primary cellular components of T-cell–dependent antibody response to a variety of viral pathogens. Upon re-exposure to virus, activated MBCs rapidly differentiate into ASCs that produce high-affinity antibodies, and in the steady state could also replenish long-lived ASCs throughout life [1, 2]. Virus-specific ASCs transiently circulate in the peripheral tissues at the acute phase of infection and survive in the bone marrow from months to years [3, 4]. It seems that the germinal center reaction allows the clonal expansion and selection of high-affinity virus-specific B cells, which leads to the establishment of antibody immunity. However, it has long been a controversial issue whether acute B-cell response is responsible for protection as a correlate against viral infections in humans.

While a significant correlation was noted between acute ASC response and the serological or neutralizing antibody level in viral infection and vaccination [5, 6], evidence was accumulating that acute ASC response might otherwise associate with the viremic status and severe immunopathology in infected patients [7–9]. Persistence of circulating ASCs was also noted in those patients with severe illnesses [2]. These circulating ASCs were believed to derive from recently proliferated and terminally differentiated preexisting MBCs [2, 10].
In animal studies, the adoptive transfer of virus-specific MBCs contributed to the viral control and survival rate in challenged mice [11, 12]. In contrast, only limited data characterizing the MBC response to acute viral infection in humans were available, and its correlation with the serological response remained disputable [13, 14].

Influenza virus causes endemics annually and occasionally global outbreaks, which pose a major threat to human health. The antibody level with HI titer over 1:40 has been established as an immune correlate of protection against acute infection in adults [15]; however, the antibody response might be short-lived, and impaired in immunologically naive individuals and immunosenescent and immunocompromised populations [16–20]. In addition, most individuals would lack antibody immunity to newly emerging influenza subtypes, such as H5N1 and H7N9 viruses. The cellular mechanisms underlying the influenza virus–specific antibody response in humans have not been fully elucidated. The human challenge model mimics a natural virus infection and allows careful longitudinal study of experimental and controlled infection and immune response in a quarantine environment. In contrast to the study of clinical infection in a hospitalized setting, the enrolled subjects, inoculum dose, type of challenge virus, time of infection, and sampling time can be tightly controlled.

Recently, we have reported the use of the influenza virus challenge model to define the roles of influenza virus–specific CD4+ T lymphocytes in the control of virus shedding and disease severity in humans [21]. It remains unclear if acute influenza infection can induce a rapid recall of B-cell response and its relationship with the virus load and the clinical course in seronegative individuals. In the present study, we measured the frequencies, phenotypes, and survival niches of circulating influenza virus–specific ASCs and MBCs from baseline and followed the clinical and immunological parameters for 28 days during the experimental influenza infection.

**MATERIALS AND METHODS**

**Human Influenza Challenge Model**

Infected subjects reported here were from 2 challenge studies with influenza A/Brisbane/59/07 virus, as described previously [21]. Briefly, subjects were intranasally inoculated with Good Manufacturing Practice–grade virus on day 0. Clinical symptoms (cough, sore throat, runny nose, nasal stuffiness, sneezing, earache, breathing difficulty, muscle ache, general malaise, headache, hoarseness, chest discomfort, and overall discomfort) and oral temperature were recorded throughout the study period. Symptom assessments were performed by subjects twice daily on a 4-point scale (0–3 corresponding to absent to severe). The total symptom score for each day was obtained by adding the individual symptoms scores for that particular day.

Nasal wash samples were collected daily during the quarantine period for virus isolation. Fresh whole-blood samples were taken on days -2, 3, and 7, and day 28 after challenge.

In this study, the establishment of infection was defined by the development of influenza-like illness and/or virus shedding and/or seroconversion by day 28. Viral load was determined by half-maximal tissue culture infectious dose assay (TCID50), as described previously [21]. Seroconversion was defined as either a hemagglutination-inhibition titer before challenge of less than 1:10 and a titer after challenge of 1:40 or more, or a titer before challenge of 1:10 or more and at least a 4-fold increase after challenge.

The studies were in compliance with both Good Clinical Practice guidelines (CPMP/ICH/135/95) and the Declaration of Helsinki. The protocol was also approved by the local ethics review committee. All subjects provided written informed consent.

**Cellular Assays**

Freshly separated peripheral blood mononuclear cells (PBMCs) were used to set up the ex-vivo B-cell enzyme-linked immunosorbent spot (ELISpot) assay [22] and memory B-cell assay as described by Crotty et al [23]. The assays were performed with purified influenza split vaccine antigen (ie, A/California/07/09 (CA07)–like, A/Brisbane/59/07 (BR59)–like, and A/Brisbane/10/07 (BR10)–like vaccine antigen), and recombinant H1N1 A/England/195/09 (Eng195) hemagglutinin (HA).

PBMCs were stained with a panel of antibodies specific for surface markers to identify different B-cell populations, including PB anti-CD3 (clone UCHT1, BD), FITC anti-CD19 (clone HIB19, BD), PE-Cy7 anti-CD27 (clone M-T271, BD), PE-Cy5 anti-IgM (clone G20-127, BD), FITC anti-IgD (clone IA6–2, BD), APC-H7 anti-CD20 (clone L27, BD), and PE-Cy5 anti-CD38 (clone HIT2, BD). FITC anti-Ki-67 (clone B56, BD) was used for proliferation marker detection.

To identify the cellular source of memory B cells making influenza-specific antibodies, PBMCs were stained and CD3negCD19pos B cells were sorted into CD27pos IgMneg IgDneg (isotype-switched), CD27neg IgMneg IgDneg (isotype-switched), CD27pos IgMpos IgDpos, and CD27neg IgMpos IgDPpos (naive) subsets by fluorescence-activated cell sorter (FACS). Sorted B-cell subsets were cocultured with CpG oligonucleotide (5′ TCG TCG TTT GTT CGT TTT GTC GTT 3′, Invivogen), *Staphylococcus aureus* Cowan Strain (SAC) (Calbiochem), Pokeweed mitogen (PWM) (Sigma-Aldrich), and heterologous irradiated PBMCs in the memory B-cell assay [23, 24]. Following a 5.5-day polyclonal stimulation of sorted B cells, the B-cell ELISpot was performed to detect B-cell subsets that have differentiated into ASCs in vitro. Briefly, cultured cells were harvested, washed, and resuspended in medium to a final concentration of 2 million cells per mL. For influenza virus–specific and negative control wells, 100 μL cell suspension was added, while for total
Ig wells, 10 μL cell suspension was added in the first well followed by the 2-fold serial dilutions. Plates were incubated for 18–24 hours at 37°C, 5% CO₂. After washing, plates were incubated with alkaline phosphatase conjugates antihuman immunoglobulin (IgG, IgM, or IgA (Calbiochem) for 2 hours at room temperature. Plates were washed and then developed with alkaline phosphatase substrate kit (Bio-Rad) at room temperature. Spot-forming cells were measured and counted with automatic ELISpot reader (AID iSPOT reader) and AID software version 5.0 (Cadama Medical Ltd).

Hemagglutination-Inhibition Assays
The serum samples were evaluated by hemagglutination-inhibition (HI) assay, as previously described [22]. HI assay was performed with antigens or viruses of BR59.

Enzyme-Linked Immunosorbent Assay
To measure the HA-specific IgG antibody titer in plasma samples, the enzyme-linked immunosorbent assay (ELISA) was performed with recombinant HA of H1N1 A/Brisbane/59/07 (Sino Biological), H1N1 A/England/195/09 and H5N1 A/Vietnam/1203/04 (VN1203) kindly provided by A. Townsend. In preliminary experiments, the coating concentration of each HA antigen was optimized to yield adequate reproducibility and a significant signal/noise ratio with post-vaccination sera/plasma and monoclonal antibodies. With the established HA coating concentration, the linear part of sample dilution curve was noted from 1/100 to 1/100 000 dilution. Then flat-bottomed 96-well ELISA plates (NUNC) were coated with 50 μL recombinant HA at 10 μg/mL in carbonate buffer overnight at 4°C, washed 5 times with phosphate-buffered saline (PBS)–0.05% Tween 20 and then blocked with the PBS-3% bovine serum albumin (BSA). Wells coated with only carbonate substrate kit (Bio-Rad) at room temperature. The reaction was stopped by adding 50 μL stop solution (2N sulphuric acid), then read at 450 nm. All the plasma samples were tested in duplicate and the mean absorbance was calculated. The ELISA titer was determined by the positive (antigen-coating well)-over-negative (buffer-coating well) method, in which the endpoint was the highest dilution that gave a positive-over-negative ratio of equal to or greater than 2.

Statistical Analysis
Graphs were presented by GraphPad Prism software (version 5) and statistical analysis was done by GraphPad Prism and SPSS. Spearman rank correlation test and Kruskal–Wallis tests were used. A P value <.05 was considered to be significant.

RESULTS

Clinical and Virological Features of Influenza-Infected Subjects
Twelve infected subjects from 2 challenge studies with H1N1 BR59 virus were analyzed. Six out of 12 subjects were female and the median age was 25 ± 3 years (range, 20–35). Prior to influenza virus challenge, all subjects were influenza-virus-culture and polymerase-chain-reaction negative in nasal lavage. Eleven out of 12 subjects (92%) were HI seronegative (<1:10), and only 1 had an HI titer of 1:10 to the challenge virus.

After H1N1 BR59 virus challenge, all infected subjects developed influenza-like illness, and presented with cough, sore throat, headache, runny nose, muscle ache, or general malaise with or without a documented fever, and 10 of 12 (83%) had either positive viral load or seroconversion by day 28 (Table 1). The mean duration of virus shedding was 2.9 ± 2.4 (range, 1–6) days, but 2 infected subjects had prolonged virus shedding for as long as 6 days.

Influenza-Specific ASCs
Before challenge, no influenza virus-specific ASCs were detected in the peripheral blood of any subjects. Following acute H1N1 BR59 infection, total and influenza virus-specific ASC populations became detectable and rapidly expanded in magnitude on day 7 postchallenge. We detected 166 ± 55 BR59-specific IgG ASCs per million PBMCs among infected subjects (Figure 1A). The BR59-specific IgG ASCs averaged 23.3% ± 4.4% (range, 5%–50.4%) of total IgG ASCs. The day 7 BR59-specific ASC response significantly correlated both with the viral load and the duration of viral shedding (Figure 1B), indicating that the response was antigen specific. A significant correlation between the ASC response and total symptom scores was also noted (Figure 1C). The fold increase in BR59 HA-specific IgG antibody titer was 1.5 ± 0.2 on day 7, and significantly increased to 4.2 ± 1.2 on day 28 postchallenge (P = .0013, Student t test) in an ELISA and the day 7 BR59-specific ASC response correlated with the fold rise of the day 28 (P = .0061) but not the day 7 ELISA response (P = .2403) (Table 1). Moreover, neither seroconversion nor positive HI response was detected on day 7 postchallenge, and the size of BR59-specific ASC response did not correlate with serum HI titer on day 28 (P = .9921).

We noted mild antibody response to H1N1 Eng195 and H5N1 VN1203 HA by ELISA with the fold increase of 1.1 ± 0.1
and 1.0 ± 0.1 on day 7 and 1.9 ± 0.2 and 1.5 ± 0.2 on day 28, respectively (Supplementary Table 1). However, the fold rise in Eng195 HA and VN1203 HA-specific antibody titer was significantly lower than that in BR59 HA-specific titer on day 7 (P = .0183) and day 28 (P = .0021), indicating that the cross-reactive antibody response was feeble.

By ex-vivo FACS staining, at baseline, the ASC population was barely detectable and accounted for around 0.01% of lymphocytes in the peripheral blood. On day 7 postchallenge, a significant increase of ASCs (an average of 10-fold) was observed and the ASCs appeared as a distinct population on the FACS (P < .0001 for all time point comparisons). Ki-67 protein was expressed in the majority of day 7 ASCs, indicating the nature of recent proliferation. The frequency of day 7 ASC response by FACS significantly correlated with the BR59-specific and total IgG ASC response as measured by ELISpot (Figure 1D).

The exact duration of influenza virus-specific ASC response was uncertain due to the lack of sampling between days 7 and 28. However, on day 28, no influenza virus–specific ASC was detected and the total ASC frequency returned to preinfection level (Figure 1A and 1D).

Influenza-Specific MBCs
At baseline, 50% of subjects had preexisting BR59-specific IgG cells, which accounted for around 0.2% of total IgG cells. After acute H1N1 BR59 infection, a significant increase of BR59-specific IgG cells was observed. By day 3, the frequency averaged 0.67% ± 0.17%; and by day 7, the average response was 0.94% ± 0.18%. On day 28, the BR59-specific IgG cells averaged 1.86% ± 0.29% of total IgG cells (P < .0001 for all time point comparisons) (Figure 2A). The frequency of day 28 BR59-specific IgG cells significantly correlated with both the viral load and the duration of viral shedding (Figure 2B). In contrast, the BR59-specific IgG cell response among uninfected control subjects was similar across the study period (P = .5760), indicating that the change in influenza virus–specific IgG B cells was responding to virus challenge. However, neither day 7 nor day 28 BR59-specific IgG cell response correlated with day 28 serum HI titer (P = .1518). No significant correlation was noted between the BR59-specific IgG cell response and total symptom scores, either (P = .3390).

In the 2010 challenge study, we observed preexisting H1N1 CA07–specific and H3N2 BR10–specific IgG cells at 0.40% ± 0.14% and 0.52% ± 0.11% of total IgG cells, respectively. An average of approximately 50% of H1N1 CA07–specific IgG cells was HA-specific. After infection, the frequency of H1N1 CA07– and H3N2 BR10–specific B cells increased (0.61% ± 0.24% and 0.85% ± 0.09% of total IgG cells on day 28, respectively), but the increase was statistically insignificant for all time point comparisons, suggesting that heterovariant cross-reactive B-cell activation was minimal and the response was strain-specific.

To identify the cellular precursors of the MBC-producing influenza virus–specific antibodies, PBMCs were sorted into naive, CD27pos IgMpos IgDpos, and isotype-switched memory

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Table 1. Summary of Infected Subjects in the H1N1 BR59 Challenge Studies

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<th>Subject</th>
<th>IFIa</th>
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<th>IgG ELISA Titer (BR59)</th>
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Abbreviations: ASCs, antibody-secreting cells; BR59, A/Brisbane/59/07; D, day after challenge; ELISA, enzyme-linked immunosorbent assay; HI, hemagglutination inhibition; IFI, influenza-like illness; IgG, immunoglobulin G; NDA, nondetectable activity; PBMCs, peripheral blood mononuclear cells; TCID50, half-maximal tissue culture infectious dose.

a Influenza-like illness was defined as acute onset of cough, sore throat, headache, runny nose, muscle ache, or general malaise, etc.
b Fever was defined as oral temperature 38°C degree or more.
c The ASC response was expressed as the frequency of BR59-specific IgG ASCs per 10^6 PBMCs.
B-cell subsets based on expression of IgD, IgM, and CD27 in the 2010 challenge study (Figure 2C). B-cell subsets were stimulated in vitro with a cocktail of SAC, CpG, and PWM and the frequency of antigen-specific cells was measured by ELISpot.

Influenza virus–specific IgG cells were identified among CD27pos IgMneg IgDneg and CD27neg IgMneg IgDneg isotype-switched B-cell subsets (Figure 2D). H1N1 BR59–specific cells accounted for 1.45% ± 0.23% of CD27pos IgMneg IgDneg
and 0.52% ± 0.08% of CD27neg IgMneg IgDneg B-cell subsets (P = .1000), while H1N1 CA07–specific cells accounted for 0.39% ± 0.11% of CD27pos IgMneg IgDneg and 0.08% ± 0.04% of CD27neg IgMneg IgDneg B-cell subsets (P = .1000). There was no detectable influenza-specific IgG cell within the CD27pos IgMpos IgDpos and naive B-cell subsets.

Across the study period, the frequency of total T cells, B cells, CD27pos B cells, and CD27neg B cells did not change significantly, regardless of infection status. During the study period, the frequency of B-cell subsets had fluctuated but had not changed significantly, confirming that the changes were only observed in antigen-specific responses.
DISCUSSION

The present study characterized the development of virus-specific B-cell response in acute influenza infection, in relationship to clinical, virological, and serological responses.

In this model of experimental virus infection, healthy adult subjects were infected with a Good Laboratory Practice–grade virus under controlled conditions. Predictable numbers of subjects became infected and shed virus [21]. Many clinical features of the response were very similar to typical influenza infection in healthy adults [25]. Seronegative individuals were selected for susceptibility to challenge infection. We fully accept that it is a model and may lie at variance from clinical disease, and we have highlighted these issues in the discussion.

The detection of significant IgG antibody–secreting cells in the first week following experimental infection clearly indicates a quick elicitation of secondary immune response and thus the strain-specific antibody immunity in susceptible adults. It has been suggested that the preexisting MBCs could have contributed to the ASC response that carries a highly mutated immunoglobulin gene and produces high-affinity antibodies, indicating the recall of memory [2, 26, 27]. However, in the study, the HI antibody response was undetectable on day 7 postchallenge. We noted that 50% of infected subjects had no increase in BR59 HA–specific antibody titer by ELISA on day 7. It seemed unlikely that day 7 ASCs primarily produced antibodies against the conserved HA head and stem region, because the fold increase in Eng195 HA– and VN1203 HA–specific titer was barely detectable on day 7. One possible explanation is that the secreted antibodies on day 7 have not yet built up to the high enough level in the serum to register a change in the HI or ELISA assay. Additional time points (ie, day 8 and more postchallenge) for collection of blood samples might be helpful for detailed analysis of the acute ASC and antibody responses. Our serological data hardly provide the information on the actual range and the relative abundance of the antibodies that bind to the BR59 HA and are positive on ELISA, but neither inhibit hemagglutination nor neutralize in the serum and plasma samples. Further research into the understanding of the breadth and function of virus-specific B-cell and antibody repertoires at the single-cell level will be crucial to delineate their role in the antibody immunity against reinfection and to assist in optimizing the protective efficacy of new vaccines in the near future.

It was noted that the virus-specific ASC response following experimental influenza infection was lower than that previously described. An average of over 1000 influenza virus–specific IgG ASCs per million PBMCs was detected after natural infection by Wrammert et al [2]. First, this discrepancy could be related to the difference in clinical severity. In this study, all infected subjects presented influenza-like illness and only 25% had fever. However, in Wrammert’s study, all enrolled patients presented fever and one-third of them developed severe pneumonia. Second, the magnitude of the ASC response might correlate with the viral load and the duration of viral shedding following infection. In Wrammert’s study, patients with severe illness had prolonged viral shedding, and their ASC response could be detected for several weeks. In the study, subject 4032–2010 with the high viral load and prolonged viral shedding also generated a strong ASC response. Therefore, it has to be pointed out that the removal of this single ASC data set would affect the statistical correlation between the ASC response and symptoms scores and viral shedding in the challenge study. These findings suggested that ongoing and severe influenza infection might continuously induce the generation and accumulation of influenza-specific ASCs and result in the detection of a stronger and prolonged ASC response.

A correlation between the virus-specific MBC response and the viral load and virus shedding duration was noted post influenza infection. It has been shown that through antigen-independent polyclonal stimulation, MBCs could differentiate into ASCs ex vivo, which indicates their potential for providing long-term antibody immunity. Although there was no correlation between virus-specific MBC frequency and serum antibody levels at baseline or any time postinfection in the present study and in the influenza vaccination [28], strong correlation had been reported in other bacterial or viral antigens [1, 29, 30]. Interestingly, it was found that immunization of previously primed adults with a booster dose of adjuvanted H5 vaccine could induce an extremely strong MBC response, as high as 20% of total peripheral IgG cells on day 21, which significantly correlated with the antibody titer [31]. It was suggested that the priming of CD4+ effector and memory T cells might help the induction of dominant B-cell and antibody responses upon antigen re-exposure, or the adjuvant effect on the antigen presentation might be involved. Further studies addressing the interaction between specialized B-cell populations and CD4+ T cells in the germinal center to promote the development of long-lived antigen-specific B-cell pools should be explored.

In summary, we showed that experimental influenza A H1N1 BR59 infections induced a significant ASC response in the first week and the expansion of influenza virus–specific MBCs until day 28 postinfection in susceptible adults. Although no correlation between the influenza virus–specific B-cell response and serological HI antibody level was found, these data clearly supported that acute infections could quickly induce the recalling response of virus-specific B cells, even where the preexisting antibody level is undetectable. Our study provided additional indicator for prevention and evaluation of new influenza infections such as H7N9 in humans.

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 copypedited. 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. T. W., A. G., and J. O. report personal fees from Retroscreen Virology during the conduct of the study. A. G., J. O., and R. I.-W. are employees of Retroscreen Virology. A. G. reports ownership of stock options. All other authors report no potential conflicts. 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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