Rapid Communication

Characteristics of neutralizing antibodies to adenovirus capsid proteins in human and animal sera

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\textbf{A B S T R A C T}

Although it is known that Ad5-specific neutralizing antibodies (NAbs) against three major viral capsid components (hexon, penton and fiber) are generated, differences in the frequency and nature of these pre-existing NAbs remain unclear. The results emphasized the contribution of anti-fiber antibodies to Ad5 neutralization responses generated during natural viral infection. Additionally, Ad5-specific NAbs against the fiber knob protein were present in over 90% of the positive serum samples while 42% of the sera had NAbs against hexon in this study based on neutralization assay of anti-HVR and anti-knob subtracted sera and Western blotting analysis. We also found that the trimeric knob was preferentially recognized by fiber-induced NAbs and it was serotype-specific in human adenovirus species C. Results indicated that the trimeric knob protein would be a good candidate antigen for detecting adenovirus serotype-specific NAbs in naturally infected sera.

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\textbf{Introduction}

Adenoviruses (Ads) are non-enveloped DNA viruses that have been extensively studied and used as vectors for vaccine and gene delivery. A limitation that has become apparent with the most well-studied adenovirus type 5 (Ad5) vaccine vector is the high titers of naturally occurring Ad5 neutralizing antibodies (NAbs) in human populations, particularly in the developing world (Yu et al., 2012b).

There are three major capsid proteins on Ad particles: fiber, penton base and hexon. Both the fiber and hexon proteins contain group-specific as well as type-specific epitopes (Norrby, 1969; Rux and Burnett, 2000). Previous studies have reported that fiber- and penton-specific antibodies against Ad5 exert synergistic neutralizing activities, which are functionally significant, and Ad5-specific NAbs directed against the Ad5 hexon protein are found in both humans and mice (Gahery-Segard et al., 1998; Sumida et al., 2005). It is clear from these studies that Ad5-specific NAbs against multiple capsid components are generated. Interestingly, recent studies have emphasized that the specificity of Ad5 NAbs is directed towards hexon and fiber, but it is unclear which protein is dominant in the NAb response and whether the route of exposure (e.g., natural infection versus rAd5 vaccination) may lead to a difference between the humoral immune response to these two proteins (Bradley et al., 2012; Cheng et al., 2009). Clarify the characteristics of NAbs to adenovirus capsid proteins in human and animal sera will help to design the novel chimeric Ad5 vectors evaded Ad5-specific NAbs.

Currently, 57 human Ad serotypes are defined and distributed into seven species from A to G (Walsh et al., 2011). Infection-induced humoral immunity to a particular serotype does not provide cross-immunity to an Ad serotype of another subgroup (Hierholzer et al., 1991). Serotype-specific NAbs are detected by viral vector-based neutralization assays (NA) and have been used in epidemiological studies on the prevalence of pre-existing NAbs to several conceptually interesting vaccine candidate types (Mast et al., 2010). Although neutralization assays are efficient means for detecting specific antibodies against each Ad serotype, they are cumbersome and expensive to perform. Therefore, more simple typing methods for NAbs are needed.

In this study, we demonstrated that NAbs against fiber are commonly generated in natural adenovirus infection in humans and rAd immunization in animals. We also showed that the trimeric knob protein was specifically recognized by Ad NAbs in species C, rendering it would a good candidate antigen to detect serotype-specific NAbs in natural Ad infection and rAd vaccination.

\textbf{Abbreviations:} Ad5, adenovirus type 5; NAbs, neutralizing antibodies; HVR, hypervariable regions; Luc, luciferase; IM, intramuscular; IN, intranasal

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Results

Analysis of capsid-specific humoral immune response in human sera from natural Ad infection

Human serum samples were analyzed at different titers (≤ 200, 201–1000, > 1000) by Western blotting to determine whether immune responses to the three major components (hexon, penton and fiber) of the viral capsid in the sera correlated with the titers calculated using previous methods (Abbink et al., 2007; Yu et al., 2012b). As shown in Fig. 1A, higher serum titers corresponded to increased antigen–antibody responses, and vice versa. Substantially higher levels of anti-penton and anti-fiber antibodies were detected than anti-hexon antibodies in subjects with natural Ad infection (titer < 1000) in our study. However, antibodies to Ad5 in some high titered (> 1000) samples were also partly against the hexon protein. To precisely identify the anti-fiber and anti-hexon antibody responses in human sera, the recombinant Ad5 knob domains of the fiber protein and the complete peptide including all seven short HVRs on the surface of the Ad5 hexon protein were used in Western blotting analysis. The results showed that anti-fiber antibodies and anti-hexon antibodies from the seropositive sera weakly recognized the monomeric form of the knob (22 kDa) and HVR (40 kDa) proteins. We also showed that both anti-fiber and anti-hexon antibodies from the sera preferentially recognized the native form of the knob (66 kDa) and HVR (over 700 kDa) proteins (Fig. 1B).

To analyze the natural frequency of immune responses to fiber and hexon proteins in sera with natural Ad5 infection, a total of 149 human serum samples with moderate and high NAb titers (> 200) were analyzed by virus or native protein-based Western blotting. Fig. 1C shows that 98% of the sera had fiber-specific antibodies, and 93% of the sera (including nearly all of the sera with the fiber-specific band) specifically recognized the native knob protein. In contrast, 48% and 42% of the sera had a hexon-specific band and reacted with native HVR protein, respectively. The results indicated that over 90% of the positive human sera had anti-fiber antibodies and could also detect the native knob protein in our study. To characterize the nature of the antibodies induced by the capsids in human sera of different titers, the sera diluted 1:100 were partially depleted of anti-fiber and anti-hexon antibodies by incubation with knob and HVR proteins. The results showed that less of the neutralizing activity was depleted by knob (p < 0.05) in all tested sera, while a larger part of the serum neutralizing activity was depleted by HVR (p < 0.001) in sera with anti-hexon antibodies, especially those with high titer NAbs (titer > 1000) (Fig. 1D and E). These results indicated that NAb against fiber are commonly generated in natural adenovirus infection in humans, although the neutralizing activity detected by the knob was weak.

Fig. 1. Capsid-specific humoral immune responses in human sera after natural Ad infection. (A) Ad5 positive sera from participants with low (titer < 200), moderate (200 < titer < 1000) or high (titer > 1000) anti-Ad5 antibodies were analyzed by Western blotting recognizing purified rAd5. Bands corresponding to hexon, penton and fiber are indicated. Ab6982, anti-adenovirus type 5 antibody (Abcam, Cambridge, UK) was a positive control. (B) Sera with high titer anti-Ad5 antibodies were assessed against HVR and knob proteins by native or SDS-denatured Western blotting as described in Materials and methods. Ad negative sera were used as negative control (con). (C) Percentages of anti-hexon (HVR) and anti-fiber (knob) antibodies contained in 149 human sera with baseline NAb titers were analyzed by Western blotting as in (A) and (B). (D) Ad5 neutralization activity of anti-knob subtracted sera. Different concentration of knob proteins were mixed with anti-Ad5 serum and incubated with cells before the addition of rAd5. S=human positive serum, S+5/10/25/50/100 means serum mixed with different concentration of the proteins (1 unit=1 μg/ml); (E) NAb titers of human sera cleared of selected antibodies by pre-incubation with HVR and (or) knob proteins detected. S=serum; S+K=serum after incubation with knob; S+H=serum after incubation with HVR; S+K+H=serum after incubation with both knob and HVR. ***p < 0.0001, **p < 0.001 and *p < 0.05.
Analysis of capsid-specific humoral immune responses in mouse and rabbit sera

To analyze the humoral immune responses to Ad5 capsid proteins in animal sera, rabbit and BALB/c mice were immunized using different rAd5 immunization protocols. One and two immunizations in rabbits or IM and IN immunizations in mice showed different immune responses from that with human natural infection in that antibodies to Ad5 were directed mainly to the hexon and fiber proteins and seldom to the penton protein (Fig. 2A). Moreover, results of the Ad5 neutralization assay with anti-HVR and anti-knob depleted sera were in good agreement with the viral capsid protein immune responses and indicated that NAbS to these two proteins accounted for most of the neutralization activity in the immune sera \( (p < 0.0001) \) (Fig. 2B).

Interestingly, Western blotting analysis showed that only sera from IM immunized mice had Ad5 NAbS which could bind with trimeric knob proteins, while sera of IN immunized mice were negative for Ad5 NAbS, although similar patterns of antibody specific to Ad5 were observed in sera of IM and IN inoculated mice. These results indicated that the trimeric knob was preferentially recognized by fiber-induced NAbS (Fig. 2C and D).

Trimeric knob proteins could induce and were recognized by serotype-specific NAb

Since NAbS induced by different Ad serotypes did not show cross-immunity, trimeric knob proteins of Ad2 and Ad5 within human adenovirus species C (HAdV-C; Ad1, 2, 5 and 6) were expressed as His-tagged fusion proteins in *Escherichia coli* to analyze that if the trimeric knob protein can serve as an antigen for distinguishing serotype specific responses. As shown in Fig. 3A, after immunization in rabbits with the purified trimeric proteins, NAbS against adenoviruses were found in the hyper-immune sera and showed no cross-neutralization between the two rAd types (rAd2 and rAd5) tested in our study. These results indicated that trimeric knob proteins could induce serotype-specific Ad NAbS.

Based on human natural viral infection sera against Ad2 and Ad5, no cross-immunity of the sera was observed by reactivity with trimeric knob antigens in Western blotting assays. However, weak cross-reactivity of the monomeric knob proteins was shown with the human sera (Fig. 3B).

Discussion

Ads are the cause of common upper respiratory infections, and thus most individuals have circulating antibodies to these viruses. Pre-existing immunity to Ad is an important issue in clinical trials with rAd5 vector-based vaccines for HIV-1 and other pathogens as it can limit in vivo delivery of Ad vectors and gene transfer (Hong et al., 2003). Thus, understanding the nature of anti-Ad5 immunity and mapping the targets of dominant Ad5-specific NAbS in human sera is important for the development of vaccines against HIV-1 and other infections or diseases. In this study, we examined in detail the Ad5-specific humoral immune response in healthy humans by natural infection and in mice and rabbits with rAd5 immunizations. These results confirm and extend the findings of previous studies showing that antibodies generated by natural infection in individuals are directed primarily to fiber and penton components and not to hexon or HVR antigens (Hong et al., 2003). However, antibodies to Ad5 in some high titered (> 1000) NAb samples were also directed largely to the hexon

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Fig. 2. Capsid-specific humoral immune responses in mouse and rabbit sera after Ad5 immunization. (A) Anti-Ad5 antibodies in rabbit and mouse serum were analyzed by Western blotting. Left: lane 1, after one immunization; lane 2, after two immunizations. Right: M, prestained protein marker; IM: intra-muscular; IN: intra-nasal. (B) NAb titers of rabbit and mouse serum after incubation with HVR and (or) knob proteins detected by neutralization assays. (C) Mouse serum after IM or IN Ad5 immunization were assessed with knob protein by SDS-denatured or NDS Western blotting. Lanes 1 and 3, SDS-PAGE; lanes 2 and 4, NDS-PAGE and (D) Ad5 NAb titers of mouse serum detected by neutralization assays.
protein. Ad5-specific NAb s against the fiber knob was also shown to be present in most of the seropositive samples and appeared to be functionally relevant, although secondary in nature to NAb s against HVR when anti-hexon antibodies were present. This result may reflect differences in the exposure to fiber and hexon antigens during natural infection. First, a mild Ad infection commonly generates antibodies against fiber and penton antigens, while severe and repeated infections over a short period induce antibodies directed largely to the hexon protein. In contrast to the immune responses with natural infection in humans, rAd5 immunizations either in rabbits or mice resulted in antibodies to Ad5 that were directed mainly to the hexon and fiber proteins with few responses to the penton antigen. Moreover, Ad5-specific NAb s were directed primarily against the HVRs and relatively weak against the knob domain. This observation may explain why the hexon HVR chimeric rAd5 vectors can circumvent pre-existing anti-vector immunity in mice with high titer NAb s induced by Ad5 immunization (Roberts et al., 2006).

The results emphasized the contribution of anti-fiber antibodies to neutralizing activity generated by natural viral exposure and Ad immunization. Prior studies have demonstrated a direct correlation between the capacity of anti-fiber antibodies to recognize the native trimeric form of the fiber protein and recognition of the native trimeric knob protein, which contain mainly species-specific epitopes (Bauer et al., 2005; Gahery-Segard et al., 1998). This study showed that the NAb s induced by fiber in rAd5 immunized mice preferentially recognized the trimeric knob protein. We assumed that the knob protein from different Ad serotypes would specifically recognize NAb s of the same Ad serotype since NAb s induced by different adenovirus serotypes commonly show no cross-immunity, although some previous studies have concluded that knob mainly contains species-specific epitopes (Bauer et al., 2005; Havenga et al., 2002; Liebermann et al., 2002). In fact, in a crystal structure model, some important features such as valleys formed by the R-sheets and the HI loops were found on the surface of the Ad5 knob protein (Xia et al., 1994). Therefore, type-specific epitopes may be located in highly variable regions on the surface of the knob domain, and several type-specific antigenic epitopes on knob can be identified by ELISA and other methods (Fender et al., 1995; Liebermann et al., 2001; Liebermann, Lotz, and Seidel, 2002). To verify our hypothesis, we expressed two knob proteins from HAdV-C in E. coli and found that the trimeric knob protein could induce type-specific Ad NAb s in rabbits and mice, which were different from those found in humans induced by rAd5 immunization (Gahery-Segard et al., 1998). This difference may result from the different antigens or immune response to Ad in human and in animal body, as our results shown that almost no antibody was produced against penton after Ad5 immunization in mice and rabbits. The pattern of reactivity of the human sera indicated no cross-immunity against trimeric knob proteins of the HAdV-C species. By comparing with the corresponding amino acid sequences from selected serotypes, we proposed that neutralizing epitopes were located in the region that may be more limited, such as LSSMTGTV (445–452) in many Ad2 fiber epitopes, and that the trimeric form of knob was necessary for the recognition to NAb s (Fender et al., 1995; Liebermann et al., 2002).

Of the more than 50 human adenovirus serotypes occurring worldwide, the most commonly used rAd vector is Ad5; however, considering its high seroprevalence in the human population, other serotypes are being investigated. The evaluation of serotype-specific NAb s as a means of selecting the adequate adenovector(s) is important for both clinical applications and epidemiological studies (Piedra et al., 1998; Sanchez et al., 2001). Currently, the quantitative neutralization assay based on rAds carrying luciferase or secreted alkaline phosphatase (SEAP) reporter gene with the inhibition of transgene expression as a readout is used to determine type-specific NAb titers (Aste-Amezaga et al., 2004; Sprangers et al., 2003). Recent international epidemiological studies of several Ad serotypes were carried out using this method due to its good accuracy and sensitivity (Mast et al., 2010). However, the lower replication and difficult construction of some rAds will limit the application of this method for additional Ad serotypes. In this study, we suggested that trimeric fiber knob may be a good candidate antigen to detect serotype-specific NAb s, although more knob proteins from other Ad serotypes need to be detected the cross-immunity with the sera raised against Ads in different human adenovirus species.

In conclusion, this study highlights the contribution of anti-fiber antibodies to neutralization generated by natural infection. Furthermore, we have shown that the trimeric knob might be used to test the prevalence of several Ad serotypes.

Materials and methods
Cell lines and serum

Human embryonic kidney (HEK) 293 and human lung epithelial A549 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). All cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, (FBS; Hyclone; Logan, UT),...
The sequence of Ad knob including the last shaft of the fiber (Yeh et al., 1994) was cloned into the pRSSET-B plasmid (Invitrogen) at the Nco I and Xho I restriction sites. The plasmid containing an N-terminal His tag was expressed in BL21 cells overnight at 16 °C. The pellet from a 2-1 culture was treated with 1 mg of lysozyme/ml on ice and sonicated 10 times for 10 s each time. After centrifugation at 40,000g for 30 min, 10 mM imidazole was added to the soluble fraction at 4 °C with gentle stirring. The resin was filled in a small polyethylene column and washed with 20 mM imidazole in a buffer containing 25 mM Tris–HCl (pH 7.5) and 150 mM NaCl. The protein was eluted with 500 mM imidazole in the same buffer and analyzed by SDS-PAGE. The Ad5 hexon hypervariable regions (HVRs) protein was expressed as described previously (Yu et al., 2012a).

Mice and immunizations

Six- to eight-week-old BALB/c mice were given two intramuscular (IM) injections, separated by a 4-week interval, with 1 × 1010 viral particles (vp) of replication incompetent rAd5-blank vectors in 100 µl of sterile PBS divided equally between both quadriceps muscles. For intranasal (IN) inoculation, 5 µl rAd5-blank (5 × 107 vp) was instilled in both nostrils of each animal using a micropipette tip. Five mice were used in each group and the serum used for the Western blotting and neutralization assay was pooled from all the 5 mice. All animal studies were approved by University Committee on the Use and Care of Animals of Jilin University.

Human serum

Human serum samples were collected randomly in 2010–2011 in China by the Provincial Centers for Disease Control and Prevention. Donors were aged from 18 to 60 years old of both sexes, with no history of any febrile illness during the few weeks preceding sample collection. Informed consent was obtained from all participants and the serum samples utilized in adenovirus neutralization assays were approved by the Human Ethical Committee Institutional Review Board, Jilin University, China.

Virus neutralization assays

Ad-specific NAb titers in human serum samples were assessed by luciferase-based assays as described previously (Sprangers et al., 2003). HEK293 cells were plated at a density of 1 × 104 cells per well in 96-well plates and infected with replication-incompetent rAd-Luc reporter constructs at a multiplicity of infection (MOI) of 500 with 2-fold serial dilutions of serum in 200 µl reaction volumes. Following a 24-h incubation, luciferase activity in the cells was measured using the Steady-Glo Luc reagent system (Promega, Madison, WI) with a Victor 1420 multilabel counter (Perkin Elmer, Wellesley, MA). Neutralization titers were defined as the maximum serum dilution that inhibited 90% of luciferase activity.

Ad5 neutralization assay of anti-HVR or anti-knob subtracted sera

HEK293 cells were plated at a density of 1 × 104 cells per well in 96-well plates and incubated for 24 h at 37 °C and 5% CO2. The following day, 50 µg/ml HVR or knob proteins were incubated with serially diluted Ad5-seropositive sera for 2 h at 37 °C in 100 µl reaction volumes. Next, 50 µl of 5% nickel nitrilotriacetic acid (Ni-NTA) beads suspension (Invitrogen, Carlsbad, CA) were added and incubated for 1 h with gently shaking at room temperature before centrifugation at 12,000 rpm for 5 min to remove the proteins and protein–antibody complexes as the knob proteins would block the infection of rAd5 in 293 cells. A fixed amount of rAd5-Luc was then incubated for 1 h at 37 °C either with no serum and removal of protein solution, corresponding to 100% luciferase activity, or with the serum solution after removal of protein–antibody complexes. As another positive control, virus only with serially diluted serum was added to cells, and as a negative control, cells were cultured in the absence of virus. Following 24 h of incubation, luciferase activity in the cells was measured as for the neutralization assays above.

Western blotting analysis

Protein samples were electrophoresed by SDS-PAGE, NDS-PAGE or Native-PAGE as described previously (Yu et al., 2012a), and transferred to nitrocellulose membranes for Western blotting. SDS was not added into the transfer buffer (39 mM glycine, 48 mM Tris–HCl, 20% methanol, pH 9.2) when proteins were separated by Native-PAGE. Membranes were incubated with different human, rabbit and mouse sera overnight at 4 °C in PBS containing 1.0% milk and then subsequently incubated with ImmunoPure alkaline phosphatase-conjugated goat anti-rabbit IgG secondary Abs (Jackson ImmunoResearch Laboratories, Inc.) at a 1/10,000 dilution for 1 h. The color reaction was developed with 0.1 M Tris–HCl (0.1 M NaCl, 5 mM MgCl2, pH 9.5) containing 0.66% NBT solution and 0.33% BCIP solution.

Statistical analysis

Values are expressed as means ± SEM. Differences between groups were examined by Student’s t-test (significant when p < 0.05).

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