Localization of neutralization epitopes on adenovirus fiber knob from species C

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Although potential neutralization epitopes on the fiber knob of adenovirus (AdV) serotype 2 (Ad2) and Ad5 have been revealed, few studies have been carried out to identify neutralization epitopes on the knob from a broader panel of AdV serotypes. In this study, based on sequence and structural analysis of knobs from Ad1, Ad2, Ad5 and Ad6 (all from species C), several trimeric chimeric knob proteins were expressed in Escherichia coli to identify the locations of neutralization epitopes on the knobs by analysing their reactivity with mouse and rabbit polyclonal sera raised against AdVs and human sera with natural AdV infection. The dominant neutralization epitopes were located mainly in the N-terminal part of knobs from Ad1, Ad2 and Ad5, but they seemed to be located in the C-terminal part of the Ad6 knob, with some individual differences in rabbit and human populations. Our study adds to our understanding of humoral immune responses to AdVs and will facilitate the construction of more desirable capsid-modified recombinant Ad5 vectors.

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INTRODUCTION

Recombinant adenovirus (rAdV) serotype 5 (rAd5)-based vectors are widely utilized for vaccine and gene therapy; however, their efficacy is decreased in both pre-clinical studies and clinical trials by the pre-existing Ad5 immunity (Majhen et al., 2014). Therefore, understanding the functionally relevant determinants of adenovirus (AdV) immunity and developing novel AdV vectors to circumvent Ad5-specific neutralizing antibodies (NAbs) are important goals. AdV NAbs are serotype specific, and minimal or no cross-reactivity in these antibodies exists between different AdV species (McCoy et al., 2007; Sharma et al., 2010; Yu et al., 2014). Our laboratory and others recently reported that Ad5-specific NAbs are directed primarily against both the hexon and fiber following natural infection (Bradley et al., 2012; Cheng et al., 2010; Yu et al., 2013). Bradley et al. (2012) also showed that capsid chimeric Ad5 vectors in which both the hexon hypervariable regions and the fiber knob were exchanged could almost completely evade Ad5-specific NAbs both in vitro and in vivo. However, the fiber knob is responsible for the specific high-affinity binding of the virion to the cell receptor (e.g. CAR, CD46 and DSG2) and thus determines the AdV infection and tissue tropism (Arnberg, 2012). Therefore, knob-exchanged Ad5 vectors may have altered infection patterns and biodistribution that result in reduced transduction and pose a health security risk (Coughlan et al., 2010). Characterization of serotype-specific neutralization epitopes on the fiber knob will add to our understanding of humoral immune responses to AdVs and will contribute to the construction of novel and more desirable capsid-modified rAd5 vectors.

Many previous studies have mapped linear epitopes on the fiber knobs of Ad2, Ad3, Ad5, Ad8 and Ad15, and several type-specific antigenic epitopes on the knob can be identified by ELISA and other methods (Fender et al., 1995; Liebermann et al., 1998, 2001, 2002). However, few studies have been carried out to identify the neutralization epitopes on the AdV fiber knob. Potential neutralization epitopes on the fiber knob of Ad2 and Ad5 were revealed by Liebermann et al. (2001), who showed that peptides near the C terminus of the knob (FK2PIII, FK2PIV, FK5PII and FK5PIII) were able to induce moderately NAbs, while those more distant from the C terminus of the knob (FK2P6/P7 and FK2PI) induced weakly NAbs in rabbits. These authors also demonstrated previously that peptides P14 and the C-terminal P16 of the Ad3 fiber knob were able to inhibit Ad3 infection of cells (Liebermann et al., 1998). These studies indicated that the C-terminal region of the fiber knob may contain dominant neutralization epitopes. In this study, several chimeric
knob proteins based on fiber knobs from species C (Ad1, Ad2, Ad5 and Ad6) were expressed in *Escherichia coli*. The reactivity of these recombinant proteins was analysed with mouse and rabbit antisera raised against AdVs and with human sera from natural AdV infection to further clarify the location and immunogenicity of neutralization epitopes on the fiber knob.

**RESULTS**

**Expression of chimeric trimeric knob proteins**

We first analysed the structures, sequences and hydrophilicity (Fig. S1, available in the online Supplementary Material) of fiber knobs from Ad1, Ad2, Ad5 and Ad6. Five continuous or portions of discontinuous sequences were presumed to be neutralization epitopes on the fiber knob, which overlapped the epitopes or regions of neutralization epitopes published previously (Liebermann et al., 2001), including three regions in the N terminus and two in the C terminus (Fig. 1, green shading). To obtain trimeric chimeric knob proteins, the knob domains were selected based on amino acid and nucleic acid sequence analysis and expressed as described previously (Yu et al., 2014). The identical sequence in the middle region of the fiber knob from Ad2 and Ad5 (Fig. S2, shown in red) was used to construct two chimeric knobs (K2-5 and K5-2) by using an overlapping PCR method, as shown in Fig. 2(a). The *HindIII* site in the

![Fig. 1. Amino acid sequence alignment of the fiber protein knob domain from Ad1 (GenBank accession no. AB361378), Ad2 (AB361379), Ad5 (AB361382) and Ad6 (AB361383). Dashes in the alignment are identical amino acids, and spaces are deletions. Ad2 knob- and Ad5 knob-derived peptides representing epitopes are highlighted in yellow and potential neutralization epitopes are underlined (Liebermann et al., 2001). Green shading shows putative neutralization epitopes and brown shading shows region of identical sequence in the Ad2 and Ad5 fiber protein knob domain (this region is shown as it was chosen as a site for exchange between serotypes); blue shading shows *HindIII* sites in the sequences of the Ad1 and Ad6 fiber protein knob domain (this region was also used as a site for exchange between serotypes).](image-url)
sequences of both fiber knobs from Ad1 and Ad6 (Fig. S2, shown in green) was used to construct the other two chimeric knob (K1-6 and K6-1) plasmids with this restriction enzyme and ligation, as shown in Fig. 3a. All four chimeric proteins were expressed with the last repeat in the shaft of the fiber and an N-terminal His tag, as described previously (Seiradake et al., 2006; Yeh et al., 1994; Yu et al., 2014). As shown in Figs 2(b) and 3(b), all of the chimeric knob proteins migrated as trimers in non-denaturing SDS (NDS)-PAGE and were generally stable in the presence of 1% SDS in this study.

### Reactivity of animal sera raised against AdV displaying knob antigens

A previous study by us showed that the trimeric knob was preferentially recognized by fiber-induced NABS and could be used to detect AdV serotype-specific NABS in sera from AdV-immunized and naturally infected subjects by Western blotting following NDS-PAGE or by ELISA (Yu et al., 2014). To demonstrate the abilities of the trimeric chimeric knob proteins (K2-5 and K5-2) to recognize NABS, the chimeric proteins and relevant original proteins (as control) were analysed by Western blotting of NDS-PAGE with animal immune sera. As shown in Fig. 2(c), anti-Ad2 sera from all five individual C57BL/6 mice specifically recognized K2-5 rather than K5-2, suggesting that Ad2 NABS in the mouse sera preferentially recognized the N-terminal region of trimeric knob (L401–L522). Similarly, anti-Ad5 sera from all five individual mice recognized K5-2 rather than K2-5, which showed that Ad5 NABS in mouse sera also preferentially recognized the N-terminal part of the trimeric knob (L401–L522). These consistent results unexpectedly determined that, for Ad2 and Ad5, the fiber knob neutralization epitopes may be located in the N-terminal part of knob.

### Schematic of the construction and expression of chimeric knob proteins K2-5 and K5-2

(a) Schematic of the construction and expression of chimeric knob proteins K2-5 and K5-2, and their reciprocal assays with antisera. (b) Expression of trimeric chimeric knob proteins K2-5 and K5-2. Chimeric knob proteins were analysed by SDS-PAGE and non-denaturing SDS (NDS)-PAGE. M, size marker (kDa). (c) Reactivity analysis of knob proteins with anti-AdV mouse serum by Western blotting following NDS-PAGE. MS, Mouse serum. (d) Reactivity analysis of knob proteins with anti-AdV rabbit sera by Western blotting following NDS-PAGE. RS, Rabbit serum. All sera were tested at a dilution of 1:100.

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**Fig. 2.** Expression of trimeric chimeric knob proteins K2-5 and K5-2, and reactivity of these chimeric proteins to mouse and rabbit polyclonal sera raised against AdVs. (a) Schematic of the construction and expression of chimeric knob proteins K2-5 and K5-2, and their reciprocal assays with antisera. (b) Expression of trimeric chimeric knob proteins K2-5 and K5-2. Chimeric knob proteins were analysed by SDS-PAGE and non-denaturing SDS (NDS)-PAGE. M, size marker (kDa). (c) Reactivity analysis of knob proteins with anti-AdV mouse serum by Western blotting following NDS-PAGE. MS, Mouse serum. (d) Reactivity analysis of knob proteins with anti-AdV rabbit sera by Western blotting following NDS-PAGE. RS, Rabbit serum. All sera were tested at a dilution of 1:100.
As the experimental mice used in this study were an inbred strain, we then performed the same analysis using anti-AdV sera of outbred rabbits. As shown in Fig. 2(d), of the five anti-Ad2 rabbit serum samples, three recognized K5-2 rather than K2-5, one recognized K2-5 rather than K5-2, and one recognized K2-5 preferentially over K5-2. However, in the Western blotting following NDS-PAGE analysis with anti-Ad5 sera, all five samples recognized K5-2 rather than K2-5. These results indicated that both the N-terminal and C-terminal parts of the fiber knob contain the neutralization epitopes, but they may be located mainly in the N-terminal part; however, this localization pattern may be different in some individuals.

**Reactivity of knob antigens to serum from humans with natural AdV infection**

To validate the results gained from animal sera, K2-5, K5-2, K1-6 and K6-1 were analysed by Western blotting following NDS-PAGE with human sera harbouring Ad1, Ad2, Ad5

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**Fig. 3.** Expression of trimeric chimeric knob proteins K1-6 and K6-1, and their reactivity to human sera with natural AdV infection. (a) Schematic of the construction and expression of chimeric knob proteins K1-6 and K6-1, and their reciprocal assays with antisera. (b) Expression of trimeric chimeric knob proteins K1-6 and K6-1. Chimeric knob proteins were analysed by SDS-PAGE and NDS-PAGE. M, size marker (kDa). (c) Reactivity analysis of knob proteins with human sera by Western blotting following NDS-PAGE. HS, human serum. All sera were tested at a dilution of 1:100, and serial identification numbers of the human serum samples are indicated on the left.
and Ad6 serotype-specific NAbs, which were collected in our previous study (Yu et al., 2014). As shown in Fig. 4, six out of 10 anti-Ad2 human serum samples recognized K2-5 rather than K5-2, three (SH-30, SH-70 and SH-99) recognized K2-5 preferentially over K5-2, and one (SH85) recognized K5-2 rather than K2-5. Similarly, seven of 10 anti-Ad5 human serum samples recognized K5-2 rather than K2-5, two (SH-28 and SH53) recognized K5-2 preferentially over K2-5, and one (SH47) recognized K2-5 rather than K5-2.

As shown in Fig. 3(c), six of eight anti-Ad1 human serum samples recognized K1-6 rather than K6-1, one (11-32) recognized K1-6 preferentially over K6-1, and one (11-39) recognized K6-1 rather than K1-6. However, only two of seven anti-Ad6 human serum samples (11-12 and 11-29) recognized K6-1 rather than K1-6, while five of them recognized K1-6 rather than K6-1. These results showed that, for Ad1, Ad2 and Ad5, NAbs preferentially recognized the N-terminal part of the fiber knob, while for Ad6, NAbs preferentially recognized the C-terminal part of the fiber knob.

**Induction of NAbs by chimeric knob proteins**

To further detect the antigenicity of the chimeric knob and guide the construction of knob-chimeric rAdV vectors, virus neutralization assays were performed with rabbit polyclonal sera against chimeric knob proteins as described previously (Yu et al., 2014). As only rAd5–GFP was available in our group, the neutralization assay performed in this study was based on Ad5. After immunization with K2-5 and K5-2 in rabbits, NAbs against K2-5 and K5-2 were found in rabbit serum by Western blotting following NDS-PAGE analysis (Fig. S3). In neutralization assays, we found that rabbit serum raised against K5-2 but not against K2-5 antigens could block the infection of Ad5 in human embryonic kidney 293 (HEK293) cells (Fig. 5). These results showed that chimeric knob proteins could induce serotype-specific NAbs in rabbits and further confirmed that neutralization epitopes were located mainly in the N-terminal part of the fiber knob of Ad5.

**DISCUSSION**

Recent studies have suggested that the route of exposure (e.g. natural infection versus rAd5 vaccination) and the genus of the infected organism may lead to a differential specificity and immunogenicity of Ad5 NAbs (Bradley et al., 2012; Cheng et al., 2010). Many other studies have
also suggested that the trimeric form of fiber is necessary for making contact with the receptor, and that discontinuous neutralizing epitopes on the knob may be involved in fiber–cell interactions (Fender et al., 1995; Gahe´ ry-Se´ gard et al., 1998). Our previous studies detected fiber-specific NAbs that could preferentially recognize the trimeric knob protein (Yu et al., 2013). Therefore, trimeric chimeric knob proteins maintaining the conformation of their domains are ideal for analysing the location and reactivity of neutralization epitopes.

In the current study, four related chimeric knob proteins were expressed and migrated on PAGE in the form of trimers. The reactivity of the chimeric knob proteins was analysed with anti-AdV sera by Western blotting following NDS-PAGE, as we previously showed that Western blotting following NDS-PAGE may give more precise results than ELISA (Yu et al., 2014). The results demonstrated that serotype-specific NAbs of Ad1, Ad2 and Ad5 preferentially recognized the N-terminal part of the corresponding knob protein, which suggested that neutralization epitopes are located mainly in the N-terminal part of the fiber knob. However, the converse seemed to be true for Ad6 fiber knob, although only seven human serum samples were tested. Our results are different from those of Liebermann et al. (2001), who showed that the C-terminal region of the knob appeared to contain the dominant neutralization epitopes. The number of regions with potential neutralization epitopes in the N-terminal part was greater than that in the C-terminal part of the fiber knob. In addition, the CAR-binding site on K5 has been reported to involve positions S408, P409, K417, K420 and Y477 (Roelvink et al., 1999). Specifically, S408, P409, Y477 and L485 were determined to be contact residues and A406, R412 and R481 to be indirectly involved in CAR binding (Kirby et al., 2000). All these positions are located in the N-terminal part of the fiber knob. These two points may explain why the dominant neutralization epitopes were localized mainly in the N termini of the Ad1, Ad2 and Ad5 fiber knobs. The difference observed with the Ad6 fiber knob may be due to the limited number of samples, although serotype differences still may exist, and further studies are needed to clarify this. Another noteworthy problem is that results of the analysis with inbred mice sera were different from those with Japanese White rabbit sera or human sera, in that no individual differences were found. The fact that different

![Fig. 5. Virus neutralization assays with rabbit polyclonal sera against chimeric knob proteins. The neutralizing activity of the anti-knob polyclonal sera was analysed by virus neutralization assays based on rAd5–GFP. Anti-K5, anti-K2-5 and anti-K5-2 indicate sera obtained from rabbits immunized with the Ad5 knob, chimeric protein K2-5 and chimeric protein K5-2, respectively. All sera were used at a dilution of 1 : 200.](image-url)
regions of the fiber knob provide the dominant neutralizing epitopes in different outbred individuals, while all individuals of an inbred mouse strain mount NABs to the same fiber knob region probably reflects the critical role of MHC antigens in selecting peptides for presentation. This observation indicated that rabbits or rats with more complex immune backgrounds would be more appropriate as an animal model than inbred animals when used for evaluating the ability of capsid chimeric AdV vectors to evade pre-existing AdV immunity. Our findings also indicate that construction of novel knob-modified Ad5 vectors to circumvent fiber-specific NABs would be quite feasible, although further studies to identify the precise neutralization epitopes are needed.

**METHODS**

**Cell lines and viruses.** HEK293 cells obtained from the American Type Culture Collection were cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% FBS (Hyclone), 2 mM l-glutamine, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ (all from Invitrogen). The rAd5–GFP recombinant plasmid was constructed using a two-plasmid rescue method as described previously (Ng et al., 2006). rAd2 vectors were provided by L. Chen (Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China).

**Construction and expression of chimeric trimeric knob proteins.** An identical sequence exists in the middle region of the fiber knob of Ad2 and Ad5. Based on this sequence, two chimeric knob plasmids were constructed using an overlapping PCR method as shown in Fig. 2(a). As the HindIII site is present in the sequences of the fiber knobs of both Ad1 and Ad6, two chimeric knob plasmids were constructed by digestion with this restriction enzyme and ligation as shown in Fig. 3(a). The chimeric knob protein sequence, including the last repeat in the shaft of the fiber (Seiradake et al., 2006; Yeh et al., 1994), was cloned into the pSET-B plasmid (Invitrogen) at the NcoI and XhoI restriction sites. This plasmid with an N-terminal His tag was expressed as described previously (Yu et al., 2014).

**Hyperimmune sera.** Rabbit polyclonal sera against the chimeric knob proteins (K2-5 and K5-2) were obtained from rabbits immunized three times with purified proteins (300 μg per injection) together with Freund’s complete and incomplete adjuvants. Anti-Ad2 and Ad5 rabbit polyclonal sera were from Japanese White rabbits immunized three times with purified virus (1 × 10¹⁰ viral particles per injection) together with Freund’s complete and incomplete adjuvants. Anti-Ad2 and Ad5 mouse polyclonal sera were from 6–8-week-old C57BL/6 mice given two intramuscular injections, separated by a 4-week interval, of 1 × 10¹⁰ viral particles of Ad2 or Ad5 in 100 μl sterile PBS in the quadriceps muscle. AdV NABs induced by Ad2 and Ad5 in sera of rabbits and mice were determined by neutralization assays. Five mice or rabbits were used in each group, and sera used for the Western blotting and neutralization assays were pooled from all five animals of each group. All animal studies were approved by the University Committee on the Use and Care of Animals of Jilin University.

**Human serum.** Ad1-, Ad2-, Ad5- and Ad6-positive human serum samples with serotype-specific NABs were gathered randomly during the period of 2010–2011 in China by the Provincial Centers for Disease Control and Prevention and tested by neutralization assays and ELISA (Yu et al., 2014). No history of febrile illness was noted during the few weeks before the collection in donors of both genders who were between the ages of 1 day and 78 years. Informed consent was obtained from all participants or their guardians in written form.

**Virus neutralization assays.** HEK293 cells were plated at a density of 5 × 10⁴ cells per well in 24-well plates. Following cell adhesion, rAd5–GFP was added at an m.o.i. of 200 with twofold serial dilutions of serum in 750 μl reaction volumes. Sera were inactivated at 56 °C for 30 min prior to testing. After 24 h of incubation, virus infection was observed by fluorescence microscopy.

**Protein analysis.** Proteins samples were separated by means of SDS-PAGE or NDS-PAGE and were analysed by staining with Coomassie Brilliant Blue or were transferred to nitrocellulose membranes for Western blotting. SDS-PAGE was performed on 13.5% acrylamide gels. Samples were denatured by boiling at 99 °C in Laemmli buffer [62.5 mM Tris/HCl (pH 6.8), 2% SDS, 1% β-mercaptoethanol, 25% glycerol, 0.01% bromophenol blue] before electrophoresis. NDS-PAGE was similar to SDS-PAGE except that samples were free of β-mercaptoethanol and not boiled. For Western blotting, the nitrocellulose membranes with proteins were blocked in PBS with 3% milk and then incubated with different human, mouse and rabbit sera overnight at 4 °C in PBS with 1% milk. After washing, the membranes were incubated with secondary antibodies (Jackson ImmunoResearch Laboratories) at a 1:1000 dilution for 1 h. The colour reaction was carried out with 0.1 M Tris/HCl (pH 9.5) containing 0.1 M NaCl, 5 mM MgCl₂, 0.66% nitro blue tetrazolium solution and 0.03% BCIP solution.

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**REFERENCES**


