Construction and characterization of human adenovirus serotype 3 packaged by serotype 7 hexon

Xingui Tian, Xiaobo Su, Haitao Li, Xiao Li, Zhichao Zhou, Wenkuan Liu, Rong Zhou*

State Key Lab of Respiratory Disease, The First Affiliated Hospital of Guangzhou Medical College, Guangzhou Medical University, Guangzhou 510120, China

ABSTRACT

Human adenovirus serotype 3 (Ad3) and serotype 7 (Ad7) are important pathogens causing respiratory tract diseases such as acute respiratory disease in pediatric and adult patients, but the immunodominant targets of Ad3- and Ad7-specific neutralizing antibodies (NAbs) remain unclear. A chimeric Ad vector, Ad3/H7, was constructed by replacing the Ad3 hexon gene (H3) with the hexon gene (H7) of Ad7. The chimeric viruses were successfully rescued in HEp-2 cells, and the Ad7 hexon was able to encapsidate the Ad3 genome, and functioned as efficiently as the Ad3 hexon. Furthermore, we tested the host neutralization responses against the viruses using BALB/C mice. Up to 97% of the NAbs produced by mice that were infected with these viruses were specific for the hexon protein in vitro. Preimmunization of mice with one of Ad7 and Ad3/H7 significantly prevented subsequent intranasal infection of the other type in vivo. In contrast, preimmunization of mice with one of Ad3 and Ad3/H7 did not remarkably prevent subsequent infection of the other type. We next evaluated the functional significance of hexon and other structural proteins specific NAbs to suppress the immunogenicity of Ad3/H3 and Ad3/H7 vectors expressing EGFP in mice preimmunized with wild type Ad. Preimmunization of mice with Ad7 evidently suppressed EGFP-specific humoral immune responses elicited by Ad3/H7, and did not exert suppressive effects on Ad3/H3. But contrary to the in vitro neutralization results, EGFP-specific humoral immune responses elicited by Ad3/H7 was remarkably inhibited in Ad3-preimmunization mice. The whole genome of the Ad7 strain was sequenced and aligned with Ad3. The major differences between Ad3 and Ad7 were only observed in the fiber and hexon among all structural proteins, and the variation between the hexons only located in four hypervariable regions (HVRs), HVR-1, -2, -5, and -7. These results thus suggest that Ad3- and Ad7-specific NAbs are directed primarily against the hexon proteins both in vitro and in vivo. But high titer Ad3 fiber-specific NAbs may also play an important role in blunting Ad3 immunogenicity in vivo. These studies contribute to a more profound understanding of Ad immunogenicity and have relevance for the design of novel Ad vaccine.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Human adenoviruses (HAdVs) in the alimentary canal and respiratory tract were discovered in 1953, and have been found to cause a broad spectrum of diseases in pediatric and adult patients (Aoki and Tagawa, 2002; Arnold et al., 2010; Kunz and Ottolini, 2010; Louie et al., 2008; Rowe et al., 1953). HAdV can be classified into six species (A–F) consisting of 53 serotypes, based on serum neutralization antibodies and nucleotide sequence. Recently, a new serotype, HAdV52, was reported and defined as a seventh species, by the US military from 1971 to 1996 (Gooch and Mogabgab, 1972; Hierholzer, 1992; Li et al., 2005; Tang et al., 2011). As yet, there is no effective medicine or vaccine except for live, oral vaccines used in America (Erderman et al., 2002; Frantzidou et al., 2005; Gooch and Mogabgab, 1972; Lyons et al., 2008). For all these reasons, development of an effective adenovirus vaccine is required.

Recently, a broad recombination phenomenon has been discovered between different serotypes of virus strains leading to lethal strains, some of which may be new serotypes (Kajon et al., 2010; Lukashev et al., 2008; Rebello-de-Andrade et al., 2010). Members of species B, HAdV-3 and -7, have caused severe respiratory disease, such as acute respiratory disease (ARD) pediatric pneumonia epidemics and outbreaks in Asia, Europe and America (Erderman et al., 2002; Frantzidou et al., 2005; Hierholzer, 1992; Li et al., 2005; Tang et al., 2011). As yet, there is no effective medicine or vaccine except for live, oral vaccines used by the US military from 1971 to 1996 (Gooch and Mogabgab, 1972; Lyons et al., 2008). For all these reasons, development of an effective vaccine directing HAdV-3 and -7 is required.

An adequate knowledge of the immunodominant regions of adenovirus NAbs will be helpful for the development of novel adenovirus vaccine. The Ad capsid consists of three major structural proteins: hexon, fiber, and penton base. Research carried out on adenovirus serotype 2 (Ad2) and Ad5 have identified the major
coat protein of the HAdV hexon as the main target for neutralizing antibodies (NAbs) (Pichla-Gollon et al., 2007; Sumida et al., 2005; Wu et al., 2002; Youil et al., 2002); however, it is not clear whether this is the case for other HAdV serotypes. Some studies have found that other structural proteins, such as fiber and penton base, also could induce neutralizing antibodies (Fender et al., 1995; Hong et al., 2003; Liebermann et al., 1998). Little is currently known about the neutralizing epitopes of the more than 50 HAdV serotypes (Pichla-Gollon et al., 2007; Rux and Burnett, 2000), although it is possible to locate HVRs using the multiple sequence alignment method (Crawford-Miksza and Schnurr, 1996; Ebner et al., 2005).

Replacement of the immunogenic capsid proteins with those of other serotypes is one of the approaches used to circumvent the problem of pre-existing immunity to an adenovirus delivery vector (Gall et al., 1998; Roberts et al., 2006; Rux et al., 2003; Wu et al., 2002; Youil et al., 2002). Some studies based on the HAdV5 vector have suggested that replacement of the hexon may or may not affect the packaging and gene transfer efficiency (Gall et al., 1998; Wu et al., 2002; Youil et al., 2002). An Ad3 vector has been developed previously as a candidate for vaccine design and gene transfer (Zhang et al., 2009). In the present study, we attempted to construct a chimeric adenovirus, Ad3/H7, by replacing the Ad3 hexon gene with the hexon gene of Ad7 (H7) in the hope that this study could help understand the functional significance of NAbs against major structural proteins, and the strategy used to modify the hexon from Ad3 could be applied to adenovirus vaccine development and gene therapy.

2. Materials and methods

2.1. Cells, virus strains and vectors

The wild type Ad3 strain HAdV3 GZ-01 (w-Ad3) and the E3-defective replication-competent EGFP expression plasmid, pBRAdV3dE3egfp (pAd3egf), corresponding virus Ad3/H3 (AdV3-egf) was got from Zhang et al. (2009). The Ad7 used in this study was HAdV7-g208 strain, originally isolated from a child with ARD and cultured in HEp-2 cells. The HEp-2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% (v/v) fetal calf serum. The viral DNA was extracted by the modified protease K digestion method without virus purification (Hardy et al., 1997).

2.2. Construction of recombinant plasmid

The shuttle vector, pBRH3S, for the Ad3 hexon replacement was constructed via two sub cloning steps. The left segment, L1 (4.34 kb), containing nucleotides (nt) 16,764–21,140 of pAd3egf and the right segment, L2 (3.44 kb), containing nt 21125–24569 of pA3egf, were amplified separately by polymerase chain reaction (PCR). L1 was cloned into pBR322 between the EcoRI and BamHI restriction endonuclease sites, with L2 ligated between the BamHI and SalI sites to obtain pBRH3S.

To create the shuttle vector, pBRH7S, H7 was obtained by PCR from Ad7 with the primers Hexon-F (5′-ACA GGA GAA AGA GAC-3′) and Hexon-R (5′-CCA CCC TTC GTG GAT CCC-3′). The H7 PCR product was digested with Clal and BamHI and ligated into pBRH3S that had also been treated with Clal and BamHI. The resulting shuttle vector, pBRH7S, contained 2681 nt of H7 from nt 12–2692. The resultant hexon was not different from the wild-type full-length H7.

The pBRH7S shuttle vector was digested with EcoRI and SalI, and the fragment containing the homologous recombination regions and the hexon genes was purified and recombined with AvrII- and PciI-digested pAd3egf in Escherichia coli strain BJ5183. The resultant clones containing H7 and EGFP were designated pAd3egf/H7 and selected by PCR using primers a7u (5′-ACA GCA GTA GAA GAA AAG GGA AGA G-3′), a7r (5′-CCA CCC TTC GTG GAT CCC-3′), efgu (5′-GAA ATC GTG ATG AAC AGG GCG GAG CAG C-3′) and efgr (5′-CTA GAT CGG GTG TAG GAT CCC-3′). Constructs were confirmed by restriction digestion and sequencing analysis.

2.3. Virus rescue and preparation

To rescue modified Ad3 viruses, pAd3egf/H7 was digested with Asil. Linearized genomic DNA was transfected into HEp-2 cells grown in 30 mm dishes using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). The transfected cells were cultured at 37 °C/5% CO2 for 6–10 days and were examined for evidence of cytopathic effect daily. Cells were frozen and thawed for three cycles and fresh cultures of HEp-2 cells were infected with viral suspension. At 96 h post-infection, the virus was harvested and designated Ad3/H7. Finally a total of ten 100-mm dishes containing HEp-2 cells infected with Ad3/H7 were harvested. Viruses were purified by standard CsCl gradient centrifugation as described by Wu (Wu et al., 2002). The virus particle (VP) titers were determined by spectrophotometry using a conversion factor of 1.1 × 1012 VP per absorbance unit at 260 nm.

2.4. Growth characteristics and heat stability of Ad3/H7

To evaluate DNA replication efficiency of Ad3/H3 or Ad3/H7, quantitative PCR (qPCR) was performed with HAdV using a Q-PCR kit (South China JOV, Guangzhou, China). HEp-2 cells were seeded into 24-well plates at a density of 100,000 cells/well the day before infection. The cells were infected with 1 × 106 DNA copies of Ad3/H3, Ad3/H7 or Ad7, and the infected cells including the medium for each virus were harvested every 12 h. Viral genomic DNA copy numbers could then be determined by qPCR.

At the same time, HEp-2 cells were seeded into 96-well NUNC Optical bottom plates (ThermoFisher Scientific, Rochester, NY, USA) and infected with 1 × 105, 1 × 106 and 1 × 107 copies of Ad3/H3 or Ad3/H7, and cultured in RPMI Medium 1640 (GIBCO, Carlsbad, CA, USA) without phenol red and without serum for 48 h. The fluorescence was measured with a Varioskan Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA) by exciting at 488 nm and recording the emitted light at 507 nm. Background fluorescence was equalized to wells containing cells only.

To test the heat stability of Ad3/H7, Ad3/H3 and Ad7 viruses, 1 × 107 copies/ml of viral DNA were incubated at 45 °C for different periods before infecting HEp-2 cells. Viral genomic DNA copy numbers in infected cells were analyzed at 48 h post-infection.

2.5. In vitro neutralization

Viruses were injected into mice by the intraperitoneal (i.p.) route at a concentration of 1010 VPs/mouse. Control mice were injected with phosphate-buffered saline (PBS). There were five mice in each group. Mice were subjected to booster injections every two weeks for a total of three injections. At day 42 after the first immunization, blood samples were collected from the mice and sera were isolated and kept frozen for serology tests. Procedures for these animal experiments complied with all relevant federal guidelines and institutional policies.

Neutralization tests in vitro were performed to test the neutralizing effect of three antisera against Ad3/H3, Ad3/H7 and Ad7 viruses. All the antisera and preimmune serum (negative control) were serially diluted 2-fold in DMEM, and 50 μl aliquots of each dilution were mixed with 50 μl of virus with 100 TCID50. The antibody–virus mixtures were incubated for 1 h at 37 °C and then transferred to 96-well plates containing 85–95% confluent mono-
layers of HEp-2 cells. Monolayers were incubated for 72 h in the presence of 5% CO₂. The serum neutralization titer was defined as the reciprocal of the serum dilution that inhibited 50% of the cytopathic effect.

2.6. In vivo neutralization

Each mouse was injected with PBS or 2 × 10¹⁰ VPs of w-Ad3 or Ad7, Ad3/H7. Each group included 9 mice. Mice received a booster injection after two weeks. Twenty days later, each group of mice was subjected to an intranasal immunization of 2 × 10¹⁰ VPs of w-Ad3 or Ad7, Ad3/H7 per mouse, resulting in three mice for each subgroup. Three days later, the mice were sacrificed with CO₂ inhalation and the lungs were harvested, homogenized on dry ice, lysed in lysis buffer, and measured for Ad genome copies by qPCR using a Q-PCR kit. Procedures for these animal experiments complied with all relevant federal guidelines and institutional policies.

2.7. ELISA

For another in vivo neutralization experiment, each mouse also received twice i.p. injection of PBS or 2 × 10¹⁰ VPs of w-Ad3 or Ad7. Each group included six mice. Two weeks after the second injection, serum Ab titers from immunized mice specific for EGFP were measured by ELISA. Ninety-six well plates coated overnight with 100 μl/well of 1 μg/ml recombinant GFP (Millipore) in PBS were blocked for 2 h with PBS containing 2% BSA and 0.05% Tween 20. Sera were then added in serial dilutions and incubated for 1 h. The plates were washed four times, developed with tetramethylbenzidine, stopped with 2 M H₂SO₄, and analyzed at 450 nm with ELISA plate reader (Thermo Scientific Multiskan MK3).

2.8. Comparison of Ad7 and Ad3 genome

The whole genome of HAdV7-gz08 strain was sequenced by PCR and annotated using a previously described method (Purkayastha et al., 2005). The genome of HAdV3-GZ01 was sequenced before (GenBank Accession No. DQ099432.4) (Zhang et al., 2006, 2009). The whole genome comparisons and the main structural protein sequence alignments were performed with blastn or blastp from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/), and multiple sequence alignment was done with software MEGA4.1 and ClustalX (1.83).

3. Results

3.1. Generation of hexon chimeric vector pAd3egf/H7

The H7 gene obtained by PCR was used to replace the corresponding region of H3 in pBRH3S to obtain pBRH7S. This resulted in the replacement of H3 for amino acid residues 5–901. Because the N- and C-terminal regions of H3 are the same as that of H7, the resultant hexon gene was 100% identical to the native H7 gene. The chimeric hexon plasmids, pAd3egf/H7, were obtained by homologous recombination and confirmed by restriction enzyme analysis with BamHI, EcoRV, AsisI and KpnI (Fig. 1A). Viruses Ad3/H7 were rescued in HEp-2 cells and confirmed by PCR with the Ad7-specific primers, a7u and a7r (Fig. 1B). The hexon gene of virus Ad3/H7 was 100% identical to the native H7 gene by sequencing. Two clones were determined and shown in Fig. 1, the clone Ad3/H7-4 was used in the following work.

3.2. Growth characteristics of recombinant adenovirus

The replication of Ad3/H3, Ad7 and Ad3/H7 were compared by quantifying genomic DNA using a quantitative PCR method (Fig. 2A). The replication efficiency of the chimeric Ad3/H7 was approximately the same as that of Ad3/H3. We then examined the efficiency of gene transfer for Ad3/H7 in HEp-2 cells with the aid of the EGFP reporter. There was no apparent difference between

---

**Fig. 1.** Confirmation of the Ad3/H7 chimera. (A) Restriction enzyme analysis of the recombinant plasmid pAd3egf/H7. (1) pAd3egf/H7-4/BamHI, (2) pAd3egf/H7-4/EcoRV, (3) pAd3egf/H7-4/KpnI, (4) pAd3egf/H7-7/BamHI, (5) pAd3egf/H7-7/EcoRV, (6) pAd3egf/H7-7/KpnI, (7) pAd3egf/H7-4/AsisI and (8) pAd3egf/H7-7/AsisI. (B) Serotype-specific PCR analysis with H7 specific primers, a7u and a7r. (9) Virus Ad3/H7-4, (10) virus Ad3/H7-7, (11) negative control virus Ad3/H3, and (12) positive control virus HAdV7-gz08. M1: DL-5000 DNA marker; M2: DL-15000 DNA marker; and M3: DL-2000 DNA marker.
Ad3/H3 and Ad3/H7, suggesting that the gene transfer ability of Ad3/H3 was not reduced by hexon replacement with H7 (Fig. 2B). By comparing the heat stabilities of Ad3/H3 and Ad3/H7 we tested whether hexon replacement could affect the structural integrity of the virions. The viruses were incubated at 45 °C for different durations before infecting HEp-2 cells, and their replication efficiencies were compared. The replication efficiency of Ad3/H7 was not significantly reduced following a 10 min incubation at 45 °C, and its activity was retained at about 20% after a 30 min incubation at 45 °C, which was nearly the same as that of Ad3/H3 (Fig. 2C). This suggested that the chimeric Ad3/H7 virus was as stable as Ad3/H3.

Table 1 Neutralizing antibody titers from adenovirus-immunized mice.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Anti-Ad3/H3</th>
<th>Anti-Ad7</th>
<th>Anti-Ad3/H7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad3/H3</td>
<td>2048–4096</td>
<td>0*</td>
<td>64</td>
</tr>
<tr>
<td>Ad7</td>
<td>0*</td>
<td>4096</td>
<td>4096</td>
</tr>
<tr>
<td>Ad3/H7</td>
<td>64</td>
<td>4096</td>
<td>4096</td>
</tr>
</tbody>
</table>

* No detectable neutralization.

3.3. Ad3/H3 and Ad3/H7 were cross-neutralized in vitro

To determine whether Ad3/H3 and Ad3/H7, Ad7 could escape the host neutralization response, we performed in vitro neutralization assays using serum obtained from mice experimentally immunized with $10^{10}$ VPs/mouse of Ad3/H3, Ad7 or Ad3/H7. As shown in Table 1, the antisera were specific for the corresponding virus with titers ranging from 2048 to 4096. Serum from Ad3/H3-immunized mice had much lower but clearly detectable cross-reactive NAb titers against Ad3/H7. Serum from Ad3/H7-immunized mice had comparable NAb titers to Ad3/H7 and Ad7, but exhibited 30-fold lower NAb titers to Ad3/H3. Serum from Ad7-immunized mice had comparable NAb titers to Ad3/H7 and Ad7. As expected, there was no cross-neutralization between Ad3/H3 and Ad7. These data clearly demonstrated that in vitro Ad3- and Ad7-specific NAbs were principally directed against the hexons, although Ad3 fiber-specific NAbs were also present but at much lower titers than Ad3 hexon-specific NAbs.

3.4. Ad3/H3 and Ad3/H7 were cross-neutralized in vivo

We next examined the in vivo cross-neutralization using mice that were pre-immunized twice with $2 \times 10^{10}$ VPs of w-Ad3, Ad7, Ad3/H7 or PBS. As shown in Fig. 3A, the NAb titer of these mice serum arrived about 2048–4096 in vitro. Because Ad3 and Ad7 were ordinarily infected through respiration tract, the in vivo cross-neutralization of w-Ad3, Ad7 and Ad3/H7 was firstly examined by measuring the Ad genome DNA copies in lungs of mice immunized through intranasal infection. As shown in Fig. 3C, preimmunization of mice with one of Ad7 and Ad3/H7 significantly prevented subsequent infection of the other type in vivo. In contrast, preimmunization of mice with one of w-Ad3 and Ad3/H7 did not remarkably inhibit subsequent infection of the other type. These data suggest hexon-specific NAbs play a major role in the protective effect against infection of Ad3 and Ad7 in vivo.

Ad3/H3 and Ad3/H7 were also used as vectors expressing EGFP. We assessed EGFP-specific humoral immune responses following Ad3/H3 or Ad3/H7 infection using ELISA. Two weeks after the third injection with $2 \times 10^{10}$ VPs of Ad3/H3 or Ad3/H7, EGFP-specific serum Ab titers were measured by ELISA. As shown in Fig. 3B, anti-Ad7 immunity did not suppress Ad3/H3-elicited immune responses in mice. Ad3/H3 immunogenicity was greatly inhibited by w-Ad3 pre-immunization. However, Ad3/H7 vector-elicited immune responses were greatly suppressed not only by anti-Ad7 immunity, but also by anti-w-Ad3 immunity, although the NAb titers in vitro against Ad3/H7 of the mice preimmunized with w-Ad3 were much lower than that preimmunized with Ad7 (Fig. 3A). These data suggest that high titers of Ad3 fiber-specific NAbs may have an important role in suppressing Ad vector immunogenicity in vivo, which cannot be predicted by in vitro neutralization assays.
Fig. 3. Cross-neutralization in vivo of Ad3/H3 and Ad3/H7. BALB/c mice received two injections of \(2 \times 10^{10}\) VPs of w-Ad3, Ad7, Ad3/H7 or PBS. (A) Serum was obtained from these mice following a third infection and assessed for NAbs to Ad3/H3 or Ad7, Ad3/H7. (B) EGFP-specific ELISAs for serum obtained from mice immunized with Ad3/H3 or Ad3/H7 by i.m. injection. (C) Three days after booster injection with \(2 \times 10^9\) VPs of Ad3/H3 or Ad7, Ad3/H7 via intranasal infection, the mice were sacrificed and their lungs were harvested for the qPCR assay. Results are shown as the means (and standard deviations) for samples from three individual mice.

3.5. HAdV7-gz08 DNA sequence and comparison with HAdV3-GZ01

The complete genome of HAdV7-gz08 (GenBank Accession No. GQ478341) was 35,306 bp with a base composition of 25.2% G, 25.8% C, 25.4% A and 23.6% T. The genome of Ad7 had a G + C content of 51%, similar to the G + C content of other members of species B adenoviruses (50–52%). The genome of HAdV7-gz08 had only one or two amino acid changes in the major structural proteins compared with the prototype HAdV-7 Gomen strain (Purkayastha et al., 2005). The genomes of strains HAdV7-gz08 and HAdV3-GZ01 share 97% identity. The hexons, fibers, and all other structural proteins coding sequences of strains HAdV7-gz08 and HAdV3-GZ01 were 96, 57 and >99% identical at amino acid level, respectively. There were four single amino acid substitutions in the penton base, three substitutions in pV1, four in pVIII, five in pV1a2, a single change in pVII and no change in pIX. Hexon protein sequences of HAdV3-GZ01 and HAdV7-gz08 were analyzed by multiple sequence alignment. Four regions of variability were identified; they were in amino acids 139–143, 175–180, 268–275 and 416–441 of HAdV3-GZ01, which were respectively located in HVR-1, -2, -5 and -7 in the hexon structures, and may contribute to immunogenicity (Crawford-Miksza and Schnurr, 1996; Rux et al., 2003) (Fig. 4). In the rest of the hexon sequences, there were six single amino acid substitutions.

The fiber sequences of HAdV3-GZ01 and HAdV7-gz08 were also analyzed using the blastp program. It was found that the identity of them was low (58%), but the fibers of HAdV7-gz08 and some strains of subspecies B2, such as HAdV11, HAdV14 and HAdV34a, were greater than 92% identical.

4. Discussion

For adenovirus vaccine development, it is important to determine the neutralizing antigens of pathogenic adenovirus types, such as Ad3, Ad4 and Ad7. Any of the major capsid proteins may be recognized by NAbs (Fender et al., 1995; Hong et al., 2003; Liebermann et al., 1998). It has been previously reported that hexon is the major target of adenovirus NAbs (Pichla-Gollon et al., 2007; Sumida et al., 2005; Wu et al., 2002; Youil et al., 2002). The present experimental data in vitro showed that up to 97% of the NAbs produced by mice that were infected with Ad3 and Ad7 were hexon-specific. The known genome sequences of the two strains made it simple to analyze the results of neutralization experiments. Comparative analysis of Ad3 and Ad7 structural proteins showed that major differences were only observed in the fiber and hexon. The fiber of Ad3 led to weak neutralization and no cross-reaction with Ad7. The penton base protein did not lead to neutralization and was unable to react with NAbs. The fiber-specific NAbs accounted for less than 3% of the total NAb pool in vitro, which partly explains the results of other investigators who have reported that fiber-specific NAbs could be detected at high titers in human sera, but not in sera with lower neutralizing titers (Nanda et al., 2005). In the current experiments it could not be concluded whether the fiber of the Ad7 virus can induce neutralizing antibodies. To construct the recombinant type 3 virus entirely escaping the host neutralization response, the fiber domain needs to be reconstructed to delete such activity. The fiber of virus Ad3/H7 could be replaced with the fiber of Ad7 in the next stage, to produce a recombinant virus with almost identical structural proteins of the Ad7 virus.

Inconsistent with the in vitro neutralization results and previous study using rAd5 vectors (Sumida et al., 2005), preimmunization of w-Ad3 could markedly suppress Ad3/H7-elicited EGFP-specific Ab response in vivo. The first possible reason is that Ad3 fiber-specific NAbs may play a more important role in vivo than in vitro, perhaps reflecting different mechanisms of virus neutralization from Ad5. Unlike members of other adenovirus species that bind to the cell surface receptor, coxsackie and adenovirus receptor (CAR), members of species B recognize the membrane co-factor proteins CD46, CD80 or CD86 as a cellular receptor (Short et al., 2006; Sirena et al., 2004). The second possible reason is the method. Different from the previous studies using adoptive transfer studies, we used mice preimmunized with Ad following injection. The suppression might
be led to not only by Ad-specific humoral immune responses but also by Ad-specific cellular immune responses in this study. Different immunization routes may also contribute to different results. Ad3/H3 or Ad3/H7 vectors expressing EGFP were injected to mice via i.m. A previous study also suggested that in vitro neutralization assays might not reliably predict the effects of virus-specific antibodies on adenovirus vectors in vivo, and the mice were injected via i.m. with AdC68 or AdCDQ vectors in the study (Pichla-Gollon et al., 2009). These data suggest that Ad3 and Ad7 neutralization in vivo is a more complex process than in vitro neutralization. In another experiment of Ad genome copies measurement in mice, hexon-specific NAbs significantly prevented subsequent infection of Ad via intranasal tract in vivo, but limited to the number of each group mice, it was not well-defined whether preimmunization of mice with w-Ad3 and Ad3/H7 could prevent subsequent infection of the other type. More profound researches may be necessary for understanding the Ad3 and Ad7 fiber-specific neutralization mechanism in vivo. However, the capability of the fiber of virus particles inducing neutralization is still weak compared with hexon in vivo, therefore it may be more efficient to use hexon as a target in vaccine development.

Although specific sites recognized by NAbs have been proposed to reside within small regions of the hexon that are exposed to the capsid surface and possess sequences that vary among serotypes, they have only been identified in a few adenoviruses (Rux and Burnett, 2000). The main difference was seven highlighted HVRs, in which HVR7 could be further subdivided into three highly variable regions (Crawford-Miksza and Schnurr, 1996; Rux et al., 2003). Among the HVRs of adenoviruses, only a few are type-specific NAb recognition sites (Pichla-Gollon et al., 2007; Rux and Burnett, 2000). Sequence alignment (Fig. 3) clearly demonstrated that there was no difference within HVR-3, -4 and -6 between the Ad3 and Ad7 hexon, with variation only in HVR-1, -2, -5 and -7. Types 3 and 7 adenovirus hexon-neutralizing epitopes should be determined by one or more of the four HVRs. Yuan et al. (2009) used homology modeling and an epitope mapping method to determine five neutralizing epitopes of the adenovirus type 3 Harbin04B strain hexon (GenBank Accession No. EU078562). A comparison with hexon sequences of the strains used in this study indicate they may be the same hexon HVRs contained in strain Harbin04B serotype 7. Work is currently progressing to exchange the HVR-1, -2, -5 and -7 between Ad3 and Ad7 to distinguish the neutralizing epitopes and construct a chimeric hexon embodying neutralizing epitopes of both serotypes.

Previous studies have suggested a strategy for engineering novel Ad5 vectors through replacement of the hexon to evade dominant Ad5-specific NAbs (Gall et al., 1998; Wu et al., 2002). In this study, the chimeric recombinant Ad3 virus whose hexon was replaced with the same subspecies Ad7 hexon could be generated, and the replacement had no apparent effect on the replication efficiency, thermostability and gene transfer ability. In our another experiment, a recombinant adenovirus plasmid pAd3egf/H4 was successfully constructed by replacing Ad3 hexon gene of pAd3egf with Ad4 hexon (Group E), but chimeric virus Ad3/H4 could not be rescued, despite several attempts with several different plasmid clones (data not shown). This may be the major difference among the virus structural proteins of various species, resulting in defective packaging. The incompatibility to hexon of other Ad species agrees with a study based on the Ad5 vector (Youil et al., 2002). The potential incompatibility of various species of Ad may influence Ad evolution mode. We also constructed a recombinant Ad3 expressing H7 which gene was inserted into the E3 region of A3/H3, but we did not know whether H7 was embeded in the Ad3 capsid. Roberts et al. (2006) obtained a modified Ad5 that successfully evaded the role of Ad5 neutralizing antibodies by using Ad48 hexon HVRs to replace the original HVRs of the Ad5 hexon. Our study and the results of others (Matthews et al., 2010; Roberts et al., 2006) indicate that the HVRs are the major targets of Ad-specific NAbs, and modification of specific HVRs may be a more effective hexon gene-switch strategy. This strategy can be used to develop adenovirus vectors able to evade pre-existing humoral immunity. A multivalent adenovirus vaccine inducing NAbs against several serotypes could also be constructed in this way.

Acknowledgments

This work was supported by the National Nature Science Foundation of China (NSFC, 30770102), School of Guangzhou Research Projects (No. 10A006D) and the Mérieux Foundation in France. We thank Zifeng Yang, Wenda Guan, Tianhua Zhong, Rong Liu and Sheng Qin of State Key Lab of Respiratory Disease for their kind help with this work.
References