Virus Research 208 (2015) 66–72

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Mapping the epitope of neutralizing monoclonal antibodies against human adenovirus type 3

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ARTICLE INFO

Article history:
Received 24 March 2015
Received in revised form 30 May 2015
Accepted 1 June 2015
Available online 9 June 2015

Keywords:
Human adenovirus type 3
Neutralizing
Monoclonal antibody
Epitope
Hypervariable region 4

ABSTRACT

Human adenovirus type 3 (HAdV-3) has produced a global epidemic in recent years causing serious diseases such as pneumonia in both pediatric and adult patients. Development of an effective neutralizing monoclonal antibody (MAb) and identification of its neutralizing epitope is important for the control of HAdV-3 infection. In this study, three neutralizing MAbs were generated, of which MAb 3D7 had a high neutralization titer of 4096 (approximately 0.5 μg/ml) against HAdV-3 infection. In indirect enzyme-linked immunosorbent assays, all three MAbs specifically recognized HAdV-3 virus particles and hexon protein, but did not react with the virus particles or the hexon protein of HAdV-7. Analyses using a series of peptides and chimeric adenovirus particles of epitope mutants revealed that all three MAbs bound to the same exposed region (amino acid positions 244–254 of hexon) in hypervariable region 4 (HVR4), which is highly conserved among global HAdV-3 strains. The amino acids T246 and G250 may be the critical amino acids recognized by these MAbs. MAb 3D7 reduced the recombinant enhanced green fluorescent protein-expressing HAdV-3 (rAd3EGFP) load recovered in the lungs of mice at 3 days post-infection. The generation of MAb 3D7 and the identification of its neutralizing epitope may be useful for therapeutic treatment development, subunit vaccine construction, and virion structural analysis for HAdV-3.

1. Introduction

Human adenoviruses (HAdV) can cause a broad spectrum of diseases in both pediatric and adult patients, such as acute respiratory infection, acute gastroenteritis, and epidemic keratoconjunctivitis (Lenaerts et al., 2008; Sandkovsky et al., 2014). To date, seven species including more than 68 genotypes have been characterized and defined by genomics and bioinformatics (Dehghan et al., 2012; Robinson et al., 2013). Specific species genotypes are often associated with particular clinical manifestations. HAdV species C (HAdV-1, -2, -5, and -6), species B (HAdV-3, -7, -14, and -55) and species E (HAdV-4) are most commonly found in patients with respiratory infection. Among these, HAdV-3 strains of subspecies B1 are the major epidemic strains responsible for severe respiratory disease epidemics and outbreaks worldwide (Yun et al., 2014; Lu et al., 2014; Barrero et al., 2012; Alkhalaf et al., 2015; Ampuero et al., 2012; Guo et al., 2012; Deng et al., 2013; Lai et al., 2013; Lee et al., 2015; Zhang et al., 2006).

Currently, there is no effective treatment or vaccine against HAdV-3 infection. Neutralizing monoclonal antibodies (MAb) may be a promising prophylactic or therapeutic medicine against viral disease. The creation of neutralizing MAb could also be useful for identifying neutralizing epitopes, which is of great importance in the molecular design of vaccines. The adenovirus capsid icosahedron is composed of three major structural proteins: hexon, penton base, and fiber. The hexon protein is the major antigenic determinant recognized by neutralizing antibodies (NAbs) (Tian et al., 2011; Yu et al., 2013; Wu et al., 2002). Type-specific epitopes on hexons have been proposed to reside within seven highly variable regions (HVRs), of which HVR7 can be further subdivided into three

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http://dx.doi.org/10.1016/j.virusres.2015.06.002
0168-1702/

additional highly variable regions (Rux et al., 2003; Bradley et al., 2012; Yuan et al., 2009). Our previous study demonstrated that HVR1, 2, 5, and 7 of HAdV-3 contained neutralizing epitopes (Qiu et al., 2012).

In this study, we report three neutralizing MAbs against HAdV-3, including MAb 3D7, which has a strong neutralizing capacity. Epitope mapping revealed that all three MAbs recognize the HVR4 of HAdV-3 hexon. The findings of this study will be useful for the development of prophylactic or therapeutic treatments against HAdV-3 infection and for adenovirus structural analysis.

2. Materials and methods

2.1. Virus strains and cells

The following strains of adenoviruses used in this study were obtained as previously described (Tian et al., 2011; Qiu et al., 2012; Zhang et al., 2009): recombinant adenovirus rAd3EGFP encoding a HAdV-3 GZ-01 genome and an enhanced green fluorescent protein (eGFP) with an E3 region deletion, hexon chimeric adenovirus rAd3egf/H7 generated by replacing the HAdV-3 hexon gene of the rAd3EGFP with the hexon gene from HAdV-7 GZ08 strain, epitope mutants (rAd3H7R1, rAd3H7R2, rAd3H7R5, and rAd3H7R7) from rAd3egf/H7 replaced with corresponding HAdV-3 epitopes, and epitope chimeric mutant rAdMHE3 from rAd3EGFP replaced with HVR5 of HAdV-7. All the adenoviruses were cultured in HEp-2 cells or AD293 cells as previously described (Tian et al., 2011). Adenovirus particles were purified by standard CsCl gradient centrifugation as previously described (Wu et al., 2002). The virus particle (VP) titers were determined by spectrophotometry using a conversion factor of 1.1×10^{12} VPs per absorbance unit at 260 nm (Tian et al., 2011).

2.2. Generation of the HVR4 mutants rAd3A7R4 and rAd3H7A3R4

The plasmid pBRAdΔE3GFP encoding a HAdV-3 GZ-01 genome (Genbank accession no. DQ099432) and eGFP with an E3 region deletion was constructed, as previously described (Zhang et al., 2009). The hexon-chimeric adenovirus vector pAd3egf/H7, in which the HAdV-3 hexon gene in the pBRAdΔE3GFP vector was replaced with the hexon gene from HAdV-7, was constructed as previously described (Tian et al., 2011). The shuttle vector pBRLR was also constructed as previously described (Qiu et al., 2012). In this study, the HVR4 mutants rAd3A7R4 and rAd3H7A3R4 were obtained using the same strategy as previously described (Qiu et al., 2012). Briefly, the mutated fragment H7A3R4 was produced by overlapping PCR extension mutagenesis with primer pairs A3HVR4u/HexD, A3HVR4r/HexU, and HexU/HexD, using pAd3egf/H7 as the DNA template. The mutated fragment H3A7R4 was produced by overlapping PCR extension mutagenesis with primer pairs A7HVR4u/HexD, A7HVR4r/HexU, and HexU/HexD, using pBRAdΔE3GFP as the DNA template. Then, the fragments H7A3R4 and H3A7R4 were cloned into pBRLR to generate shuttle vectors pBRLR-H7A3R4 and pBRLR-H3A7R4. Finally, the LR-H7A3R4 fragment and the LR-H3A7R4 fragment were cloned into the pAd3egf/H7 vector to generate the HAdV-7 hexon HVR4 mutagenesis vector pBRAd3-H7A3R4 and the HAdV-3 hexon HVR4 mutagenesis vector pBRAd3-A7R4, respectively, using homologous recombinant technology in *Escherichia coli* strain BJ5183. The successful creation of these constructs was confirmed by restriction digestion and sequencing analyses.

To rescue viruses, these modified plasmids were digested with AsisI to linearize genomic DNA, then transfected into AD-293 cells grown in 30-mm dishes using Lipofectamine LTX with Plus reagents (Invitrogen, USA) according to the manufacturer's instructions. The transfected cells were cultured at 37 °C with 5% CO_2 for 6–10 days and were examined daily for evidence of cytopathic effect. The 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 viruses were harvested and designated rAd3A7R4 and rAd3H7A3R4. Finally, the mutant viruses were cultured with AD-293 cells in a total of twenty 100-mm dishes, then harvested and purified by standard CsCl gradient centrifugation as described above. The full-length modified hexon genes of the viruses were identified by sequencing.

2.3. Virus neutralization test

Purified rAdMHE3 virions were used to immunize BALB/c mice and to screen the resulting MAbs. Production and selection of mouse monoclonal antibodies against rAdMHE3 were performed as described previously (Liu et al., 2014). For *in vitro* adenovirus neutralization experiments, MAbs were serially diluted 2-fold in Dulbecco's modified eagle's medium (DMEM) (Gibco, China) and 50-μl aliquots of each dilution were mixed with 50-μl recombinant adenoviruses with 2×10^5 VPs. The antibody-virus mixtures were incubated for 1 h at 37 °C and then transferred to 96-well plates containing 85–95% confluent monolayers of HEp-2 cells. Monolayers were cultured in RPMI Medium 1640 (Gibco) without phenol red and serum for 72 h. Infected cells were analyzed using a Varioskan Flash Multimode Reader (Thermo Scientific) to measure the eGFP expression. Neutralizing titers were defined as the minimum concentration of MAb that inhibited 90% of the eGFP expression.

2.4. Indirect enzyme-linked immunosorbent assay (ELISA) analysis

HAdV-3 and HAdV-7 hexon peptides with a hexahistidine tag (designated A3H and A7nH, respectively), and the recombinant short peptides (HAdV-3 HVRs) with an N-terminal glutathione S-transferase (GST) tag were expressed and purified as described previously (Tian et al., 2013).

For ELISAs, 96-well plates (Nunc Maxisorp) were coated overnight at 4 °C with fusion peptides (about 2 μg/ml) or virus particles (about 10^{10} VPs/ml) in PBS (pH 7.4) and were washed once with 0.05% Tween-20 in phosphate-buffered saline (PBST) and blocked for 2 h with 2% bovine serum albumin (BSA) in PBST. Then, 100-μl/well MAb ascites at a dilution of 1:5000 were added to each well and incubated for 1 h at 37 °C. The plates were washed three times with PBST and incubated for 1 h with a 1:10,000 dilution of goat anti-mouse IgG (H + L)-HRP conjugated affinity-purified secondary antibody (Bio-Rad). After washing four times with PBST, the plates were developed with tetramethylbenzidine (TMB) substrate, the reaction was stopped with 2 M H_2SO_4, and the results were analyzed at 450 nm using an ELISA plate reader (Thermo Scientific Multiskan MK3).

2.5. Peptide competition ELISA

The epitope detected by each MAb was confirmed by competitive inhibition ELISA. Optimized concentrations of the MAbs were determined by serial dilution. Briefly, the HAdV-3 GZ01 virions in PBS (pH 7.4) were used to coat 96-well plates overnight at 4 °C. In separate tubes, constant concentrations of MAb at a final dilution of 1:5000 were added to increasing concentrations of competitor peptide (0, 3.125, 12.5, 50, or 200 μg/ml) in PBST with 2% BSA and incubated for 30 min at 37 °C. The virion-coated plates were washed once with PBST and incubated with 2% BSA in PBST for 2 h at 37 °C. Then, each of the MAb-peptide mixtures was added to duplicate wells, and the plates were incubated for 1 h at 37 °C. The