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Adenovirus 36 antibody detection: Improving the standard serum neutralization assay

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Adenovirus 36 (AdV36) causes weight gain in animal models, including non-human primates. In humans, AdV36-neutralizing antibodies are associated with adiposity; however, longitudinal studies in large populations are needed to clarify AdV36's contribution. The current gold standard for detection of AdV36-specific antibody is the serum neutralization assay (SNA), which requires long incubation times and highly trained personnel. The standard SNA was modified using an immunocytochemical (ICC) approach, which allows for a more rapid and objective assessment of AdV36 antibodies. Using the ICC assay, virus-infected cells were detected as early as day 1 (D1) and by D5 were detected in 100% of microtiter wells versus 20.3% of wells detected by observing the cytopathic effect. Further, human sera tested with the ICC assay at D5 had a sensitivity and specificity of 80.0% and 95.7%, respectively, when compared to the standard SNA read at D11. Thus, the ICC assay decreased assay incubation time, provided a more objective and easily interpreted assessment, and had a high degree of sensitivity and specificity in determining serological status. The more rapid and objective ICC method will make large population studies feasible, improve comparability among laboratories, and contribute to understanding the role of AdV36 in obesity.

1. Introduction

Adenovirus-36 (AdV36) infection is responsible for the development of adiposity in several animal species, including mice, rats, chickens, and non-human primates (marmosets) (Dhurandhar et al., 2000, 2002; Pasarica et al., 2006). Natural exposure to AdV36 as determined by the presence of Adv36-specific neutralizing antibodies has also been linked to increased adiposity in children (Atkinson et al., 2010; Na et al., 2010; Gabbert et al., 2010) and adults (Dhurandhar et al., 1997; Atkinson et al., 2005; Trovato et al., 2009, 2010, 2012; Pasarica et al., 2008; Salehian et al., 2010; Lin et al., 2013). These studies typically show a higher prevalence of serum antibodies to AdV36 in overweight or obese individuals. Even among persons of normal weight, the AdV36-positive individuals were significantly heavier than those without AdV36 (Atkinson et al., 2005). In addition, two studies which also showed an association between the presence of Adenovirus antibodies and increased adiposity were based on the ELISA (Almgren et al., 2012; Aldhoon-Hainerová et al., 2014), which is not specific for AdV36 (Dubuisson et al., 2015). In contrast, three notable exceptions found no association between AdV36 and adiposity. These studies were done with military personnel (Broderick et al., 2010), as well as Korean (Na et al., 2012) or Dutch/Belgian adults (Goossens et al., 2011). Finally, to date three meta-analyses (Yamada et al., 2012; Shang et al., 2014; Xu et al., 2015), including the most recent using 10,000 subjects,

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show that exposure to Ad36 is associated with a two-fold or greater risk of obesity in humans.

Although natural exposure to Ad36 is significantly linked with human obesity, experimental infections with AdV36 and their adiposity related outcomes cannot be directly demonstrated in humans due to ethical constraints. Thus, the accumulation of circumstantial evidence regarding AdV36 effects will rely on large, longitudinal studies designed to detect naturally-occurring infection and its subsequent outcomes. Such studies will necessarily require testing hundreds of subjects over time to monitor for newly-acquired AdV36 infections—a goal that will be impractical unless a more rapid, specific assay system can be developed.

The SNA for AdV36 is the current gold standard in detecting neutralizing antibodies to the virus. This standard approach to detecting neutralizing (specific) antibody was modified for AdV36, principally by extending the incubation time to allow for slow viral growth and subsequent recognizable damage to cells (Dhurandhar et al., 2000). This method used the AdV36-permissive, A549 cell line to detect the cytopathic effect (CPE) of virus infection. The assay requires an especially long incubation of 11–13 days without changing the medium, a procedure which stresses cells and risks microbial contamination. Also, recognition of CPE, which is subjective and often subtle, requires highly-trained personnel. Thus, the complexity, time and expense of the assay may preclude many laboratories from undertaking AdV36 studies. Further, the inherent difficulties of the assay and subjectivity of the assessment pose problems in comparing results from different laboratories (Goossens et al., 2011; Atkinson, 2011). Thus, an improved and reproducible method is critically needed to conduct population studies essential in moving the AdV36 field forward.

A method to more rapidly determine plaque-forming units was described (Bewig and Schmidt, 2000) and adapted to a commercially-available kit (Rapid RCA Assay Kit, Cell Biolabs, Inc., San Diego, CA). This kit was recently used along with the standard AdV36 SNA assay (11–14-day incubation) to visualize virus-infected cells in addition to assessment of CPE (Dubuisson et al., 2015). The AdV36 status of human sera (n = 31) was determined by the standard 13-day assay and compared to the presence of virus-stained cells after 2, 5, 8 and 11 days of incubation. Concordance between the assays was reported as 87.2% at day 5 (D5), a percentage which increased with longer incubation times. Both assays reached >97% concordance by D9 of cell culture incubation.

While this immunocytochemical (ICC) method appears to have some obvious advantages, it has not been fully characterized nor have sensitivity and specificity of the assay been reported. A similar virus-staining method has been used to: expand the number of subjects tested; provide a working definition for positive wells; increase the number of experiments done to establish the optimal cell culture incubation time; and determine the virus detection limit of the standard versus ICC assay. The ICC assay has also been adapted for use in other types of AdV36 experiments by using a soluble substrate, which can be read spectrophotometrically. Finally, the detection limits of the non-soluble and soluble substrates have been compared.

The present data show that the ICC method decreases the cell culture incubation time to as few as six days (compared to 11 to 14 days for the standard SNA), while maintaining a high level of specificity with only minor differences in the virus detection limit. Further, stained cells (ICC) allowed for a more objective and more easily interpreted endpoint, which should improve comparability among laboratories. Lastly, both ICC substrates (soluble and insoluble) performed equally well in detecting virus-infected cells and can be used in a variety of experimental designs. Thus, the ICC assay should make the study of AdV36 more easily accessible to investigators and improve the capacity of laboratories to engage in large population studies.

2. Materials and methods

2.1. Serum samples

Sera were collected from New Zealand White rabbits (n = 3) immunized with three injections of *Adenovirus-36* (total of approximately 3×10^9 virus particles). Briefly, virus was mixed with Freund's Complete Adjuvant for the first injection and Freund's Incomplete Adjuvant for the two additional injections. Control rabbits (n = 3) were injected with adjuvant alone and were housed separately from rabbits injected with virus. Injections and clinical monitoring of rabbits were done by the veterinary staff. Neutralizing antibodies were determined with the SNA before and after each immunization and reached high titers by weeks 8 and 9 (data not shown). Sera collected at each time point were aliquoted and stored at −80 °C. The rabbit immunization protocol (#14-018) was approved by the Animal Welfare Committee at the University of Texas Health Science Center at Houston.

As part of a previous study (PI, S. Day), human sera were collected and then tested using the standard SNA (Dhurandhar et al., 2000) in the laboratory of Nikhil Dhurandhar (Pennington Biomedical Research Center, Baton Rouge, LA). In the present study, a subset of these sera (n = 123) were selected to represent individuals who were serologically-positive or negative for AdV36 in the SNA. The study was approved by the Committee for Protection of Human Subjects at the University of Texas Health Science Center (HSC-SPH-10-0240). Serum titer was defined as the highest dilution yielding no evident cytopathic effect (CPE), and a titer of 1:8 or greater was considered positive. Sera were aliquoted at the time of collection and stored at −80 °C. For the present study, sera were thawed and used immediately; all sera were tested in duplicate (or more) wells. All laboratory work was approved (#HSC-10-066 and IBC-15-085) by the Biosafety Committees at the University of Texas Health Science Center and Pennington Biomedical Research Center.

2.2. Virus growth and immunocytochemical staining

Tissue culture plates (Costar Cell Culture Plate, Corning, Inc., Corning, NY) were set up as in the standard SNA. Briefly, AdV36 stock was diluted in complete DMEM (DMEM containing 10% fetal bovine serum + 1% antibiotic solution) and adjusted to a desired concentration. Virus (100 μl containing 100 tissue culture infectivity dose (TCID)) was added to the first well of a 96-well tissue culture plate and two-fold serial dilutions were made in the remaining wells. A549 cells (CCL-185, ATCC, Manassus, VA) were grown overnight in a T-75 flask (Becton Dickinson Labware, Franklin Lakes, NJ) in complete DMEM, harvested with 0.05% trypsin-EDTA (Gibco, Invitrogen, Grand Island, NY), and suspended in 50 ml complete DMEM. Approximately 2×10^4 cells (100 μl) were added to each well of the tissue culture plate. Control wells contained cells without virus. The plate was then incubated in 5% CO_2 at 37 °C. After incubation, 100 μl of cold methanol (4 °C) was added to each well for 10 min before being replaced by 200 μl of 0.15 M phosphate buffered saline, pH 7.2, containing 0.1% Tween 20 (PBS-T, Fisher Scientific, Fair Lawn, NJ). Plates were sealed and stored at 4 °C for up to one month prior to staining.

To stain virus-infected cells, PBS-T was removed, and wells were blocked with 100 μl of 1% fetal bovine serum (FBS, Sigma-Aldrich Chemicals, St. Louis, MO) or undiluted Superblock (SB; Thermo Scientific, Rockford, IL) for one hour at room temperature. Plates were emptied, and 100 μl of unlabeled goat anti-hexon (from *Adenovirus* 5) antibody (AbD Serotec, Oxford, UK; diluted 1:1000 in