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Adenovirus 36 Antibody Detection: Improving the Standard Serum Neutralization Assay

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Abstract

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. We have modified the standard SNA using an

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ROLES AND RESPONSIBILITIES OF THE AUTHORS

Author roles and responsibilities in the project and manuscript preparation are given. Each author is in agreement with the manuscript's submission and has participated in revisions prior to submission.

Cynthia Chappell—designed and carried out all of the experiments described herein; analyzed the data and created the tables and figures presented; prepared the draft manuscript and revisions.

Mary Dickerson—immunized rabbits and collected serum used in the study.

Sue Day—the PI responsible for the collection of the human sera used in the study.

Olga Dubuisson—Conducted the technical aspects of the standard SNA on all human sera used in the study.

Nikhil Dhurandhar—supervised the SNA conducted by Dr. Dubuisson and certified Dr. Dubuisson's results for the standard SNA for all sera.

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.

Keywords

serological assay; immunochemical staining; ELISA; obesity; adiposity; infectobesity

1.0 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-Hainerova 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 [Goosens 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, 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. In the present study, we have used a similar virus-staining method 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. We have also adapted the ICC assay 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.

Our 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.

2.0 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 minutes before being replaced by 200 μl of 0.15M 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 μ l of unlabeled goat anti-hexon (from *Adenovirus 5*) antibody (AbD Serotec, Oxford, UK; diluted 1:1000 in blocker) was added to each well and incubated on a shaker at room temperature for one hour. The plate was then washed three times using a MultiWash III automated plate washer (TriContinental Scientific, Inc., Grass Valley, CA). Horseradish peroxidase (HRP)-conjugated anti-goat IgG (Pierce, Thermo Fisher Scientific; diluted 1:2000 in blocker) was added to wells and incubated as above. Wells were emptied, and 100 μ l of 3,3'-diaminobenzidine (DAB) substrate (5 mg/ml, Amresco Inc, Solon, OH) activated with 0.03% hydrogen peroxide (Fisher Scientific) was added to each well. Plates were incubated at room temperature for 3–5 minutes until dark staining was visualized microscopically in virus-exposed cells. Substrate was then replaced with 100 μ l of PBS-T, each well was assessed visually for stained cells using an EVOS $\times$ 1 inverted microscope (AMG, Bothwell, WA), and digital photomicrographs were taken. One investigator read and scored all wells and was blinded to the results of the standard SNA. Wells were scored as positive if ten or more stained cells were seen in the well.

As an alternative to the insoluble substrate, pre-activated 2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; MP Biochemicals, Solon, OH) was added to each well and incubated at room temperature for 10–30 minutes. Reactions were stopped with 5% sodium dodecyl sulfate (Ambion, Thermo Fisher Scientific), respectively. Plates were then read spectrophotometrically (SpectraMax i3, Molecular Devices, Sunnyvale, CA) at A414 nm (ABTS).

3.0 RESULTS

3.1 Detection of Adenovirus 36-infected cells

Time course—Cells with or without 100 virus particles (VP) per well were incubated for 2, 7 and 20 hours to determine how early cell infection could be detected. No staining was seen in any of 18 replicate wells at 2 or 7 hours (data not shown). By 20 hours, a few cells in each well had light staining in the cytoplasm (Figure 1A), and 3–5 stained cells per well had a characteristic rounding and dark cytoplasmic staining indicative of infection by virus (data not shown). The progression of viral growth was examined at days 3, 5 and 13 post inoculation with 100 VP (Figure 1). On day 3 (D3), most cells appeared to be healthy with some subtle cytopathic changes in one or two areas of the well. Most of the monolayer was indistinguishable from control wells (i.e. without virus). However, after DAB was added, several darkly-stained cells in the well were easily distinguished from the background monolayer (Figure 1B). By D5, the number of stained cells increased dramatically and were typically spread widely across the still-intact monolayer (Figure 1C). Non-specific staining remained low in wells containing cells but no virus (Figure 1D). By D13, CPE was evident in the majority of wells and was enhanced by staining (Figure 1E). Stained cells were also numerous in areas of the monolayer where CPE was not evident. Of note, small DAB precipitates could occasionally be seen in control wells containing cells but no virus. Most were easily distinguished by size, shape and number from positively-stained cells in wells inoculated with virus at the same time period. Nevertheless, we were conservative in judging

a well positive for virus and defined positive as the presence of ten or more characteristically rounded and stained cells per well.

3.2 Detection limit of the ICC method

The lengthy incubation time for the standard SNA ensures that even a small number of virus particles in the well will have ample time to replicate to the level of detection. This method, although long and technically challenging, is sensitive to low inocula in the range of 10 VP or less (Table 1). SNA positivity depends on the development of a recognizable CPE caused by viral replication; whereas, the ICC indicates viral proteins in a cell detected with a chromophore label. Hence, the detection of visible CPE requires longer time to develop compared to the ICC, which can be readily detected after virus particles replicate in cells.

We hypothesized that using ICC to stain infected cells prior to CPE formation would decrease the incubation time needed without compromising accuracy. To this end, we inoculated different numbers of virus particles per well and incubated plates for various periods of time before fixing and staining cells. In wells receiving 100VP, the ICC assay was 100% positive by D3 and 94% in wells receiving as few as 25 VP. In contrast, only 21.3% (10 of 47) of wells receiving 100 VP had detectable CPE at D5 (data not shown).

The two assays also differed in virus detection limit. In the ICC assay, while ~3 inoculated VP per well could be detected in some wells at D3, the percentage of positive wells was low (23%), a number that increased with incubation time. By D6, the majority of wells (50–87%) inoculated with 6–12 VP were positive. In comparison, 11 days of incubation were needed for CPE to develop in most wells (65%) receiving low inocula (~3 VP) (Table 1). Thus, the detection limit of the D11 SNA was 2–4 times lower than in the ICC assay at D6, a trade-off of some sensitivity for a more rapid endpoint. Together, these data show that the ICC assay is reproducible and allows for detection of virus replication in half the incubation time recommended for the standard SNA, even at VP inocula of 12 or less.

3.3 Validation of the ICC assay with human sera

The ICC assay was compared to the standard SNA by testing 123 human sera from individuals who were previously tested using the conventional SNA (Figure 2). For the ICC assay, plates were fixed and stained as described at day 5 or day 6 post inoculation and assessed microscopically. Each serum was run in duplicate, and 26 (21%) sera were confirmed by a repeat experiment. The serostatus of four sera could not be determined due to discrepancies among ICC assays repeated 2–3 times. All of these indeterminate sera were positive by the SNA; however, positive and negative results were seen in repeated ICC assays of the four sera. Of the 119 sera which yielded interpretable results, 106 (89%) were in agreement between the SNA and ICC assays. Thirteen sera were discrepant, and ten of these were SNA-positive but negative in the ICC assay. Interestingly, seven of these ten sera had the lowest positive SNA titer (1:8) with the remaining three having a titer of 1:16. Overall, the positive predictive value of the ICC assay was 93%, and the negative predictive value was 86.8% relative to SNA.

Alternative endpoint using soluble substrate—For some experiments, parametric data may be valuable since means and standard deviations can be calculated. To compare the non-soluble versus soluble substrates, plates were divided in half and each half was inoculated with the same number of cells and virus titrations. At the end of the incubation period, one side of the plate was developed with DAB and the other side with ABTS (Figure 3). In ABTS wells, the presence of virus correlated in a dose-dependent manner with the absorbance value. Both substrates performed equally well in identifying virus-infected wells and were positive in wells originally inoculated with low numbers of virus particles. Detection limits showed a median of ~8 VP (range 3–16 VP) among five replicate experiments, each with virus concentrations tested in triplicate. Of note, replicate wells varied in their absorbance values given the degree of cellular damage in the well. Extensive damage and loss of infected cells from the monolayer as that seen with lengthy incubations yielded absorbances that were falsely low and not indicative of infection. Thus, this endpoint had a greater utility with shorter cell culture incubations (Day 3–6), i.e. at a time when virus had replicated but the monolayer had been largely preserved.

4.0 DISCUSSION

We undertook this study to develop a more rapid, objective and reproducible assay for the detection of serum antibody to AdV36. Our results demonstrate that the ICC assay can decrease cell culture incubation time by approximately 50% (six versus the standard eleven days). Indeed, at early time points (D3–D6), we were able to easily identify infected cells in a monolayer where no obvious CPE had yet developed. While infected cells could be detected at Day 3, assessing wells was tedious given the few infected cells in each. It was much easier to discern stained cells quickly at D5 or 6. By D6 of the ICC assay, wells could be read in rapid fashion, greatly decreasing the time necessary to assess each plate, thus increasing the number of sera that could be evaluated per unit time. Even in plates that were incubated for 11 days, staining was often helpful in identifying or verifying infected wells (data not shown). This more rapid assessment along with the shorter cell incubation time could have a significant impact on the ability to study large populations.

Given the findings, we recommend that virus-inoculated cell cultures be incubated for six days before being fixed with MEOH. At this point, plates could be stored in PBS at 4°C for up to one month before being stained (data not shown). While we did not do an exhaustive comparison of commercial anti-adenovirus hexon antibody preparations, we did note that not all products stained cells to an equal extent. Thus, investigators should adopt the method outlined herein or verify the quality of the antibody preparation used. The ABTS endpoint can also be useful in a variety of experimental designs where continuous data is needed. In our study, DAB and ABTS were equally sensitive in detecting low levels of virus. ABTS was particularly useful with short cell incubation times; by D3 there were ample virus particles and components in cells to react with antibody and yield high absorbances. Of note, the substrate was best used at D3–5 since cell loss at later time points, especially by D11, yielded false-negative results in some wells.

There appears to be substantial variability among laboratories carrying out SNA studies of AdV36. AdV36 prevalence estimates have ranged from less than 10% to over 60%. While

these variations may be the result of different study populations, they also could, in part, arise from lab-to-lab variability in carrying out the SNA, especially given the requirement for well-trained and experienced readers. In at least one instance, widely different prevalence estimates have been reported from the same set of sera [Goossens et al., 2011; Atkinson, 2011]. Thus, the increased objectivity in assessing stained cells should make the ICC less prone to variation among laboratories.

Finally, we validated the ICC method by testing 119 sera using both assays and found a high level of concordance (89.1%) between the two. The ICC assay (D6) had a high degree of specificity (95.7%) when compared to the standard SNA (D11–13). While some degree of sensitivity was sacrificed, the ICC (D6) was still adequate to detect virus in cells receiving a low initial inoculum (6–12 VP).

It should be noted that the ICC assay and SNA were compared under the most stringent conditions. That is, the assays were carried out in two different locations by different technicians. The SNA data were generated two to three years before the ICC data, and the additional storage time and freeze/thaw cycle of sera may have contributed to some loss of ICC sensitivity. Further, ICC assay sensitivity may have been underestimated since the data represent the compilation of results from different experiments, virus lots, cell passage numbers, and reagent lots.

5.0 CONCLUSIONS

In summary, the possibility that an adipogenic virus is an etiological agent for human obesity represents an exciting prospect for cause-specific treatment and prevention strategies. Accumulation of evidence for the role of AdV36 in the development of obesity will rely on testing large populations over time. While SNA is the gold standard for determining Adv36 neutralizing antibodies, we report the development of a more rapid assay which uses an alternative endpoint (ICC) for assessing AdV36 antibodies in human sera, especially for screening a large number of samples in a short time. The ICC assay provides an objective and specific method that should be more comparable among laboratories.

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CONFLICT OF INTEREST DECLARATION

NVD declares several patents in viral obesity and Adenovirus 36 including uses for E1A, E4orf1 gene and protein, and AKT1 inhibitor. He also declares ongoing grant support from Vital Health Interventions for determining anti-diabetic properties of E4orf1 protein. "The following Patents are granted or have been applied for:

A.	United States Patents approved:
1.	Number 6,127,113. Viral obesity methods and compositions.
2.	Number 6,664,050. Viral obesity methods and compositions.
3.	Number US 8,008,436B2: Adenovirus 36 E4orf1 gene & protein & their uses

- B.** Patents filed:
1. Adenovirus Ad36 E4orf1 protein for prevention and treatment of non-alcoholic fatty liver disease. US patent application 61/362,443; Taiwan Patent number 100124173.
 2. Enhanced glycemic control using Ad36E4orf1 and AKT1 inhibitor
- C.** Provisional patent filed:
1. Compositions and methods for improving glucose uptake"

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HIGHLIGHTS

- Adenovirus 36 is linked to human obesity but evidence for causation is needed.
- The serum neutralization assay for specific antibody is the lengthy and laborious.
- An ICC assay is more objective and detects virus in half the incubation time.
- Sensitivity and specificity of ICC-tested sera vs SNA were 80.0% and 95.7%.
- The more rapid and objective ICC assay will make large population studies feasible.

Figure 1. Time course of *Adenovirus 36* (AdV36) replication and detection

Cells inoculated with 100 virus particles were fixed at various times post infection (p.i.) and immunostained with HRP-conjugated anti-*Adenovirus* antibody and 3,3' diaminobenzidine (DAB). Photos are representative of the replicate experiments. Arrows show stained cells infected with *Adenovirus 36*. Panels illustrate typical virus growth at the following days post infection: A = Day 1; B = Day 3; C = Day 5; D = Day 5 “no virus” control; E = Day 13

		SNA	
		Positive	Negative
ICC	Positive	40	3
	Negative	10	66

Figure 2. Human sera (n=) tested for antibodies to *Adenovirus 36* (AdV-36)

The immunocytochemical (ICC) method at Day 5–6 is compared to the standard serum neutralization assay (SNA) at Day 13.

Figure 3. Detection limits for *Adenovirus 36* in A549 cells using the immunocytochemical method Cells were inoculated with various concentrations of virus and incubated for six days. After fixation, cells were probed with horseradish peroxidase-labelled anti-*Adenovirus* antibody and developed with either substrate, DAB (+ or –) or ABTS read at 414 nm. Data shown are the mean values for triplicate wells; results are representative of five separate experiments.

Table 1**Detection limits of the immunocytochemical assay at various time points after infection with *Adenovirus 36***

Values represent the percent of positive wells at the day post inoculation (DPI). The number of wells tested (compiled from experiments with 2 or more wells per virus concentration) and the number of separate plates (in parentheses) is indicated

Number of virus particles inoculated into each well										
DPI	# wells (# plates)	100	50	25	12.5	6.3	3.1	1.6	0.8	0.4
3	30 (3)	100	96.7	96.7	53.3	30.0	23.3	3.3	0	0
5	48 (9)	100	100	93.8	87.5	50.0	35.4	22.9	8.6	0
6	38 (6)	100	100	94.7	81.2	50.0	39.5	17.9	10.7	0
11-13*	20 (2)	100	100	100	90.0	85.0	65.0	ND	ND	ND

* Wells on Days 11-13 post-infection were assessed for cytopathic effect without staining.