

Adipogenic potential of multiple human adenoviruses in vivo and in vitro in animals

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Whigham, Leah D., Barbara A. Israel, and Richard L. Atkinson. Adipogenic potential of multiple human adenoviruses in vivo and in vitro in animals. *Am J Physiol Regul Integr Comp Physiol* 290: R190–R194, 2006. First published September 15, 2005; doi:10.1152/ajpregu.00479.2005.— Human adenovirus-36 (Ad-36) increases adiposity in chickens, mice, and nonhuman primates and is associated with human obesity. Ad-36 paradoxically reduces serum cholesterol and triglycerides in animal models. Ad-36 increases adipocyte differentiation and triglyceride accumulation in 3T3-L1 cells in vitro. This study evaluated whether three other human adenoviruses increase adiposity in chickens and in 3T3-L1 cells in vitro. We inoculated chickens with human Ad-2, Ad-31, Ad-37, or media at age 3 wk. Food intake and weights were recorded for 3.5 wk, and then chickens were killed and visceral fat, body composition, serum lipids, and viral antibody status were determined. Visceral fat and total body fat were significantly elevated ($P < 0.001$) in the Ad-37 group compared with all other groups. Final body weights were higher in chickens inoculated with Ad-37 compared with Ad-2, but not significantly higher than in control or Ad-31 groups. Food intake did not differ among groups. Serum cholesterol was elevated in Ad-37 chickens compared with control ($P < 0.01$) but was not affected by other viruses. Triglycerides were reduced in Ad-37 chickens ($P < 0.0001$) but were not affected by other viruses. In 3T3-L1 cells in vitro, Ad-31, Ad-36, and Ad-37, but not Ad-2, increased adipocyte differentiation and triglycerides accumulation. In summary, Ad-37 is another human adenovirus that increases adiposity and reduces serum triglycerides in an animal model. However, the response of serum cholesterol is opposite that of Ad-36. Evaluation of other human adenoviruses to determine their effects on adiposity and serum lipids is warranted, but in vitro assays may not be definitive for this purpose.

obesity; cholesterol; triglycerides; 3T3-L1 preadipocytes; body weight; body fat

THE WORLD HEALTH ORGANIZATION has declared that there is a global epidemic of obesity (24). The prevalence of obesity has doubled in adults in the United States in the last ~30 years and has tripled in children (10, 14, 19). With the exception of infectious diseases, no other chronic disease in history has spread so rapidly, and the etiological factors producing this epidemic have not been clearly identified (12, 16). There has been little consideration given to the possibility that the epidemic of obesity could be due to an infectious agent. However, in animals, six viruses and a scrapie agent have been shown to produce obesity (2, 3, 5–7, 9, 13, 17, 18, 20). SMAM-1, an avian adenovirus, and Ad-36, a human adenovirus, have been associated with human obesity (1, 8).

There are currently 51 known serotypes of human adenoviruses divided into six subgroups (species) based on biochemical characteristics and hemagglutination properties (4). We previously reported (1, 8, 9) that human adenovirus-36 (Ad-36) increases adiposity and produces a paradoxical reduction in serum cholesterol and triglycerides when inoculated into chickens, mice, and nonhuman primates. Human adenovirus-5 has been shown to produce obesity in mice (20). In humans, the presence of antibodies to Ad-36 in serum is associated with increased body mass index (BMI) and reductions in serum cholesterol and triglycerides (1). In human twin pairs discordant for Ad-36 antibodies, the antibody-positive twins were heavier and fatter (1).

While evaluating mechanisms of adiposity with Ad-36, our group exposed 3T3-L1 preadipocytes to this virus (5, 21). 3T3-L1 preadipocytes are a murine cell line that differentiates into adipocytes when exposed to a differentiation mixture, which has been used extensively as a model for adipocyte metabolism. We found that Ad-36 increased differentiation of 3T3-L1 preadipocytes and increased the rate of triglyceride accumulation but that Ad-2 did not (5, 21). These data, coupled with the in vivo observations that there were no gross histological changes in the brains of Ad-36-infected animals (5) make it likely that the Ad-36 virus works directly on adipose cells.

Effects of the other 49 human adenoviruses on adiposity and serum lipids in vivo and on adipogenic potential in vitro have not been assessed. In the current study, we wanted to evaluate several human adenoviruses to determine whether they produced increased adiposity in vivo or in vitro. In addition, we wanted to determine whether the in vitro effect of an adenovirus can predict its effects on enhancing adiposity in vivo. Because such in vitro studies are much easier to perform than in vivo studies, we postulated that we could use the in vitro studies to screen other human adenoviruses for adipogenic potential.

MATERIALS AND METHODS

Ad-36 is classified as a subgroup (species) D adenovirus, so adenoviruses in this subgroup are of special interest. We chose Ad-37 as another subgroup D virus for evaluation (22). To ensure that increased adiposity is not a phenomenon of all human adenoviruses, we evaluated Ad-31 from subgroup A, and from subgroup C, we evaluated Ad-2, one of the most commonly occurring human adenoviruses (4, 15, 22, 23). We chose chickens as our in vivo model because these animals respond very rapidly to infection with Ad-36. The study was divided into in vivo and in vitro experiments:

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In Vivo Experiment

Animals. One-day-old male specific pathogen-free white leghorn broiler chickens (Specific Pathogen Free Avian Supply, Roanoke, IL) were housed under biosafety level 3 conditions ($n = 54$). Protective hooded body suits, gloves, and masks were worn when dealing with the animals. The treatment groups were housed in separate rooms with a separate air supply for each room and provided with food (Purina Starter Grow, Purina Mills, St. Louis, MO) and water ad libitum throughout the course of the study. Food intake (corrected for spill) and body weight were measured three times weekly. The protocol was approved by the University of Wisconsin Animal Care and Use Committee.

Procedures. Upon arrival, chickens were housed in a brooder cage at 35°C (95°F) with a 12:12-h light/dark cycle. At age 3 wk, they were weight matched into four groups and housed in individual cages at 25–27°C (78–80°F) with a 12:12-h light/dark cycle. Chickens were then inoculated intranasally with 10^5 plaque forming units (0.2 ml of a suspension of virus in growth media) of Ad-2 ($n = 14$), Ad-31 ($n = 14$), or Ad-37 ($n = 14$), or with 0.2 ml of control media ($n = 12$). Blood was drawn from the wing vein before inoculation to confirm the absence of antibodies and at the end of the experiment to confirm seroconversion after viral inoculation. Blood was centrifuged, serum separated, and frozen until assay, as reported previously (1, 8, 9).

Chickens were killed with carbon dioxide asphyxiation 3.5 wk after inoculation. Final weights and visceral fat pad weights were recorded. Visceral fat, representing all of the visible dissectible abdominal adipose tissue, was collected and weighed, and an aliquot was frozen until analysis for viral DNA. The remainder of the visceral fat was returned to the carcass for body composition analysis.

Body composition analysis. Body composition analysis was performed in all chickens. Digestive tracts of the carcasses were cleaned and returned to the carcass. After autoclaving and homogenization of the carcasses, aliquots were taken for analysis of water, ash, and fat content. All measurements were performed in triplicate. Water content was determined by heating aliquots overnight at 90°C until a constant weight was achieved. Ash content was determined by incineration at 600°C for 4 h. Total body fat was assayed using the Folch method of extraction with methanol-chloroform and subsequent weighing (11).

Tissue culture and viral preparations. Viral preparations were performed in tissue culture, as previously described, using A549 human bronchial carcinoma cells (1, 5, 6, 9, 17, 21). Viruses and A549 cells were obtained from American Type Culture Collection (Manassas, VA). MEM (cat no. M-0643, Sigma, St. Louis, MO) and nonessential amino acids, Earle's salts, and L-glutamine were used for growing A549 cells. Work stocks for each virus were grown in A549 cells using MEM with 10% FBS and 2.9% NaHCO_3 (vol/vol) at pH 7.4.

Serum neutralization assays. The presence of antibodies to individual viruses in the serum of chickens was determined by serum neutralization assay using A549 cells, as previously described (5, 17). In this assay, serial dilutions of serum are tested for the ability to protect A549 cells from cytopathic effect (CPE) of added virus. Samples were assayed in duplicate, and controls for serum (serum and cells, no virus), cells (cells only), and virus (cells and virus, no serum) were included in each assay. For this study, serum samples showing no CPE in dilutions of 1:8 or higher, in at least one of the duplicates, were considered positive for neutralizing antibodies to the respective virus. If both duplicates had CPE at titers of $< 1:8$, these serum samples were defined as "antibody negative."

Nested PCR assay for detection of Ad-36 DNA. Ad-36 is known to produce obesity and serum lipids changes in animals (5, 6, 9, 17), so it was necessary to eliminate the possibility of Ad-36 infection in the chickens in this study. Ad-36 infection may be diagnosed by the presence of serum-neutralizing antibodies and/or the presence of Ad-36 DNA in tissues using nested PCR assay (5, 6, 9, 17). We

assayed adipose tissue samples from all of the chickens in the present experiment by nested PCR to ensure the absence of Ad-36 DNA. Four primers were designed for unique regions of the Ad-36 fiber protein gene for use in a nested PCR assay for detection of viral DNA. Sequences of primers were outer forward primer (5'-GTCTG-GAAACTGAGTGTGGATA-3'), outer reverse primer (5'-ATC-CAAAATCAAATGTAATAGAGT-3'), inner forward primer (5'-TTAACTGGAAAAGGAATAGGTA-3'), and inner reverse primer (5'-GGTGTGTTGTTGGCTTAGGATA-3'). DNA was isolated using a QIAamp Tissue Kit (cat. no. 29304; Qiagen, Valencia, CA). Negative PCR controls used were water and DNA from uninfected A-549 cells. Positive PCR control used was DNA from Ad-36-infected A-549 cells. DNA was denatured for 2 min at 95°C and subjected to 35 cycles of PCR (94°C for 1 min, 55°C for 1 min, 72°C for 2 min), followed by incubation at 72°C for 5 min. PCR products were visualized on a 1% agarose gel with a size marker.

Assays for serum cholesterol and triglycerides. Serum cholesterol was assayed using the cholesterol oxidase-peroxidase method. Colorimetric determinations were made using Sigma kits (cat. no. 352), and the absorbance was read at 500 nm. Cholesterol calibrator (cat. no. C 7921; Sigma) and cardiolidipid control (cat. no. C 4571; Sigma) were used.

Triglycerides were determined using the glycerol phosphate-peroxidase method. Colorimetric determinations were made using Sigma kits (cat. no. 339–50), and the absorbance was read at 540 nm. Glycerol (cat. no. 339–11, Sigma) was used as a standard.

In Vitro Experiment

3T3-L1 cells (American Type Culture Collection) were cultured as preadipocytes (fibroblasts) in DMEM (Sigma) containing 10% calf serum until cells were 60% confluent. Cells were then exposed to Ad-2, Ad-31, Ad-36, Ad-37, or control media at a multiplicity of infection equal to 1.0. Once cells were 2 days past confluence, they were cultured in DMEM containing 10% FBS and a differentiation cocktail of 0.5 mM methylisobutylxanthine, 0.1 μM dexamethasone, and 5 $\mu\text{g/ml}$ insulin. Cells were stained with Bodipy-FL-488 (Molecular Probes, Eugene, OR), a triglyceride-specific fluorescent compound, and fixed with 4% formalin on days 2, 3, and 4. The fluorescence score (marker of differentiation) of each well was determined with laser scanning confocal microscopy (model MRC 1024; Microsciences Division, Bio-Rad, Hercules, CA) and quantified using NIH Image software (Wayne Rasband, NIH, version 1.62).

Statistical Analysis

Data were analyzed using the SAS (Cary, NC) General Linear Model procedure. Both one-way (treatment) and two-way (treatment and antibody status) ANOVAs were performed to test for differences on reported parameters.

RESULTS

In Vivo Experiment

Body weight. Mean final body weights of the control, Ad-31, and Ad-37 groups did not differ (Fig. 1). The mean weight of the Ad-2 group was significantly lower than that of the Ad-37 group ($P < 0.02$) but not of the other two groups.

Body fat. The Ad-37 group had almost threefold more visceral fat and over twofold more total body fat vs. the control group (Fig. 2). Also, the amounts of visceral and total fat of the Ad-37 group were significantly greater than the other two groups (for each group compared with Ad-37: $P < 0.0001$ for visceral fat, $P < 0.001$ for total fat; Fig. 2). The mean visceral fat and total body fat were not different among control, Ad-2, and Ad-31. Least squared means and SE for visceral fat for

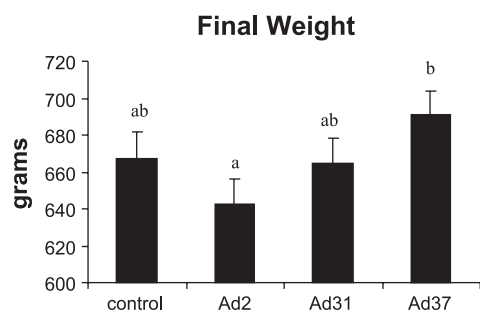


Fig. 1. Final body weights in grams of chickens inoculated with adenoviruses (Ad) Ad-2, Ad-31, Ad-37, or control media. Unlike letters are significantly different.

control, Ad-2, Ad-31, and Ad-37 groups were 2.30 ± 0.59 , 2.54 ± 0.54 , 1.93 ± 0.54 , and 6.03 ± 0.54 g, respectively. Least squared means and SE for total fat for control, Ad-2, Ad-31, and Ad-37 were 30.2 ± 4.9 , 41.9 ± 4.6 , 39.4 ± 4.6 , and 63.8 ± 4.6 g, respectively.

Food intake. Average cumulative food intakes were not statistically significantly different between groups (841.4 ± 9.0 , 853.3 ± 8.3 , 860.6 ± 8.3 , and 846.6 ± 8.3 g, for control, Ad-2, Ad-31, and Ad-37, respectively).

Serum lipids. The Ad-37 group had a significant increase in serum cholesterol from baseline to death ($P < 0.01$) (Fig. 3A). Increase in serum cholesterol in the Ad-37 group compared with the changes in the control and Ad-2 groups also was significant ($P = 0.01$; Fig. 3A). There was a trend for a difference in change in serum cholesterol between Ad-37 and Ad-31 ($P = 0.08$). Ad-37 had significantly decreased serum triglycerides levels at the end of the study compared with all

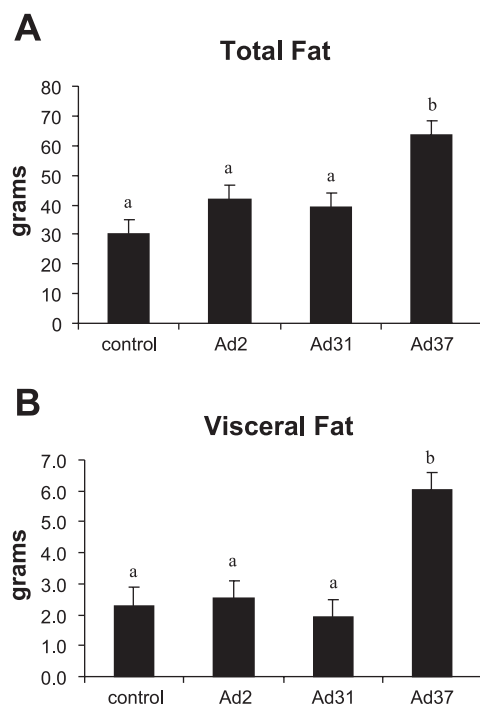


Fig. 2. A: final total fat weight in grams from methanol-chloroform extraction method of chickens inoculated with Ad-2, Ad-31, Ad-37, or control media. Unlike letters are significantly different. B: final visceral fat weight in grams of chickens inoculated with Ad-2, Ad-31, Ad-37, or control media. Unlike letters are significantly different.

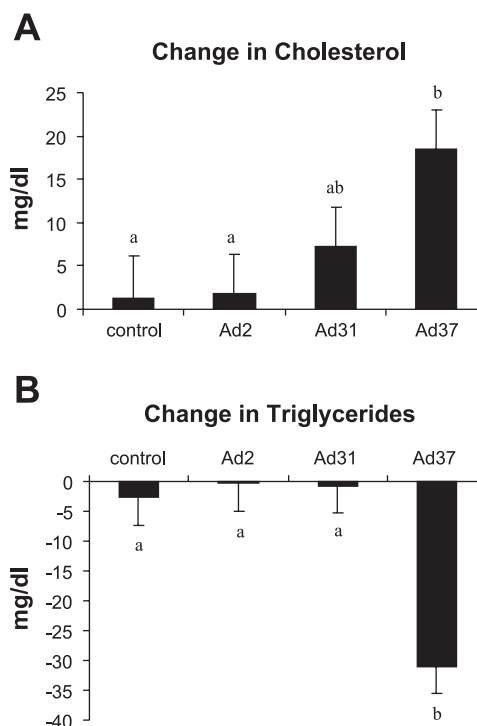


Fig. 3. A: change in serum cholesterol levels from baseline to 3.5 wk in chickens inoculated with Ad-2, Ad-31, Ad-37, or control media. Unlike letters are significantly different. B: change in serum triglyceride levels from baseline to 3.5 wk in chickens inoculated with Ad-2, Ad-31, Ad-37, or control media. Unlike letters are significantly different.

the other groups ($P < 0.0001$ for each group compared with Ad-37, Fig. 3B). The changes in serum cholesterol and triglyceride levels from baseline to the end of the study were not significantly different for the control, Ad-2, and Ad-31 groups, respectively.

Viral antibodies. Evaluation for prior viral infection was tested by serum neutralization in each treatment group, and all groups were negative. Specifically, at baseline before inoculation, none of the chickens tested positive for antibodies to Ad-2, Ad-31, Ad-37, or Ad-36. At the end of the trial, none of the chickens from the control group or any of the treatment groups tested positive for antibodies to Ad-36. Also, none of the chickens from the control group tested positive for any of the test viruses (Ad-2, Ad-31, or Ad-37). At the end of the trial, each group had the following serum neutralization results for the respective inoculated viruses: Ad-2, six positive, eight negative; Ad-31, 10 positive, four negative; Ad-37, 10 positive, four negative.

PCR for Ad-36 DNA. No Ad-36 DNA was present in the visceral fat of any of the chickens in any of the groups as confirmed by nested PCR.

In Vitro Experiment

3T3-L1 cells were exposed to Ad-36 and the three viruses used in the in vivo experiment described above (Ad-2, Ad-31, and Ad-37). The 3T3-L1 cells from each viral group and the control group were checked at days 2, 3, and 4 after the differentiation cocktail was added. Two wells per group were scored, and nine fields per well were counted. Data are representative of four different experiments repeated under similar

conditions. At *day 2* there were no significant differences in triglyceride-related Bodipy fluorescence scores among the different treatment groups and the control (data not shown). At *day 3*, Ad-2 infected cells were not significantly different from controls. Ad-31, Ad-36, and Ad-37 infected cells had a fivefold ($P < 0.01$), a fourfold ($P < 0.05$), and a 2.5-fold ($P < 0.06$) higher fluorescence score, respectively, compared with control (Fig. 4). Ad-2 and Ad-37 were not significantly different from each other on *day 3* (Fig. 4). At *day 4*, Ad-2-infected cells were still not different compared with controls. The cells infected with Ad-31, Ad-36, and Ad-37 all had more than a twofold higher fluorescence score compared with control cells ($P < 0.0001$ for each group compared with control).

DISCUSSION

Our previous studies (5, 6, 9, 17) with human adenovirus-36 demonstrated that Ad-36 increases adiposity in several animal models and So et al. (20) demonstrated that human adenovirus-5 increases adiposity in mice. Results of the current study demonstrate that Ad-37 increases adiposity in a chicken model, but Ad-2 and Ad-31 do not. Thus an adiposity-promoting effect is specific and is not a characteristic of infection with all human adenoviruses. Ad-37 joins the list of seven other pathogens reported to increase adiposity in animal models, and it is the third human virus to do so.

Our results on body fat and body weight with Ad-37 cannot be explained by changes in food intake. Similar to previous experiments with Ad-36 (5, 6, 9, 17), food intake was not higher in the Ad-37 group compared with control, and actually was slightly lower than food intakes of the Ad-2 and Ad-31 groups (P value not significant). Although possible, it is unlikely that insufficient accuracy of the food intake measurements could explain these results. A reduction in metabolic rate might explain the increased fat deposition, but the small changes over time would be impossible to detect with current technology. The greater body fat deposition, despite similar

Table 1. *Clinical and laboratory values*

	Mean	SE	<i>P</i> value	Group Differences
Final weight, g				
control	667.33	±14.70	NS	ab
Ad-2	642.21	±13.61	0.015	a
Ad-31	664.79	±13.61	NS	ab
Ad-37	690.64	±13.61		b
Visceral fat, g				
control	2.30	±0.59	<0.0001	a
Ad-2	2.54	±0.54	<0.0001	a
Ad-31	1.93	±0.54	<0.0001	a
Ad-37	6.03	±0.54		b
Total fat, g				
control	30.16	±4.94	<0.0001	a
Ad-2	41.88	±4.57	0.0014	a
Ad-31	39.43	±4.57	0.0004	a
Ad-37	63.77	±4.57		b
%Total body fat				
control	4.55	±0.65	<0.0001	a
Ad-2	6.62	±0.60	0.0045	b
Ad-31	6.01	±0.60	0.0005	ab
Ad-37	9.15	±0.60		c
Cummulative food intake, g				
control	841.4	±9.0	NS	a
Ad-2	853.3	±8.3	NS	a
Ad-31	860.6	±8.3	NS	a
Ad-37	846.6	±8.3		a
Change in cholesterol, mg/dl				
control	1.19	±4.8	0.01	a
Ad-2	1.74	±4.5	0.01	a
Ad-31	7.25	±4.5	NS	ab
Ad-37	18.45	±4.5		b
Change in triglycerides, mg/dl				
control	2.51	±4.9	<0.0001	a
Ad-2	-0.34	±4.6	<0.0001	a
Ad-31	-0.80	±4.6	<0.0001	a
Ad-37	-31.0	±4.6		b

Data are least squared means, SE, and P values compared with Ad-37. Different letters denote differences among groups; NS, not significant ($P > 0.05$).

Effect of Viruses on Adipocytes *In Vitro*

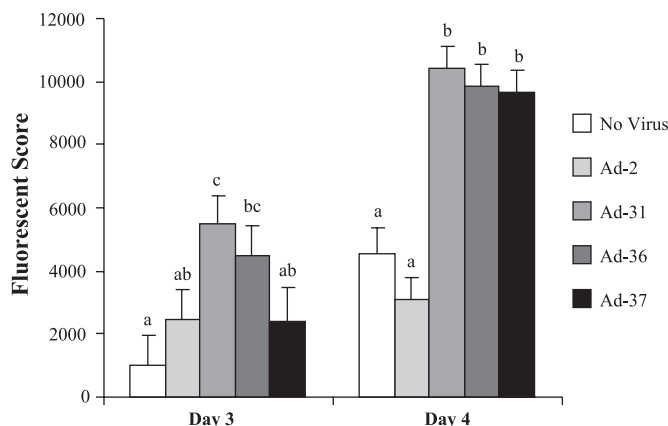


Fig. 4. Effect of viruses on adipocytes in vitro. The fluorescence score (marker of differentiation) of 3T3-L1 cells exposed to Ad-2, Ad-31, Ad-36, Ad-37, or control media. Cells were stained with Bodipy, a triglyceride-specific fluorescent compound, and fixed with formalin. The fluorescence score of each well was determined with laser scanning confocal microscopy and quantified using NIH Image software. Cells infected with viruses (left to right) on days 3 and 4: no virus (control), Ad-2, Ad-31, Ad-36, and Ad-37. Unlike letters are significantly different.

body weight, suggests a shift from lean body mass deposition to fat deposition during the growing period of Ad-37-infected chickens. Further research will be necessary to understand the mechanisms of these changes.

In contrast to the other viruses that produce obesity in animals, Ad-37 has a different effect on serum lipids. The four nonadenoviruses reported to produce obesity in animals (canine distemper virus, Rous-7 virus, Borna virus, and scrapie agent), in general, produce increases in serum triglycerides, a finding that is commonly associated with weight gain (2, 3, 13, 18). These viruses are thought to produce obesity by causing damage to the central nervous system (2, 3, 13, 18). SMAM-1 avian adenovirus and human Ad-36 decreased both serum cholesterol and triglycerides in chickens, but no brain damage was noted. These adenoviruses reduced levels of both cholesterol and triglycerides by about 30 mg/dl compared with controls (5–7, 9, 17). Serum lipids were not measured in the study with Ad-5 by So et al. (20). In the current study, Ad-37 decreased serum triglycerides similar to Ad-36 and SMAM-1, but instead of a decrease in serum cholesterol, Ad-37 caused a significant increase of 18 mg/dl. These data suggest that at least two different mechanisms are responsible for the increased adiposity and changed serum lipid levels in animals inoculated with Ad-36 and Ad-37.

Our in vitro data in 3T3-L1 cells demonstrated that the effects of the four viruses on differentiation of preadipocytes and increased triglycerides accumulation vary by virus, just as increased adiposity does in vivo. Ad-2 did not increase preadipocyte differentiation nor produce adiposity in vivo. Although Ad-37 increased adiposity in vivo and Ad-31 did not, both increased preadipocyte differentiation and triglycerides accumulation in the 3T3-L1 cells. This is a disappointing finding because it suggests that the in vitro assay will not be a good screening test to predict response to human adenoviruses in whole animals. Both of the human adenoviruses that produce obesity in vivo increased preadipocyte differentiation, but Ad-31 had a dichotomous effect in vivo vs. in vitro. It seems possible that an in vitro effect on preadipocytes may be a tool to detect candidate viruses for an adiposity-promoting effect, but in vivo studies will be necessary to determine which of the remaining human adenoviruses are capable of increasing adipose tissue in animals.

In summary, Ad-37 is the third human adenovirus to increase adiposity in animals, but not all adenoviruses produce obesity. The evidence from the animal studies, the high prevalence of Ad-36 antibodies in obese people compared with nonobese (1), and the increased BMI and body fat of Ad-36 antibody-positive twins (1) suggest that some cases of human obesity are due to adenovirus infection. The nearly simultaneous increase in the prevalence of obesity in most countries of the world is difficult to explain by changes in food intake and exercise alone, and suggests that adenoviruses could have contributed. The role of adenoviruses in the worldwide epidemic of obesity is a critical question that demands additional research.

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GRANTS

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DISCLOSURES

Dr. Atkinson owns all shares of Obetech, LLC, a company that markets assays to detect infection with human adenovirus-36 and owns patent rights for these assays.

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