Comparison of Immunization Methods for Producing Reference Adenovirus Antisera in Horses

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Horses were immunized by a variety of inoculation procedures designed to determine the most efficient method of producing antisera to adenovirus types 25 to 31. The procedures evaluated included immunization by (i) direct intravenous (iv) injection, (ii) iv infusion, (iii) intramuscular (im) injection of virus with and without Freund's incomplete adjuvant, (iv) combined iv and im injections, and (v) combined iv infusion and im injection. The im schedule (no. 3) was superior to the others in terms of immunizing antigen and time required, and hemagglutination-inhibition (HI) and serum-neutralizing (SN) antibody levels produced. HI and SN tests performed with sera before and after heating at 56°C for 30 min showed that heat-inactivation was not necessary for tests with equine antisera.

Adenovirus (AV) antisera have usually been prepared in rabbits by multiple intravenous (iv) inoculations. This method has worked well for the first 18 AV serotypes (14, 15, 18-22) but has been less efficient for the remaining 13 types (16-18, 21). In addition, the necessity of pooling the serum of many rabbits to obtain a sufficient volume of antiserum to a single type is laborious and results in a product which may be of less than optimal quality. To circumvent these problems, Lucas et al. (13) prepared antisera to types 1 to 18 in horses by giving multiple iv injections, usually five inoculations of 40 ml each, at weekly intervals and exsanguinating the animals 14 days after the last injection. This procedure resulted in large volumes (approximately 10,000 ml per horse) of specific antisera with acceptable homologous antibody titers.

In continuing the production of equine antisera to the human AV, we have found that iv injection does not result in acceptable levels of antibodies to the higher-numbered types (types 25 to 31). In this report we describe a procedure which is superior to the iv schedule and which should be applicable for production of equine antisera to other viruses as well.

MATERIALS AND METHODS

Production of immunizing antigen (IA). Prototype strains of AV types 25 through 31 were obtained from the Research Resources Branch (RRB) of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), Bethesda, Md. Stock viruses were propagated in HEp-2 human epithelium cell cultures grown on Eagle's minimum essential medium (MEM) with 10% fetal calf serum, and maintained on MEM without serum. For production of immunizing antigens, cell monolayers in 32-oz (ca. 0.946 liter) prescription bottles were washed twice with Hanks balanced salt solution and inoculated dry with 3 to 4 ml of stock virus containing approximately 10^5 median tissue culture infective doses (TCID₅₀) per 0.1 ml. After a 2-hr adsorption period at 35°C, 26 ml of serum-free, antibiotic-free maintenance medium was added to each bottle. The cultures were observed daily and then frozen 2 days after development of complete cytopathology (CPE). The virus and viral components were harvested after four freeze-thaw cycles followed by a low-speed centrifugation (1,000 × g, 30 min) to remove cellular debris. The supernatant fluid served as the immunizing antigen.

Determination of purity. The product prepared for horse immunization was comprehensively tested for bacterial, fungal, and mycoplasmal sterility; 1.5 ml of the product was inoculated into Trypticase soy and thioglycolate broths, neopeptone infusion agar streak/pour plates with 5% defibrinated sheep blood, Sabouraud glucose agar, and PPLO agar. All media were kept for 21 days at 37°C. Breakthrough neutralization tests to detect contamination by other complete viruses were performed as described by Hampil and Melnick (6). In this test, the culture was consid-
ered pure if all breakthrough viruses were neutralized at serum dilutions equivalent to the known homologous titers of reference NIH (RRB) antisera. Complement fixation (CF) tests to detect contamination by the AV-associated viruses (AAV types 1–4) were performed with prototype antisera (2, 11).

**Infectivity titrations.** Virus titrations were performed in HEP-2 and primary human embryonic kidney cell cultures using a 10-fold dilution series. CPE was read at 3, 7, 10, and 14 days, and the titer was recorded as the log_{10} TCID_{50} per 0.1 ml.

**SeroIogical tests.** CF tests were performed by the standardized LBCP microtiter method; five units of complement were used and fixed overnight at 4 C (3). The group-specific CF (hexon) activity of the immunizing antigens was measured against a human convalescent serum (AV type 4) and compared with a purified type-2 hexon antigen (5).

Hemagglutination (HA) and hemagglutination-inhibition (HI) tests were performed in microtiter by the standardized HA-HI procedures (10), with standardized 0.4% suspensions of rat, rhesus, and human "O" red blood cells (RBC) and 0.01 M phosphate-buffered saline (PBS) as diluent (9). Sera to be tested by HI were absorbed with the RBC to be used in the test but were not routinely heat-inactivated or further treated.

Serum neutralization (SN) tests were performed in primary rhesus monkey kidney cell cultures as described by Stevens et al. (18) except that the "infectivity" titrations and tests proper were read at 3 or 4 days. When the HI and SN tests were performed to confirm identity of the virus, the same RRB reference rabbit antisera were utilized that were used in the breakthrough neutralization tests.

**Protein determinations.** Protein measurements were made by the method of Lowry et al. (12) and read in a Beckman DB spectrophotometer at 750 nm against a bovine serum albumin standard curve.

**Immunization methods.** A variety of immunization preparations and methods were used for intramuscular (im) and iv routes. In method A (iv), 40 ml of immunizing antigen was given by direct intrajugular inoculation. In method B (iv-drip), 40 ml of IA was diluted into 4,000 ml of cold, sterile PBS in an aspirator bottle packed in wet ice. The solution was passed under restricted gravity flow over an 18-hr period through an indwelling catheter in the jugular vein. If necessary, horses were tranquilized with an im injection of 250 mg of promazine-hydrochloride.

In method C (im), 20 ml of IA was given im, 5 ml in each of two sites in the hindquarters (gluteus medius muscles) and in each of two sites in the pectoral muscles. In method D (im-FICA), 20 ml of IA was vigorously mixed with an equal volume of Freud's incomplete adjuvant (FICA; Difco Laboratories, Detroit). The emulsion was mixed in an International PR-II centrifuge equipped with an IEC shaker head (no. 6007) and operated at 1,000 rev/min for 30 min at 4 C. Although the emulsion thus prepared was completely stable for weeks, fresh material was prepared for each injection. An injection consisted of 40 ml of the mixture, 10 ml in each of two sites in the muscles of the hindquarters and in each of two sites in the pectoral muscles.

**RESULTS**

**Evaluation of immunizing antigens.** The immunizing antigens of AV types 25 to 31 were thoroughly tested for purity, potency, and specificity as described above. All products were free from bacterial, fungal, and mycoplasmal contaminants. Contamination of the IA by other complete viruses or by AAV was not detected by breakthrough neutralization and CF tests, respectively.

Biological data on the IA are presented in Table 1. The 14-day infectivity titers are somewhat lower than usual because the IA cultures were incubated well beyond the point of optimal infectivity to attain maximal concentrations of CF and HA antigens. Identity of the IA was shown by homologous HI and SN tests with reference antisera. In all cases, HI and SN titers obtained with the IA were equivalent to the known serum titers obtained with reference virus stocks. Identification of the breakthrough viruses from breakthrough neutralization tests was also confirmed by HI and SN tests performed in parallel with reference virus stocks. Again, equivalent titers were obtained. All IA were thus shown by our criteria to be acceptable for horse inoculation.

Total protein concentration of the 7 IA preparations was 697 to 833 (average 758) μg/ml. Of this, 85 to 115 (average 100) μg/ml was estimated to be virus and viral component protein.

**Evaluation of immunizing schedules.** All horses were pretested for absence of antibody to all 31 human AV types. Antibody-free horses were then randomly divided into five immunization schedules or were assigned to one or another schedule for a particular AV type.

The schedules are summarized in Table 2. Schedule 1 was the control schedule and consisted of five iv inoculations (method A) of 40 ml each, at 1- or 2-week intervals; if necessary, these inoculations were followed by an 80-ml booster after 7 weeks. Schedule 2 used the iv-drip (method B) only; the inoculations were given on days 0, 3, 7, and 10.

Schedule 3 called for im method D on day 0, and im method C on days 14 and 21. Schedule 4 was the combined im/iv schedule: an im-FICA inoculation (method D) on day 0, 20 ml im (method C) and 40 ml iv (method A) on day 14; 40 ml iv on day 17; 20 ml im and 40 ml iv on day 21; and 40 ml iv on day 24.

Schedule 5 was the combined im/iv-drip schedule: an im-FICA inoculation (method D) on day 0; 20 ml im (method C) and 4,000 ml iv-drip (method B) on day 14; 4,000 ml iv-drip
on day 17; 20 ml im and 4,000 ml iv-drip on day 21; and 4,000 ml iv-drip on day 24.

Since it was not feasible to evaluate all schedules with all seven AV types, types 25 and 28 were first used to compare schedules 1, 3, and 5 (Table 3). The HI and SN titers listed show several unequal qualities for the three schedules. The HI titers for AV 25 and 28 developed first with schedule 5 and were quite delayed in schedule 3; yet the final HI titers were virtually identical for all three schedules. SN titers for both AV 25 and 28 also developed

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**Table 1. Potency and specificity of immunizing antigens**

| Type | Strain | 14-day infectivity titer (log_{10} TCID_{50}/0.1 ml) | CF titers | HA titers<sup>a</sup> | Monkey kidney infectivity titer (2<sup>+</sup> CPE, 3 days) | Homologous titer of reference antiserum<sup>a</sup> | Protein<sup>a</sup> (μg/ml) |
|------|--------|---------------------------------|---------|----------------|---------------------------------|-----------------------------|-----------------
|      |        |                                 | HEp-2   | HEK*           |                                 |                             |                 |
| 25   | BP-1   | 2.7                             | 4.3     | 32             | 16                               | 16                           | <1              |
| 26   | BP-2   | 3.9                             | 5.5     | 32             | 1,024                            | 1,024                        | <1              |
| 27   | BP-4   | 3.7                             | 5.5     | 64             | 1,024                            | 1,024                        | 4               |
| 28   | BP-5   | 3.0                             | 4.0     | 32             | 8                                | 16                           | <1              |
| 29   | BP-6   | 3.0                             | 5.2     | 64             | 64                               | 64                           | <1              |
| 30   | BP-7   | 3.2                             | 5.0     | 32             | 4,096                            | 4,096                        | 32              |
| 31   | 1315/63| 2.5                             | 4.5     | 64             | <1                               | 4                            | <1              |

<sup>a</sup> HEK, Human embryonic kidney cells.

<sup>b</sup> Complement-fixation (CF) titer of group-specific hexon component, expressed as reciprocal of the optimal antigen dilution as determined by the optimal dilution of human convalescent AV-4 antiserum tested in block titrations.

<sup>c</sup> Hemagglutination (HA) titer expressed as reciprocal of the highest dilution of antigen producing complete hemagglutination with rat, rhesus, and human "O" erythrocytes in PBS diluent, and with rat cells in heterotypic serum (HS) diluent (PBS containing 1% AV-6 equine antiserum) (10).

<sup>d</sup> Reference rabbit antiserum obtained from the RRB, NIH.

**Table 2. Outline of immunization schedules evaluated**

| Immunization<sup>a</sup> | Vol of inoculum (ml) | Schedule<sup>a</sup> |
|--------------------------|----------------------|----------------------|
| Method | Route | 1 | 2 | 3 | 4 | 5 |
| A | iv | 40 | 0 | 14 |   |   |
|   |   | 40 | 7 | 17 |   |   |
|   |   | 40 | 14 | 21 |   |   |
|   |   | 40 | 28 | 24 |   |   |
|   |   | 40 | 35 |   |   |   |
|   |   | 80 | 85<sup>a</sup> |   |   |   |
| B | iv-drip | 4,000 | 0 | 14 |   |   |
|   |   | 4,000 | 3 | 17 |   |   |
|   |   | 4,000 | 7 | 21 |   |   |
|   |   | 4,000 | 10 |   |   |   |
| C | im | 20 | 14 | 14 |   |   |
|   |   | 20 | 21 | 21 |   |   |
| D | im-FICA | 40 | 0 | 0 |   |   |

<sup>a</sup> See text (Materials and Methods) for description of route and inoculum preparation.

<sup>b</sup> Numbers in body of table are the days on which the inoculations were given, starting with the first inoculation on day 0.

<sup>c</sup> Booster inoculation, if needed.
TABLE 3. Homologous HI and SN titers on interval sera from horses inoculated with AV types 25 and 28 by different schedules

| Antiserum | Day | Schedule 1 iv | Schedule 3 im | Schedule 5 im/iv-drip |
|-----------|-----|---------------|---------------|-----------------------|
|           |     | HI* | SN* | HI | SN | HI | SN |
| Type 25   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
|           | 7   | 0   | 0   | 0  | 40 | 20 | 320|
|           | 14  | 20  | 0   | 20 | 80 | 160| 320|
|           | 21  | 0   | 40  | 80 | 160| 320| 80 |
|           | 24  | 80  | 0   | 20 | 80 | 640| 160|
|           | 28  | 160 | 80  | 80 | 640| 320| 80 |
|           | 35  | 160 | 160 | 320| 640| 320| 80 |
|           | 42  | 160 | 160 | 320| 640| 80 |
|           | 92  | 320 | 160 |    |    |    |    |
| Type 28   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
|           | 7   | 0   | 10  | 40 | 20 | 160|
|           | 14  | 0   | 20  | 40 | 20 | 160|
|           | 17  | 0   | 40  | 160| 80 | 320|
|           | 21  | 80  | 20  | 20 | 160| 80 | 320|
|           | 24  | 80  | 20  | 20 | 160| 160| 320|
|           | 28  | 160 | 80  | 40 | 640| 80 | 320|
|           | 35  | 160 | 80  | 40 | 640| 80 | 320|
|           | 42  | 160 | 80  |    | 80 | 320|
|           | 92  | 160 | 80  |    |    |    |

* Hemagglutination-inhibition (HI) titers are listed as reciprocal of the highest serum dilution exhibiting complete inhibition of hemagglutination. HI titers for types 25 and 28 antisera were determined with rat RBC (7).

* Serum neutralization (SN) titers are listed as reciprocal of the highest serum dilution exhibiting a 2+-reduction in CPE in 4 days in monkey kidney cells (18).

* 0 = <10.

earliest with schedule 5, but were clearly highest at the end with schedule 3. Since the neutralization test is the ultimate test in AV type identification, schedule 3 was considered to be superior to schedules 1 and 5. Schedule 1 was dropped from further study.

The results with schedule 3 supported our long-term experience in immunizing rabbits with various AV serotypes by the im route (7, 8). It therefore appeared that the im route alone was superior to either iv method previously used. To confirm this hypothesis, we compared the efficiency of im and iv routes, used alone and in combination, in eliciting HI and SN antibody to AV-29. Type 29 was chosen because of its poor antibody response in rabbits and its significant cross-reactions with types 15 and 23 in the SN test (17, 18, 22). We felt that the deliberate choice of one of the more “difficult” AV types would provide a better test of the im hypothesis than a type which traditionally has given good antibody responses and which is free of troublesome cross-reactions.

Comparative HI and SN antibody titers in eight horses inoculated with AV-29 by schedules 2, 3, 4, 5 are presented in Table 4. Two horses were assigned at random to each of the four schedules. Schedule 2 was unsuccessful as seen by the poor HI response and by the absence of SN response. Schedules 3, 4, and 5, all of which contained the im schedule (no. 3), were approximately equal by both HI and SN. Schedule 3, however, consistently gave the highest HI and SN antibody responses in both horses. Reference equine antisera to AV-29 was therefore obtained from one of the schedule 3 horses (no. 6), which was exsanguinated on day 35. Continuing interval bleedings of the remaining horses up to 70 days after initial inoculation showed a gradual decline in antibody levels. Hence an immunization period of 28 to 38 days (Tables 3,4) appeared to be optimal for the production of high-titered antisera.

The im schedule (no. 3) was then used to produce the remaining antisera in the AV-25 through AV-31 series (Table 5). The HI and SN antibody levels obtained in the final serum samples are all equal to or, in most cases, higher than the comparable titers in the reference rabbit antisera (Table 1). This supports the superiority of the im route over the iv route, especially since the equine serum titers of the first 18 AV types (13) were generally lower than the equivalent titers in rabbits (18, 19).

Throughout this study, HI and SN titers on final serum samples were identical in unheated sera and in sera which had been heat-inactivated at 56 C for 30 min. Only 7- to 14-day serum samples showed differences in titer for unheated and heated sera; the unheated serum often exhibited higher titers than the heated serum (Table 4). This effect was shown previously to be the result of broadly-specific immunoglobulin M (IgM) or “early” antibody (1, 7).

DISCUSSION

The production of reference equine antisera to the human AV has been a function of our laboratory for many years. The earlier AV types (1 to 18) elicited suitable HI and SN antibody responses in horses when the antigens were injected iv in multiple 40-ml volumes
(13). Antisera to types 19 to 24 were also successfully produced by this method. The higher-type AV (types 25 to 31), on the other hand, failed to elicit acceptable antibody levels.

Investigation of the iv-infusion or "drip" method was prompted by the suggestion that the slow continual application of antigen over a long period of time might provide better stimulation of antibody-forming sites. However, the method was not satisfactory in our hands with AV-29 in horses.

The use of an im immunization schedule with an initial adjuvant injection was suggested by the effectiveness of im-FICA immunizations in rabbits (7, 8). Adjuvant-induced sterile abscesses are a severe problem in horses and other large animals if multiple adjuvant injections are given (K. D. Quist et al., Amer. Ass. Lab. Anim. Sci. Abstr. 130, 1968). Our choice of a single initial im injection of a 50:50 emulsion of virus in FICA followed by two im injections of virus did not produce any serious inflammatory reactions or side effects that would be considered undesirable for the welfare of the research animal. However, even single injections of FICA can produce a suppressive local reaction in the tissues. The site of inoculation, therefore, is an important con-

### Table 4. Homologous HI and SN titers on interval sera from horses inoculated with AV-29 by different schedules

| Day | Schedule 2 iv-drip | Schedule 3 im | Schedule 4 im/iv | Schedule 5 im/iv-drip |
|-----|-------------------|---------------|-----------------|----------------------|
|     | HI* | SN* | HI | SN | HI | SN | HI | SN | HI | SN | HI | SN | HI | SN |
| 11e | 15  | 6   | 8  | 8  | 10 | 12 | 10 | 12 |
| 0   | 0   | 0   | 0  | 0  | 0  | 0  | 0  | 0  |
| 7   | 10  | 10  | 20 | 20 | 10 | 10 | 10 | 10 |
| 14  | 40  | 160 | 1,280 | 40 | 40 | 640 | 320 | 0  | 10 | 640 | 320 | 20  |
| 17  | 80  | 160 | 1,280 | 40 | 40 | 640 | 320 | 0  | 10 | 1,280 | 320 | 10  |
| 21  | 80  | 160 | 1,280 | 40 | 40 | 640 | 320 | 0  | 10 | 2,560 | 320 | 10  |
| 28  | 80  | 80  | 1,280 | 40 | 40 | 2,560 | 1,280 | 10 | 40 | 1,280 | 640 | 10  |
| 35  | 80  | 80  | 1,280 | 40 | 40 | 2,560 | 1,280 | 10 | 40 | 1,280 | 1,280 | 20  |
| 42  | 80  | 40  | 1,280 | 80 | 2,560 | 1,280 | 1,280 | 0  | 20 | 1,280 | 640 | 0   |
| 56  | 80  | 80  | 1,280 | 40 | 1,280 | 1,280 | 0  | 10 | 640 | 640 | 0  |
| 70  | 80  | 80  | 1,280 | 40 | 1,280 | 1,280 | 0  | 10 |

a See Table 3, footnote a.
b See Table 3, footnote b.
c Horse.
d See Table 3, footnote c.

### Table 5. Homologous HI and SN titers of alternate interval sera from horses inoculated with adenovirus types 25-31 by schedule 3

| Day     | Antiserum type* |
|---------|-----------------|
|         | 25              | 26 | 27 | 28 | 29 | 30 | 31 |
|         | HI | SN | HI | SN | HI | SN | HI | SN | HI | SN |
| 0       | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| 17      | 40 | 160| 2,560| 40 | 640| 80 | 40 | 160| 1,280| 20 | 160| 80 | 160 |
| 28      | 80 | 640| 1,280| 80 | 1,280| 80 | 40 | 640| 5,120| 40 | 640| 80 | 640 |
| 35-38   | 320| 640| 2,560| 80 | 1,280| 160| 80 | 640| 5,120| 80 | 640| 80 | 640 |
| Lyophilized | 320| 320| 2,560| 80 | 1,280| 160| 80 | 160| 2,560| 80 | 320| 80 | 640 |

a HI titers of antisera 25 through 30 were determined with rat RBC; AV-31 does not agglutinate any RBC and therefore the homologous antiserum does not have a detectable HI titer. All HI and SN titers are listed as reciprocal of end-point dilution (see Table 3, footnotes a and b).
b 0 = <10.
c Underlined numbers are titers of the frozen, pre-lyophilized antiserum.
d Italicized numbers are final titers of the lyophilized reference antiserum.
sideration, and should not be in proximity to the joints.

The consistent observation that the combined im/iv and combined im/iv-drip methods produced slightly lower HI and SN titers than the im method alone may be a consequence of the time of serum sampling in relation to the last iv injection. A portion of the specific serum antibodies might be bound by the iv virus and subsequently removed from the circulating blood by the host’s immunological clearance systems. This would tend to lower the serum antibody titers temporarily, and might permanently depress the host’s response to the antigen.

Heat-inactivation (56 C, 30 min) of the equine antisera evaluated in this study had no apparent effect on HI or SN antibody titers. As expected, early sera drawn 7- to 14-days after primary inoculation often contained elevated IgM levels which were detectable in unheated sera by both tests. But sera drawn later than the 14th day after primary inoculation contained identical titers in heated and unheated serum samples. This supports our extensive observations that equine antisera do not contain nonspecific inhibitors or complement components in sufficient levels to affect HI or SN antibody titers. Hence heat-inactivation of equine antisera for AV serology appears to serve no useful purpose, and may in fact destroy heat-labile factors that could be essential (4, 23).

The immunization schedule (no. 3) described in this report is superior in many ways to the iv routes evaluated. (i) It is simpler to perform since it uses only three inoculations—one with adjuvant, two without. (ii) It requires smaller amounts of antigen than do the iv routes: 60 ml at an average of 100 μg of viral protein per ml (approximately 6,000 total μg of viral protein per horse) compared with 200 to 280 ml (20,000 to 28,000 total μg of viral protein) for the iv schedule. (iii) It requires only 28 to 38 days and could thereby minimize heterotypic antibody responses. (iv) It results in high-titered specific antisera. We hope that this procedure will be applied to the production of antisera to new AV serotypes as well as to other viruses and organisms with low immunizing potential in large animals.

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LITERATURE CITED

1. Altemeier, W. A., F. K. Mundon, F. H. Top, and P. K. Russell. 1970. Method for extracting viral hemagglutination-inhibiting antibodies from the non-specific inhibitors of serum. Appl. Microbiol. 19:785-790.

2. Blacklow, N. R., M. D. Hoggan, M. S. Sereno, C. D. Brandt, H. W. Kim, R. H. Parrott, and R. M. Chanock. 1971. A seroepidemiologic study of adenovirus-associated virus infection in infants and children. Amer. J. Epidemiol. 94:359-366.

3. Casey, H. L. 1965. Standard diagnostic complement fixation method and adaptation to micro test. In Public Health Service Monogr. no. 74. Washington, D.C.

4. DeMeio, J. L., and A. N. DeSanctis. 1971. Heat-labile factor necessary for hemagglutination-inhibition testing of horse sera. Appl. Microbiol. 21:860-861.

5. Dowdle, W. R., M. Lambriex, and J. C. Hierholzer. 1971. Production and evaluation of a purified adenovirus group-specific (hexon) antigen for use in the diagnostic complement fixation test. Appl. Microbiol. 21:718-722.

6. Hampil, B., and J. L. Melnick. 1969. Method of testing virus stocks for viral contaminants. Appl. Microbiol. 17:17-20.

7. Hierholzer, J. C., and W. R. Dowdle. 1970. Hemagglutination properties of adenovirus types 20, 25, and 28. Proc. Soc. Exp. Biol. Med. 134:482-488.

8. Hierholzer, J. C., and W. R. Dowdle. 1970. Immunological basis of the adenovirus 8-9 cross-reaction. J. Virol. 6:782-787.

9. Hierholzer, J. C., and M. T. Suggs. 1969. Standardized viral hemagglutination and hemagglutination-inhibition tests. I. Standardization of erythrocyte suspensions. Appl. Microbiol. 18:816-823.

10. Hierholzer, J. C., M. T. Suggs, and E. C. Hall. 1969. Standardized viral hemagglutination and hemagglutination-inhibition tests. II. Description and statistical evaluation. Appl. Microbiol. 18:824-833.

11. Hoggan, M. D., N. R. Blacklow, and W. P. Rowe. 1966. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. Proc. Nat. Acad. Sci. U.S.A. 55:1467-1474.

12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

13. Lucas, J. B., G. J. Johnston, H. S. Kaye, M. A. Bucca, and R. Q. Robinson. 1965. Production of adenovirus antisera in horses. Pub. Health Rep. 80:647-652.

14. Rafajko, R. R. 1964. Production and standardization of adenovirus types 1 to 18 reference antisera. Amer. J. Hyg. 79:310-319.

15. Rosen, L. 1960. A hemagglutination-inhibition technique for typing adenoviruses. Amer. J. Hyg. 71:120-128.

16. Rosen, L., S. Baron, and J. A. Bell. 1961. Four newly recognized adenoviruses. Proc. Soc. Exp. Biol. Med. 107:434-437.

17. Rosen, L., J. F. Hovis, and J. A. Bell. 1962. Further observations on typing adenoviruses and a description of two possible additional serotypes. Proc. Soc. Exp. Biol. Med. 110:710-713.

18. Stevens, D. A., M. Schaeffer, J. P. Fox, C. D. Brandt, and M. Romano. 1967. Standardization and certification of reference antibodies and antisera for 30 human adenovirus serotypes. Amer. J. Epidemiol. 86:617-633.

19. Uchida, S., T. Hoshika, H. Yamamoto, K. Koike, S. Koseki, and A. Furuno. 1959. Relationships between types of adenovirus indicated by heterologous neutralization. Jap. J. Exp. Med. 29:121-129.
20. Wigand, R. 1968. Serologische Beziehungen der Adeno-
viren der Gruppe II. Arch. Ges. Virusforsch. 23:40–47.
21. Wigand, R., H. Bauer, F. Lang, and W. Adam. 1965. 
Neutralization of the adenoviruses types 1 to 28: spec-
ificity and antigenic relationships. Arch. Gesamte 
Virusforsch. 15:188–199.
22. Wigand, R., and D. Fliedner. 1968. Serologically inter-
mediate adenovirus strains: a regular feature of group 
II adenoviruses. Arch. Gesamte Virusforsch. 24:245–256.
23. Zolotarnkaya, E. E., and R. S. Dreizin. 1968. Inhibitors 
of adenovirus hemagglutination and spontaneous 
hemagglutinins in human and animal sera. Vop. Vi-
rusol. 13:84–88.