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Production and Reactivity of Immune Sera Specific for HADEN Virus Polypeptide Antigens

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Antisera were prepared against the three structural polypeptides of HADEN virus dissociated with sodium dodecyl sulfate. These immunological reagents were used in immunofluorescence tests to study the kinetics and location of polypeptide antigen appearance in infected cells. These sera did not neutralize virus infectivity, did not cross-react with adenovirus-associated virus-infected cells, and reacted in complement fixation tests with sodium dodecyl sulfate-dissociated virus, but not with complete virus antigen. The polypeptide antigens were heat labile, and all appeared in infected cells at least 2 h prior to whole-virus antigen.

The hemadsorbing enteric virus of calves (HADEN) was isolated in 1961 by Abinanti and Warfield (1). This virus was shown to be heat stable (14) and to cause mild diarrhea in calves with development of both hemagglutination inhibition and neutralizing antibody (15). It has been demonstrated that HADEN virus contains a DNA genome, and it has been suggested that this virus be classified as a parvovirus (17). As is the case with the other parvoviruses, HADEN virus has a high relative specific density in CsCl (5). The particle has an average diameter of 22 nm as determined by electron microscopy and is serologically distinct from other parvoviruses (5, 16).

Purified HADEN virus particles have been shown to contain three structural polypeptides (8). The major polypeptide has an estimated molecular weight of 67,000 and accounts for more than 75% of the virion protein. The other two protein components have higher molecular weights.

The present study was undertaken to produce immunological reagents against each of the structural polypeptides, to study the kinetics of antigen formation during infection, and to compare the properties of the native antigens on the complete virion with the antigens revealed on the polypeptides subsequent to sodium dodecyl sulfate (SDS) treatment.

MATERIALS AND METHODS

The original strain of HADEN virus used was obtained from F. R. Abinanti. The virus was grown in primary cultures of bovine embryonic kidney (BEK) cells obtained from either Grand Island Biological Co., Grand Island, N. Y., or Flow Laboratories, Inc., Inglewood, Calif. Production of virus pools and purification in isopycnic CsCl gradients were previously described (8).

The adenovirus type-2 strain was originally described by Rowe et al. (12) as the adenoid 6 strain of adenovirus type 2. The isolation and characterization of the H strain of adenovirus-associated virus (AAV) type 3 has been previously described (3, 6). The AAV was grown, along with its adenovirus helper, in HEp-2 cells obtained from Flow Laboratories, Inc., Inglewood, Calif.

Polyacrylamide gel electrophoresis was carried out with purified virus in 0.01 M sodium phosphate buffer, pH 7.2. These particles were disrupted in 1% SDS and 0.01 M dithiothreitol at 100 C for 2 min. Protein concentrations were estimated by the method of Lowry et al. (10) by using bovine serum albumin as the standard protein. The polypeptides of the disrupted virus were separated on 12-cm 7.5% SDS-polyacrylamide gels by the method of Maizel (11) and as previously described (8, 9).

Antisera were prepared in guinea pigs found to be free from preexisting antibody to HADEN virus when tested by complement fixation (CF) and fluorescent antibody (FA) tests. Production of antibody against whole virus was previously described (5). Antiserum to a mixture of HADEN structural polypeptides, that is, purified virus disrupted in 1% SDS with no electrophoretic separation, was prepared by inoculating each animal with 35 μg of viral protein in Freund complete adjuvant. This mixture was distributed between the two rear footpads. Each animal was bled 20 days after the primary inoculation. At 40 days postinoculation the animals were bled and boosted by footpad injection of 35 μg of SDS-dissociated viral proteins contained in incomplete Freund adjuvant. The guinea pigs were bled at 2-week intervals thereafter.

As was reported earlier (8) HADEN virus contains three structural polypeptides. The major one was designated HVP1, and the other two were designated HVP2 and HVP3 in order of increasing molecular weight. Guinea pig antisera were prepared against each of these polypeptides in a manner similar to that outlined by Johnson et al. (7) for the AAV structural polypeptides. Purified virus was disrupted by the SDS procedure and separated on 12-cm 7.5% acrylamide gels. One of the 12 gels run in parallel was stained with Coomassie brilliant blue to locate the positions of the bands in the gels. The unstained bands were then dissected with a razor blade from the remaining gels. Figure 1 shows the three polypeptide-densitometric profiles on the gel which was stained, and the arrows indicate the position at which the gels were cut. Three small segments of acrylamide gel were thus obtained from each gel column, each containing one of the separated viral polypeptides. That the gel pieces actually contained the desired polypeptide bands after cutting and that there was no overlap of a band in a neighboring gel piece was further verified by staining the three gel pieces obtained from such a gel column. Each gel piece contained a single polypeptide band.

The unstained replicate gel pieces were broken up by forcing them through a syringe and 18-gauge needle in preparation for immunization. This gel pulp was suspended in Freund complete adjuvant and inoculated into the rear footpad of guinea pigs. Two gel pieces were used for each immunization, with the animals receiving about 14 μg of HVP1 and 2 μg each of HVP2 and HVP3. The antigen quantities were estimated by determining the quantity of protein on the gels and knowing the relative percentage of each polypeptide species (8). This dosage was repeated 40 days after primary injection, at which time the antigen was combined with incomplete Freund adjuvant and was distributed between the rear footpads of the guinea pigs. The animals were bled at 2-week intervals thereafter.

The neutralization assay of HADEN virus was performed in culture tubes containing BEK cell monolayers. One-tenth milliliter dilutions of heated antisera (whole virion, mixed polypeptide, HVP1, HVP2, and HVP3) were mixed with 0.1 ml of virus containing 10^2 mean tissue culture infective doses ($TCID_{50}$) and allowed to react at room temperature for 20 min. A 0.1-ml portion of the mixture was then added to each of duplicate tubes and allowed to adsorb for 2 h in 1.0 ml of Eagle minimal essential medium minus serum. Then 0.5 ml of medium containing 6% fetal calf serum was added to each tube and incubated at 37 C. This latter step was taken to avoid unwanted neutralization by possible antibodies in the fetal calf serum. The tubes were observed daily for cytopathic effects. Upon reaching 100% cytopathic effect, the cultures were frozen. After 21 days all remaining tubes were frozen. The freeze-thawed cultures were tested in CF to reconfirm the presence or absence of HADEN virus.

RESULTS

The antibody response which developed in the animals immunized with the purified HADEN virus polypeptides is depicted in Fig. 2. The indirect method of immunofluorescence as described elsewhere (2) was used in all the fluorescence tests. Prior to immunization, no detectable FA antibody was present in sera from the animals. After primary immunization a very low antibody titer developed against HVP1, the major virus polypeptide. No detectable antibody appeared in the serum of HVP2 and HVP3 immunized animals until secondary inoculation. Maximum titers of antibodies to HVP2 and HVP3 were observed 20 to 30 days after the boost. The maximum titer of antibody to HVP1 appeared at about 2 weeks after the secondary inoculation. These maximum titers were maintained for a limited period of time and soon began to decrease (Fig. 2). These results demonstrate that immune sera can be prepared against the purified SDS-treated HADEN virus polypeptides.

These antibodies raised against the HADEN polypeptides were found to cause positive flu-

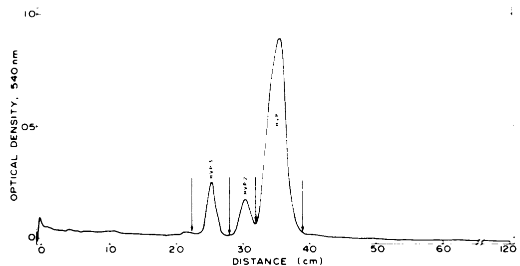

FIG. 1. *Densitometric scan of the Coomassie blue-stained acrylamide gel which was a replicate of those used for preparation of purified HVP immunogens. The individual polypeptide bands were removed at the points indicated by the arrows.*

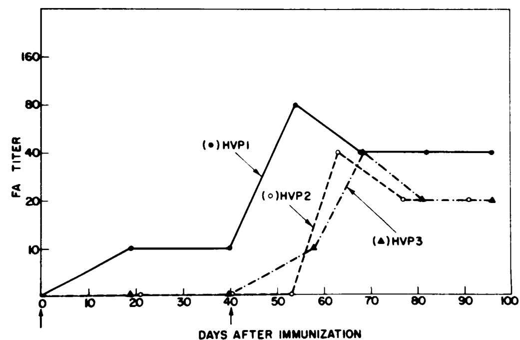

FIG. 2. *Kinetics of antibody formation to purified sodium dodecyl sulfate-treated HADEN virus polypeptides. The antibody titers are expressed as the reciprocal of the highest dilution of antiserum giving positive fluorescence in the indirect immunofluorescence test. The arrows along the abscissa indicate times of immunogen inoculation.*

orescence in HADEN-infected BEK cells, but not in uninfected cells. These reagents were then used in a study of infection kinetics to determine whether antigens appeared in infected cells prior to the time when antigen stainable with whole-virion serum appeared. Monolayers of BEK cells on cover slips were infected with HADEN virus at a multiplicity of infection of 5 to 7 $TCID_{50}$ per cell. At various times after infection, the cover slips were fixed in cold acetone. Such infected cells were then tested with each of the various types of antisera. Infected cells fixed 16 h postinfection and stained with anti-whole-virion serum appeared as shown in Fig. 3a. Although some nuclear stain was observed, probably nucleoli, most of the antigen appeared in paranuclear areas of the cells. Cells fixed before 16 h did not stain with this serum. By 18- to 20-h postinfection the stain was localized only in the nuclei (Fig. 3b), and no paranuclear staining was observed.

Infected cells fixed 14 h postinfection and stained with anti-HVP1 serum showed cytoplasmic and paranuclear staining (Fig. 3c). Cells fixed prior to this time showed no fluorescence. Solid nuclear staining was not observed. In infected cells fixed 16 h postinfection and stained with HVP1 antiserum, fluorescence was localized in the nuclei, and by 18 h the stain was brilliant and of exclusively solid nuclear morphology (Fig. 3d).

Anti-HVP2 serum-stained infected cells fixed 10 to 14 h postinfection showed only fluorescent nuclear specks (Fig. 3e). These nuclear specks (possibly nucleoli) stained positively when compared with the background nonspecific nucleolar stain in uninfected control cover slips and in negative cells on the same cover slip. The nucleoli of infected cells treated with nonimmune preserum were also negative. When infected cells fixed at 16 h postinfection were stained with anti-HVP2 serum, a mixture of nuclear specks and solid fluorescent-stained nuclei occurred (Fig. 3f).

Cells fixed at 14 h and stained with HVP3 antiserum showed cytoplasmic and paranuclear fluorescence. Cells fixed prior to this time did not stain with this serum. By 24 h postinfection, the staining observed with this serum was primarily paranuclear (Fig. 3g) with some cells showing positive flecks in the nuclei. Thus, the staining morphologies of antisera to HVP1 and HVP3 polypeptide antigens appeared to be quite similar at early time points, but later became distinguishable. Early in time the HVP2 antigen was distinct, but later came to appear similar to the HVP1-stained solid fluorescing nuclei.

These studies on kinetics of formation demonstrated the presence of HVP2 polypeptide antigen about 10 h postinfection, whereas the HVP1 and HVP3 polypeptide antigens appeared at about 14 h, and whole virion antigen appeared at 16 h postinfection.

The heat stability of the HADEN antigens was tested in BEK cells fixed 30 h postinfection. These cells were heated at 56 C for 10, 20, and 30 min in pH 7.4 phosphate-buffered saline solution. They were examined by indirect immunofluorescence and compared with nonheated infected control cells. The whole-virion antigen was not inactivated by heat under these conditions after 30 min. However, the HADEN antigens which stain with antisera to HVP1, HVP2, and HVP3 were inactivated by heat after 10 min of exposure.

It was reported by Johnson et al. (7) that the AAV polypeptide antisera prepared against AAV type-3 polypeptides cross-reacted in FA with the polypeptide antigens of AAV types 1 and 2, whereas whole virion antiserum did not significantly cross-react. It was, therefore, of interest to determine if any cross-reactivity would occur on the polypeptide level among the antigens of widely differing parvoviruses. To determine HADEN polypeptide antisera specificity, cover slips with monolayers of HEp-2 cells infected with AAV-3 were treated with mixed polypeptide, HVP1, HVP2, and HVP3 antisera. In all cases the cover slips were negative, showing there was no cross-reactivity between HADEN polypeptide antisera and AAV-3 antigens.

CF tests were carried out to determine whether antisera to the virus polypeptides would react with whole-virus antigen, because positive reactions were noted by FA in virus-infected cells. The microtiter CF test was carried out by using the procedure outlined by Sever (13). The polypeptide antigen preparations were disrupted in 0.1% SDS and prepared as previously described (7). The results of these tests are shown in Table 1. The antiserum to the polypeptides was specific for the antigens on the dissociated polypeptides, and the whole-virion antiserum reacted only with the purified virion antigen. Thus, it appears that treatment of the virus with SDS reveals antigens on the polypeptides which are not immunoreactive on the complete virus particle.

Neutralization of HADEN virus infectivity was tested by using antiwhole-virion serum, antimixed polypeptide serum, anti-HVP1, anti-HVP2, and anti-HVP3 sera. Antiwhole-virion serum neutralized the virus at a titer greater than 2,560. However, no neutralizing

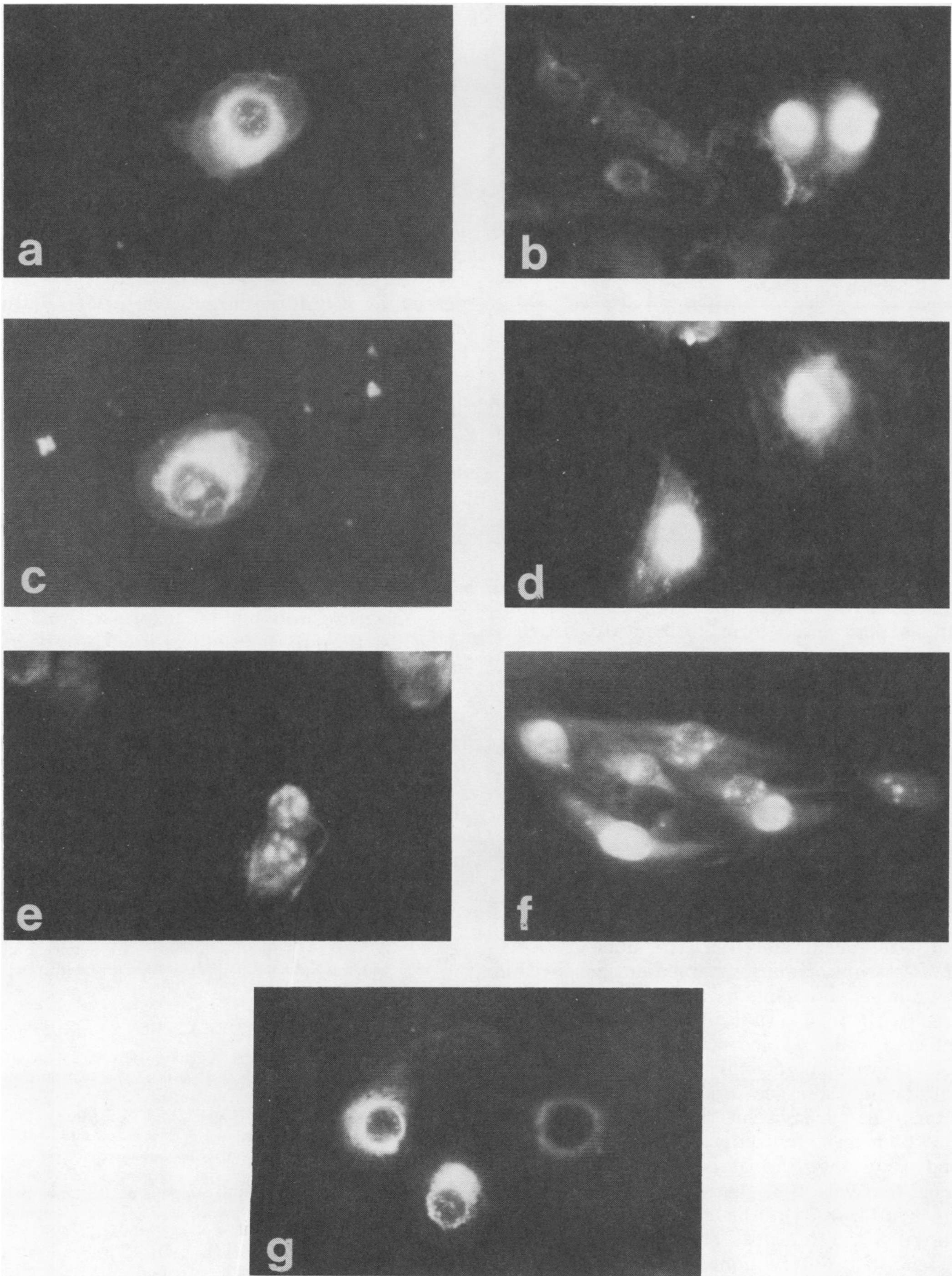

FIG. 3. *Immunofluorescence photomicrographs of HADEN virus antigens in infected bovine embryonic kidney cells. (a) Cells stained with whole-virion antiserum 16 h postinfection (PI) showing predominent paranuclear staining with some nuclear antigen. (b) Cells stained with whole-virion antiserum 20 h PI exhibiting nuclear staining. (c) Cells stained with anti-HVP1 serum 14 h PI showing cytoplasmic staining. (d) Cells stained with anti-HVP1 serum 18 h PI showing nuclear staining. (e) Cells stained with anti-HVP2 serum 10 h PI showing nuclear specks. (f) Cells stained with anti-HVP2 serum 16 h PI showing homogeneously stained nuclei and other nuclei with positive specks. (g) Cells stained with anti-HVP3 serum 24 h PI showing cytoplasmic-paranuclear staining.*

TABLE 1. *Specificity of antisera to HADEN virus purified virions and sodium dodecyl sulfate-dissociated virus (mixed polypeptides) tested by complement fixation*

Antigens	Antisera[a]	
	Virion	Mixed polypeptides
Virion[b]	8	0
Mixed polypeptides[c]	0[d]	16

[a] Data are expressed as antigen titers using 4 to 8 units of antibody.
[b] Virus purified by banding in CsCl gradients.
[c] Purified virus disrupted in SDS by methods described elsewhere (7).
[d] No detectable complement fixation at lowest concentration tested (1:2).

activity was observed with the antisera prepared against the virus polypeptides.

DISCUSSION

The results of this study demonstrated that guinea pigs would form antibody to HADEN virus structural polypeptides dissociated in SDS. Although we have prepared analogous reagents to the defective AAVs, this is the first such study of an autonomously replicating parvovirus. These reagents in comparative studies may yield information regarding the reasons for the defectiveness of AAV.

The antiserum to the major structural polypeptide of HADEN virus (HVP1) in indirect FA tests stained the cytoplasm and paranuclear area of cells actively infected with the virus. The antigen first appeared in the cytoplasm, but later accumulated in the nucleus. Anti-HVP2 serum primarily stained antigens within the nucleus, but some paranuclear staining was seen with this serum at much later times. Infected cells stained with HVP3 antiserum first showed cytoplasmic-paranuclear staining with some fluorescent nuclear flecks appearing later. The observation that both HVP1 and HVP3 antigens first appeared in the cytoplasm and then later appeared in the nucleus is consistent with the suggestion that the proteins are synthesized in the cytoplasm and transported to the nucleus. This suggestion is consistent with observations made in adenovirus-infected cells (4, 18, 19) and in cells infected with AAV (7). It is not clear, from these data, if HVP2 is synthesized cytoplasmically because it first appeared in the nucleus, but rapid transport of newly synthesized molecules could take place so that it would not be detected in the cytoplasm by these techniques until sufficient accumulation could occur, which apparently happened by 24 h. On the same note, it should be emphasized that the information on the kinetics of formation of these viral polypeptides reflects the time of detection, which probably is preceded in time by actual synthesis, and the polypeptides are not detected until accumulations exceed the threshold of sensitivity of the FA test. All of the polypeptide antigens appeared at a minimum of 2 h prior to the appearance of whole-virus antigens. When infected cells were stained with antiwhole-virion serum fluorescence first appeared in the paranuclear area of the cell, and later only homogeneously stained nuclei were observed. Because the infected cells stained positively with the antipolypeptide sera earlier in time than did the cells stained with whole-virion antiserum and because the CF tests and neutralization tests showed that the antigens on the polypeptides are different from the antigens on the whole virus particles, then the FA stainable polypeptide antigens probably are expressed on the molecules prior to their folding into the tertiary structure they possess as virus subunits. It would appear, therefore, that detection of the polypeptides by FA prior to formation of the tertiary structure of the virus proteins is possible and that formation of the tertiary protein structure begins in the paranuclear area of the infected cell, and the protein is then transported to the nucleus where virus assembly takes place.

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