Arch Virol (1992) 126: 195–213

Archives of Virology

Characterization of the structural proteins of hemorrhagic enteritis virus

J. V. van den Hurk

Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan, Canada

Accepted January 27, 1992

Summary. The structural proteins of hemorrhagic enteritis (HEV), a turkey adenovirus, were analyzed by polyacrylamide gel electrophoresis (PAGE) and Western blotting using polyspecific, monospecific and monoclonal antibodies for detection. In purified HEV preparations, eleven polypeptides with apparent molecular weights ranging from 96,000 to 9,500 (96k to 9.5k), were specifically recognized by convalescent turkey serum. Six of these polypeptides were further characterized by PAGE, Western blotting, ELISA, sucrose gradient centrifugation and electron microscopy. The 96k polypeptide was identified as the hexon polypeptide which is a monomer of the major outer capsid or hexon protein. The 51/52k and 29k polypeptides, identified as the penton base and fiber polypeptides respectively, were the components of the vertex or penton protein. The 57k polypeptide was identified as a homologue of the human adenovirus type 2 (Ad 2) IIIa protein with which it shares a common epitope. Two core proteins with molecular weights of 12.5 and 9.5k were present in purified HEV nucleoprotein cores. The proteins of two HEV isolates, one apathogenic (HEV-A) and one virulent (HEV-V), resembled each other in most respects. However, differences between HEV-A and HEV-V were found in electrophoretic migration of the penton base protein both under native and denatured conditions, and in the electrophoretic migration of the 43/44k polypeptide. Moreover, homologous antiserum against the fiber protein reacted stronger than heterologous antiserum in an ELISA. Single fibers were detected by electron microscopy attached to the penton base proteins of HEV virions and in isolated pentons. The feature of having single fibers is shared with the mammalian adenoviruses and the avian egg drop syndrome 1976 virus (EDS 76 V), but not with the fowl adenoviruses which have double fibers attached to their penton base proteins.

Introduction

The family *Adenoviridae* is divided into the mammalian adenoviruses (genus *Mastadenovirus*) and the avian adenoviruses (genus *Aviadenovirus*). This division

is based upon a difference in host range and the absence of an antigenic relationship between mammalian and avian adenoviruses [31]. Within the genus *Aviadenovirus* there are at least two groups; the fowl adenoviruses [45] and a second group comprised of hemorrhagic enteritis virus (HEV) of turkeys [5, 20, 38], marble spleen disease virus (MSDV) of pheasants [18, 19] and splenomegaly virus (SV) of chickens [10, 11]. It has been suggested that these be referred to as group I and group II avian adenovirus, respectively [7]. A major difference between fowl adenoviruses and mammalian adenoviruses is the composition of the penton protein which consists of a penton base and two fibers in the case of fowl adenoviruses and a penton base and one fiber in the case of mammalian adenoviruses [15, 26]. The fowl adenoviruses are distantly related to the human adenoviruses with which they share a limited amount of DNA sequence homology [1].

HEV causes an acute infectious disease in turkeys [8, 17]. It is classified as an adenovirus on the basis of its morphology, mode of replication, and physical-chemical properties [5, 20, 32, 38]. HEV, MSDV and SV are serologically identical viruses [7–9, 19, 39, 42]. To date, no serologic relationship has been found between these viruses and the fowl adenoviruses [10, 11, 21, 36]. However, the lack of suitable cell culture system for HEV propagation has hampered a thorough investigation of its properties [40].

Our overall study of HEV involved developing a vaccine for turkeys [42] and defining the role of viral components in eliciting protective immunity. Therefore, the identification and characterization of the structural proteins of HEV was required. Until recently, none of the structural proteins of HEV, with the exeption of the hexon, has been well characterized [30, 42]. The best studied adenoviruses in both genera are the human adenoviruses type 2 (Ad 2) and type 5 (Ad 5), and chick embryo lethal orphan (CELO) virus (fowl adenovirus type 1, FAV 1). These viruses have been shown to consist of outer capsid proteins (hexons and pentons), proteins associated with the capsid, and core proteins associated with double-stranded DNA.

In the present study, the structural proteins of an apathogenic (HEV-A) and a virulent (HEV-V) strain of HEV were analyzed using polyacrylamide gel electrophoresis (PAGE) under non-denaturing and denaturing conditions, and Western blotting using polyspecific, monospecific, and monoclonal antibodies. Furthermore, the hexon and penton proteins of both HEV strains were purified by immunoaffinity chromatography and characterized by sucrose gradient sedimentation, PAGE, Western blotting, and electron microscopy. The data presented in this report are discussed and compared with those of human and fowl adenoviruses.

Materials and methods

Viruses and virus propagation

The characteristics of HEV-A and HEV-V and their propagation in young turkeys are described elsewhere [39]. Ad 2 was obtained from the American Type Culture Collection and propagated in HEp-2 cells.

Virus purification

Spleens of HEV-A or HEV-V infected turkeys were homogenized in 0.01 M Tris-HCl, pH 8.1, in the presence of 0.1% phenylmethylsulfonyl fluoride (Sigma), and the supernatants (crude spleen extracts) were collected after centrifugation for 10 min at 10,000 g [39]. Further purification was carried out by a modification of the method described by Green and Pina [16] in which the supernatants were repeatedly extracted by trichlorotrifluoroethane, whereafter the HEV present in the aqueous phase was concentrated by centrifugation onto a dense CsCl cushion (1.40 g/cm^3). The virus band was collected and further purified by CsCl density centrifugation, and the layer above the virus band (soluble protein fraction [3]) was used for the analysis of soluble viral proteins and for affinity chromatography. HEV was dialyzed against 0.01 M Tris-HCl, pH 8.1 containing 20% glycerol and stored at − 70 °C. Ad 2 was purified from infected HEp-2 cells in a similar way.

Production and screening of monoclonal antibodies

Balb/c mice were immunized with 0.2 ml (2 mg/ml) of purified HEV-A emulsified in Freund's complete adjuvant. The primary injection was followed by a second injection of HEV-A in Freund's incomplete adjuvant 2 weeks later. Final booster inoculations with 0.1 ml HEV-A in PBS were given intravenously 7 and 3 days prior to fusion. Mouse spleen cells were fused with NS-1 myeloma cells as described by Kennett et al. [22]. The supernatants of the hybridoma cells were initially screened for HEV-specific antibody production by an indirect immunofluorescent antibody (FA) test using control and HEV-infected turkey spleen leukocytes, and by an indirect ELISA using purified HEV-A or HEV-V to coat the microtiter plates. The hybridoma cells were subcloned in microtiter plates by the limiting dilution method. Ascites fluids were obtained from pristane-(2,6,10,14-tetramethyl pentadecane; Aldrich Chemicals) primed Balb/c mice intraperitoneally injected with approximately 10^7 hybridoma cells.

Fluorescent antibody (FA) test

Leukocytes were obtained from turkey blood by centrifugation through Ficoll-Paque (Pharmacia) [40]. The leukocyte suspension cultures were infected with HEV-A or HEV-V and cell smears were made with a cytocentrifuge 2–3 days postinfection. HEp-2 cells, grown in Lab-Tek tissue culture chambers (Miles Laboratories), were infected with Ad 2 virus. The cells were fixed in either acetone or methanol for 5 min. Infected or control cells were incubated with hybridoma supernatant media for 1 h at 41 °C followed by an incubation with affinity-purified, fluorescein-labelled goat immunoglobulin prepared against mouse immunoglobulins (diluted 1 : 100 with PBS; Boehringer) for 1 h at 41 °C. The cells were mounted with PBS-glycerine (1 : 1, v/v) and observed with a Zeiss IM 35 microscope equipped with epifluorescent illumination.

ELISA

Indirect ELISAs were used for analysis of HEV proteins using antibodies of turkey, rabbit, or mouse origin and appropriate peroxidase conjugates as described previously [39, 42].

Polyacrylamide gel electrophoresis

Electrophoresis of the HEV proteins under native conditions was performed on 6% polyacrylamide gels [2]. Samples were applied in electrophoresis sample buffer (0.0625 M Tris-HCl (pH 6.8), 10% glycerol, and 0.002% bromophenol blue). Pentons were dissociated by heat treatment for 1 min at 56 °C in the presence of 0.05% deoxycholate. For analysis under denaturing conditions purified HEV and HEV proteins were dissociated by boiling in

electrophoresis sample buffer containing 1% sodium dodecyl sulfate (SDS), and 0.15 M 2-mercaptoethanol and analyzed on 10 or 13% SDS-polyacrylamide gels [24].

Western blotting

A modification of the "Western" blotting technique described by Burnette [4] was used to analyze the interaction between HEV antibodies and HEV proteins. HEV proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% or 13% slab gels, or by PAGE in 6% slab gels under native conditions. They were then transferred electrophoretically to nitrocellulose in a Bio-Rad transblot cell (Bio-Rad Laboratories) at 32 V for 4 h in 25 mM sodium phosphate buffer, pH 6.8. Subsequently, strips cut from the nitrocellulose sheets were either stained in amidoblack dye (0.6% amidoblack in 45% methanol, 10% acetic acid in H_2O) or processed for antigen detection following the instructions for the use of the Bio-Rad immunoblot assay kit. Briefly, strips were incubated for 1 h in Tris-buffered saline (TBS: 0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5) containing 3% gelatin. Thereafter, the strips were incubated overnight with the first antibody solution of turkey, rabbit or mouse origin containing 1% gelatin. After washing with TBS-T (TBS containing 0.05% Tween 20) the strips were incubated with the second antibody solution of rabbit anti-turkey IgG or rabbit anti-mouse IgG in TBS containing 1% gelatin where appropriate. Following washing, strips were incubated with horseradish peroxidase-conjugated protein A. Finally, after washing of the strips, bands were visualized by incubation with substrate (0.05% 4-chloro-1-naphtol, 0.015% H_2O_2 in TBS) for 15–30 min. All incubation steps were carried out at room temperature on a rocking platform.

Immunoaffinity chromatography

The IgG fraction of monoclonal antibodies was purified from mouse ascites fluids using protein A-Sepharose CL-4 B (Pharmacia) [14]. Purified IgG, dialyzed against 0.1 M sodium carbonate buffer, pH 8.0, was linked to activated Affigel-10 (Bio-Rad Laboratories) at 5 mg/ml gel following the manufacturer's instuctions. The immunobeads were packed into a column, washed and equilibrated with TNE (0.01 M Tris-HCl, 0.5 M NaCl, and 0.001 M Na_2 EDTA, pH 7.5). The soluble antigen fraction in TNE obtained during virus purification [3] was recycled three times through the column. After washing the column with TNE, protein fractions were eluted with 0.05 M diethylamine, pH 11.5. During collection the protein fractions were neutralized with 1 M Tris-HCl, pH 6.8. The HEV protein-containing fractions were pooled and dialyzed against PBS. Subsequently, HEV soluble proteins were separated by centrifugation on a 10 to 30% (w/v) linear sucrose gradient for 23 h at 35,000 rpm in a Beckman SW 41 rotor at 4 °C [6]. After testing the fractions by ELISA, the appropriate fractions were pooled, dialyzed against PBS containing 10% glycerol (v/v) and stored at − 70 °C. This method was used to purify hexon and penton proteins.

Preparation of immune sera

Purified penton base and fiber proteins were obtained after immunoaffinity chromatography and preparative PAGE under native conditions, followed by electroelution of the proteins from the gels. Rabbits were immunized subcutaneously three times with 1 ml of purified penton, penton base, or fiber protein of either HEV-A or HEV-V. The first immunization was given in complete Freund's adjuvant and the second and third ones (each 2 weeks apart) in incomplete Freund's adjuvant.

Electron microscopy

Virus preparations for electron microscopy collected from CsCl gradients were applied on carbon coated grids, washed with H_2O and stained with 1% uranyl acetate [15]. Hexon,

penton, penton base and fiber preparations were negatively stained with 1% uranyl acetate or 1% Na-silica-tungsten. The specimens were screened and photographs were taken at an initial magnification of 38,000 to 76,000 using a Philips 410 electron microscope at 80 kV. The size of the fibers on isolated pentons was measured on prints usually at a magnification of 200,000 with a micrometer graduated to 0.1 mm. The length of two hundred fibers was measured for each virus and the mean and standard deviation were calculated.

Results

HE virus

To date there is no suitable cell culture system for HEV, which will produce sufficient quantities of virus for structural protein characterization. Therefore, HE virus and soluble proteins were purified from the spleens of turkeys infected with HEV. After purification of HEV two virus bands (with densities of 1.30 and 1.34 g/cm^3) were present in the CsCl gradients. The virus band with the higher density contained complete, infectious virus. The diameter of both HEV-A and HEV-V particles was 72 nm (Fig. 1). Groups of nine (GON) hexons were observed in disrupted virions of both HEV strains (Fig. 1). Virus with a density of 1.34 g/cm^3 was used for the characterization of the structural proteins.

HEV structural proteins

The HEV polypeptides were analyzed by SDS-PAGE followed by Western blotting in order to differentiate HEV-specific polypeptides from potential host cell polypeptides. Following transfer, the polypeptides of HEV-A, HEV-V, and

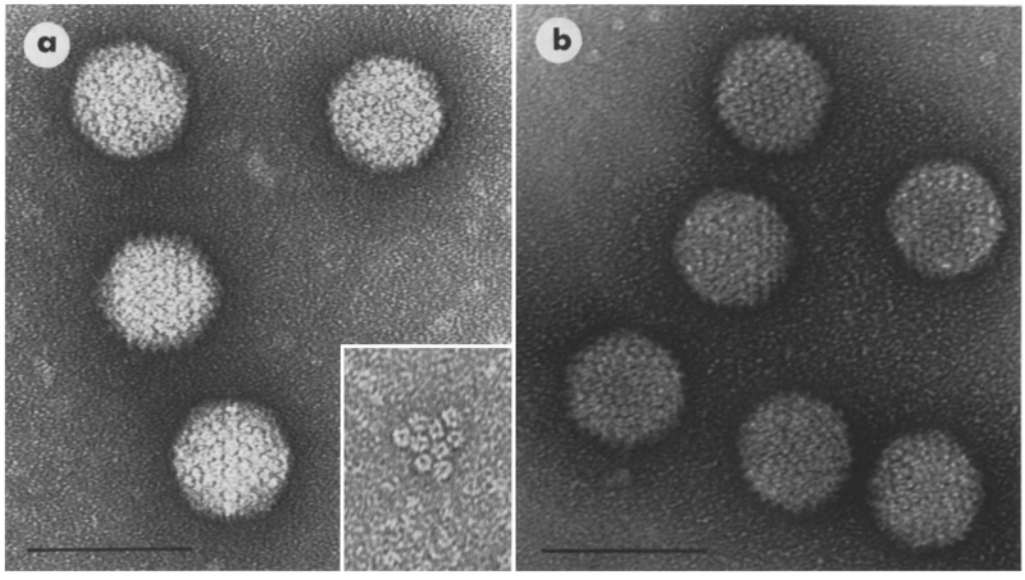

Fig. 1. Electron micrographs of CsCl purified HEV-A (**a**) and HEV-V (**b**) (ρ = 1.34 g/cm^3). A value of 72 nm was estimated for the diameter of HEV particles. Insert shows a GON hexons of disrupted HEV-A. Negative staining with uranyl acetate. Bars: 100 nm

Ad 2 were visualized by amido black staining (Fig. 2 A). Eleven of the polypeptides found in stained blots were recognized specifically by antibodies present in convalescent sera from HEV infected turkeys (Fig. 2 B). The apparent molecular weights of the HEV polypeptides were calculated using Ad 2 polypeptides as standards [33]. The molecular weights of the HEV-A polypeptides were estimated to be 96k, 57k, 52k, 44k, 37k, 34k, 29k, 24k, 21k, 12.5k, and 9.5k,

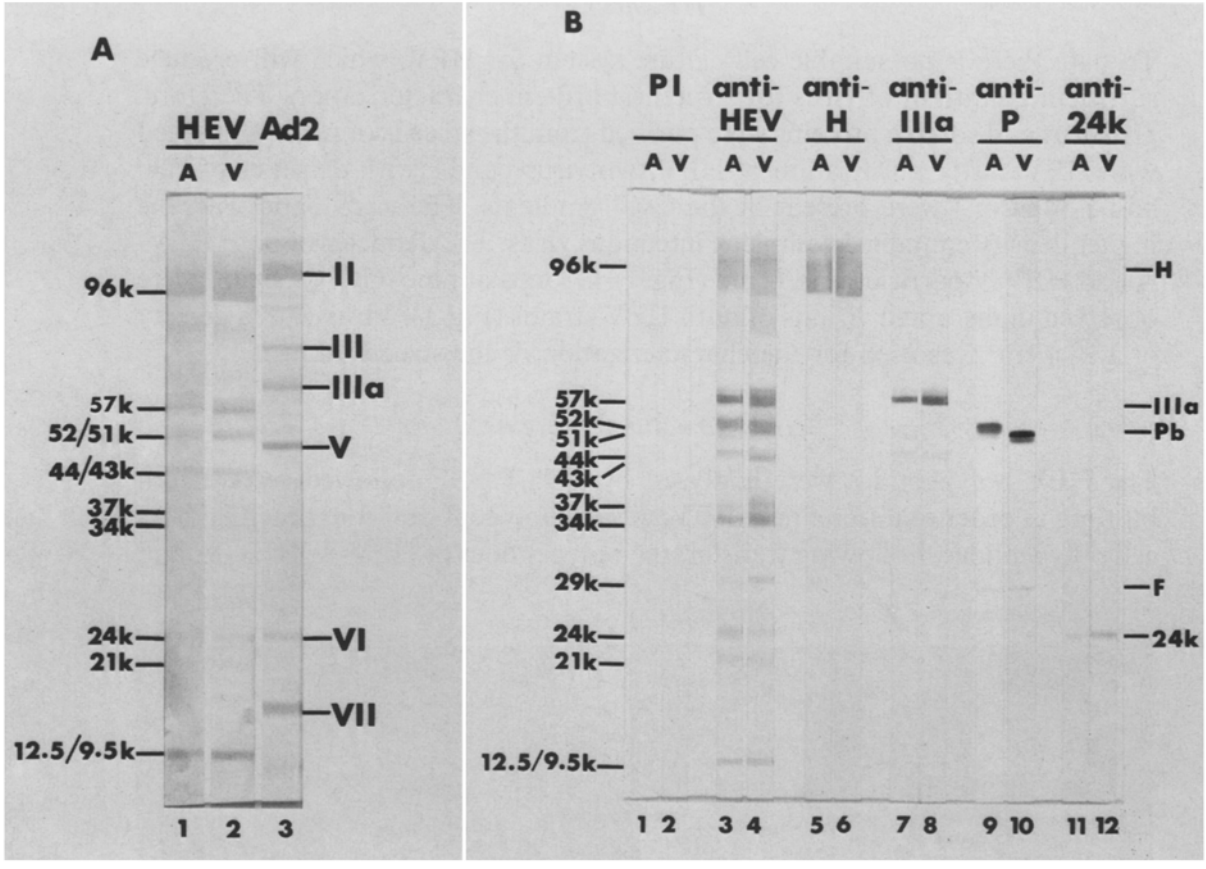

Fig. 2. Analysis of HEV polypeptides by SDS-PAGE and Western blotting. **A** Polypeptides of HEV-A (*1*) or HEV-V (*2*), separated by SDS-PAGE, were transferred onto nitrocellulose and visualized by amido black staining. The molecular weights of the HEV polypeptides, indicated in the lefthand margin, were estimated using the polypeptides of Ad 2 (*3*) as molecular weight standards (righthand margin) (polypeptide II, hexon, 108k; polypeptide III, penton base, 85k; polypeptide IIIa, 66k; polypeptide V, 48k; polypeptide VI, 24k; polypeptide VII, 18.5k). **B** Polypeptides of HEV-A (*1, 3, 5, 7, 9,* and *11*) and HEV-V (*2, 4, 6, 8, 10,* and *12*) separated as described for **A** were analyzed by Western blotting using preimmune (*PI*) serum of turkeys (*1* and *2*), turkey anti-HEV serum (*3* and *4*), monospecific turkey anti-HEV hexon protein (*5* and *6*), monoclonal antibody 4B3-10D2 reactive with the HEV IIIa protein (*7* and *8*), monospecific rabbit anti-HEV penton protein (*9* and *10*), and monoclonal antibody 6C1, reactive with 24k protein (*11* and *12*). HEV molecular weights are indicated in the lefthand margin and identified polypeptides are indicated in the righthand margin (*H* hexon; *Pb* penton base; *F* fiber, *IIIa* protein; *24k* protein)

and those of the HEV-V polypeptides 96k, 57k, 51k, 43k, 37k, 34k, 29k, 24k, 21k, 12.5k, and 9.5k. The 12.5k and 9.5k polypeptides migrated as one band on 10% polyacrylamide gels but migrated as two bands on 13% gels; both were detected after Western blotting using convalescent turkey serum. Further studies, described in the following sections, resulted in the identification of six polypeptides as the hexon (96k), penton base (52/51k), fiber (29k), IIIa protein (57k), a 24k protein, and two nucleoproteins (12.5 and 9.5k) (Fig. 2 B).

HEV-specific monoclonal antibodies

A panel of monoclonal antibodies, prepared against HEV, was reactive with the virus by FA staining and in an ELISA (Table 1). Most antibodies were only reactive under non-denaturing conditions. In order to define the specificities of these monoclonal antibodies, HEV soluble proteins and HEV core proteins were prepared as described in the following sections.

HEV soluble proteins

The hexon, penton, and fiber proteins of human adenoviruses are produced in large excess during viral replication. These viral proteins occur in the soluble protein fraction of cell extracts. Analogously, HEV soluble proteins were purified from spleens of infected turkeys. Two HEV-specific bands were detected on Western blots of the soluble protein fraction with serum of infected turkeys

Table 1. Properties of monoclonal antibodies used for the characterization and identification of HEV structural proteins

Monoclonal[a] antibody	Virus	FA staining[c]		Reactivity in ELISA[d]				Protein specificity
		cytoplasm	nucleus	Virus		Soluble proteins		
				− SDS	+ SDS	− SDS	+ SDS	
15G4	HEV-A or HEV-V[b]	+ + + +	+ + + +	+ + + +	−	+ + + +	−	hexon
2D4	HEV-A or HEV-V	+ + +	+ + + +	+ + +	−	+ + +	−	fiber
4C3	HEV-A or HEV-V	+ + + +	+ + +	+ + +	−	+ + +	−	penton base
6C1	HEV-A or HEV-V	+ +	+ +	−	+ + +	−	−	24k
4B3-10D2	HEV-A or HEV-V	±	+ +	±	+ +	+	+ +	IIIa
4B3-10D2	Ad 2	±	+ +	±	+	±	+	IIIa

[a] Each monoclonal antibody is representative of a group of monoclonal antibodies with identical specificities

[b] Data obtained for HEV-A and HEV-V were the same

[c] Fluorescent antibody staining intensity: + + + + Very strong; + + + strong; + + medium; + weak; ± very weak; − no staining

[d] Optical density: + + + + ⩾ 1.0; + + + 0.5–1.0; + + 0.25–0.50; + 0.1–0.25; ± 0.05–0.1; − < 0.05

(Fig. 3 A). Both bands were electroeluted and examined by electron microscopy. One group of monoclonal antibodies recognized the faster moving band, which was identified as the hexon protein and the other group of monoclonal antibodies recognized the slower moving protein, which appeared to be the penton protein. Soluble protein suspensions were heat-treated in the presence of deoxycholate to dissociate penton proteins into penton base and fiber proteins. In addition to the penton two new protein bands, both migrating faster than the hexon

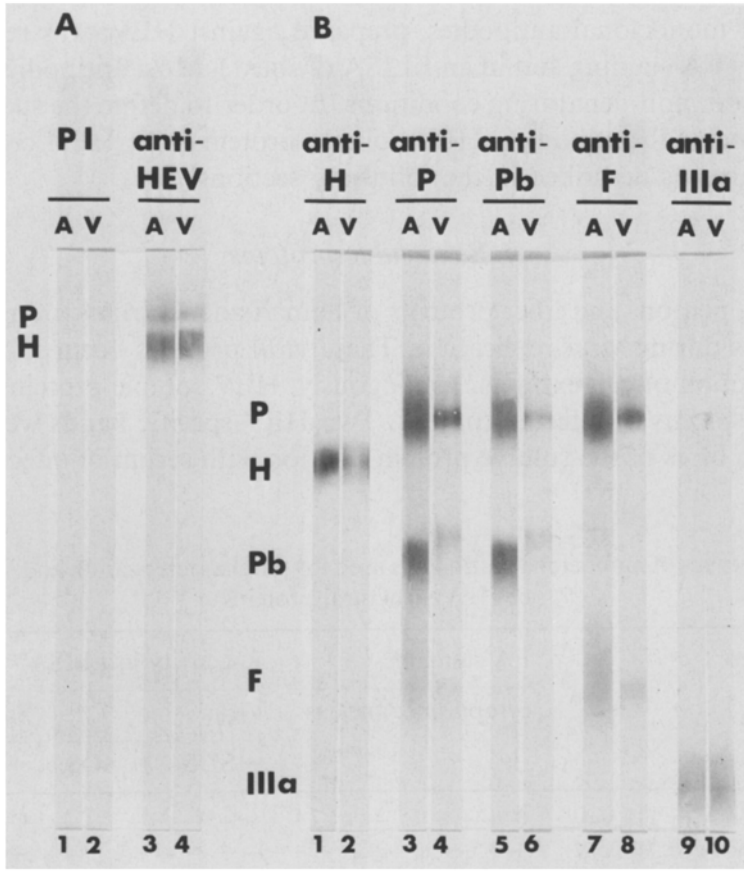

Fig. 3. Analysis of HEV soluble proteins separated by PAGE (6%) under non-denaturing conditions and detected by Western blotting. **A** Western blot analysis of HEV-A (*1* and *3*) or HEV-V (*2* and *4*) soluble proteins using turkey preimmune (*PI*) serum (*1* and *2*) or turkey anti-HEV serum (*3* and *4*) for detection. The position of the hexon (*H*) and penton (*P*) proteins are marked in the lefthand margin. **B** Western blot analysis and identification of HEV-A (*1, 3, 5, 7,* and *9*) or HEV-V (*2, 4, 6, 8,* and *10*) soluble proteins after heat treatment in the presence of deoxycholate. The HEV hexon protein was detected using monoclonal antibody 15G4 (*1* and *2*). The penton (*P*), penton base (*Pb*) and fiber (*F*) proteins were detected using rabbit anti-HEV-A penton serum (*3*), rabbit anti-HEV-V penton serum (*4*), monoclonal antibody 4C3 reactive with the HEV penton base (*5* and *6*), or monoclonal antibody 2D4 reactive with the HEV fiber protein (*7* and *8*). The IIIa polypeptide was detected using monoclonal antibody 4B3-10D2 (*9* and *10*)

protein, were detected after Western blotting using rabbit antipenton protein serum for analysis (Fig. 3 B). Electron microscopic observation of these proteins obtained after electroelution, revealed that the slower migrating protein was the penton base protein while the faster migrating proteins was the fiber protein. The HEV-A penton base protein migrated faster than the HEV-V penton base in 6% polyacrylamide gels under non-denaturing conditions (Fig. 3 B). The penton-specific monoclonal antibodies could now be divided into a group reacting with the fiber protein and a group reacting with the penton base protein. Finally, the IIIa protein was detected in the soluble protein fraction using monoclonal antibody 4B3-10D2 for identification. This protein migrated faster than the fiber protein (Fig. 3). The identification of the IIIa protein was based upon the cross-reactivity of this monoclonal antibody with the IIIa protein of Ad 2. The same electrophoretic pattern of the penton, hexon, penton base, fiber, and IIIa protein was observed when these proteins were obtained from purified HEV dissociated by four freeze-thaw cycles followed by heat treatment for 1 min at 56 °C in the presence of 0.05% deoxycholate (data not shown).

The HEV soluble proteins were further analyzed and characterized by sucrose gradient centrifugation. The profiles of the HEV-A and HEV-V soluble proteins on the gradients are shown in Fig. 4. Western blot analysis of the proteins in the two peaks using fiber-specific monoclonal antibody 2D4 (Table 1) showed that fractions 6–8 contained free fiber proteins and fractions 14–15 contained penton proteins. The free fiber peak of HEV-A was always larger than that of HEV-V under comparable conditions, regardless of whether the antibody source for detection was monoclonal antibody recognizing the HEV fiber protein, rabbit serum against the penton protein of HEV-A or HEV-V, or convalescent serum from turkeys infected with HEV-A or HEV-V. These data suggest that the soluble protein fractions contain more free fiber protein of HEV-A than of HEV-V. The shoulder of the penton protein peak (fraction 13), analyzed by Western blotting using penton base-specific monoclonal antibody 4C3 (Table 1), contained free penton base protein as well as penton protein. Sedimentation coefficients of the HEV soluble proteins were determined by centrifugation in sucrose gradients using Ad 2 soluble proteins as standards [6, 33]. The following values were determined for both HEV strains: 12 S for the hexon protein, 10 S for the penton protein, 9 S for the penton base protein, 6 S for the fiber protein, and 6 S for the IIIa protein.

Purified penton, penton base, and fiber proteins

Penton, penton base, and fiber proteins were purified to: (*i*) study their structure by electron microscopy; and, (*ii*) immunize rabbits to generate specific antibodies for the identification of the penton base and fiber polypeptides of HEV under denaturing conditions. Pentons of HEV-A and HEV-V were purified from soluble protein fractions by immunoaffinity chromatography using monoclonal antibody 2D4 and 4C3 linked to Affi-Gel 10 followed by sucrose gradient centrifugation. The penton proteins of the sucrose gradient fractions were de-

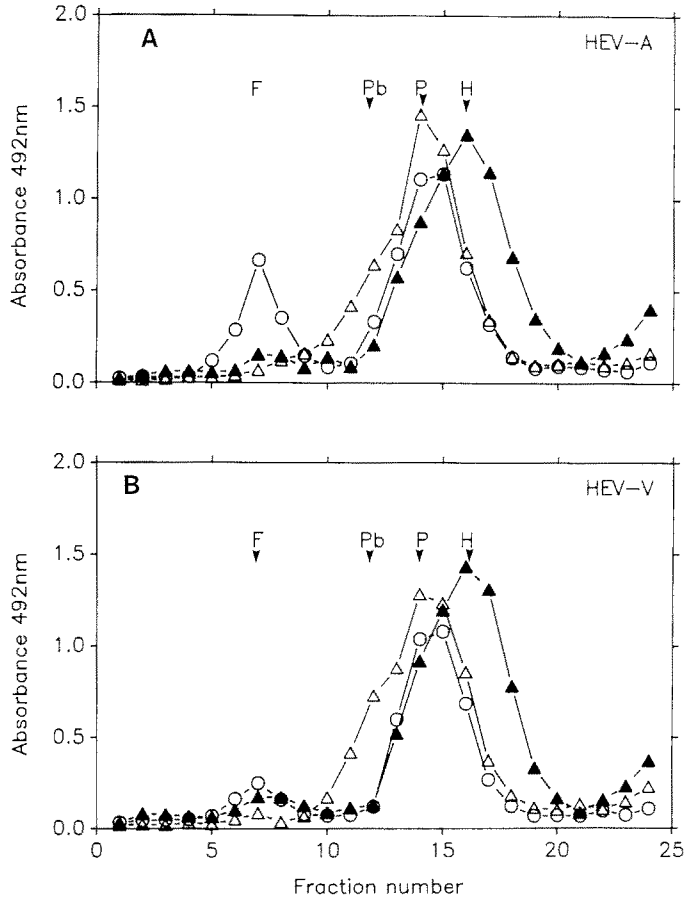

Fig. 4. Soluble protein fractions of HEV-A (**A**) and HEV-B (**B**) were centrifuged through linear sucrose gradients (10–30%, w/v) for 23 h at 38,000 rpm in a Beckman SW 41 rotor at 4 °C. Fractions were collected and analyzed by an indirect ELISA for the presence of fiber antigen (monoclonal antibody 2D4, ○), penton base antigen (monoclonal antibody 4C3, △), and hexon protein (monoclonal antibody 15G4, ▲). The positions of the fiber (*F*), penton base (*Pb*), penton (*P*), and hexon (*H*) proteins are indicated

tected by ELISA (fractions reacting with monoclonal antibody 2D4 and 4C3). The pentons were dissociated by heat treatment in the presence of deoxycholate and the resulting proteins were separated by PAGE on 6% gels. Two HEV protein bands were detected after Western blotting of which the slower migrating protein was identified as the penton base protein and the faster migrating one as the fiber protein. Again, the penton base protein of HEV-A migrated slightly faster than the penton base protein of HEV-V. The fiber protein of both HEV strains migrated at the same rate (Fig. 5 A). The penton base and fiber proteins were recovered by electroelution from 6% gels and analyzed by SDS-PAGE. The molecular weight of the penton base polypeptide was 52k for HEV-A and 51k for HEV-V, whereas the molecular weight of the fiber polypeptide was 29k for both HEV strains (Fig. 5 B) confirming the apparent molecular weight values

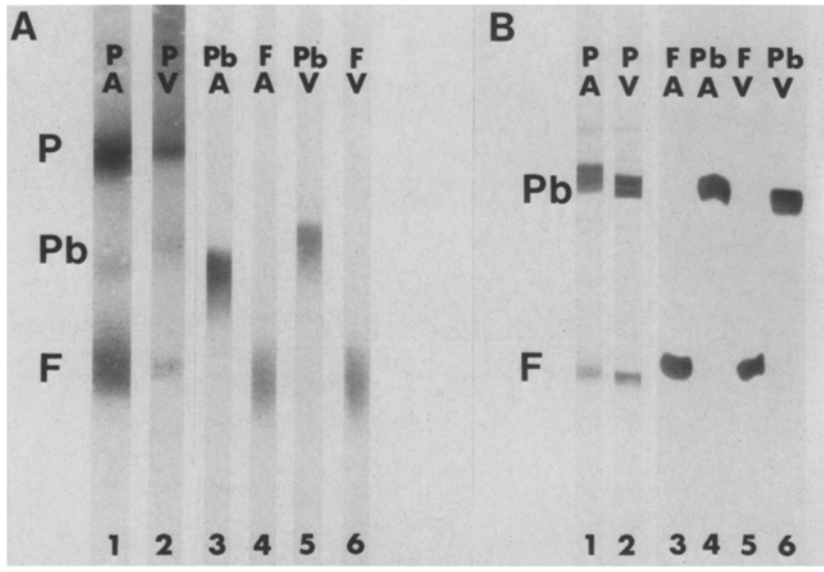

Fig. 5. Western blot analysis of HEV penton proteins purified by affinity chromatography, sucrose gradient centrifugation, and preparative PAGE. **A** Penton base and fiber proteins of HEV were obtained from penton proteins, purified by affinity chromatography and sucrose gradient centrifugation , dissociated by heat treatment in the presence of deoxycholate, and separated by preparative PAGE on 6% gels under non-denaturing conditions. Western blot analysis of the penton proteins of HEV-A (*1*) and HEV-V (*2*), purified penton base proteins of HEV-A (*3*) and HEV-V (*5*), and purified fiber proteins of HEV-A (*4*) and HEV-V (*6*) was carried out after separation of the proteins by PAGE (6%) under non-denaturing conditions using monospecific rabbit anti-penton serum for detection. **B** Western blot analysis of purified penton proteins (*1* and *2*), fiber proteins (*3* and *5*), penton base proteins (*4* and *6*) of HEV-A (*1, 3,* and *4*) and HEV-V (*2, 5,* and *6*) separated by SDS-PAGE (13%) and detected by rabbit anti-penton serum

obtained following Western blot analysis of HEV structural proteins. The same molecular weights are obtained when affinity purified pentons were directly analyzed by SDS-PAGE and Western blotting (data not shown).

Antisera from rabbits immunized with immunoaffinity-purified fiber protein of HEV-A or HEV-V reacted stronger in ELISA with the homologous than with the heterologous fiber protein (data not shown). This specificity was not found in antisera from rabbits immunized with purified penton or penton base protein.

Purified penton proteins were observed with the electron microscope. One fiber protein per penton protein was found for both HEV strains (Fig. 6). Virus particles and penton proteins of disrupted virions also showed single fiber proteins attached to their penton base proteins (Fig. 6). Length measurements of the HEV fiber proteins were performed on free pentons using negatively stained preparations. A fiber length of 17 ± 1.8 nm was measured for HEV-A and of 17 ± 2.0 nm for HEV-V using negatively stained preparations of purified

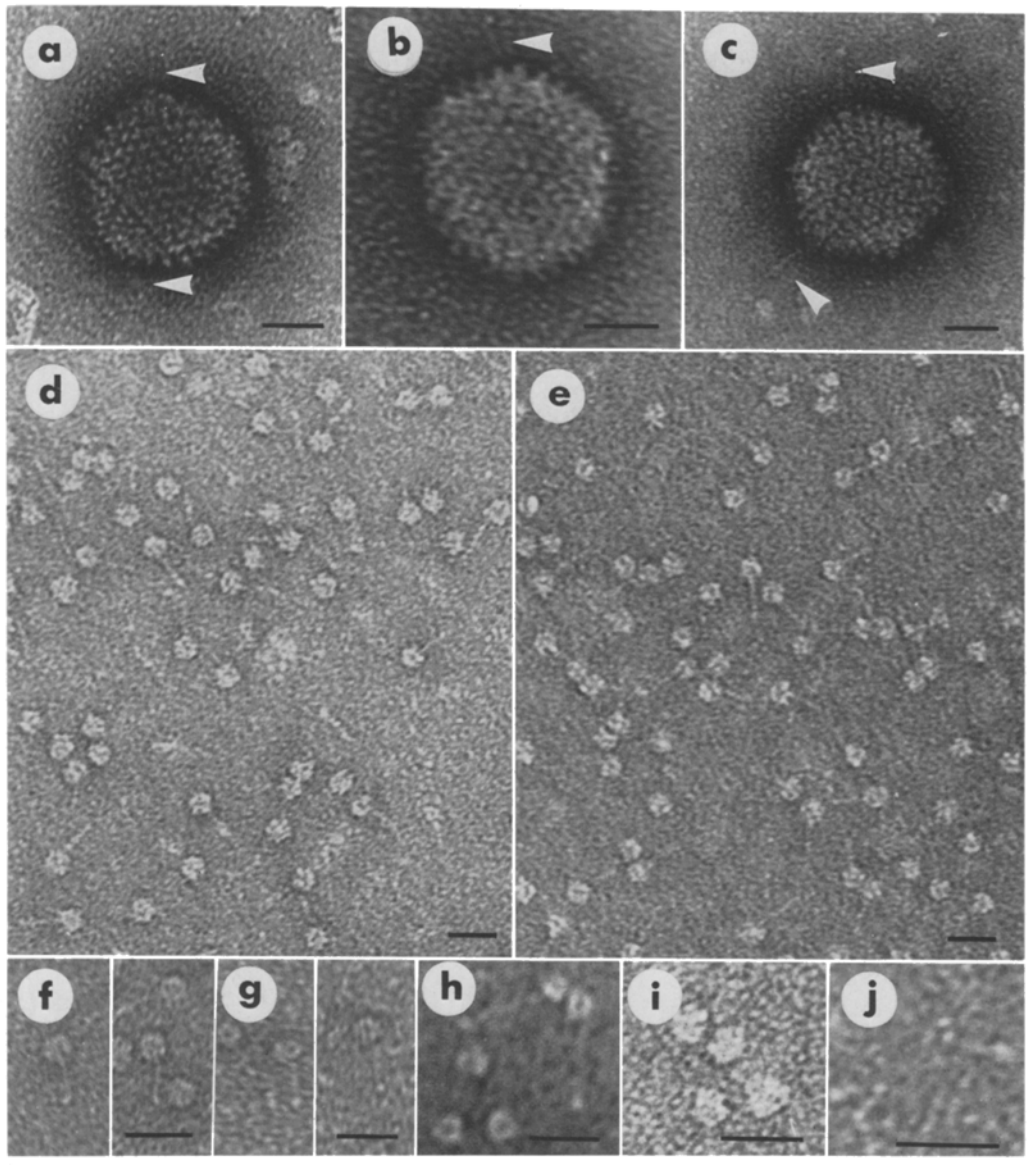

Fig. 6. Electron micrographs of CsCl purified HEV-A (**a**), HEV-B (**b**), and Ad 2 (**c**) virions showing single fibers protruding from the capsid (▷). Single fibers were also observed on pentons of HEV-A (**d**) and HEV-V (**e**) purified by immunoaffinity chromatography and sucrose gradient centrifugations, and on pentons of HEV-A (**f**) and Ad 2 (**g**) from disrupted virions. The fiber length measured from the pentons of HEV-A (**d**) and HEV-V (**e**) was estimated to be 17 nm. Pentons of HEV-A obtained by immunoaffinity chromatography from the soluble protein fraction (**f**) are compared with pentons of HEV-A (**g**) and Ad 2 (**h**) both from dissociated virions. Penton bases obtained from pentons of Ad 2 (**h**) from dissociated virions. Penton bases (**i**) obtained from pentons after dissociation, separation by PAGE, and electroelution, and fibers (**j**) obtained after immuno-affinity chromatography and sucrose gradient centrifugation are also shown. Note the knob and anchorage of the fiber in the pentons (**f**). Negative staining with uranyl acetate. Bars: 25 nm

penton proteins. A value of 31 ± 2.3 nm was measured for Ad 2 fiber proteins under the same conditions.

Protein IIIa

Monoclonal antibody 4B3-10D2 reacted specifically with both HEV strains in ELISA's of dissociated HEV virions and HEV soluble proteins, and in FA tests of HEV infected cells (Table 1). In addition, this monoclonal antibody also reacted specifically with Ad 2 in ELISA's of dissociated Ad 2 virions and soluble proteins, and in FA tests of Ad 2 infected cells. Western blot analysis revealed that this monoclonal antibody recognized the IIIa protein of Ad 2 (Fig. 7). Consequently, the 57k HEV protein recognized after Western blotting by this monoclonal antibody could be identified as the IIIa protein of HEV. No differences in migration were observed between the IIIa protein of HEV-A and HEV-V on either 6% non-denaturing polyacrylamide gels or on 10% and 13% denaturing polyacrylamide gels after Western blot analysis using monoclonal antibody 4B3-10D2 for detection.

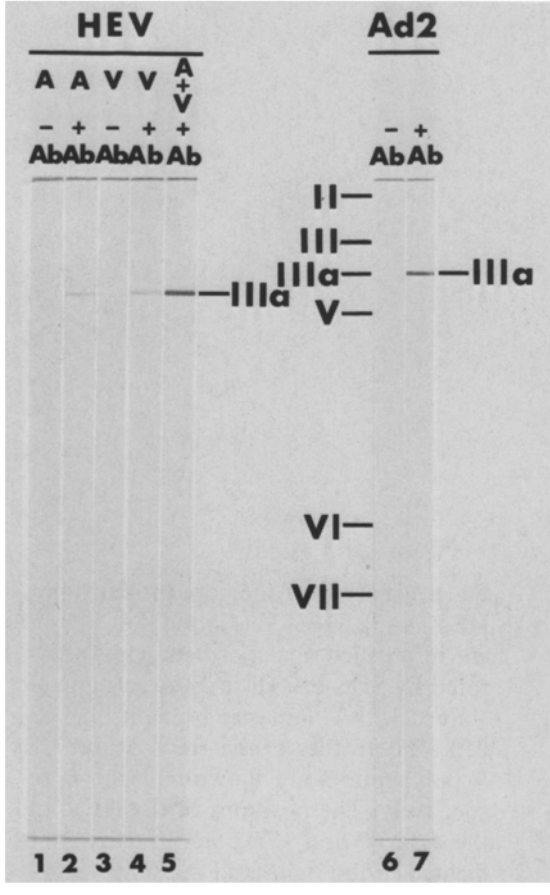

Fig. 7. Analysis of the IIIa polypeptide of HEV-A (*1*, *2*, and *5*), HEV-V (*3*, *4*, and *5*) and Ad 2 (*6* and *7*) separated by SDS-PAGE on 13% gels and detected by Western blotting in the presence (*2*, *4*, *5*, and *7*) or absence (*1*, *3*, and *6*) of monoclonal antibody 4B3-10D2. The positions of the IIIa polypeptide of HEV-A and HEV-V are identical. The positions of the marker polypeptides of Ad 2 are indicated by roman numerals

Protein 24k

The 24k protein was only detected by monoclonal antibody 6C1 in HEV virions after dissociation with SDS or by repeated freezing and thawing, but it was not detected in the soluble protein fraction by ELISA (Table 1).

Core proteins

HEV nucleoprotein cores obtained after disruption of virions were separated from capsid components by centrifugation through a linear glycerol gradient [12]. Two proteins with molecular weights of 12.5k and 9.5k were detected in the nucleoprotein fraction of both HEV strains after analysis on 13% SDS-polyacrylamide gels (Fig. 8). PAGE analysis of virus and nucleoprotein core showed a difference in stochiometry of the 12.5 k and 9.5 k polypeptides (Fig. 8). Weaker attachment of the 12.5 k protein to the DNA, resulting in a partial loss during the purification, might explain the appearance of a less prominent 12.5 k polypeptide in nucleoprotein preparations than expected.

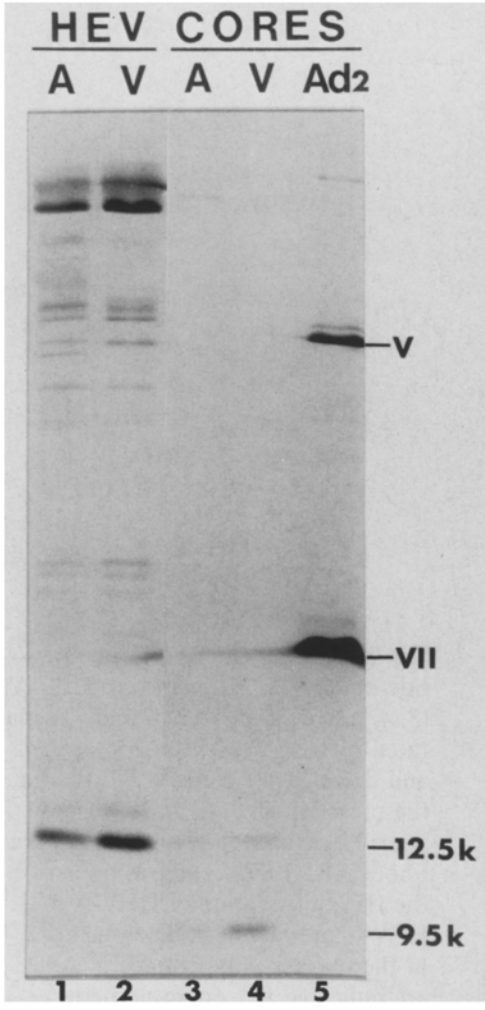

Fig. 8. Analysis of the core proteins of HEV-A (*3*), HEV-V (*4*), and Ad 2 (*5*) present in purified nucleoprotein cores separated by SDS-PAGE in 13% gels and stained with Coomassie brilliant blue R-250. The profiles of the HEV-A and HEV-V polypeptides are shown in *1* and *2*, respectively. The positions of the HEV and the Ad 2 (*V* and *VII*) core proteins are indicated in the righthand margin

Discussion

In the present study we found that purified HEV consisted of at least eleven structural proteins. The polypeptide patterns of HEV-A and HEV-V were similar with the exception of two polypeptides with apparent molecular weights of 52k vs. 51k (penton base) and 44k vs. 43k for HEV-A and HEV-V, respectively. A total of 11 polypeptides have also been described for turkey cells infected with a virulent strain HEV and a strain of MSDV using immunoprecipitation and immunoblotting [30]. Since in this study no distinction has been made between structural and non-structural proteins and no molecular weights have been described, it is difficult to compare these data with ours, although similarities in PAGE migration were observed. The electrophoretic migration pattern of the native HEV soluble proteins was different from the pattern of Ad 2 proteins. The migration order of the penton, penton base, and IIIa proteins of HEV and Ad 2 was similar, but it was inversed in the case of the hexon, and fiber proteins [2, 27]. Separation of proteins under non-denaturing conditions by PAGE depends on charge and size. Assuming that differences in size are more important than charge, an explanation as to why the HEV fiber and penton base proteins migrate faster than the HEV hexon protein might be the smaller size of these two proteins. This is in contrast to the larger size of the Ad 2 fiber and penton base proteins relative to Ad 2 hexon protein. However, the differences in migration rate might also be caused by differences in charge or a combination of both charge and size.

The hexon protein was identified on the basis of quantitative analysis and morphological characteristics. It was the most prominent protein in the outer capsid and in the soluble protein fraction, it was the structural protein with the highest molecular weight, and its sedimentation coefficient was similar to that of the hexons of other adenoviruses [33, 42]. In addition, it is an important neutralizing antigen [30, 42]. No differences were observed in electrophoretic mobility between the hexons of HEV-A and HEV-V in native or denatured conditions. In addition, the hexons of both strains had a high degree of antigenic homology because they could not be distinguished by homologous or heterologous antibodies from HEV-infected turkeys (van den Hurk, manuscript in prep.).

The penton of HEV was identified on the basis of its characteristic shape observed by electron microscopy. Single fibers attached to penton bases were observed in preparations of purified virions and pentons. Hence, HEV, in common with the mammalian adenoviruses and the avian EDS 76 V [15, 23] has pentons with single fibers, and this is in contrast to the double fibers present on penton bases of fowl adenoviruses [15, 26, 28]. In addition, the quality of one fiber was confirmed by Western blot analysis of purified HE virus and pentons in which only one fiber protein was detected.

The fibers of both HEV strains shared the following characteristics: (*i*) the same electrophoretic mobility in native and denatured condition, (*ii*) the same

electrophoretic mobility in crude and purified soluble protein fractions, as well as in purified virus preparations, and (*iii*) relatively short fibers of the same size (17 nm). The molecular weight of 29k was lower than that found for the long fibers of Ad 2 (62k), FAV-1 (65k), and EDS 76 V (67k) but resembled more closely those found for the shorter fibers of Ad 3 (34.8k), and FAV-1 (44.5k) [28, 33, 34, 37]. The observation of relatively short fibers with a low molecular weights is in agreement with the suggested relationship between the length of the native fiber protein and the size of the polypeptide [34]. However, differences between the HEV-A and HEV-V fibers were observed in serological tests where higher titers were obtained with homologous than with heterologous antiserum. In addition, a difference in the recognition of fibers of both strains by monoclonal antibodies was observed (J. V. van den Hurk, manuscript in prep.). This difference in antigenicity between two HEV strains is in agreement with the concept that the fiber protein is the most variable adenovirus component, both in size and antigenicity [15, 28, 44].

In contrast to the many properties shared by HEV-A and HEV-V, there are differences in pathogenicity, in migration of the penton base in polyacrylamide gels, and in amount of free fiber present in spleen extracts. Although speculative, there might be a relationship between pathogenicity caused in the turkey by the penton base of HEV-V and cytopathic effect caused by the penton (base) observed in vitro for human adenovirus [13, 33]. The difference in pathogenicity of the two virus isolates might be caused by: (1) a qualitative difference in toxicity of the penton base; (2) a quantitative difference in toxicity meaning a higher concentration or a more active HEV-V penton base, and (3) a combination of (1) and (2). The presence and possible role of free pentons in peripheral blood of several fatal cases of adenovirus pneumonia in young children might be supportive for this hypothesis [24].

The identification of the 57k protein of HEV as the IIIa protein was based upon recognition by monoclonal antibody 4B3-10D2 which also reacted with the IIIa protein of Ad 2. Similar results for HEV-A, HEV-V, and Ad 2 were obtained by FA staining, ELISA of soluble proteins and purified virus, and Western blotting. The recognition of the IIIa protein of HEV and Ad 2 is the first evidence of a shared antigenic determinant on a structural protein in avian and mammalian adenoviruses. The epitope shared by both viruses probably is a continuous epitope because it is still recognized after denaturation, and it is probably cryptic because it is only recognized by monoclonal antibody 4B3-10D2 after dissociation. Concealment in the virion could be expected of an epitope shared by viruses with such phylogenetically remote hosts. This conserved site might have a function in the adenovirus architecture. Hypothetically, conservation of this site might be important for linkage of the penton or peripentonal hexons to the core, a function that is probably filled by the IIIa protein [43].

The identity of the 24k protein could not be determined with certainty from the present information. However, it might be an analogue of the 24k (VI,

hexon associated) protein of Ad 2 because it had the same molecular weight, was not detected in either the soluble protein fraction or in the core protein fraction, and is probably located internally in the virion. No protein with a similar molecular weight was reported for FAV-1, but the 26k protein of EDS 76 V might also be analogous [37].

The 12.5k and 9.5k proteins were identified as core proteins of HEV. They are smaller than those of Ad 2 (48k and 18.5k), more closely resemble those of FAV-1 (20k, 12k, and 9.5k) [29, 33], and might be the two smallest polypeptides reported for group II avian adenoviruses [30]. The 12k and 9.5k proteins of FAV-1 might be the counterparts of the 12.5k and 9.5k proteins of HEV, respectively. A feature shared between HEV, FAV-1 and Ad 2 is that the smallest core protein (9.5k, 9.5k, and 18.5k respectively) seems to be more tightly attached to viral DNA than the larger one(s) [29, 33].

This report strengthens the arguments for the classification of HEV in the family *Adenoviridae* for two reasons: (*i*) the common properties of the structural proteins of HEV and other adenoviruses, and (*ii*) the homology in the IIIa protein of HEV and Ad 2. In addition, the presence of single or double fibers respectively supports the division of the aviadenoviruses into group I (fowl adenoviruses) and group II (HEV, MSDV, SV).

Furthermore, since hexon, penton, and fiber proteins can be purified by immunoaffinity chromatography, the role of these proteins as immunogens can be investigated.

Acknowledgements

I thank S. D Acres and L. A. Babiuk for helpful and constructive criticism, J. E. Gilchrist for preparation of some of the monoclonal antibodies, L. McDougall, D. Dent, and U. Medrek for technical assistance, and M. Hagen for typing this manuscript. Published with the permission of the Director as Journal Series No. 62.

References

1. Allestrom P, Stenlund A, Li P, Bellett A, Pettersson U (1982) Sequence homology between avian and human adenoviruses. J Virol 42: 306–310
2. Boudin M-L, Moncany M, D'Halluin JC, Boulanger PA (1979) Isolation and characterization of adenovirus type 2 vertex capsomer (penton base). Virology 92: 125–138
3. Boulanger PA, Puvion F (1973) Large-scale preparation of soluble adenovirus hexon penton and fiber antigens in highly purified form. Eur J Biochem 39: 37–42
4. Burnette WM (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 112: 195–203
5. Carlson HC, Al-Sheikhly F, Pettit JR, Seawright FL (1974) Virus particles in spleens and intestines of turkeys with hemorrhagic enteritis. Avian Dis 18: 67–73
6. Chee-Sheung CC, Ginsberg HS (1982) Characterization of a temperature-sensitive fiber mutant of type 5 adenovirus and effect of the mutation on virion assembly. J Virol 42: 932–950

7. Domermuth CH (1980) Hemorrhagic enteritis of turkeys In: Hitcher SB, Domermuth CH, Purchase HG, Williams JE (eds) Isolation and identification of avian pathogens. American Association of Avian Pathologists, College Station, TX, pp 106–107
8. Domermuth CH, Gross WB (1984) Hemorrhagic enteritis and related infections. In: Hofstad MS, Barnes HJ, Calnek BW, Reid WM, Yoder HW Jr (eds) Diseases of poultry. Iowa State University Press, Ames, pp 511–516
9. Domermuth CH, Gross WB, DuBose RT, Mallison ET (1975) Experimental reproduction and antibody inhibition of marble spleen disease of pheasants. J Wildl Dis 11: 338–342
10. Domermuth CH, Harris JR, Gross EB, DuBose RT (1979) A naturally occurring infection of chickens with a hemorrhagic enteritis/marble spleen disease type of virus. Avian Dis 23: 479–484
11. Domermuth CH, Weston CR, Cowen BS, Colwell WM, Gross WB, DuBose ET (1980) Incidence and distribution of adenovirus group II splenomegaly of chickens. Avian Dis 24: 591–594
12. Ennever JF, Love SM, Harpst JA (1985) Tonic effects on the structure of nucleoprotein cores from adenovirus. Biochim Biophys Acta 826: 67–79
13. Everitt SF, Ginsberg HS (1958) A toxic-like material separable from type 5 adenovirus particles. Virology 6: 770–771
14. Ey PL, Prowse SJ, Jenkins CR (1978) Isolation of pure IgCl, IgG 2a and IgG 2b immunoglobins from mouse serum using protein A-Sepharose. Immunochemistry 14: 429–436
15. Gelderblom H, Maichle-Lauppe J (1982) The fibers of fowl adenoviruses. Arch Virol 72: 289–298
16. Green M, Pina M (1963) Biochemical studies on adenovirus multiplication. IV Isolation, purification and chemical analysis of adenovirus. Virology 20: 199–207
17. Gross WB, More WEC (1967) Hemorrhagic enteritis of turkeys. Avian Dis 11: 533–547
18. Iltis JP, Daniels SB (1977) Adenovirus of ring-necked pheasants: purification and partial characterization of marble spleen disease virus. Infect Immun 16: 701–705
19. Iltis JP, Jakowski RM, Wyand DS (1975) Transmission of marble spleen disease in turkeys and pheasants. Am J Vet Res 36: 97–101
20. Itakura C, Carlson HC (1975) Electron microscopy findings of cells with inclusion bodies in experimental hermorrhagic enteritis of turkeys. Can J Comp Med 39: 299–304
21. Jakowski AM, Wyand DS (1972) Marble spleen disease in ringnecked pheasants: demonstration of agar gel precipitation antibody in pheasants from an infected flock. J Wildl Dis 8: 261–263
22. Kennett RH, Dennis KA, Tung AS, Klinman NR (1978) Hybrid plasmacytoma production: fusion with adult spleen cells, monoclonal spleen fragments, neonatal spleen cells and human spleen cells. Curr Top Microbiol Immunol 81: 77–91
23. Kraft V, Grund S, Monreal G (1979) Ultrastuctured characterization of isolate 127 of egg drop syndrome 1976 virus as an adenovirus. Avian Pathol 8: 353–361
24. Ladish S, Lovejoy HF, Hierhozen JC, Oxman MN, Strieder D, Vowter GF, Finer N, Moore M (1979) Extrapulmanary manifestations of adenovirus type 7 pneumonia simulating Reye syndrome and the possible role of adenovirus toxin. J Pediatr 95: 348–355
25. Laemmli UK (1972) Cleavage of structural proteins during the asembly of the head of bacteriophage T 4. Nature 227: 680–685
26. Laver WG, Younghusband HB, Wrigley NG (1971) Purification and properties of chick embryo lethal orphan virus (an avian adenovirus). Virology 45: 598–614

27. Lemay P, Boudin M-L, Millevile M, Boulanger P (1980) Human adenovirus type 2 protein III a. 1. Purification and characterization. Virology 101: 131–143
28. Li P, Bellett AJD, Parish CR (1984) Structural organization and polypeptide composition of the avian adenovirus core. J Virol 52: 638–649
29. Li P, Bellett AJD, Parish CR (1984) The structural proteins of chick embryo lethal orphan virus (fowl adenovirus type 1). J Gen Virol 65: 1803–1815
30. Nazerian K, Lee LF, Payne WS (1991) Structural polypeptides of type II avian adenoviruses analysed by monoclonal and polyclonal antibodies. Avian Dis 35: 572–578
31. Norrby E, Bartha A, Boulanger T, Dreizin R, Ginsberg HS, Kalter SS, Kawamura H, Rowe WP, Russell WC, Schlesinger W, Wigand R (1976) Adenoviridae. Intervirology 7: 117–125
32. Ossa JE, Bates RC, Schurig GG (1983) Hemorrhagic enteritis in turkeys: purification and quantification of the virus. Avian Dis 27: 235–245
33. Pettersson U, Hoglund S (1969) Structural proteins of adenoviruses: III. Purification and characterization of the adenovirus type 2 penton antigen. Virology 39: 90–106
34. Philipson L (1983) Structure and assembly of adenoviruses. Curr Top Microbiol Immunol 109: 1–52
35. Signas C, Akusjarvi G, Pettersson U (1985) Adenovirus 3 fiber polypeptide gene: implications for the structure of the fiber protein. J Virol 53: 672–678
36. Silim A, Thorsen J, Carlson HC (1978) Experimental infection of chickens with hemorrhagic enteritis virus. Avian Dis 11: 106–114
37. Todd D, McNulty MS (1978) Biochemical studies on a virus associated with egg drop syndrome 1976. J Gen Virol 40: 63–75
38. Tolin SA, Domermuth CH (1975) Hemorrhagic enteritis of turkeys. Electron microscopy of the causal virus. Avian Dis 19: 118–125
39. van den Hurk JV (1986) Quantitation of hemorrhagic enteritis virus antigen and antibody using enzyme-linked immunosorbent assays. Avian Dis 30: 662–671
40. van den Hurk JV (1990) Propagation of group II avian adenoviruses in turkey and chicken leukocytes. Avian Dis 34: 12–25
41. van den Hurk JV (1990) Efficacy of avirulent hemorrhagic enteritis virus propagated in turkey leukocyte cultures for vaccination against hemorrhagic enteritis in turkeys. Avian Dis 34: 25–35
42. van den Hurk JV, van Drunen Littel-van den Hurk (1988) Characterization of group II avian adenoviruses with a panel of monoclonal antibodies. Can J Vet Res 52: 458–467
43. van Oostrum J, Burnett RM (1985) Molecular composition of the adenovirus type 2 virion. J Virol 56: 439–448
44. Waddell G, Hammarskjold M-L, Winberg G, Varsany TW, Sundell G (1980) Genetic variability of adenoviruses. Ann New York Acad Sci 354: 16–42
45. Winterfield RW (1984) Adenovirus infections of chickens. In: Hofstad MS, Barnes HJ, Calnek BW, Reid WM, Yoder HW Jr (eds) Diseases of poultry. Iowa State University Press, Ames, pp 498–506

Author's address: J. V. van den Hurk, Veterinary Infectious Disease Organization, 124 Veterinary Road, Saskatoon, Saskatchewan S7N 0W0, Canada.

Received January 22, 1992