Development of a vaccine for the prevention of hemorrhagic enteritis in turkeys

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
in het openbaar te verdedigen
op dinsdag 13 december 1988
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

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STELLINGEN

1. Het is onwaarschijnlijk dat de resultaten, die in dit proefschrift zijn beschreven, zouden zijn bereikt zonder gebruik te maken van monoklonale antibodies.
2. De bewering dat de lange fiber van fowl adenovirus type 1 is opgebouwd uit een octameer van polypeptide IV is waarschijnlijk onjuist.
   Li, P., A.J.D. Bellett, en R. Parish. J. Gen. Virol. 65:1803-1815. 1984.
3. Een recombinant hexon polypeptide van een adenovirus zal waarschijnlijk geen immuniteit induceren tegen een wild type virus infektie in de natuurlijke waard.
4. De konklusie van Perrin et al. dat hemorrhagic enteritis virus in vitro repliceert in miltcellen van de kalkoen kan niet gemaakt worden op grond van hun experimentele resultaten.
   Perrin, G., C. Louzis, en D. Toquin. Bull. Acad. Vet. Fr. 54:231-235. 1981.
5. De specifieke selektie van monoklonale antibodies die reageren met virus-specifieke antigenen heeft de ontdekking en studie van gemeenschappelijke determinanten in virus en waard negatief beinvloed.
6. Het gebruik van een gekontamineerde celkultuur, zoals RP19 cellen met Mareks virus, voor het produceren van vaccins zou niet moeten worden toegestaan.
7. De bewering van Davenas et al. dat basofielen kunnen worden gedegranuleerd door anti-IgE antiserum dat 10^{30} tot 10^{120} keer verdund is, is voorbarig.
   Davenas, E., F. Beauvais, J. Amara, M. Oberbaum, B. Robinzon, A. Miadonna, A. Tedeschi, B. Pomeranz, P. Fortner, P. Belon, J. Sainte-Laudy, B. Poitevin, and J. Benveniste. Nature 333:816-818. 1988.
8. De natuurlijke staat van het landschap in Canada wordt beschermd door de aanwezigheid van bloedzuigende insekten.
9. Het tweetalig karakter van Canada bevat meer negatieve dan positieve kanten.
10. De vervanging van de bison door het rund voor vleesproduktie in de prairie provincies van Canada is niet gebaseerd op ekonomische overwegingen.

Jan V.J.M. van den Hurk
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Wageningen, 13 december 1988

Aan Sylvia
Remko
Marcel

VOORWOORD

Graag wil ik bij het verschijnen van dit proefschrift allen danken die aan de totstandkoming ervan hebben bijgedragen.

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CONTENTS

LIST OF ABBREVIATIONS

A	absorbance
Ad2	adenovirus type 2
AGP	agar-gel precipitin
CBA	competitive antibody binding assay
CMI	cell-mediated immunity
d	days
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double-stranded
E	early
EDS76V	egg drop syndrome 1976 virus
EDTA	ethylene diamine tetraacetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
F	fiber
FA	fluorescent antibody
FAV-1	fowl adenovirus type 1
FITC	fluorescein isothiocyanate
g	gram
g	acceleration of gravity
GON	group of nine
h(r)	hour(s)
H	hexon
HE	hemorrhagic enteritis
HEV	hemorrhagic enteritis virus
HEV-A	avirulent hemorrhagic enteritis virus
HEV-V	virulent hemorrhagic enteritis virus
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
k	kilo
kV	kilovolt
l	liter
L	late

M	molar
MDV	Marek's disease virus
mg	milligram
ug	microgram
min	minute(s)
ml	milliliter
mM	millimolar
mRNA	messenger-ribonucleic acid
MSDV	marble spleen disease virus
NK	natural killer
nm	nanometer
OD	optical density
P	penton
PAGE	polyacrylamide gel electrophoresis
Pb	penton base
PBS	phosphate-buffered saline
PI	preimmune
r	correlation coefficient
	density (g/cm^3)
RNA	ribonucleic acid
rpm	revolutions per minute
s	sedimentation coefficient in Sverdberg units
TBS	tris-buffered saline
$TCID_{50}$	tissue culture infectious doses 50
TID_{50}	turkey infectious doses 50
TN	tris-NaCl
TNE	tris-NaCl-EDTA buffer
tris	tris(hydroxymethyl)aminomethane
v	volume
V	volt
VIDO	Veterinary Infectious Disease Organization
w	weight
wk	week(s)

CHAPTER 1

GENERAL INTRODUCTION

1.1 General characteristics of adenoviruses.

Adenoviruses are able to infect man and a wide variety of animal species in which they may cause respiratory, ocular, gastrointestinal, and urinary infections. These viruses are classified into a single family based upon common physical, chemical, morphological, and structural properties (95,144). The members of this family all have a remarkable uniform morphology and size (70-90nm) when observed by electron microscopy. Adenoviruses have an icosahedral shell of 252 capsomers (71) and a nuclear core containing double-stranded DNA. The icosahedral shell comprises 240 hexons, each surrounded by six neighbouring capsomers, and pentons each surrounded by five peripentonal hexons (61,142). The hexons are found at the 20 triangular facets and 30 edges, and the pentons at the 12 vertices of the icosahedral capsid. A penton consists of two structural units: a penton base and single or double fibers protruding from the vertex (59,88,110,142).

The family of the Adenoviridae is divided into the genera Mastadenovirus, consisting of 90 or more serotypes, and Aviadenovirus, consisting of 15 or more serotypes (144). This division is based upon the presence of a group-specific antigen in most mammalian adenoviruses which is absent in the avian adenoviruses (144). The adenoviruses have been classified into subgroups and types in various ways according to biological, chemical, immunological, or structural properties, including natural host, antigenic relationship, neutralization, and DNA sequence homology (144). The classification of the avian adenoviruses is less well defined than that of the mammalian adenoviruses, because the members of the former group have been less extensively studied and characterized, and have a wider host range than those of the latter group (83,144). A subdivision of the avian adenoviruses into two groups or types, group I avian adenovirus and group II avian adenovirus, has been tentatively proposed based upon an antigenic relationship within but not between the two groups and a difference in host-cell tropism (37,45,84). The group I avian adenoviruses includes twelve serotypes of the fowl adenoviruses (FAV-1 to FAV-12) and probably two serotypes of turkey adenoviruses which all share a group-specific antigen and replicate in homologous kidney cell cultures (33,99). Egg drop syndrome 1976 virus (EDS76V) might also be a member of the group I avian adenoviruses although serological crossreactivity between EDS76V and FAV serotypes

was marginal (1b,8,100). The group II avian adenoviruses, consisting of hemorrhagic enteritis virus (HEV) of turkeys (22,80,138), marble spleen disease virus (MSDV) of pheasants (23,75,76,79), and splenomegaly virus (SV) of chickens (43,44,45) are antigenically related viruses (36,40,43,44,45,77), which do not replicate in (primary) kidney, liver or fibroblast cell cultures of homologous or heterologous hosts (77,79,148). The group II avian adenoviruses are able to infect turkeys, pheasants and chickens. However, they seem to be only pathogenic for their natural host (42,43,44,45,78).

1.2 Virus-host interaction.

1.2.1 General aspects. Adenoviruses can cause respiratory, ocular, gastrointestinal, liver, kidney, and urinary diseases in man and animals (83,131). In addition, they may cause persistent and latent infections in many species, as well as tumors in rodents (83,131). Transmission of adenoviruses occurs mostly horizontally, but fowl adenoviruses and EDS76V can be transmitted vertically through eggs (83,101,131). Major targets of an initial infection are mucous membranes of the oral and nasopharyngeal cavity, conjunctiva, cloaca, and regional lymphatic organs. Human adenoviruses establish productive infections in gastrointestinal, respiratory, or ocular epithelial cells (131). Adenoviruses might become transiently viremic and enter lymphatic organs where they multiply in reticuloendothelial cells and produce intranuclear inclusion bodies (83,131). Clearance of the infecting virus presumably requires cellular (macrophages, lymphocytes) and humoral (antibodies, complement, and lymphokines) immune reactions (52). Live virus vaccines containing adenovirus type 4 (Ad4) or Ad7 have been used successfully to immunize recruits against acute respiratory disease (25,32,131,139,140,141).

1.2.2 Hemorrhagic enteritis in turkeys. Hemorrhagic enteritis (HE) in turkeys was first observed by Pomeroy and Fenstermacher in 1937 (122). HE is an acute disease caused by HEV (22,37,80,138). Clinical signs of illness include enteritis, splenomegaly and hemorrhages in various tissues, especially the intestine, and may lead to mortality in field outbreaks ranging from less than 1% to over 60% (37,38,69). HE is widespread and it is a disease of economical importance (37). Data from serological studies indicate that almost all adult turkeys have been infected with HEV (37,39). HE in turkeys usually occurs between 6 and 11 weeks of

age and is most common in 7- to 9-week-old birds (37,122). Younger birds are usually refractory to the disease as a result of the presence of maternal antibodies (37).

The natural hosts of group II avian adenoviruses, to which HEV belongs, are turkeys, pheasants, and chickens. Death only occurs in the natural host (37). Mortality, usually observed between 4 and 6 days after infection, may reach up to 80% in turkeys experimentally infected with pathogenic strains of HEV (37). Gross lesions are distended intestines filled with red and brownish blood, and enlarged marbled spleens (22,68,69,81). Intranuclear inclusions containing HEV are present in reticuloendothelial cells of many organs especially the spleen (22,68,80,81,146).

1.2.3 Prevention of hemorrhagic enteritis in turkeys by vaccination.

1.2.3.1 Vaccine requirements. A vaccine should be safe and effective (1,1a). In this context, safety indicates the need to avoid harmful side effects following vaccination, and effectiveness indicates the need to induce a protective immune response in most recipients. Protective immune responses can be subdivided into three categories. The first is the induction of neutralizing antibodies (e.g. antibodies which prevents viral infection) which should be long lasting. The second is the induction of an appropriate form of cell-mediated immunity (CMI). The value of such a response is mostly to limit rather than to prevent an infection. Furthermore, the CMI response is important because it is usually crossprotective between subtypes in contrast to serological response. The last is the induction of a non-specific immune response consisting of the activation of macrophages, NK cells, and increase in the production of cytokines. This immune response is important in the face of an epidemic.

1.2.3.2 Hemorrhagic enteritis vaccines. Until recently, only two vaccines were available for prevention of HE in turkeys. The first one is a crude vaccine prepared from spleens of turkeys infected with avirulent HEV (HEV-A) (38,136). The second vaccine contains HEV-A grown in a lymphoblastoid cell line (RP19) derived from a Marek's disease virus (MDV)-induced tumor (49,50,104,105,106). Both are live virus vaccines administered in the drinking water and both vaccines elicit protective immunity in turkeys. However, the safety features of these vaccines have to be carefully evaluated. Spleen extract vaccines might contain extraneous pathogens due to the manner they are obtained from spleen without further

purification, and to immunosuppression caused by HEV replicating in immune cells which lowers resistence against infectious agents. In addition, infectious MDV is present in RP19 cell cultures (104), and products from tumor cells, like RP19 cells, are only recenty allowed for vaccine production.

It is evident from these data that the safety of these two vaccines leaves to be desired. Hence, there was a need for a safer vaccine to prevent HE in turkeys. Therefore, one of the major goals was to develop an appropriate cell culture-propagated live virus vaccine for the preparation of HE in turkeys.

1.3 Virus-cell interaction.

The interaction between adenovirus and host cell may result in a lytic, semipermissive or abortive infection. The outcome of the virus infection depends on the nature of the infecting virus and the type of host cell.

1.3.1 Lytic infection. Lytic infections of cell cultures are convenient for virus propagation and characterization, and have been described for most mammalian and group I avian adenoviruses. Primary and continuous epithelial cell lines such as HEK, Hela, HEp-2, or KB cells are suitable for human adenovirus propagation (9,73,131). Characteristic cytopathic alterations include rounding and ballooning of the cells, cellular aggregation, and detachment of the cells from the surface (9,73,131). The first steps during infection are attachment and penetration of the virus into the cell. Two mechanisms for the internalization of Ad2 in cells have been described. The most likely mechanism is receptor-mediated endocytosis (53,63,134,135), but the mechanism of direct penetration can not be excluded (16,94,103). Receptor-mediated endocytosis of adenoviruses is a process in which virus-ligands (fibers at physiological pH) bind to cellular receptors (26,53,90,120,134,135). Subsequently, virus-receptor complexes enter coated pits by random diffusion, and become trapped in vesicles (endocytic vesicles or receptosomes) formed from the coated pits, which are then internalized in the cell. Attachment and endocytosis destabilizes the virions and renders them sensitive to DNase. Then the hydrophobic area of the penton base, alone or in combination with fiber and hexon, is thought to destabilize the receptosomes and cause virus release in the cytosol (114,127,128). Subsequent steps involve transfer to the nuclear membrane and uncoating of the virion resulting in a free nucleocapsid which penetrates into the nucleus (27,103). Final uncoating occurs in the nucleus when

viral DNA is released for replication (94). Six to eight h after infection progeny DNA can be detected in the nuclei and after 24h 10^5 to 10^6 molecules of viral DNA are synthesized of which only 20% is packaged to form mature virions (65).

1.3.2 Semipermissive and abortive infection. In semipermissive infections virus production is reduced (58,123). A number of factors may cause this reduction including virus type, virus mutant, slower uptake and uncoating of virus in the cell, a lower rate of viral DNA replication and a less efficient translation of viral proteins (64,70,72). In abortive infections the virions enter the cell but viral DNA replication does not take place. Abortive infections, which can be caused by many mammalian and group I avian adenoviruses, sometimes result in transformation (5,6,7,24,62,97,98). Only the left end of the genome of adenoviruses, including the E1 gene, is required for transformation. Whether HEV can give rise to semipermisive or abortive infections resulting in transformation is not known.

1.3.3. Propagation of HEV. Group II avian adenoviruses replicate well in their hosts, but difficulties were encountered with the in vitro propagation of these viruses. In contrast, most mammalian and group I avian adenoviruses can be propagated easily in cell cultures. Attempts to propagate HEV in embryonated chicken and turkey eggs and in chicken and turkey embryo fibroblast cultures have all been unsuccessful (22,41). Furthermore, several attempts have been made to infect spleen cells of turkeys in vitro (51,115). Although infection has been reported, actual virus replication in these cells has not been demonstrated. Similar negative results have been reported for the propagation of MSDV in cell cultures of embryo fibroblasts and kidneys of chickens, pheasants, and turkeys, and in embryonated eggs of turkeys (77,79,148). The only cell lines in which HEV replicate are the MDV-transformed B lymphoblastoid cell lines RP16 and RP19 obtained from turkey tumors (104,105). However, the presence of MDV in these cell lines made them unattractive for HEV propagation and vaccine production.

Therefore, a different approach had to be taken for the in vitro propagation of HEV. Instead of trying to grow HEV in epitheloid cells, attempts were made to propagate this virus in turkey leukocytes, a cell population which includes the natural target cells in HEV-infected turkeys.

1.3.4 Adenovirus DNA. The size of the adenovirus genome varies from serotype to serotype with molecular weights ranging from 17 x 10^6 for simian adenoviruses to 30

x 10^6 for FAV-1 (20,88). Basic features of the DNA shared by all adenoviruses including FAV-1 are (57,83,85,133): 1) the linearity of the double strands; 2) terminal proteins (TP's), covalently linked to the 5' ends of both DNA strands in the virions, which play a role in the initiation of DNA replication; 3) the presence of inverted terminal repetitions; 4) a DNA sequence homology of at least five base pairs extending from nucleotides 9-14;
5) similar mechanisms for DNA replication; and 6) the production of a virus-specific DNA-binding protein (DBP) in the cell upon infection.

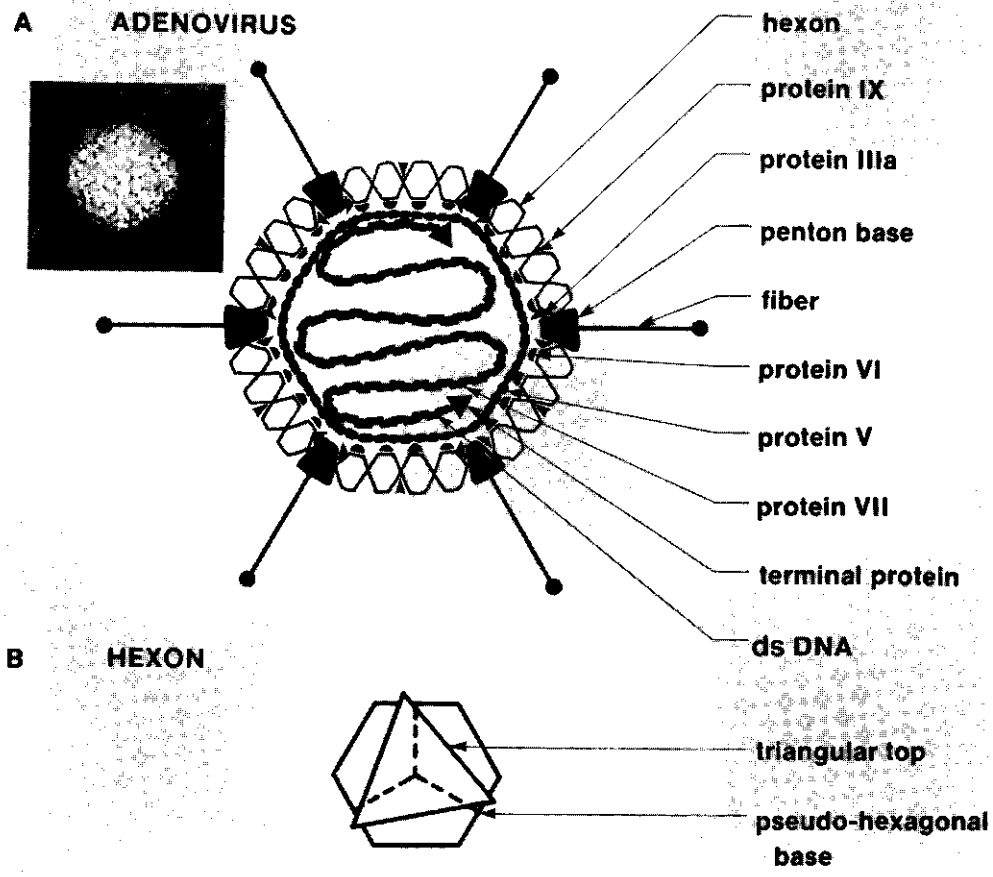
The mode of adenovirus DNA replication is semi-conservative and asymmetrical (89). Models of the replication mechanism, based on in vitro and in vivo studies in which the function of virus-coded proteins [TP, precursor TP (pTP), DNA-polymerase (DNA-pol), and DBP] and host cell factors (nuclear factor I and II) required for initiation and prolongation of DNA synthesis, have been described for the human adenoviruses type 2 (Ad2), type 5 (Ad5), and type 12 (Ad12) (56).

1.3.5 Transcription of Adenovirus DNA. The majority of the research on adenovirus transcription has been focussed on the replication cycle of Ad2 and Ad5. The adenovirus genes are subdivided into genes transcribed before the onset of viral DNA replication (early genes: regions E1a, E1b, E2a, E3, and E4), genes transcribed before and during viral DNA replication (intermediate genes: regions E2b and IVa2), and genes transcribed after the onset of viral DNA replication (late genes: regions L1, L2, L3, L4, and L5) (10,28,29,129). The positions of promoters of Ad2 from which the mRNAs are transcribed during a lytic infection have been mapped (10,28,29). The adenovirus RNA's are capped at the 5' end and polyadenylated at the 3' end (121). Regions E1a and E1b contain genes for transformation and regulation of transformation. Region E2a codes for the DBP, and region E2b for the pTP, and DNA-pol. Late transcription products from regions L1, L2, L3, L4, and L5 are processed into groups of related mRNA's that share common 5' and 3' ends, but are differently processed from the precursor-RNA molecules (28,54,102). The following regions contain coding information for the structural proteins: E1b for IX, L1 for pIIIa, L2 for III (penton base), pVIII, and V, L3 for pVI and II (hexon), L4 for pVIII, and L5 for IV (fiber).

1.3.6 Adenovirus proteins. A schematic view of the proteins in an adenovirus particle is illustrated in Fig. 1, and the properties of Ad2 and FAV-1 are summarized in Tables 1 and 2.

1.3.6.1 Hexon. Hexons are the major capsid proteins in the virion. They are localized at the 20 triangular facets and 30 edges of the icosahedral capsid. Four types of hexon polypeptides can be distinguished in adenovirus-infected cells: nascent hexon polypeptide (monomer), hexon protein (trimer), group of nine (GON) hexons, and hexons in virions. The term hexon (protein) is used to indicate the trimer. In infected cells, free hexons are present in a 10 to 100-fold excess over hexons assembled in virions (145). Free and capsid hexons are identical proteins (19) and each hexon consists of three identical polypeptides (15,31,74,124). Each monomer consists of 967 amino acids and has a molecular weight of 109,000 (109k) (2). A model of the three-dimensional structure of Ad2 hexon, based on x-ray cristallography and electron microscopy, has been described (19,124). This model shows that the hexon consists of two parts: a triangular top containing three towers and a pseudo-hexogonal base. The pseudo-hexagonal base of the trimer facilitates hexon-hexon interaction within a hexagonal array in the virion. The heterologous regions of Ad2 and Ad5 (both members of subgroup C) are found at the top of the hexons, indicating that type-specific determinants are restricted to the outside of the capsid and that hexon-hexon interactions are conserved (19,124). The fact that in the intact virion the type-specific determinant (ϵ) lies on the outside of the capsid whereas the group-specific determinant (α) is inaccessible for antibodies are in consent with this model (112).

1.3.6.2 Penton. Pentons, composed of a complex of penton base and fiber, are localized at the 12 vertices of the adenovirus icosahedron (Fig. 2) (61,142). Mammalian adenoviruses contain single fibers whereas most avian adenoviruses (fowl adenoviruses) contain double fibers with the exception of EDS76V which has single fibers (59,86,88). Pentons and fibers are also synthesized in excess in infected cells, and are present as soluble and capsid proteins (14,15). Fiber and penton base are joined by noncovalent bonds which can be dissociated by treatment with pyridine or deoxycholate (14,117). Penton base and fiber can associate in vitro into pentons which are indistinguishable from those present in the capsid (12). There is no consensus about the subunit conformation of the human adenovirus penton which is either trimeric or pentameric for the penton base (14,34,35,118,143), and dimeric or trimeric for the fiber (34,35,66,118,143). Each combination includes at least one mismatch in symmetry which occurs at the vertex. A solution might be that, based upon the observation of a pentameric penton base consisting of three shorter and two longer polypeptides, a trimeric fiber could bind to the three


A ADENOVIRUS
hexon
protein IX
protein IIIa
penton base
fiber
protein VI
protein V
protein VII
terminal protein
ds DNA
B HEXON
triangular top
pseudo-hexagonal base
C GROUP OF NINE HEXONS
hexon
protein IX

shorter penton base polypeptides (143). How two fibers, probably coded by different genes, are associated with one penton base as described for the fowl adenoviruses is not known (92). It has been suggested that the penton base of FAV-1 exists as a trimer, the long fiber as an octomer, and the short fiber as a trimer (92).

Fibers vary in length from 9 - 47nm (59,118), and in molecular weight from 35k (Ad3) to 67k (EDS76V) (130,137). A fiber consists of a knob, a shaft with a repeating motif of 15 residues (Ad2, Ad3), and a tail (66,108,130). The 15 residue motif is repeated 22 times in the longer Ad2 fiber (30nm), and six times in the shorter Ad3 fiber (10-11nm). The fiber is the only adenovirus protein that is glycosylated (82). Each polypeptide carries two molecules of N-acetylglucosamine. The fiber mediates the early recognition between virus and cell (120). Furthermore, it carries a species-specific determinant (γ) in its knob which might interact with red blood cells causing hemagglutination (111,142).

The penton can induce an early cytopathic effect on cells, including a rounded cell morphology and detachment of the cells from the surface (46,142). Although it has been definitely demonstrated that the penton base is responsible for this phenomenon the mechanism involved is still unclear (14,117,147).

1.3.6.3 Protein IIIa. Protein IIIa is localized in the peripentonal region, might be accessible on the surface of the virion, and appears to connect the peripentonal

Fig. 1. Architecture of adenovirus particle and hexon protein based on data from Nermut, Philipson, and Van Oostrum and Burnette (108,119,143). A. Schematic view of an Ad2 particle illustrating the architexture and apparent topography of the structural proteins in the virion. The viral capsid is made up of the hexon, penton base, fiber, IIIa, VI, VIII and IX proteins. Protein VIII is not shown because its location in the virion is not known. The viral core consists of a core shell (protein V), and a DNA-protein complex containing protein VII associated with dsDNA and terminal proteins linked to both 5' ends of the genome. Insert shows an electron micrograph of HEV-A. B. Schematic view of an Ad2 hexon (protein) with pseudo-hexagonal base and triangular top. The three subunits of the hexon are more distinct at the base with little interpenetration of the polypeptide chains, in contrast to the triangular top in which each of the three towers is built up of all three polypeptide chains. C. Model of a group of nine (GON) hexons. The GON hexons is stabilized by protein IX.

Table 1. Composition and properties of human Ad2 proteins[A]

Polypeptide	Location	Molecular weight		Subunit composition	No. copies/ Virion	Sedimentation coefficient	Remarks
		SDS-PAGE[B]	Sequence[C]				
II	Hexon	108	109.1	Trimer	720	12.9	Major capsid protein
III	Penton base	85	63.3	Trimer or Pentomer	36 - 60	9.5	Vertex proteins
IIIa	Vertex region	66	63.3	Monomer	60 - 74	6.0	Phosphorylated
IV	Fiber	62	62.0	Dimer or Trimer	24 - 36	6.0	Protruding from penton base, glycosylated
TP	Core	55	-[D]	Monomer	2	-	Covalently-linked to 5' of DNA
V	Core	48	41.6	Monomer	157 - 180	3.5	Weakly attached to DNA, phosphorylated
VI	Hexon associated(?)	24	23.5	Dimer or Trimer	342 - 450	3.8	Phosphorylated, DNA binding
VII	Core	18.5	19.4	Monomer or Tetramer	833 - 1070	2.3	Basic, DNA binding
VIII	Hexon associated(?)	13	14.5	Monomer	211	1.6	
IX	Group of nine	14	14.3	Dimer	247 - 280	2.3	

[A] Modified from Philipson, Nermut, Pettersson, and van Oostrum and Burnett (108,116,118,143).

[B] Apparent molecular weight determined by SDS-PAGE.

[C] Molecular weight based upon polypeptide sequence determined experimentally or derived from base sequence data.

[D] Not known.

Table 2. Composition and properties of fowl adenovirus type 1 (FAV-1)[A]

Polypeptide	Location	Molecular weight[B]	No. copies/ virion	Subunit Composition
II	Hexon	100	720	Trimer
III	Penton base	92	36	Trimer
IV	Long fiber	65	96	Octamer
V	-[C]	62	66	-
VI	-	55	30	-
TP	Core	46	2	Monomer
VII	Short fiber	44.5	36	Trimer
VIII	-	30	-	-
IX	Core	20	740	-
X	-	18	1330	-
XI	Core	12	910	-
XII	Core	9.5	1910	-

[A] Data from Li et al. (91a,92)

[B] Determined by SDS-PAGE

[C] Not known

proteins to the core (143). This protein is produced in excess during the infectious cycle in the cells (90). It is phosphorylated (3,11), and cleaved during virus maturation from pIIIa into IIIa (13). There are approximately 60 - 72 copies of monomeric IIIa per virion (13,35,143). No counterpart of the human IIIa protein has been described for avian adenoviruses.

1.3.6.4 Core proteins. Viral DNA of Ad2 is associated with the core proteins V (48k) and VII (18.5k) (48,87,96) whereas the DNA of FAV-1 is associated with the core proteins IX (20k), XI (12k), and XII (9.5k) (92,93). Protein VII, derived by cleavage from pVII (4), is the major core protein (4,47). The minor core protein V is phosphorylated. It is less basic and not as strongly associated with the DNA as protein VII (17). A model for the human adenovirus core was proposed in which protein VII and viral DNA form a helical structure surrounded by a shell of protein V (17,107,108,127).

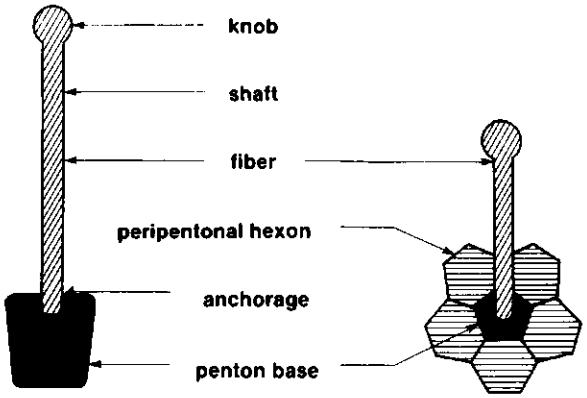


Fig. 2. Model of an Ad2 penton. The penton of this virus consists of one fiber inserted in the penton base. The pentons are present at the five fold vertices of the icosahedron and each penton is surrounded by five hexons (peripentonal hexons).

1.3.6.5 Proteins VI, VIII and IX. Less information is available about proteins VI, VIII and IX of Ad2. Protein VI (24K) (4,48,96), derived from pVI, is located inside the virion (47) and has affinity for DNA (125). Protein VIII (14.5K) (4,48,96) is weakly associated with the hexon protein, and is derived from pVIII during virus maturation (113). Protein IX (14k) is exposed at the outside of the virion, and, although it is not essential for virus assembly, it acts as a stabilizing agent for the GON hexons (30,116). Twelve copies of IX are associated with each GON hexon (18,21). An analogue of IX might not exist for FAV-1 because no GON hexons were found after dissociation of FAV-1 (88).

1.3.7 Assemblage and maturation of adenoviruses. A model for the assemblage of Ad2 based on biochemical data and electron microscopic observations has been described (119). During the late phase of an adenovirus infection host cell protein synthesis is shut off, viral mRNA is preferentially translated, and viral polypeptides are synthesized and transported to the nucleus. Empty capsids are assembled from GON hexons, single hexons, penton base, fiber, pIIIa, pVI, pVIII, and IX. Then, the core proteins V and pVII, and DNA, left-hand end of the genome first, enter the empty capsid to form immature virions. Finally, the precursor proteins are cleaved to generate mature virions.

1.4 Objective and experimental approach.

The specific aims of this investigation were: 1) the characterization of HEV and HEV proteins, 2) the development of a cell culture system for HEV propagation, and 3) the use of avirulent HEV produced in this cell culture system to prevent HE in turkeys.

First, sensitive and specific ELISAs were developed for the quantitation of HEV antigen and antibody (Chapter 2). The ELISA for the titration of antibodies was used to determine the presence and the decline of maternal antibody titers in young turkeys and to monitor seroconversion and protection in turkeys following immunization with HEV-A (Chapters 2 and 6). The ELISA for the titration of antigen was used in the potency assay to monitor protection following immunization with HEV-A and challenge with HEV-V (Chapter 6).

Second, the generation of monoclonal antibodies, also required for the antigen ELISA, was the cornerstone for further research. They were used for the characterization of HEV proteins (Chapters 3 and 4). In addition, hexon-specific monoclonal antibodies were used in a fluorescent antibody (FA) test to identify HEV-infected cells. Finally, with this test different cell cultures were screened and selected for HEV susceptability and virus production (Chapter 5).

Third, cell culture-produced HEV-A was analyzed for its potential to induce protection against HE in turkeys following immunization, initially in experimental trials at the Veterinary Infectious Disease Organization (VIDO) and subsequently in field trials on turkey farms (Chapter 6).

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CHAPTER 2

Quantitation of Hemorrhagic Enteritis Virus Antigen and Antibody Using Enzyme-Linked Immunosorbent Assays

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SUMMARY. Enzyme-linked immunosorbent assays (ELISAs) were developed to quantitate hemorrhagic enteritis virus (HEV) antibodies in turkey sera and HEV antigens in tissue extracts. These assays were more sensitive than the commonly used agar-gel precipitin tests in detecting antigen and antibody. The antibody-ELISA was used to monitor the presence and decline of passive antibodies in turkey poults and the seroconversion of turkeys infected with HEV. The antigen-ELISA was carried out using a monoclonal antibody; this test was used to quantitate HEV antigen in experimentally infected turkeys in a time-sequence experiment. Both ELISAs measured a strong antigenic relationship between an avirulent strain (HEV-A) and a virulent strain (HEV-V).

RESUMEN. Cuantificación de antígeno y anticuerpos contra el virus de la enteritis hemorrágica mediante pruebas de inmunoensayo con enzimas asociadas.

Se desarrollaron pruebas de inmunoensayo con enzimas asociadas (ELISA) para cuantificar anticuerpos en suero de pavos contra el virus de la enteritis hemorrágica y para cuantificar la presencia de antígeno viral presente en extractos de tejidos. Estas pruebas resultaron más sensibles que las pruebas de precipitación en agar para la detección de anticuerpos y de antígeno. La prueba ELISA para cuantificar anticuerpos fué utilizada para determinar la presencia y la disminución de anticuerpos pasivos en pavos jóvenes y la respuesta humoral de pavos infectados con el virus. La prueba ELISA para la detección de antígeno se realizó usando anticuerpos monoclonales y fué utilizada para la cuantificación de antígeno en pavos infectados experimentalmente. Ambas pruebas ELISA encontraron una estrecha correlación entre una cepa avirulenta y otra virulenta del virus de la enteritis hemorrágica.

Group II avian adenoviruses, which cause acute infectious diseases, include the hemorrhagic enteritis virus (HEV) of turkeys, the marble spleen disease virus (MSDV) of pheasants, and the splenomegaly virus (SV) of chickens. These three viruses have been tentatively classified as group II avian adenoviruses, because there is a strong serologic relationship among them (4,5,10) and they do not show serological cross reactivity with the group I avian adenoviruses (1,5,16).

Agar-gel precipitin (AGP) tests have been used commonly to detect HEV antibodies in sera and HEV antigens in tissue extracts of infected turkeys (3). However, this method is rather insensitive and in some cases fails to detect low to medium levels of antibody (17).

The pathogenesis of HEV in experimentally infected turkeys and the development of protective immunity following vaccination is being studied; therefore, sensitive and specific assays are needed to study the rate of decline in passive antibody titers, to monitor seroconversion after infection or vaccination, and to detect viral antigen in tissues of infected birds. Enzyme-linked immunosorbent assays (ELISAs) were used to quantitate antibody and antigen levels in poults experimentally infected with either an avirulent strain (HEV-A) or a virulent strain (HEV-V) of HEV; results are presented herein and compared with results obtained with the corresponding AGP test.

MATERIALS AND METHODS

Turkeys. Day-old small white diamond hybrid poults (Chinook Belt Hatcheries, Calgary, Alberta, Canada) raised in isolation were used in all experiments. Poults were bled at regular intervals to determine the rate of decline in passive antibody titers.

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Hemorrhagic enteritis virus. HEV-A and HEV-V were both gifts from Dr. C. Domermuth, Blacksburg, Virginia. HEV-A was originally isolated from the enlarged spleen of a pheasant suspected of having marble spleen disease. HEV-V was isolated from the spleen of a turkey that died from hemorrhagic enteritis (HE). Both strains were propagated in 6-to-9-week-old turkeys, initially by infecting the turkeys with crude spleen extracts, and subsequently by infecting them orally with 300 to 1000 median turkey infective doses (TID_{50}) of CsCl-purified HEV diluted in phosphate-buffered saline (PBS) (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4; pH 7.2). Turkeys were killed 5 days postinfection, and their spleens were removed and stored at −70 C.

Crude spleen extract. Crude virus preparations were prepared by homogenizing the spleens of HEV-A- or HEV-V-infected turkeys using a polytron (Brinkman Instruments, Rexdale, Ontario, Canada). Spleens were suspended in 0.01 M Tris-HCl buffer, pH 8.1, at a ratio of 1:10 (w/v). The resulting suspensions were sonicated three times (Braun-sonic 1510, Braun Instruments, San Mateo, California) for 20 sec at 300–400 watts and then clarified by centrifugation for 5 min at 10,000 × *g* to pellet cellular debris. The virus-containing supernatant was collected and stored at −70 C. Control extracts were prepared in the same way from spleens of turkeys that had not been exposed to HEV. All manipulations were carried out at 0 to 4 C. The extracts were tested for the presence of HEV antigens with the ELISA and the AGP test.

HEV antigen in crude spleen extracts used for evaluation in the 3-step antibody-ELISA was further purified and concentrated by an $(NH_4)_2SO_4$ precipitation at 50% saturation. The pellets obtained after a centrifugation of 10 min at 10,000 × *g* were resuspended in PBS at 0.1 × their original volume.

Crude MDTC-RP19 cell extract. MDTC-RP19 cells (13), obtained from Dr. K. Nazerian (East Lansing, Michigan), were collected by centrifugation 2 days after they were infected with HEV. The cells were resuspended in a small volume of 0.01 M Tris-HCl buffer, pH 8.1, and HEV was released from the cells by two freeze-thaw cycles followed by sonication for 15 seconds at 100 watts. After centrifuging 4 min at 10,000 × *g*, the virus-containing supernatant was collected and stored at −70 C.

ELISA for HEV antibody. An indirect ELISA for titrating HEV antibodies was developed, and the effects of several important conditions on its sensitivity were investigated. These included: (i) use of CsCl-purified virus instead of crude spleen extracts for coating, (ii) treating purified virus with 2 M $CaCl_2$ before coating, (iii) diluting serum samples in PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T), and (iv) diluting serum samples in 10× PBS-T (Table 1).

After the results of tests using these variables were evaluated, the final procedure was carried out as follows. (i) HEV used for coating microtiter plates was extracted from spleens of experimentally infected turkeys and purified on CsCl gradients (van den Hurk, manuscript in preparation). The virus band having a density of 1.34 g/cm^3 was treated with an equal volume of 2 M $CaCl_2$ and incubated for 30 min at 37 C. Thereafter, 96-well polystyrene microtiter plates (Immulon TM2, Dynatech Laboratories, Alexandria, Virginia) were coated for 2 hr at 37 C with an optimum dilution of antigen (0.2 ml per well) in 0.05 M sodium bicarbonate buffer, pH 9.6. After coating, the plates were washed three times with PBS-T, which was also used for all subsequent washes. (ii) Experimental sera, as well as positive and negative control sera, were diluted twofold in 10× PBS-T containing 1% (v/v) newborn calf serum (GIBCO, Grand Island, New York), added to the wells (0.2 ml per well), and incubated for 2 hr at 37 C. (iii) After three washes with PBS-T, 0.2 ml peroxidase-conjugated rabbit anti-turkey IgG diluted 1:1000 and containing 1% newborn calf serum was added to each well, and the plates were again incubated for 2 hr at 37 C. After three more washings with PBS-T, 0.2 ml of substrate solution containing 5-aminosalicylic acid (0.08%, w/v) and H_2O_2 (0.005%, v/v), pH 6.0, was added to each well. Absorbance (A) was measured after 30 min at room temperature with a micro-ELISA reader (MR 580, Dynatech Laboratories, Oxnard, California) at 492 nm. All tests were done in duplicate, and average titers were calculated. Each assay included two positive control sera (with known titers) and two negative control sera. The same

Table 1. Comparison of ELISA antibody titers using various test conditions.[A]

Serum no.	Purified HEV + $CaCl_2$ + 10× PBS-T	Purified HEV − $CaCl_2$ + 10× PBS-T	Purified HEV + $CaCl_2$ + 1× PBS-T	Crude spleen extract + $CaCl_2$ + 10× PBS-T
1	30	30	40	20
2	40	30	30	40
3	40	30	40	40
4	40	40	40	20
5	80	60	40	20
6	960	640	320	60
7	1920	1280	640	80
8	3840	1280	480	120
9	3840	1920	1280	640
10	5120	1920	1280	160

[A]HEV-A preparations were obtained by CsCl purification or by $(NH_4)_2SO_4$ precipitation of crude spleen extracts from HEV-A-infected turkeys. $CaCl_2$ was added to the preparations before coating the plates, and the sera were diluted in 10× PBS-T or PBS-T. Sera with antibody titers greater than 40 were considered positive.

concentrations (A_{278}) of purified HEV-A and HEV-V were used for coating the plates.

An alternative four-step ELISA for titrating HEV antibodies was also developed. In this method, the first step of the three-step ELISA described above was replaced by two steps. (i) The plates were coated with monoclonal antibody from mouse ascites fluid (diluted 1:3000 in 0.05 M sodium bicarbonate buffer). (ii) The plates were incubated with HEV-infected crude spleen extract or RP19 cell extract at optimum dilutions in PBS-T. The remainder of the assay was as described above.

ELISA for HEV antigen. This assay was carried out in four steps. (i) Polyvinyl 96-well microtiter plates (Falcon 3912 MicroTest 111®, Falcon Plastics, Oxnard, California) were coated with 0.2 ml per well of the IgG fraction of pooled immune sera of turkeys infected with either HEV-A or HEV-V. The IgG fraction was purified by Na_2SO_4 precipitation (14) and diluted to a concentration of 10 μg/ml in 0.05 M sodium bicarbonate buffer, pH 9.6. After incubation for 2 hr at 37 C, and after each of the following incubation steps, the plates were washed three times with PBS-T. (ii) Twofold dilutions of crude spleen extracts in PBS-T were added to the plates, which were incubated overnight at 4 C. (iii) A 0.2-ml amount of monoclonal tissue-culture supernatant fluid diluted 1:10 with PBS-T was added to each well, and the plates were incubated for 2 hr at 37 C. (iv) A 0.2-ml amount of affinity-purified peroxidase-linked goat anti-mouse IgG (Boehringer-Mannheim, Dorval, Quebec, Canada) diluted 1:1000 was added to the wells, and the plates were incubated for 2 hr at 37 C. The A value was measured as described above.

All tests were performed in duplicate and included extracts with known antigen titers and extracts of control turkeys that had never been exposed to HEV. The HEV antigen titers are given as the average of the duplicates. The characteristics of the monoclonal antibody used (15G4) will be described elsewhere (van den Hurk, manuscript in preparation). Briefly, it reacts specifically with a major protein of HEV. It reacts equally well with HEV-A and HEV-V and also with field isolates from HEV-infected turkeys. It does not react with the Group I avian adenoviruses.

AGP tests. Antibody and antigen titrations using the AGP test were performed as described by Domermuth *et al.* (3). For the antibody test, pooled crude spleen extracts used had high antigen titers (≥ 160 in the AGP antigen test). For the antigen test, the same pooled IgG fraction used for coating in the antigen-ELISA was used. All tests were performed in duplicate, and average titers were calculated.

Conjugate. The IgG fraction of sera from rabbits immunized with turkey IgG purified by sodium sulfate precipitation (14) was obtained by affinity chromatography on DEAE Affi-Gel Blue (described by Bio-Rad Laboratories, Richmond, Calif.). This purified IgG was linked to horseradish peroxidase type VI (Sigma, St. Louis, Missouri) according to the method of Nakane and Kawaoi (12) as modified by van den Hurk and Kurstak (18).

Experimental design for application of the ELISAs. *Decline in passive antibody titers.* In an attempt to determine how rapidly passive antibody titers decline, poults raised in isolation were bled at various times from 2 days to 7 weeks of age. Antibody titers were measured by ELISA and AGP using both HEV-A and HEV-V as antigen.

Seroconversion following exposure to HEV. Seroconversion data were obtained from a group of 10 turkeys orally infected with HEV-A when 15 weeks old. Blood samples of all turkeys were taken at the time of infection (day 0). After infection, the birds were divided into two groups of 5. Each group was bled alternately every other day until day 21. The antibody response of the birds was measured by ELISA.

RESULTS

ELISA for HEV antibody determination. *Assay conditions.* Sensitivity was greatest using $CaCl_2$-treated, CsCl-purified virus for coating the plates and 10× PBS-T as the serum diluent (Table 1). Consequently, these conditions were used in all further experiments. A linear relationship was obtained by plotting the $\log_{10}$ of the reciprocal of the serum dilution against the $\log_{10}$ of the A_{492} measured with the ELISA reader after subtracting the background absorption (i.e., wells in which turkey serum was replaced by PBS-T).

Fifty-five sera from turkeys not exposed to HEV and 50 sera from turkeys experimentally infected with HEV-A or HEV-V were tested at various dilutions in 10× PBS-T. The mean + 2 standard deviations of the A_{492} of the negative sera was the criterion used to distinguish between antibody-positive and antibody-negative sera. At a dilution of 1:40, the 55 sera from unexposed birds were all negative and the 50 sera from exposed birds were all positive (titers ranging from 200 to 80,000) (Fig. 1). At a dilution of 1:20, two of the 55 samples from unexposed birds were positive, and at a dilution of 1:10, three samples were positive. On the basis of these results, sera with titers more than 40 were considered to be positive for antibodies to HEV, and sera with titers of 40 or less were considered negative.

Decline in passive antibody titers. In an attempt to determine how rapidly passive antibody titers declined, poults raised in isolation were bled at various ages. Table 2 shows the propor-

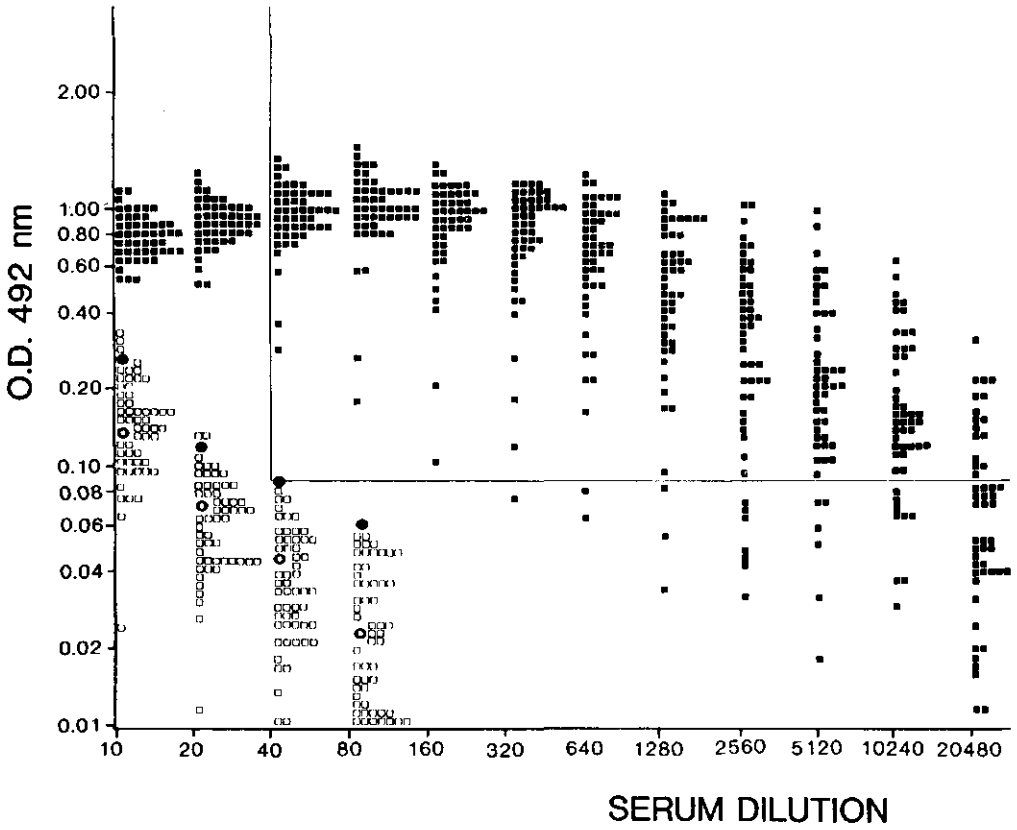

Fig. 1. Antibody ELISA of sera from 55 turkeys never exposed to HEV (□) and from 50 turkeys infected with HEV-A or HEV-V (■). Sera were collected 7–40 days postinfection. At each dilution, the mean (○) + 2× the standard deviation (●) of the absorbance ($O.D._{492}$) of sera from the unexposed birds is marked. At a dilution of 1:40 there was a clear separation between the two groups of birds.

tion of birds that were seropositive and the geometric mean antibody titers at different ages. The ELISA was much more sensitive than the AGP test. Titers declined as the birds aged. When the ELISA was used, most birds remained seropositive until 3 weeks of age. At 5 weeks, most birds were seronegative (titer of 40 or less). In contrast, when the AGP test was used, low antibody titers were detected in some birds at 2 days of age, and most were negative by 1 week of age.

The HEV-V and HEV-A antigens did not differ significantly in reactivity in either test. The Pearson moment correlation (R) for the ELISA antigen titers was 0.97 ($P < 0.01$). The ELISA detected passive antibodies in all sera from approximately 200 poults less than 3 weeks old purchased from poultry suppliers (data not shown).

Seroconversion following exposure to HEV. Seroconversion data were obtained from a group of 10 turkeys orally infected with HEV-A. All 10 birds were seronegative at the time of infection (Fig. 2). By days 3 and 4, three of the five birds in each group were positive; after this time all birds were positive. The titers peaked about 10 days postexposure and remained at this level for the remainder of the trial. No significant difference was found between plates coated with HEV-A and those coated with HEV-V (results not shown).

Antibody titers in 28 sera of turkeys at various times after experimental infection with HEV-A or HEV-V were determined using homologous and heterologous antigen (Table 3). The correlation coefficient (r) in ELISA antibody titers between plates coated with homologous and het-

Table 2. Comparison of passive antibody titers against HEV in poults at various ages using ELISA and agar-gel precipitation (AGP).

	ELISA[A]				AGP[B]			
	HEV-A		HEV-V		HEV-A		HEV-V	
Age	Titer[C]	No. positive/ total	Titer	No. positive/ total	Titer	No. positive/ total	Titer	No. positive/ total
2 days	2129 (480–7680)	10/10	2255 (320–7680)	10/10	1 (1–4)	3/10	1 (1–4)	3/10
1 week	745 (240–1920)	8/8	745 (160–1920)	8/8	<1 (<1–2)	1/8	<1 (0–2)	1/8
2 weeks	263 (80–640)	5/5	248 (80–640)	5/5	<1	0/5	<1	0/5
3 weeks	132 (40–480)	4/5	108 (40–480)	4/5	<1	0/5	<1	0/5
5 weeks	28 (20–50)	1/5	24 (15–60)	1/5	<1	0/5	<1	0/5
7 weeks	14 (5–35)	0/5	13 (5–30)	0/5	<1	0/5	<1	0/5

[A]ELISA antibody titers of poults were determined using plates coated with purified HEV-A or HEV-V. Titers greater than 40 were considered positive.

[B]AGP antibody titers were determined against crude spleen extracts of turkeys infected with HEV-A or HEV-V. A titer of less than 1 means no reaction with undiluted serum. Titers of 1 or greater were considered positive.

[C]Titers are presented as the geometric mean and the range in parentheses.

erologous antigen was 0.98 for the sera from HEV-A-infected turkeys and 0.97 for the sera from HEV-V-infected turkeys. The ELISA was at least 640 times more sensitive than the AGP test. There were no significant differences in titers due to virulence of HEV strain.

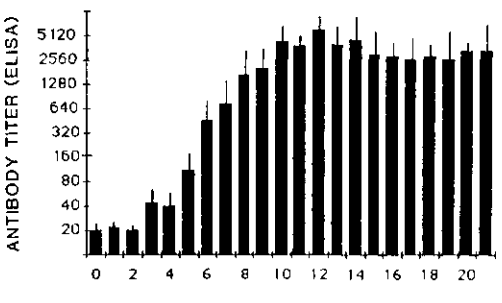

Fig. 2. Seroconversion of poults after infection with HEV-A. Ten 15-week-old turkeys were infected with HEV-A on day 0. The turkeys were split into two groups of 5, which were then bled on alternate days. The geometric mean of the ELISA titers, expressed as the reciprocal of the serum dilution, are shown for each day. Homologous virus was used as capture antigen in the ELISA. Bars represent the standard error.

A four-step ELISA for antibody titration, in which crude virus extract from either turkey spleens or MDTC-RP19 cells was used instead of purified virus, was also developed. Selective binding of HEV antigen from these crude extracts was obtained using microtiter plates coated with monoclonal antibody (15G4). The criteria for the evaluation of the four-step ELISA were the same as those for the three-step ELISA. The four-step ELISA was less sensitive than the three-step ELISA (Table 4). HEV-infected spleens and MDTC-RP19 cells yielded similar antibody titers.

ELISA for HEV antigen titration. *Test conditions.* The absorbance of spleen extracts from 50 turkeys not exposed to HEV and 50 turkeys exposed to HEV were compared. Plotting the $\log_{10}$ of the reciprocal of the extract dilution against the $\log_{10}$ of the absorbance resulted in a linear relationship. Extracts were tested at various dilutions (Fig. 3). The mean + two times the standard deviation of the A_{492} of extracts from the unexposed birds was taken as the point at which samples were considered positive. When this criterion was used, there was clear separation between the exposed and unexposed birds at di-

Table 3. Comparison of HEV antibody titers determined by ELISA and agar-gel precipitin (AGP) in sera from turkeys infected with HEV-A or HEV-V.

Serum no.	Days post-infection	Virus used for infection	ELISA titers[A]		AGP titers[B]	
			HEV-A coating	HEV-V coating	HEV-A extract	HEV-V extract
1	3	HEV-A	60	80	<1	<1
2	5		80	80	<1	<1
3	7		320	240	<1	<1
4	8		640	640	1	<1
5	10		860	640	<1	<1
6	14		1280	1920	<1	<1
7	14		1920	1280	1	<1
8	14		2560	1920	1	<1
9	14		2560	1920	4	2
10	21		2560	2560	4	2
11	14		5120	5120	8	4
12	21		7680	5120	8	4
13	14		10,240	10,240	8	4
14	35		15,360	15,360	8	8
15	4	HEV-V	120	160	<1	<1
16	5		120	160	<1	<1
17	6		160	640	<1	<1
18	7		960	960	<1	<1
19	14		2560	3840	4	4
20	14		2560	3840	8	4
21	14		2560	3840	8	4
22	21		3840	5120	4	4
23	14		3840	7680	8	4
24	21		7680	7680	8	4
25	14		7680	7680	8	8
26	14		7680	10,240	8	4
27	35		15,360	15,360	8	8
28	21		15,360	15,360	6	8

[A]ELISA antibody titers of poults were determined using plates coated with purified HEV-A or HEV-V. Titers are presented as the geometric mean. Titers greater than 40 were considered positive.

[B]AGP antibody titers were determined against crude spleen extracts of turkeys infected with HEV-A or HEV-V. A titer of less than 1 means no reaction with undiluted sera. Titers of 1 or greater were considered positive.

lutions of 1:100, 1:200, and 1:400. All 50 control extracts were negative (ELISA antigen titer ≤ 100), and all 50 spleen extracts of HEV-infected turkeys were positive (ELISA antigen titers > 100, ranging from 1200 to 100,000). Polyvinyl plates yielded more consistent results than polystyrene plates. This was tested by comparing data obtained with polystyrene plates of three different manufacturers (data not shown).

Appearance of HEV in spleen following infection. Thirty-five turkeys were orally infected with 500 TID_{50} of CsCl-purified HEV-A, and the subsequent appearance of HEV in their spleens was monitored. Using the ELISA, HEV antigen could be detected in spleen homogenates as early as 3 days after oral infection (Table 5), and all birds were positive by 4 days. Using the AGP test, no positive samples were seen until 4 days postinfection. Both tests demonstrated that the antigen titers were highest 5 days postinfection. No difference in reactivity was observed between plates coated with homologous or heterologous serum.

Antigen detection by ELISA and AGP. The presence of HEV antigen was quantitated in crude spleen extracts of 25 turkeys experimentally infected with HEV-A and in extracts of 29 turkeys experimentally infected with HEV-V. Both homologous and heterologous antisera were used as the capture antibody. The antigen titers obtained with the ELISA were 200 times higher than those obtained with the corresponding AGP test. The results of the tests conducted using

Table 4. Comparison of 3- and 4-step antibody-ELISAs for HEV.

Serum no.	Days post-exposure[A]	ELISA antibody titers: 3-step method, Purified HEV-A	4-step method, Spleen extract[B]	4-step method, RP19 extract[C]
1	0	15	<10	<10
2	0	30	10	15
3	3	30	10	10
4	7	480	240	160
5	7	320	120	80
6	7	480	240	240
7	14	5120	1280	1280
8	14	5120	1920	1280
9	14	10,240	1920	1920
10	14	40,960	5120	5120

[A]Turkeys were infected with 500 TID_{50} HEV-A.
[B]Crude spleen extract of HEV-A-infected turkeys.
[C]Extract of MDTC-RP19 cells infected with HEV-A.

HEV-A as the antigen and homologous antiserum are shown in Fig. 4 and were not significantly different when HEV-V or heterologous antiserum was used. The correlation coefficient between the ELISA titers of HEV-A extracts determined with homologous and heterologous immune serum was 0.94 (Fig. 5). The correlation coefficient between the ELISA titers of the HEV-V extracts determined similarly was 0.95 (data not shown).

DISCUSSION

The standard three-step ELISA developed for the detection of HEV antibody was at least 300 times more sensitive than the antibody-AGP test. The use of CsCl-gradient-purified HEV instead of spleen extract resulted in increased sensitivity of this assay. This was due to the elimination of the high background levels caused by nonspecific binding of serum proteins to crude antigen preparations present when HEV of lesser purity was used. This phenomenon has been observed by others (9,15).

Both the ELISA and the AGP test were used to quantitate passive antibody titers in poults. The ELISA revealed that virtually all of more than 200 poults from commercial turkey suppliers had passive antibody titers at 2 days of age. In contrast, the AGP test detected titers in only about 30% of these poults. The ELISA showed that passive antibody titers declined slowly with time and that some birds were still positive at 5 weeks of age. Since the AGP test was much less sensitive, the results of that test erroneously indicated that all birds were negative by 2 weeks of age. The rate of decline of passive antibodies is important, because it determines which turkeys are susceptible to disease, and also the age at which they can be successfully vaccinated against HE. Moreover, passive antibodies probably protect poults, because turkeys cannot be infected with HEV (8) when younger than 2 weeks old.

The AGP method appeared to be more reliable for detecting antibodies in convalescent sera than for detecting passive antibodies. Precipitins were detected in only a few of the serum samples from very young turkeys, but they were detected in nearly all convalescent sera. Malkinson *et al.* (11) suggested that the antibodies in young turkeys

Table 5. Detection of HEV antigen by ELISA and AGP in spleen extracts of turkeys infected orally with HEV-A.

Days post-infection	ELISA: Antigen titers[A] HEV-A serum	ELISA: Antigen titers[A] HEV-V serum	ELISA: No. positive/ no. tested[C]	AGP: Antigen titers[B] HEV-A serum	AGP: Antigen titers[B] HEV-V serum	AGP: No. positive/ no. tested
0	<100	<100	0/5	<10	<10	0/5
1	<100	<100	0/5	<10	<10	0/5
2	<100	<100	0/5	<10	<10	0/5
3	<100	105	1/5	<10	<10	0/5
4	1391	2433	5/5	53	53	4/5
5	5793	8006	5/5	160	92	5/5
6	1872	2531	5/5	23	20	2/5

[A]Geometric mean titers. Titers less than 100 are considered negative.
[B]Geometric mean titers. Titers less than 10 are considered negative.
[C]The number of positive turkeys per group was the same using HEV-A and HEV-V immune serum.

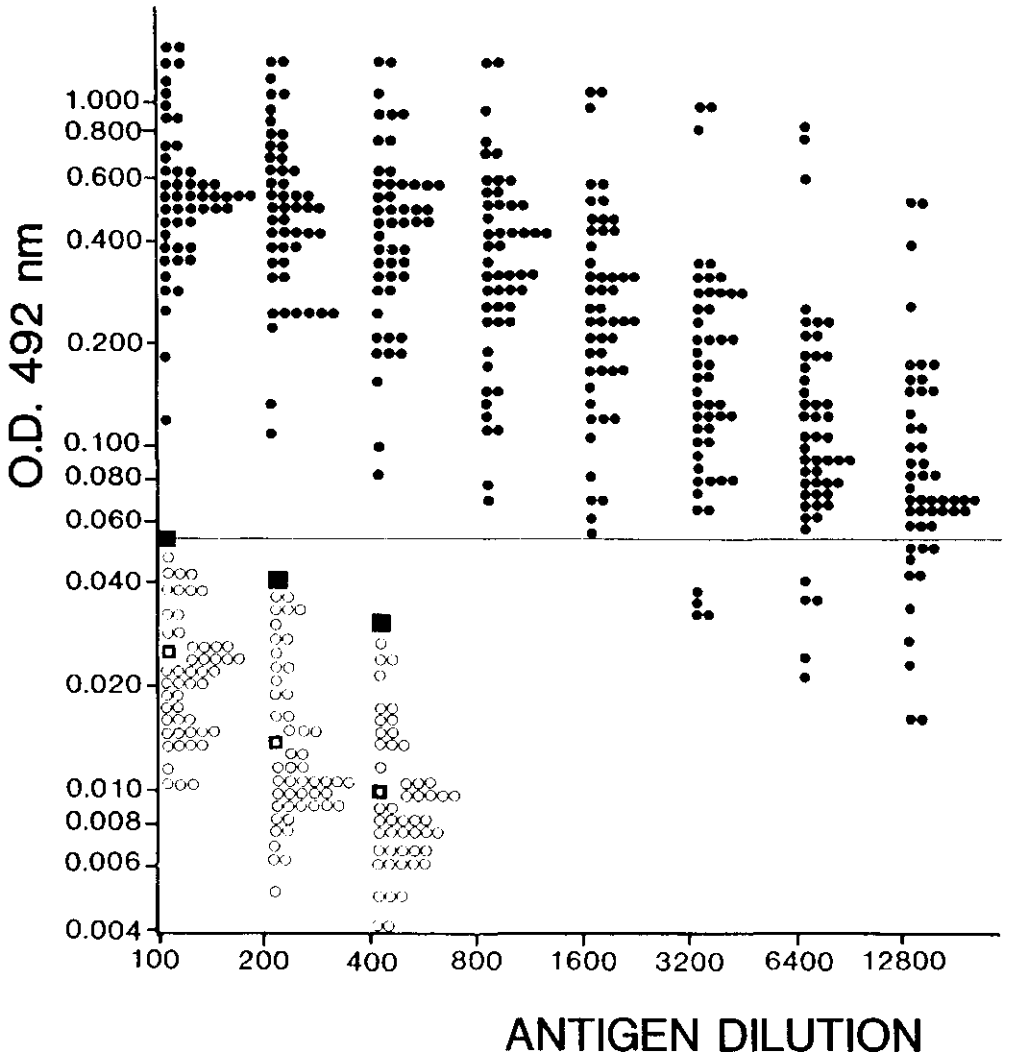

Fig. 3. Antigen ELISA of spleen extracts from 50 turkeys never exposed to HEV (○) and from 50 turkeys infected with HEV-A or HEV-V collected 4–6 days postinfection (●). The mean (□) + 2× the standard deviation (■) of the absorbance ($O.D._{492}$) of extracts from the unexposed birds is marked. At a dilution of 1:100 there was a clear separation between the two groups of birds.

are mostly passively transferred IgG, whereas the precipitin reaction is preferentially caused by IgM antibodies. This might explain why passive antibodies are poorly detected by the AGP test. However, it does not explain the improved sensitivity of this procedure for convalescent sera collected several weeks after infection, when the IgM level would be expected to be low or insignificant.

The appearance of antibodies 3 days after infection with HEV is consistent with data reported by Silim and Thorsen (15): the antibody titers were highest (5120) 12 days postinfection in the present study and highest (1000) 14 days postinfection in the earlier study (15). The slight decrease in antibody levels, which was sometimes followed by an increase 3 weeks after infection, might be caused by reinfection of birds with feces containing virus produced earlier during the acute phase of the disease.

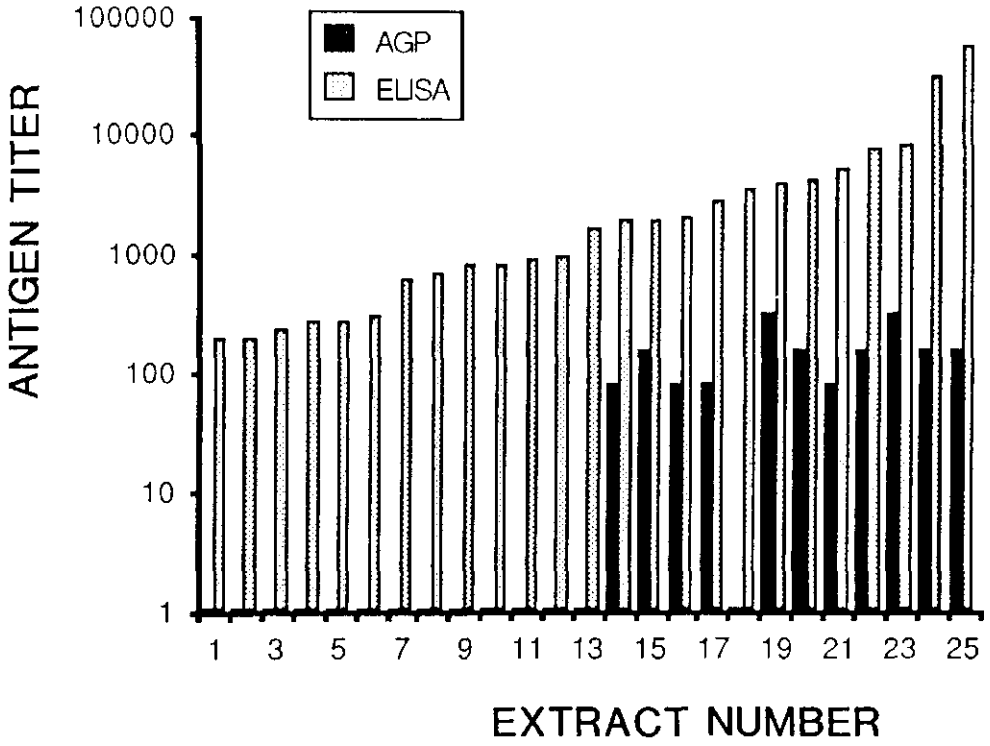

Fig. 4. ELISA antigen titers of crude spleen extracts of turkeys infected with HEV-A using homologous immune serum as the capture antibody. In addition to the ELISA titers (shaded columns), the corresponding AGP titers (dark columns) are shown.

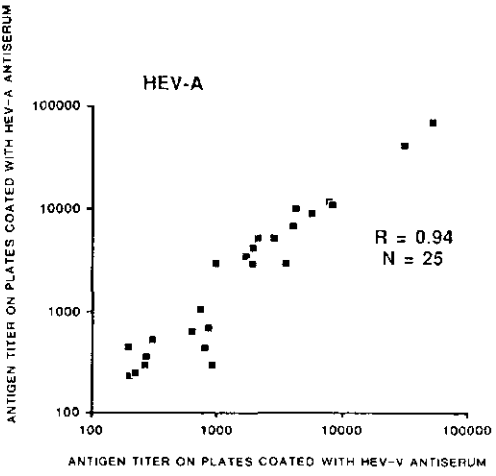

Fig. 5. Correlation between the ELISA antigen titers obtained for 25 HEV-A spleen extracts using homologous and heterologous immune sera as capture antibody.

The three-step ELISA was easier to use and more sensitive than the four-step method. However, the latter can be used with crude viral antigen, which is more readily available. The four-step method also worked using virus produced in MDTC-RP19 cells. This is in contrast to an earlier report of Ianconescu *et al.* (9), who found that MDTC-RP19-produced HEV was not suitable in an ELISA.

The antigen-ELISA was at least 10 times more sensitive than the AGP test. This ELISA has an advantage over the blocking assay developed by others (9), because it can quantify HEV antigen. This has been useful in studies on immunity to HEV and for the detection of low antigen concentrations.

Antigen was present in spleens 3 days postinfection, and values peaked 5 days postinfection. These findings agree with results of investigators who used an immunofluorescent-antibody test (6), an immunoperoxidase technique (15), and AGP test (7).

The results of the ELISAs suggest that HEV-A and HEV-V are closely related antigenically. This conclusion is supported by (i) similarity of the HEV antibody titers in convalescent sera against homologous and heterologous HEV; and (ii) similarity of the HEV antigen titers in tissue extracts against homologous and heterologous antisera. This conclusion is also supported by studies in which vaccination of turkeys with HEV-A resulted in immunity against challenge with HEV-V (2).

These ELISAs were unable to distinguish between the two virus strains. However, this might be advantageous, because one antigen preparation can be used to determine antibody titers of different strains, and similarly one antibody preparation can be used to determine viral antigens of different HEV strains.

The development of the ELISAs for the detection of HEV antigen and antibody provides suitable diagnostic and serological techniques and will facilitate further studies of HEV.

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Characterization of Group II Avian Adenoviruses with a Panel of Monoclonal Antibodies

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ABSTRACT

The interaction between a panel of ten monoclonal antibodies and hemorrhagic enteritis virus, a group II avian adenovirus, was determined. The monoclonal antibodies reacted with all nine isolates of group II avian adenoviruses, but not with any of five types of group I avian adenoviruses. All ten monoclonal antibodies recognized antigenic determinants on the hexon protein of hemorrhagic enteritis virus when analyzed by immunoprecipitation and immunoblotting. They reacted only with the native hexon protein and not with protein denatured by sodium dodecyl sulfate or guanidine-HCl/urea treatment combined with reduction and carboxymethylation. Based on the results of competitive binding assays, the panel of monoclonal antibodies could be subdivided into two groups, which recognized different antigenic domains of the hemorrhagic enteritis virus hexon protein. The monoclonal antibodies in group 1 neutralized hemorrhagic enteritis virus infectivity while the monoclonal antibodies of group 2 did not. Group 1 consisted of eight monoclonal antibodies which could be further subdivided into subgroups 1A, 1B, 1C and 1D. The subdivision of the monoclonal antibodies was based on the degree of blocking in the competitive binding assays and differences in their ability to induce enhancement. In general, the monoclonal antibodies had a higher avidity for the virulent isolate of hemorrhagic enteritis virus than for the avirulent hemorrhagic enteritis virus isolate.

RÉSUMÉ

Cette expérience visait à déterminer l'interaction entre un groupe de dix anticorps monoclonaux et le virus de l'entérite hémorragique de la dinde, un des adénovirus aviaires du groupe II. Ces anticorps monoclonaux réagirent avec les neuf isolats des adénovirus aviaires du groupe II, mais non avec l'un ou l'autre des cinq types du groupe I. Les dix anticorps monoclonaux reconnurent les déterminants antigéniques de la protéine hexonique du virus de l'entérite hémorragique, par l'immunoprécipitation et l'immunoempreinte. Ils réagirent avec la protéine hexonique intacte, mais non après sa dénaturation par le dodécyle sulfate de sodium ou par le chlorhydrate de guanidine et d'urée, combiné avec la réduction et la carboxyméthylation. D'après les résultats d'essais d'union compétitive, on pourrait subdiviser le groupe d'anticorps monoclonaux en deux groupes qui reconnurent divers domaines antigéniques de la protéine hexonique du virus de l'entérite hémorragique de la dinde. Les anticorps monoclonaux propres aux virus du groupe 1 neutralisèrent l'infectivité du virus précité, contrairement à ceux du groupe 2. Les anticorps monoclonaux du groupe 1 en comptaient huit et il s'avéra possible d'en former les sous-groupes 1A, 1B, 1C et 1D. Cette subdivision se basait sur le degré de blocage, lors des essais d'union compétitive, et les différences dans leur habileté à provoquer le renforcement de cette union. En général, les anticorps monoclonaux affichèrent plus d'avidité pour l'isolat virulent V du virus de l'entérite hémorragique de la dinde, que pour son isolat A, avirulent.

INTRODUCTION

Hemorrhagic enteritis virus (HEV) causes an economically important disease in susceptible young turkeys (1). Clinical signs of illness include enteritis, splenomegaly and hemorrhages in various tissues, especially the intestine, and may lead to mortality in field outbreaks ranging from less than 1% to over 60% (1,2).

The serologically related viruses which cause hemorrhagic enteritis (HE) of turkeys, marble spleen disease (MSD) of pheasants, and splenomegaly of chickens are tentatively classifed as group II avian adenoviruses (3-6). They do not appear to have an antigenic relationship with the group I avian adenoviruses which include 11 serotypes of fowl adenovirus (5,7-9). The fact that the group II viruses are classified as adenoviruses is

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based on their biochemical, physical and biological charcteristics which are typical for adenoviruses (10-15).

The purpose of the present study was to further investigate the serological relationship between avian adenoviruses and to characterize the biological properties and epitopes of HEV. Therefore a panel of HEV-specific monoclonal antibodies was produced and used to identify two major determinants on the hexon protein of which one was important for neutralization. These monoclonal antibodies were specific for common antigenic determinants of the group II avian adenoviruses but did not react with group I types. In addition these monoclonal antibodies proved very useful in developing tests for the detection of HEV antigen and antibody (16).

MATERIALS AND METHODS

VIRUSES

Hemorrhagic enteritis virus-A (HEV-A) is an isolate of pheasant origin which is avirulent for turkeys. HEV-D, -I, -M_1, -M_2, -V, -Y_1 and -Y_2 are isolates from turkeys which died of HEV and they are all virulent for turkeys. Isolate SV is of chicken origin, which is avirulent for turkeys. The HEV-A, -I, -V and SV isolates were a gift from C.H. Domermuth, Blacksburg, Virginia, and the other isolates were obtained from field cases of HE in Canada. All of the above serologically related group II avian adenoviruses were propagated in turkeys. The fowl adenovirus types 1, 2, 3, 4 and 8 were obtained from the American Type Culture collection and cultivated in chicken embryo fibroblasts.

VIRUS PROPAGATION AND PURIFICATION

Turkeys kept in isolation were infected orally with group II avian adenovirus when they were 6 to 10 wk old. Spleens of the sacrificed birds were collected four to five days after infection. Crude virus suspensions were prepared by homogenizing the spleens in 0.01 M Tris-HCl, pH 8.1 (ratio 1:10, w/w), and collecting the supernatants after a centrifugation of 10 min at 10,000 x *g*. Further purification of the crude virus extracts was carried out by the method of Green and Pina (17). The virus band, (P of 1.33-1.34 g/cm^3) obtained after two CsCl purification steps was collected. Hemorrhagic enteritis virus was dialyzed against 0.01 M Tris-HCl, pH 8.1, containing 20% glycerin and stored at -70°C.

PRODUCTION OF MONOCLONAL ANTIBODIES

Balb/c mice were immunized by injecting 0.05 mL purified HEV-V (2 mg/mL), emulsified in Freund's complete adjuvant, into each of two footpads. The primary injection was followed by a second footpad injection of HEV-V in Freund's incomplete adjuvant 2 wk later. Final boosts with 0.1 mL HEV-V in phosphate-buffered saline (PBS) were given intravenously seven and three days prior to fusion. Mouse spleen cells were fused with NS-1 myeloma cells as described by Kennett *et al* (18). 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. 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, Montreal, Quebec) primed Balb/c mice intraperitoneally injected with approximately 10^7 hybridoma cells.

FLUORESCENT ANTIBODY (FA) TEST

Turkey leukocytes were obtained from turkey blood by centrifugation through Ficoll-Paque (Pharmacia, Montreal, Quebec) for 15-20 min at 800 x *g*. The leukocytes were washed twice in RPMI 1640 and resuspended in the same medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, New York) at a concentration of 10^7 cells per mL. The primary leukocyte suspension cultures were infected with HEV-A or HEV-V and kept at 41°C. Cell smears were made with a cytocentrifuge two to three days postinfection. The cells were fixed either in methanol or acetone for 5 min. Hemorrhagic enteritis virus infected 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 Mannheim, Dorval, Quebec) for 1 h at 41°C. The cells were mounted with PBS-glycerin (1:1, v/v) and observed with a Zeiss IM35 microscope equipped with epifluorescent illumination. Photographs were taken with a neofluar x 40 objective.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

To identify HEV specific monoclonal antibodies, an indirect ELISA was used as described previously (16). Briefly, polystyrene microtiter plates (Immunolon 2, Dynatech Laboratories, Alexandria, Virginia) were coated with CsCl-purified HEV (2 μg/well). Hybridoma supernatant media or ascites fluids in serial dilutions were added to the wells, followed by affinity-purified peroxidase-conjugated goat antimouse Ig (diluted 1:4000, Boehringer Mannheim, Montreal, Quebec).

A four-step indirect sandwich ELISA was used to determine the reactivity of the monoclonal antibodies with group I and group II avian adenoviruses present in crude cell or spleen extracts. Polyvinyl microtiter plates (Falcon microtest III, Becton and Dickinson, Oxnard, California) were coated with a mixture of an IgG fraction of turkey anti-HEV-A serum and anti-HEV-V serum, diluted in 0.05 M $NaHCO_3/Na_2CO_3$ buffer pH 9.6 (0.2 mL/well, 10 μg/mL), for 1 h at 41°C. After three washes with the diluent, PBS-T (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.05% Tween 20; pH 7.2), crude extracts (0.2 mL/well) at an appropriate dilution in PBS-T were added to the plates and incubated overnight at 4°C. The plates were washed with PBS-T, hybridoma culture media in tenfold dilutions were added to the wells (0.2 mL/well), and the plates were incubated for 1 h at 41°C. After washing with PBS-T, affinity-purified peroxidase-conjugated goat antimouse Ig (1:4000 in PBS-T) was added to the wells. After three more washes with PBS-T, 0.2 mL of substrate solution containing 5-aminosalicylic acid (0.08%, w/v) and H_2O_2 (0.005%, v/v), pH 6.0 was

added to each well. The enzymatic activity was measured after 30 min at room temperature using a micro-ELISA reader MR 580 (Dynatec, Oxnard, California) at 492 nm. All tests were done in duplicate. The titers were calculated from titration curves in which the absorbance at 492 nm was plotted versus $\log_{10}$ of the dilution of the hybridoma culture medium that gave a reading of at least 0.1 over a control well without monoclonal antibody.

The antigenic reactivity of HEV after various treatments was measured by antibody blocking in an enzyme immunoassay (EIA) system described by Heinz *et al* (19). Various concentrations of treated and control HEV-A or HEV-V were incubated in microtiter plates for 1 h at 41°C with an equal volume of monoclonal antibody at an appropriate dilution in PBS-T containing 2% newborn calf serum (Gibco, Grand Island, New York). Subsequently, antigen-antibody mixtures were transferred to a plate coated with the homologous antigen and the antibody titers were determined as described for the indirect ELISA. The antigenic reactivity expressed as the percentage blocking was calculated from the formula 100 (C-D)/C, where C is the optical density (OD) in the absence of antigen and D is the OD in the presence of a given antigen concentration.

ANTIBODY CLASS AND SUBCLASS

Antibody class and subclass were determined in the indirect antibody ELISA, described above, by replacing the conjugate step with class and subclass specific rabbit antimouse antibodies (Miles Laboratories, Elkhart, Indiana), followed by affinity-purified peroxidase-conjugated goat antirabbit Ig (Boehringer Mannheim).

VIRUS NEUTRALIZATION

Serial tenfold dilutions of ascites fluids in RPMI 1640 were mixed with an equal volume (0.2 mL) of 1000 $TCID_{50}$ of HEV-A or HEV-V in RPMI 1640. After incubation for 1 h, 0.1 mL samples were added in quadruplicate to 10^7 primary turkey blood leukocytes cultured in 24 well plates at 41°C. Cytocentrifuge cell smears were made two days postinfection and the percentage of the infected cells was determined by the FA test.

IMMUNOPRECIPITATION OF RADIOLABELLED HEV PROTEINS

Purified HEV was disrupted by five or six repeated freeze-thaw cycles and ultrasonic treatment. Soluble HEV proteins were obtained from crude spleen extracts by freon extractions, and centrifugation of the aqueous phase onto a CsCl cushion, followed by $(NH_4)_2SO_4$ precipitation (20). Disrupted HEV and soluble HEV protein preparations were labelled with ^{125}I by the enzymobead method following the instructions of the manufacturer (BioRad, Mississauga, Ontario). The conditions for immunoprecipitation of ^{125}I labelled HEV antigen with hybridoma supernatant medium, addition of rabbit antimouse Ig, and precipitation of the immune complexes with protein A-Sepharose CL-4B (Pharmacia, Dorval, Quebec) have been described previously (21). The immune precipitates were resuspended in electrophoresis sample buffer (0.0625 M Tris-HCl [pH 6.8], 1% sodium dodecyl sulfate [SDS], 10% glycerol, 0.15 M 2-mercaptoethanol, and 0.002% bromophenol blue) and dissociated by boiling for 4 min prior to electrophoresis.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Purified HEV and immunoprecipitates were dissociated in electrophoresis sample buffer and analyzed in 10% SDS-polyacrylamide gels (22). Electrophoresis under nondenaturing conditions of the hexon proteins was performed on 6% polyacrylamide gels (23).

IMMUNOBLOTTING

A modification of the "Western" blotting technique described by Burnette (24) was used to analyze the interaction between the panel of monoclonal antibodies and HEV proteins. Hemorrhagic enteritis virus polypeptides were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% slab gels (22), and HEV proteins were separated by PAGE in 6% slab gels (23). They were then transferred electrophoretically to nitrocellulose in a Bio-Rad transblot cell (Bio-Rad Laboratories, Mississauga, Ontario) at 32 V for 4 h in 25 mM sodium phosphate buffer, pH 6.8. Subsequently, the immunoreaction was carried out as outlined in the instructions for the use of the Bio-Rad immunoblot assay kit. Briefly, strips cut from the nitrocellulose sheet 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 for 3 h with hybridoma culture media diluted 1:5 in TBS containing 1% gelatin. After washing with TBS-T (TBS containing 0.05% Tween 20) the strips were incubated with affinity-purified horseradish peroxidase-conjugated goat antimouse Ig (Boehringer Mannheim, Dorval, Quebec) diluted in TBS (1:1000) containing 1% gelatin. The strips were washed with TBS-T and 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.

PURIFICATION OF THE HEXON PROTEIN

The IgG fraction of monoclonal antibody $15G_4$ was purified using protein A-Sepharose CL-4B (Pharmacia, Montreal, Quebec) (25). Purified $15G_4$-IgG, dialyzed against 0.1 M sodium carbonate buffer, pH 8.0, was linked to activated Affigel-10 (Bio-Rad Laboratories, Mississauga, Ontario) at 5 mg/mL gel following the manufacturer's instructions. The immunobeads were packed into a column, washed and equilibrated with TNE (0.01 M Tris-HCl, 0.05 M NaCl, and 0.001 M Na_2 EDTA, pH 7.5). The soluble antigen fraction in TNE obtained during virus purification (20), was recycled three times through the column. After washing of the column with TNE, hexon 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 hexon-containing fractions were pooled and dialyzed against PBS. Hexon proteins were separated from larger complexes 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 (26). The 12S hexon fractions were pooled, dialyzed against PBS containing 10% glycerol (v/v) and stored at -70°C.

ANTIGEN TREATMENTS

The HEV treatments were essentially performed as described by Heinz *et al* (19). For the guanidine-HCl/urea treatment, HEV-A or HEV-V in 0.02 M Tris-HCl containing 0.1 M NaCl, pH 8.0 (TN) was made 5 M with respect to guanidine-HCl and incubated for 1 h at 41°C. Subsequently, the samples were dialyzed at 4°C against 2 M urea in TN or against TN only. Control samples were incubated in TN only. For the reduction and carboxymethylation treatment, dithiothreitol (BioRad, Richmond, California) at a final concentration of 0.010 M in TN, was added to each HEV sample. After incubation at 41°C, iodoacetamide (BDH, Poole, England) at a final concentration of 0.05 M was added to each sample and the incubation was continued for 30 min at 41°C. An excess of 2-mercaptoethanol (25 μL/mL), was then added to each sample before dialysis against TN at 4°C. A combination of the guanidine-HCl/urea and the reduction and carboxymethylation treatments was also carried out. For the SDS treatment, SDS at a final concentration of 1% was added to the HEV samples in TN buffer. After boiling for 3 min the samples were dialyzed against TN at 4°C.

PREPARATION OF PEROXIDASE-LABELLED MONOCLONAL ANTIBODIES

Monoclonal antibodies obtained by $(NH_4)_2SO_4$ precipitation from ascites fluids were labelled with horseradish peroxidase (type VI, Sigma, St. Louis, Missouri) using the conjugation method of Nakane and Kawaoi (27) as modified by van den Hurk and Kurstak (28).

COMPETITIVE ANTIBODY BINDING ASSAY (CBA)

The CBA was performed by the method of Kimura-Kuroda and Yasui (29), modified by van Drunen Littel-van den Hurk *et al* (30). Polystyrene microtiter plates were coated with HEV-A or HEV-V (4 μg/well). After washing, the plates were incubated with competitor antibody in ascites fluids, serially diluted in PBS-T at concentrations ranging from 10 to 10^4 ELISA units. One ELISA unit is defined as the highest dilution of an ascites fluid having an absorbance value at 492 nm of 0.1 OD above that of a control ascites. After washing, horseradish peroxidase-conjugated monoclonal antibodies were added to the plates at dilutions which gave an absorbance of 1.0 OD at 492 nm without competitive antibody. After washing and addition of substrate solution as described earlier, the absorbance at 492 nm was measured in the presence or absence of competitor antibody. The calculation of the percentage of competition was as described by Kimura-Kuroda and Yasui (29) using the formula $[100(A-n)]/(A-B)$, where A is the OD in the absence of competitor antibody, B the OD in the presence of 10^4 ELISA units of homologous antibody, and n is the OD in the presence of 10^4 ELISA units of competitor.

TABLE I. Properties of Monoclonal Antibodies Reactive with HEV

Monoclone Designation	Isotype[a]	FA Titer[b]		ELISA Titer[c]	
		HEV-A	HEV-V	HEV-A	HEV-V
$11B_6$	IgG2a	160	160	2560	2560
$11C_3$	IgG1	100	100	480	960
$12B_2$	IgG1	100	100	1280	1280
$12C_2$	IgG1	320	320	1280	1280
$14B_1$	IgG2a	160	80	160	320
$14B_{11}$	IgA	100	80	1280	2560
$14E_1$	IgG1	160	160	640	1280
$14E_8$	IgG1	100	100	640	1280
$14F_7$	IgG1	100	100	160	320
$15G_4$	IgG2b	1280	1280	5120	10240

[a]Immunoglobulin class and subclass were determined in an indirect ELISA using specific antisera for mouse IgA, IgM, IgG1, IgG2a, IgG2b and IgG3

[b]Titers of hybridoma culture media were determined in an indirect FA test using HEV-A or HEV-V infected cells; FA titers are the averages of quadruplicate tests

[c]Antibody titers of culture media were determined in an indirect ELISA using purified HEV-A or HEV-V as capture antigen

RESULTS

SCREENING AND REACTIVITY OF THE MONOCLONAL ANTIBODIES BY FA AND ELISA

Hybridoma supernatant media were screened for their capacity to react with HEV-infected cells in the FA test. In this test, 18 of the supernatant culture fluids reacted specifically with HEV-infected cells, but not with uninfected control cells. A panel of ten stable clones was selected for further characterization. Nine of the hybridomas secreted immunoglobulin G(IgG) and one secreted immunoglobulin A (IgA).The predominant isotype was IgG1, but IgG2a and IgG2b isotypes were also found (Table I). All monoclonal antibodies reacted with HEV-A and HEV-V infected cells in the FA test. Fluorescent staining was observed in both the nucleus and cytoplasm of cells fixed in either acetone or methanol (Fig. 1). The FA antibody titers of the monoclonal antibodies were similar for HEV-A and HEV-V, but the ELISA titers were generally higher against HEV-V than against HEV-A (Table I).

SPECTRUM OF REACTIVITY OF THE MONOCLONAL ANTIBODIES

The reactivity of the ten monoclonal antibodies with nine virus isolates belonging to avian adenovirus group II, and with five types of avian adenovirus group I was analyzed by a sandwich ELISA. This method was used because it allowed testing of a number of crude viral preparations of different origin without purification. The monoclonal antibodies reacted with each of the nine group II isolates, but they did not react with any of the group I types (Table II).

ABILITY OF MONOCLONAL ANTIBODIES TO NEUTRALIZE VIRUS INFECTIVITY

Eight out of the ten mouse ascites fluids containing monoclonal antibodies strongly neutralized both HEV-A and HEV-V infectivity in cell culture. The remaining two monoclonal antibodies ($14B_3$ and $15G_4$) either failed to neutralize the virus, or did so only weakly (Table III). In most cases higher neutralization titers were obtained for HEV-V than for HEV-A.

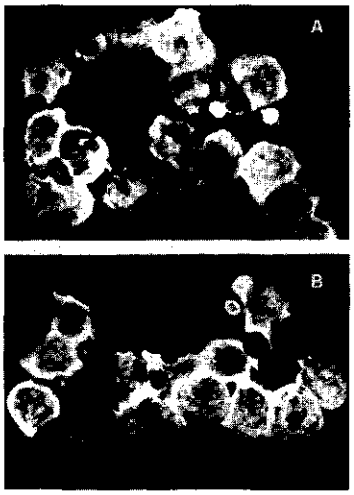

Fig. 1. Fluorescent antibody staining of turkey leukocytes infected with HEV-A (A) or HEV-V (B) with hybridoma supernatant medium of monoclonal antibody $15G_4$. The cells were fixed in methanol. Similar staining of cytoplasm and nucleus was obtained when cells were fixed in acetone. Magnification, 600x.

HEXON-MONOCLONAL ANTIBODY INTERACTION

The specific viral antigens that were recognized by the monoclonal antibodies were identified by immunoprecipitation. Hemorrhagic enteritis virus antigen preparations obtained from either disrupted purified virus or from soluble viral protein preparations were radiolabelled with ^{125}I. Analysis of the immunoprecipitates by polyacrylamide gel electrophoresis revealed that all ten monoclonal antibodies reacted specifically with the hexon, or major outer capsid protein, of both HEV-A and HEV-V. An example of immunoprecipitation of the hexon protein is shown in Fig. 2.

Hexon proteins were purified from $(NH_4)_2SO_4$ precipitates of soluble spleen extract fractions by affinity chromatography and sucrose gradient centrifugation. The interactions between these purified hexons and monoclonal antibodies were analyzed by a direct sandwich ELISA using homologous or heterologous monoclonal antibodies as capture antibody and conjugate reagent for detection. All monoclonal antibodies reacted with the hexon proteins bound to the plates by homologous antibodies. Hence, all monoclonal antibodies reacted with more than one site on the hexon protein, although to varying degrees. Higher titers were obtained when the assay was carried out with heterologous antibodies which were not competing for the same site. Examples of homologous and heterologous titration curves are presented in Fig. 3.

THE INFLUENCE OF CHEMICAL TREATMENTS ON ANTIGEN-ANTIBODY INTERACTION

The effect of conformational or chemical changes on viral epitopes was investigated by immunoblotting and blocking enzyme immunoassays using monoclonal antibodies.

None of the monoclonal antibodies reacted in immunoblots with any of the viral polypeptides separated by SDS-PAGE. However, each of the ten monoclonal antibodies recognized the native hexon protein after electrophoresis under nondenaturing conditions. An example of the immunoblotting reaction between monoclonal antibody $15G_4$ and the hexon protein of HEV-A and HEV-V is shown in Fig. 4.

Denaturation with SDS or guanidine-HCl/urea treatment combined with reduction and carboxymethylation completely destroyed the recognition sites of all monoclonal antibodies of HEV-A as well as HEV-V. This loss of antigenicity after denaturation indicates that the monoclonal antibodies recognize conformational sites on the hexon protein.

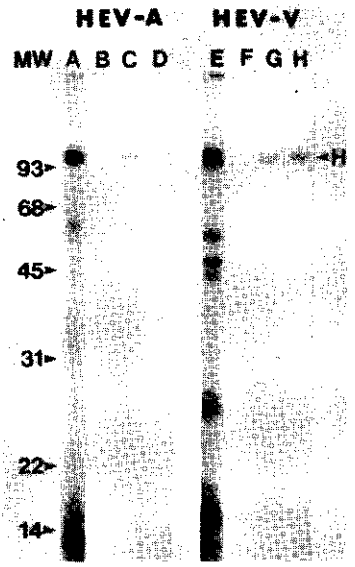

Fig. 2. Immunoprecipitation of ^{125}I-labelled disrupted HEV-A (lanes B and C), HEV-V (lanes F and G) and of soluble proteins of HEV-A (lane D) and HEV-V (lane H) with monoclonal antibody $6G_{12}$ (lanes B and F) or monoclonal antibody $15G_4$ (lanes C,D,G and H). The immunoprecipitates were analyzed by SDS-PAGE (10%). Monoclonal antibody $6G_{12}$, which reacts specifically with bovine herpesvirus type 1, was used as a negative control (lanes B and F). HEV was prepared and immunoprecipitated as described in Materials and Methods. Lanes A and E show ^{125}I-labelled polypeptides of HEV-A and HEV-V respectively. Molecular weight markers ($x10^3$) are shown in the left margin and the position of the hexon polypeptide (H) is marked in the right margin.

TABLE II. Reactivity of Monoclonal Antibodies with Isolates of Avian Adenovirus Groups I and II

		ELISA Titer of Monoclonal Antibodies[c]									
Virus[a]	Group[b]	$11B_6$	$11C_3$	$12B_2$	$12C_2$	$14B_3$	$14B_{11}$	$14E_1$	$14E_8$	$14F_7$	$15G_4$
HEV-A	II	10^4	10^2	10^3	$10^{3.7}$	10^2	10^3	10^3	10^3	10	10^4
HEV-V	II	10^4	10^2	10^3	$10^{3.7}$	10^2	10^3	10^3	10^3	10	10^4
HEV-I	II	10^3	10	10^2	10^3	10	10^2	10	10	10	10^4
HEV-M_1	II	10^4	10^2	10^4	$10^{3.7}$	10^2	10^3	10^3	10^2	10	10^4
HEV-M_2	II	10^4	10^2	10^4	$10^{3.7}$	10^2	10^3	10^2	10^2	10	10^4
HEV-D_1	II	10^5	10^2	10^4	$10^{3.7}$	10^2	10^3	10^3	10^3	10	10^4
HEV-Y_1	II	10^5	10^2	10^4	$10^{3.7}$	10^2	10^3	10^2	10^3	10	10^4
HEV-Y_2	II	10^4	10^2	10^4	$10^{3.7}$	10^2	10^3	10^2	10^3	10	10^4
SV	II	$10^{3.7}$	10^2	10^2	$10^{1.7}$	10	10^2	10^2	$10^{2.7}$	10	10^3
AAV-1,-2 -3,-4,-8	I	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

[a]Virus isolates of turkey spleens infected with HEV or SV and tissue culture cells infected with avian adenovirus group I virus

[b]Classification of avian adenovirus group I and II according to Domermuth (1,3)

[c]ELISA antibody titers for the avian adenovirus group II isolates were determined by an indirect sandwich ELISA using spleen extracts from turkeys as antigen, and the ELISA antibody titers for the avian adenovirus group I types were determined by a direct sandwich ELISA using cell extracts as antigen

TABLE III. Neutalization of HEV Infectivity by HEV-specific Monoclonal Antibodies

Monoclone Designation	Neutralization Titer[a]	
	HEV-A	HEV-V
$6G_{12}$[b]	< 10	< 10
$11B_6$	10^5	$10^{5.5}$
$11C_3$	10^6	$10^{6.5}$
$12B_2$	$10^{5.5}$	10^6
$12C_2$	$10^{5.5}$	10^6
$14B_3$	10	< 10
$14B_{11}$	$10^{5.5}$	10^7
$14E_1$	$10^{5.5}$	$10^{6.5}$
$14E_8$	10^6	$10^{6.5}$
$14F_7$	$10^{4.5}$	$10^{4.5}$
$15G_4$	10	10^2

[a]Neutralization titers, determined for ascites fluids of each of the hybridomas, are expressed as the reciprocal of the highest dilution which caused a 50% reduction of fluorescent cells
[b]Control clone reactive with bovine herpsvirus-1

Reduction and methylation of HEV-A or HEV-V without denaturation had a minimal effect upon the antigenicity, whereas guanidine-HCl/urea treatment reduced the antigenicity of both HEV-A and HEV-V, though HEV-A appeared to be more sensitive. This last result was consistently observed with all ten monoclonal antibodies and showed a clear difference in stability of the two virus isolates. An example of the effect of various chemical treatments on the antigen-antibody interaction as determined with blocking enzyme immunoassays is shown in Fig. 5.

TOPOGRAPHY OF EPITOPES ON THE HEXON PROTEIN

The spatial arrangement of epitopes on the hexon protein was investigated by using the panel of monoclonal antibodies in a CBA. Monoclonal antibodies can compete for binding to a protein when they react with the same antigenic site, or with a site in close proximity as a result of steric hindrance. Alternatively, competition can occur as a result of conformational (allosteric) changes induced after binding of one monoclonal antibody which may then lead to reduced binding of another monoclonal antibody to a distant site. The CBA was carried out as described by Kimura-Kuroda and Yasui (29). In their assay competition of antibodies with lower as well as with higher avidity was measured by: 1) first incubating with the competitor, followed by incubation with the conjugate, 2) basing their calculations on the competition of the homologous as well as the heterologous antibody and, 3) using different competitor concentrations based on ELISA units (10^1 to 10^4), rather than on antibody concentrations.

The antibody titers of the monoclonal antibodies in ascites fluids as measured by the direct and indirect ELISA are compared in Table IV. Although the antibody titers in the direct assay were lower than those in the indirect assay, their relative titers were similar with the exception of clone $14B_{11}$. This suggests that no significant changes occurred during peroxidase conjugation. The titers in direct and indirect assays of clone $14B_{11}$ did not differ much, which might be ascribed to the fact that clone $14B_{11}$ belongs to the IgA class, while the others belong to the IgG class.

On the basis of the percentage of competition, the monoclonal antibodies could be divided in two distinct groups, each reacting with a different antigenic domain of the hexon protein. A high level of competition was found between the antibodies in each group, and less competition was found between the monoclonal antibodies in different groups. Group 1 was comprised of eight clones ($11B_6$, $11C_3$, $12B_2$, $12C_2$, $14B_{11}$, $14E_1$, $14E_8$, and $14F_7$), and group 2 was comprised of two clones ($14B_3$ and $15G_4$) (Table V and Fig. 6). The monoclonal antibodies in group 1 consisted of a heterogeneous population which showed asymmetrical reciprocal competition. Therefore they were divided into four subgroups: 1A ($11B_6$ and $14B_{11}$), 1B ($11C_3$, $12B_2$ and $12C_2$), 1C ($14E_1$ and $14E_8$) and 1D ($14F_7$). The highest degree of competition was found between monoclonal antibodies within subgroup 1A, which showed competition of nearly 100% with each other, as well as with the members of subgroups 1B, 1C and 1D. The level of competition decreased for each subsequent subgroup so that subgroup 1D competed strongly only

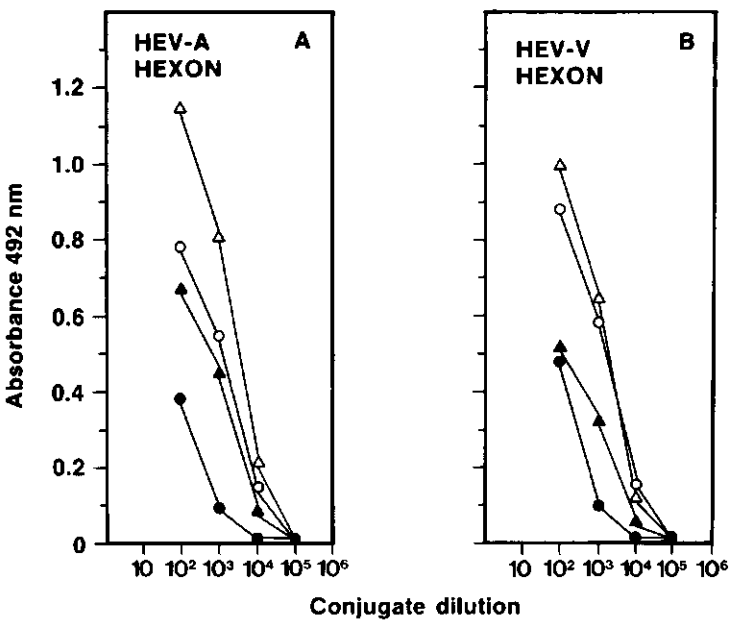

Fig. 3. Titration curves of homologous and heterologous monoclonal antibody binding to HEV-A(A) or HEV-V(B) hexons. The hexon proteins (12S) were purified by affinity chromatography and sucrose gradients. The presence of binding sites on the hexon proteins captured by monoclonal antibodies bound to microtiter plates was analyzed using homologous or heterologous monoclonal antibody-peroxidase conjugates. Symbols: ●, $11B_6$ homologous curve; ○, $11B_6$ and $15G_4$-peroxidase conjugate heterologous curve; ▲, $15G_4$ homologous curve; △, $15G_4$ and $11B_6$-peroxidase conjugate heterologous curve.

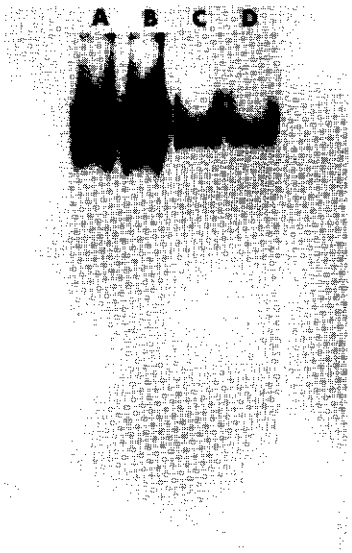

Fig. 4. Immunoblot analysis of soluble antigen extracts of HEV-A (lane A) and HEV-V (lane B), and affinity-purified hexons of HEV-A (lane C) and HEV-V (lane D). Soluble antigen extracts were obtained from spleen homogenates by freon extraction, centrifugation of the aqueous phase onto CsCl cushions, and concentration by $(NH_4)_2SO_4$ precipitation (20). Purified hexons were obtained by affinity purification of soluble antigen extracts on an Affigel-10-linked $15G_4$ monoclonal antibody column. Proteins were separated by PAGE on 6% polyacrylamide gels under nondenaturing conditions and transferred to nitrocellulose. Strips were incubated successively with monoclonal antibody $15G_4$, peroxidase-linked goat antimouse Ig, and substrate to visualize the antigen-antibody complexes.

with itself. In contrast, all other monoclonal antibodies in subgroups 1A, 1B and 1C completely blocked binding of monoclonal antibody $14F_7$.

Asymmetric reciprocal competition may be the result of differences in avidity between competing antibodies, or of differences in conformational changes due to binding of competing antibodies (31,32). Avidities of the monoclonal antibodies were determined from absorbance values (OD_{492}) in the indirect ELISA at plateau level (33). No relationship was found between the avidities of the monoclonal antibodies and the classification of the monoclonal antibodies in subgroups (data not shown).

Most of the monoclonal antibodies of group 1 also differed from those of group 2 in their ability to induce enhancement. Enhancement or negative competition has been described as a phenomenon in which the reaction of an antigen with one monoclonal antibody increases the binding of a second antibody (31,34,35). Binding of the monoclonal antibodies $14B_3$ or $15G_4$ at certain concentrations generally enhanced binding of the antibodies in group 1. Monoclonal antibody $14F_7$ of group 1 was the only one which enhanced binding of most of the conjugates of group 1 and 2, but was not enhanced by the other clones of group 1 (data not shown). This latter result supports the subdivision of group 1. Similar results were obtained in the CBA and enhancement experiments whether HEV-A or HEV-V were used. Examples of enhancement are presented in Table V and Fig. 6.

DISCUSSION

A panel of ten monoclonal antibodies was selected and characterized with respect to their interactions with HEV-A and HEV-V. All ten monoclonal antibodies reacted specifically with HEV-infected cells when analyzed with the FA test. Hemorrhagic enteritis virus antigen was observed in the cytoplasm as well as in the nucleus. In contrast, predominant nuclear (36,37) or cytoplasmic staining (38) has been reported for monoclonal antibodies reacting with the hexon protein of human adenovirus. The difference in fluorescent staining patterns might be caused by antibody-recognition of specific forms in which the hexon is present in infected cells. For example, Cepko *et al* (36,37,39) described monoclonal antibodies that recognize group-reacting antigens on the hexon of human adenovirus and show nuclear staining in infected cells. These monoclonal antibodies react only with native hexons present in the nucleus of the cell. However, they do not react with the nascent hexon polypeptide chains present in the cytoplasm. The HEV-specific panel of monoclonal antibodies recognized native hexons (ELISA, immunoblotting) and hexons in HE virions (ELISA, neutralization) which might explain the observed nuclear staining. The reason for the cytoplasmic staining might be that the panel of monoclonal antibodies also recognizes the nascent hexon polypeptide.

The panel of monoclonal antibodies reacted with all the avian adenovirus group II virus isolates but with none of the avian adenovirus group I types when tested by FA and ELISA. This implies that they react with common antigenic determinants of the group II adenoviruses which are absent on the group I viruses. It also is further evidence that the original classification of these viruses into two groups, based on serological reaction (5,7-9), is justified. Analysis by immunoprecipitation or immunoblotting of HEV soluble protein preparations, or affinity-purified hexon protein, showed that all monoclonal antibodies recognized antigenic sites on the

TABLE IV. Hemorrhagic Enteritis Virus Antibody Titers of Monoclonal Antibodies in Ascites Fluids Determined by ELISA

Monoclone Designation	ELISA Titers (x 10^3)			
	HEV-A[a]		HEV-V	
	Direct[b]	Indirect[c]	Direct	Indirect
$11B_6$	80	1600	160	1900
$11C_3$	5	30	23	170
$12B_2$	13	270	25	640
$12C_2$	12	380	26	580
$14B_3$	5	140	15	160
$14B_{11}$	80	140	130	180
$14E_1$	42	420	210	1300
$14E_8$	42	250	140	1600
$14F_7$	3	34	7	84
$15G_4$	1700	30000	2500	28000

[a]Coating of the plates with purified HEV-A or HEV-V
[b]Direct assay using monoclonal antibody-peroxidase conjugates
[c]Indirect assay using monoclonal antibodies and antimouse IgG peroxidase conjugate

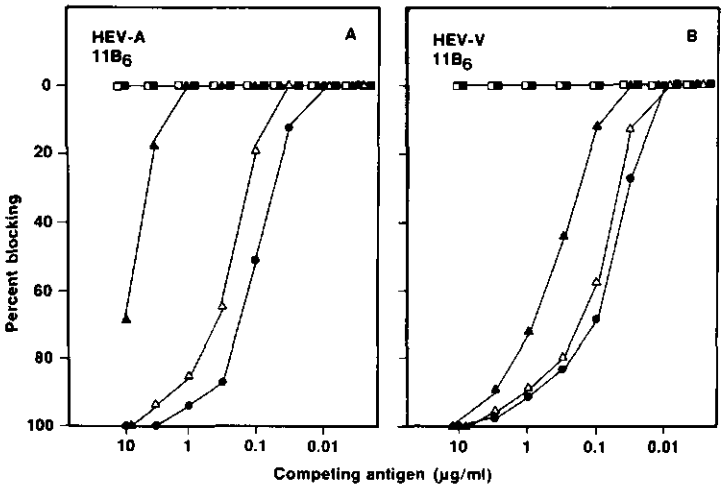

Fig. 5. The effect of chemically induced conformational changes on the antigenic activity of HEV-A (A) or HEV-V (B) was determined in a blocking enzyme immunoassay using monoclonal antibody $11B_6$. Symbols: ●, untreated HEV; △, reduced and carboxymethylated HEV; ▲, guanidine-HCl/urea treated HEV; □, SDS treated HEV; ■, guanidine-HCl/urea, reduced and carboxymethylated HEV.

hexon protein. The hexon proteins consist of three identical polypeptide chains and therefore, might have three or less identical antibody-binding sites dependent on the orientation of the polypeptides in the hexon protein. The results of the sandwich ELISA showed that all monoclonal antibodies recognized more than one site on each hexon. Consequently, the antigenic domains which are recognized by the antibodies are probably preserved on each polypeptide.

Eight of the ten monoclonal antibodies neutralized HEV infectivity in cell culture very effectively, whereas the other two did not. Hexon and fiber of mammalian adenoviruses have been reported to be the antigens responsible for virus neutralization (40-47). Monospecific antisera prepared against the human adenovirus type 2 (Ad2) fiber or hexon neutralized Ad2 virions *in vitro*, though the mechanisms of neutralization were found to be different. Virions neutralized by fiber-specific antisera were mostly present in aggregates and a strong reduction of virus-penetration was observed in the cells (47). However, when hexon-specific antisera were used, the majority of the virions were confined within vesicles (47). The mechanism(s) involved in HEV neutralization by monoclonal antibodies is presently under investigation.

Hexons of most mammalian adenoviruses contain a common group-specific determinant (α) as well as a type-specific determinant (ϵ; 48,49). The group-specific determinant is located at the inside, while the type-specific determinant is located at the outside of the virion (44-46,48,49). Since the HEV-specific monoclonal antibodies recognized intact virions and since most of them neutralized viral infectivity, they appear to represent antibodies reacting with the type-specific determinant. However, their range of activity seems to be wider because they react with turkey isolates varying in pathogenicity and also with isolates of chicken and pheasant origin.

The antigenicity of the sites that were recognized by the monoclonal antibodies was completely lost after denaturation of HEV-A and HEV-V with SDS or by guanidine-HCl/urea treatment combined with reduction and carboxymethylation. The sensitivity of the epitopes to denaturation suggests that the monoclonal antibodies recognize conformational antigenic sites. The resistance to guanidine-HCl/urea treatment was lower in HEV-A than in HEV-V which

TABLE V. Competitive Binding of Peroxidase-labelled Monoclonal Antibodies for HEV-A or HEV-V Epitopes

Virus Isolate	Peroxidase-labelled Monoclonal Antibody	Competitor[a]									
		1A		1B			1C		1D	2	
		$11B_6$	$14B_{11}$	$11C_3$	$12B_2$	$12C_2$	$14E_1$	$14E_8$	$14F_7$	$14B_3$	$15G_4$
HEV-A	$11B_6$	**100**[c]	**100**	77	70	84	66	65	40	10	-23[b]
	$14B_{11}$	**94**	**100**	47	55	63	36	34	37	-21	-17
	$11C_3$	100	100	**100**	**100**	**100**	82	88	54	-13	-54
	$12B_2$	100	100	**100**	**100**	**100**	94	91	62	**14**	-5
	$12C_2$	100	100	**87**	**84**	**100**	80	70	69	-23	-26
	$14E_1$	100	100	100	100	100	**100**	**90**	82	-17	-81
	$14E_8$	100	100	100	100	100	**92**	**100**	77	25	17
	$14F_7$	100	100	100	100	100	100	100	**100**	23	-23
	$14B_3$	20	39	30	27	48	0	8	0	**100**	**83**
	$15G_4$	21	44	43	36	31	21	20	15	**100**	**100**
HEV-V	$11B_6$	**100**	**100**	77	71	73	70	68	54	10	-26
	$14B_{11}$	**86**	**100**	48	58	60	38	30	37	**16**	-7
	$11C_3$	100	100	**100**	**100**	**100**	88	91	70	-3	-12
	$12B_2$	100	100	**100**	**100**	**100**	100	100	83	15	-25
	$12C_2$	100	100	**100**	**88**	**100**	99	99	75	4	-36
	$14E_1$	100	100	100	100	100	**100**	**100**	9	19	-43
	$14E_8$	100	100	100	100	100	**100**	**100**	76	26	8
	$14F_7$	100	100	100	100	100	100	100	**100**	32	-1
	$14B_3$	29	47	36	31	41	4	3	22	**100**	**79**
	$15G_4$	34	49	56	37	22	0	7	42	**96**	**100**

[a] The percentage of competition of the monoclonal antibodies for antigenic sites on purified HEV-A or HEV-V was determined at a concentration of 10^4 ELISA units of competitor antibody (29)
[b] Negative competition values indicate enhancement of peroxidase-labelled antibody binding
[c] Numbers in boldface indicate competition between homologous antibodies or antibodies of the same epitope specificity

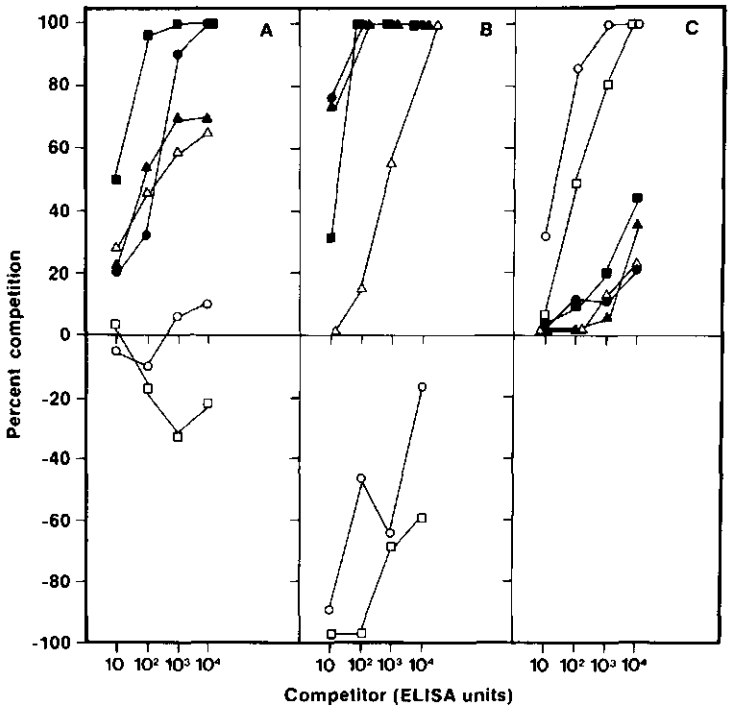

Fig. 6. Competition of monoclonal antibodies for the reaction of peroxidase-conjugated monoclonal antibody $11B_6$ (A), $14E_1$ (B), and $15G_4$ (C) with HEV-A. The specific competitor antibodies were: ●, $11B_6$, ▲, $12B_2$; ○, $14B_3$; ■, $14B_{11}$; △, $14E_1$; □, $15G_4$.

may indicate that HEV-A is less stable than HEV-V.

Based on the CBA data at least two topographically distinct antibody-binding domains appear to exist on the HEV hexon protein. The first domain is important for virus neutralization, whereas the second is not. Moreover, the monoclonal antibodies in the first group, with the exception of monoclonal antibody $14F_7$, did not enhance binding of group 1 antibodies.

The further subdivision of group 1 monoclonal antibodies into four subgroups was based upon asymmetric blocking in the CBA. Asymmetric reciprocal competition may be the result of differences in avidities of competing antibodies which recognize overlapping antigenic sites or antigenic sites in close proximity to each other. Asymmetric competition is difficult to explain in this study, because the CBA was carried out so that differences in avidity of the monoclonal antibodies were minimized. Moreover, no relationship was found between the avidities of the monoclonal antibodies and the classification of the monoclonal antibodies in subgroups. Alternatively, binding of an antibody to its epitope may allosterically affect the binding of another antibody at a topologically distant epitope. The monoclonal antibodies of the subgroups may differ in this capacity to induce conformational changes and therefore, in the CBA's result in asymmetric reciprocal competition. That conformational changes may play a role was seen in the case of monoclonal antibody $14F_7$ as competitor, which enhanced the binding of monoclonal antibodies of the subgroups 1A, 1B and 1C under certain experimental conditions. A more precise identification of the epitope recognized by the monoclonal antibodies might be obtained from the investigation of the interaction pattern of the monoclonal antibodies with fragments of the hexon protein.

Based on the results of the ELISA, the blocking enzyme immunoassay (EIA) after guanidine-HCl/urea treatment and the neutralization assays, the monoclonal antibodies in general seem to have a higher avidity for HEV-V than for HEV-A. This difference in avidity of the monoclonal antibodies might be caused by a possible conformational difference between the hexon protein of the two strains which effects binding efficiency. A higher avidity for HEV-V than HEV-A is then logical because the monoclonal antibodies were generated from mice immunized with HEV-V.

In conclusion, this study describes the characteristics of a panel of ten monoclonal antibodies produced against HEV, and the use of these antibodies to identify a major neutralizing determinant located on the hexon protein. In addition, these monoclonal antibodies proved very useful for the detection of group II avian adenovirus infection in cell cultures. The use of one of these monoclonal antibodies to titrate antigen and antibody with ELISA's is described elsewhere (16). Finally, the fact that some monoclonal antibodies enhanced each other was used to improve the sensitivity of the Ag-ELISA and FA test by selecting an appropriate combination of these antibodies (J.V. van den Hurk, unpublished observations).

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CHAPTER 4

CHARACTERIZATION OF THE STRUCTURAL PROTEINS OF HEMORRHAGIC ENTERITIS VIRUS

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Running Title: Characterization of HEV proteins
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SUMMARY

The structural proteins of hemorrhagic enteritis virus (HEV), a turkey adenovirus, were analysed 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 (Ad2) IIIa protein with which it shares a common epitope. The common antigenic site present in both viruses was cryptic in virions and was of a continuous nature. 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 in native and denatured condition, 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. In addition, only one fiber was detected in penton preparations purified by immunoaffinity chromatography. The feature of having single fibers is shared with the mammalian adenoviruses and the avian egg drop syndrome 1976 virus (EDS76V), but not with the fowl adenoviruses which have double fibers attached to their penton base proteins. The relative migration of HEV soluble proteins (penton, hexon, penton base, fiber, IIIa) after separation by PAGE under native conditions was distinct from that of the Ad2 soluble proteins (penton, fiber, penton base, hexon, IIIa).

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 (Norrby et al., 1976). Within the genus Aviadenovirus there are two groups; the fowl adenoviruses (38) and a second group comprised of hemorrhagic enteritis virus (HEV) of turkeys (5,20,35), marble spleen disease virus (MSDV) of pheasants (18,19) and splenomegaly virus (SV) of chickens (11,12). It has been suggested that these be referred to as group I and group II avian adenovirus, respectively (8). 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 (15,25). 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 (9,17). It is classified as an adenovirus on the basis of its morphology, mode of replication, and physical-chemical properties (5,20,30,35). HEV, MSDV and SV are serologically identical viruses (8,9,10,19,36). To date, no serologic relationship has been found between these viruses and the fowl adenoviruses (11,12,21,33). However, the lack of a suitable cell culture system for HEV propagation has hampered a thorough investigation of its properties.

The overall study of HEV involved developing a vaccine for turkeys 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 had been characterized. The best studied adenoviruses in both genera are the human adenoviruses type 2 (Ad2) and type 5 (Ad5), and chick embryo lethal orphan (CELO) virus (fowl adenovirus type 1, FAV1). 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 (36). Ad2 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 0.01M 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 10,000g (36). 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 ($1.40g/cm^3$). 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 0.01M Tris-HCl, pH 8.1 containing 20% glycerol and stored at $-70^{\circ}C$. Ad2 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 (2mg/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). 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 Ad2 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 IM35 microscope equiped with epifluorescent illumination.

ELISA. Indirect ELISAs were used for analysis of HEV proteins using turkey, rabbit, or mouse antibodies as described previously (36, J.V. van den Hurk and S. van Drunen Littel-van den Hurk, Can J Vet Res, 1988, manuscript accepted).

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 0.15M 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 analyse 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 and 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 32V for 4 h in 25mM 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-4B (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 instructions. 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 0.05M diethylamine, pH 11.5. During collection the protein fractions were neutralized with 1M 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.

Preparation of immune sera. Hexon and penton proteins were purified by immunoaffinity chromatography. 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. Turkeys were immunized two times with 1 ml of purified hexon protein of 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 0.1mm. 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 lower density contained incomplete, non-infectious virions (data not shown), which is a common feature of adenoviruses. The virus band with the higher density contained complete, infectious virus. The diameter of both HEV-A and HEV-V particles was 72nm (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 analysed 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 Ad2 were visualized by amido black staining (Fig. 2). Eleven of the polypeptides found in stained blots were recognized specifically by antibodies present in convalescent sera from HEV infected turkeys (Fig. 2). The apparent molecular weights of the HEV polypeptides were

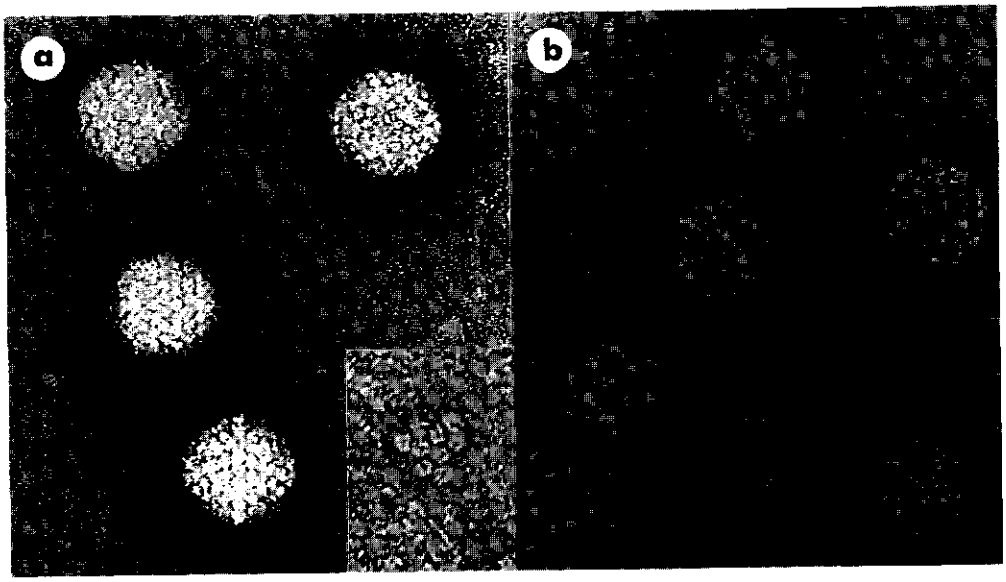

Fig. 1. Electron micrographs of CsCl purified HEV-A (a) and HEV-V (b) (ρ=1.34 g/cm^3). A value of 72 nm was estimated for the diameter of HEV particles. Insert shows a GON hexons of disrupted HEV-A. Negative staining with uranyl acetate. Bars represent 100 nm.

calculated using Ad2 polypeptides as standards (Philipson, 1983). 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, 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.

The identification of the 96k polypeptide as the hexon protein, the 57k polypeptide as the IIIa protein, and the 51/52k and 29k polypeptides as components of the penton protein, using monoclonal or monospecific polyclonal antibodies (Fig. 2), is based on data 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. HEV soluble proteins were analyzed by ELISA, Western blotting, sucrose gradient

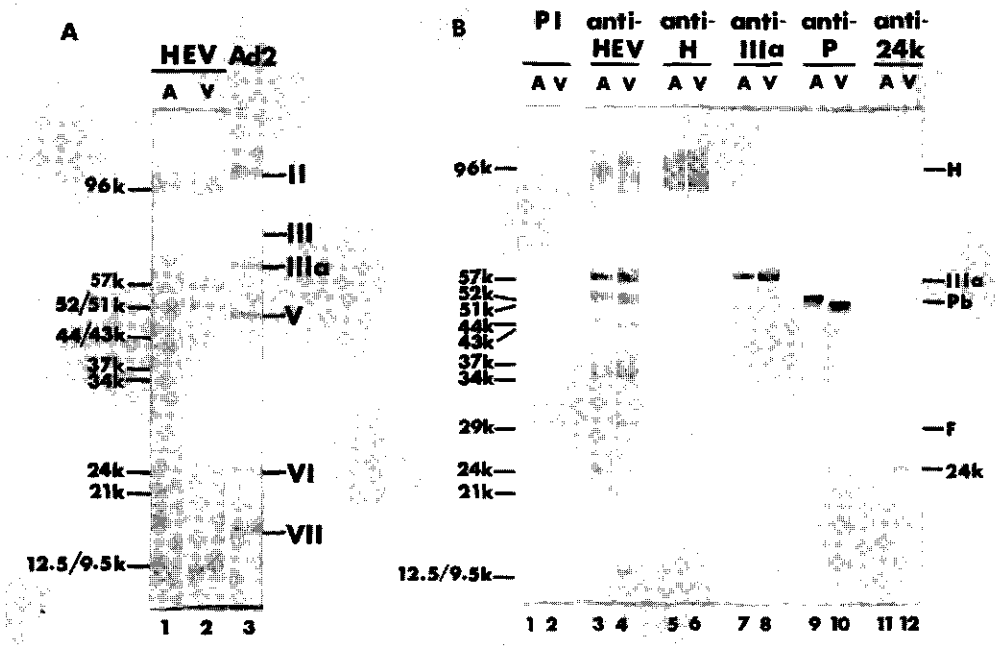

Fig. 2. Analysis of HEV polypeptides by SDS-PAGE and Western blotting. A. Polypeptides of HEV-A (lane 1) or HEV-V (lane 2), separated by SDS-PAGE, were transferred onto nitrocellulose and visualized by amido black staining. The molecular weights of the HEV polypeptides, indicated in the lefthand margin, were estimated using the polypeptides of Ad2 (lane 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 (lanes 1,3,5,7,9 and 11) and HEV-V (lanes 2,4,6,8,10 and 12) separated as described under A were analyzed by Western blotting using preimmune (PI) serum of turkeys (lanes 1 and 2), turkey anti-HEV serum (lanes 3 and 4), monospecific turkey anti-HEV hexon protein (lanes 5 and 6), monoclonal antibody 4B3-10D2 reactive with the HEV IIIa protein (lanes 7 and 8), monospecific rabbit anti-HEV penton protein (lanes 9 and 10), and monoclonal antibody 6C1, reactive with 24k protein (lanes 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).

centrifugation, and electron microscopy. Two HEV-specific protein bands were found after Western blotting when serum of infected turkeys was used for detection (Fig. 3). The faster moving protein band was identified as the hexon protein because it was recognized by monoclonal antibodies that were known to react with the hexon protein of HEV-A and HEV-V (J.V. van den Hurk and S. van Drunen Littel-van den Hurk, Can J Vet Res 1988, manuscript accepted). The slower moving protein was identified as the penton protein by electron microscopy after electroelution from the gel. 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 protein, were detected after Western blotting using rabbit anti-penton protein serum for analysis (Fig. 3). Electron microscopic observation of these proteins obtained after electroelution, revealed that the slower migrating protein was the penton base protein while the faster migrating protein 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. Monoclonal antibodies, known to react with the penton protein, could now be divided into a group reacting with the fiber protein and a group reacting with the penton base protein (Fig. 3, Table 1). This specific recognition was confirmed by ELISA with electroeluted fiber or penton base protein, and by Western blotting of dissociated penton proteins (data not shown). 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 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 monoclonal antibody 2D4 (specific for fiber protein, Table 1) showed that fractions 6-8 contained free fiber protein 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

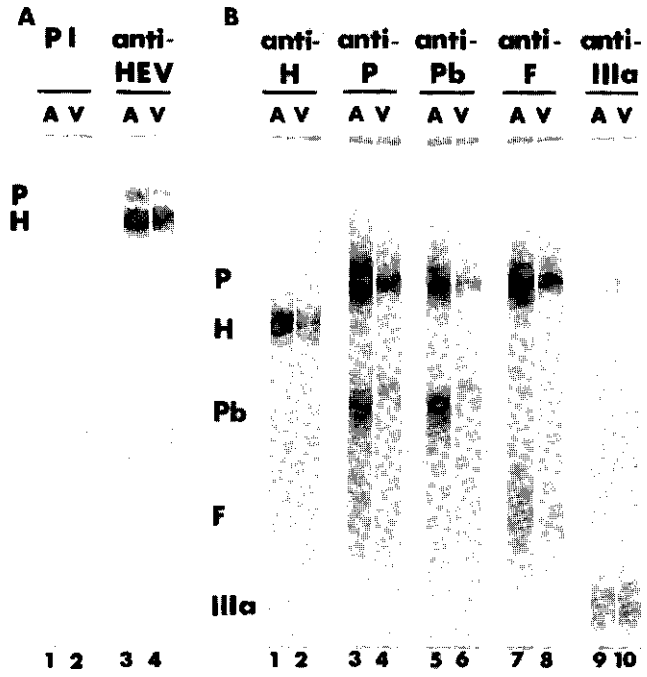

Fig. 3. Analysis of HEV soluble proteins separated by PAGE (6%) under non-denaturing conditions and detected by Western blotting. A. Western blot analysis of HEV-A (lanes 1 and 3) or HEV-V (lanes 2 and 4) soluble proteins using turkey preimmune (PI) serum (lanes 1 and 2) or turkey anti-HEV serum (lanes 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 (lanes 1,3,5,7 and 9) or HEV-V (lanes 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 (lanes 1 and 2). The penton (P), penton base (Pb) and fiber (F) proteins were detected using rabbit anti-HEV-A penton serum (lane 3), rabbit anti-HEV-V penton serum (lane 4), monoclonal antibody 4C3 reactive with the HEV penton base (lanes 5 and 6), or monoclonal antibody 2D4 reactive with the HEV fiber protein (lanes 7 and 8). The IIIa polypeptide was detected using monoclonal antibody 4B3-10D2 (lanes 9 and 10).

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

Monoclonal Antibody	Virus	Protein Specificity	FA staining[B]		Reactivity in ELISA[C]			
			Cytoplasm	Nucleus	Virus		Soluble proteins	
					-SDS	+SDS	-SDS	+SDS
15G4	HEV-A or HEV-V[A]	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	Ad2	IIIa	±	++	±	+	±	+

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

[B] Fluorescent antibody staining intensity range from ++++ to -: ++++, very strong; +++, strong; ++, medium, +, weak; ±, very weak; -, no staining.

[C] Optical density ranging from ++++ to -: ++++, ≥1.0; +++, 0.5-1.0; ++, 0.25-0.50; +, 0.1-0.25; ±, 0.05-0.1; -, <0.05

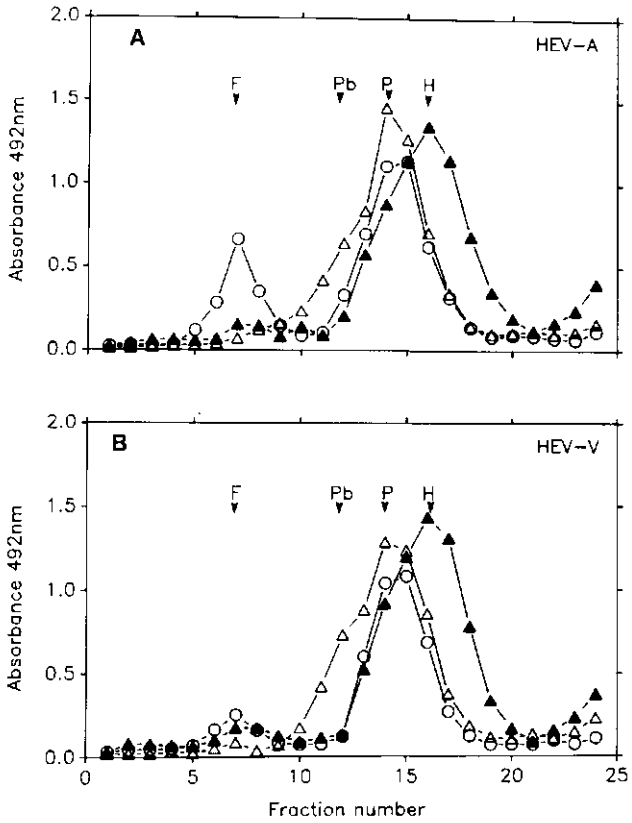

Fig. 4. Soluble protein fractions of HEV-A (A) and HEV-V (B) were centrifuged through linear sucrose gradients (10-30%, w/v) for 23 h at 38,000 rpm in a Beckman SW41 rotor at 4 C. Fractions were collected and analyzed by an indirect ELISA for the presence of fiber antigen (monoclonal antibody 2D4, O) penton base antigen (monoclonal antibody 4C3, Δ) and hexon protein (monoclonal antibody 15G4, ▲). The positions of the fiber (F), penton base (Pb), penton(P), and hexon (H) proteins, obtained by affinity chromatography are indicated.

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 monoclonal antibody 4C3 (reacts with penton base protein, 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 Ad2 soluble proteins as standards (6,31). The following values were determined for both HEV strains: 12S for the hexon protein, 10S for the penton protein, 9S for the penton base protein, 6S for the fiber protein, and 6S 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; ii) immunize rabbits to generate specific antibodies for the identification of the penton base and fiber polypeptides of HEV after SDS-PAGE and Western blotting; and iii) compare the migration of purified and unpurified proteins of both HEV strains separated by PAGE and analyzed by Western blotting. Pentons of HEV-A and HEV-V were purified from soluble protein fractions by immunoaffinity chromatography using monoclonal antibody 2D4 or 4C3 linked to Affi-Gel 10 followed by sucrose gradient centrifugation. The penton proteins of the sucrose gradient fractions were detected by ELISA (fractions reacting with monclonal 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). 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) confirming the apparent molecular weight values obtained following Western blot analysis of HEV stuctural proteins.

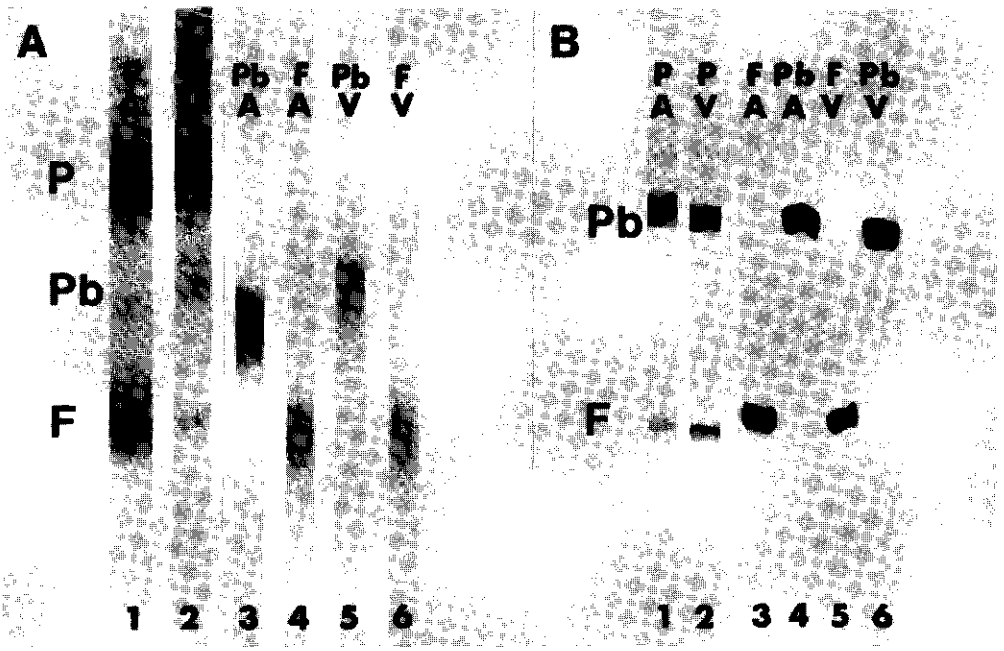

Fig. 5. Western blot analysis of HEV penton proteins purified by affinity chromatography, sucrose gradient centrifugation, and preparative PAGE. A. Penton base and fiber proteins of HEV were obtained from penton proteins, purified by affinity chromatography and sucrose gradient centrifugation, dissociated 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 (lane 1) and HEV-V (lane 2), purified penton base proteins of HEV-A (lane 3) and HEV-V (lane 5), and purified fiber proteins of HEV-A (lane 4) and HEV-V (lane 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 (lanes 1 and 2), fiber proteins (lanes 3 and 5), penton base proteins (lanes 4 and 6) of HEV-A (lanes 1,3 and 4) and HEV-V (lanes 2,5 and 6) separated by SDS-PAGE (13%) and detected by rabbit anti-penton serum.

The same molecular weights were obtained when affinity purified pentons were directly analyzed by SDS-PAGE and Western blotting (data not shown). However, sometimes fiber polypeptides with molecular weights of 27k (soluble protein fraction and purified HEV) and 20k (soluble protein fraction) were detected in addition to the 29k polypeptide after Western blotting using rabbit anti-penton, rabbit anti-fiber or turkey anti-HEV antibodies. These polypeptides are probably truncated forms of the 29k polypeptide because they all share a common antigenic site recognized by monoclonal antibody 2D4, when the PAGE and Western blot analysis were performed under conditions in which discontinuous epitopes can be recognized (7) (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 of 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 $\pm$ 1.8 nm was measured for HEV-A and of 17 $\pm$ 2.0 nm for HEV-V using negatively stained preparations of purified penton proteins. A value of 31 $\pm$ 2.3 nm was measured for Ad2 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 Ad2 in ELISA's of dissociated Ad2 virions and soluble proteins, and in FA tests of Ad2 infected cells. Western blot analysis revealed that this monclonal antibody recognized the IIIa protein of Ad2 (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.

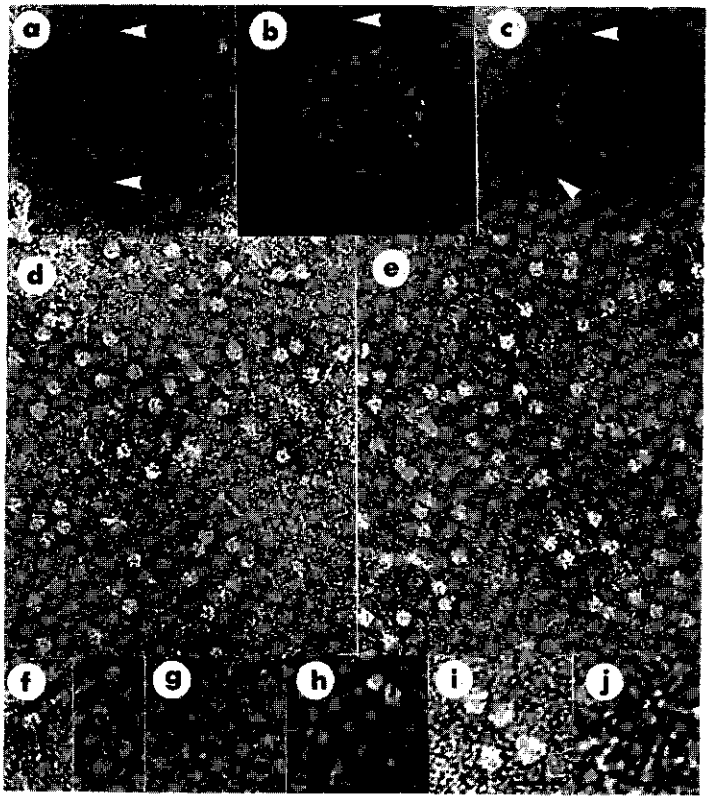

Fig. 6. Electron micrographs of CsCl purified HEV-A (a), HEV-V (b), and Ad2 (c) virions showing single fibers protruding from the capsid (arrowheads). Single fibers were also observed on pentons of HEV-A (d) and HEV-V (e) purified by immunoaffinity chromatrography and sucrose gradient centrifugations, and on pentons of HEV-A (f) and Ad2 (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 (f) are compared with pentons of HEV-A (g) and pentons of Ad2 (h) from dissociated virions. Penton bases obtained from pentons after dissociation, separation by PAGE, and electroelution, and fibers obtained after immuno-affinity chromatography and sucrose gradient centrifugation are also shown (i) and (j). Note the knob and anchorage of the fiber in the pentons of HEV-A (f,j). Negative staining with uranyl acetate. Bars represent 25 nm.

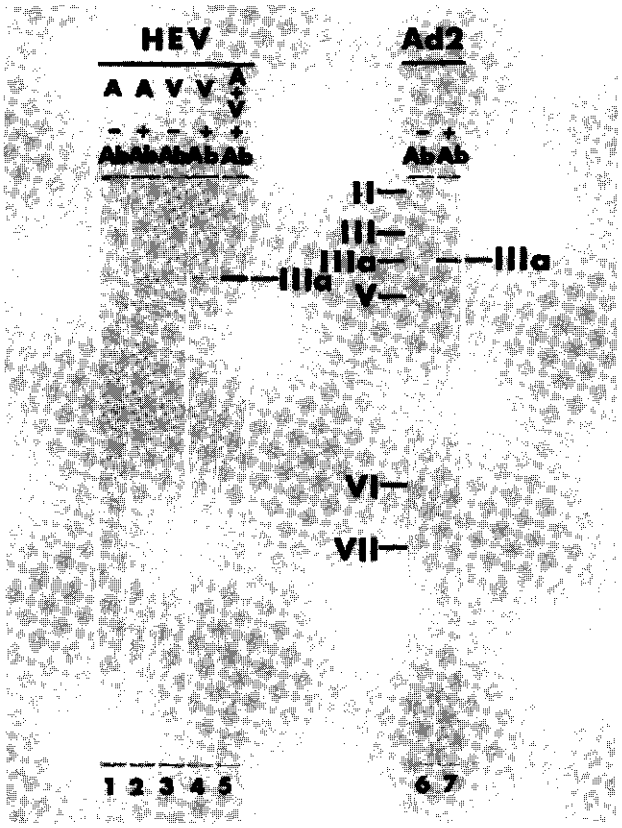

Fig. 7. Analysis of the IIIa polypeptide of HEV-A (lanes 1, 2, and 5), HEV-V (lanes 3, 4, and 5) and Ad2 (lanes 6 and 7) separated by SDS-PAGE on 13% gels and detected by Western blotting in the presence (lanes 2, 4, 5 and 7) or absence (lanes 1, 3, and 6) of monoclonal antibody 4B3-10D2. The positions of the IIIa polypeptide of HEV-A, HEV-V, and Ad2 are identical. The positions of the marker polypeptides of Ad2 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). Its counterpart in the human adenoviruses was not identified.

Core proteins. HEV nucleoprotein cores obtained after disruption of

virions were separated from capsid components by centrifugation through a linear glycerol gradient (13). 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). In stained gels, the 12.5k polypeptide band was more pronounced than the 9.5k polypeptide suggesting that the 9.5k protein is probably attached more strongly to the DNA than the 12.5k protein.

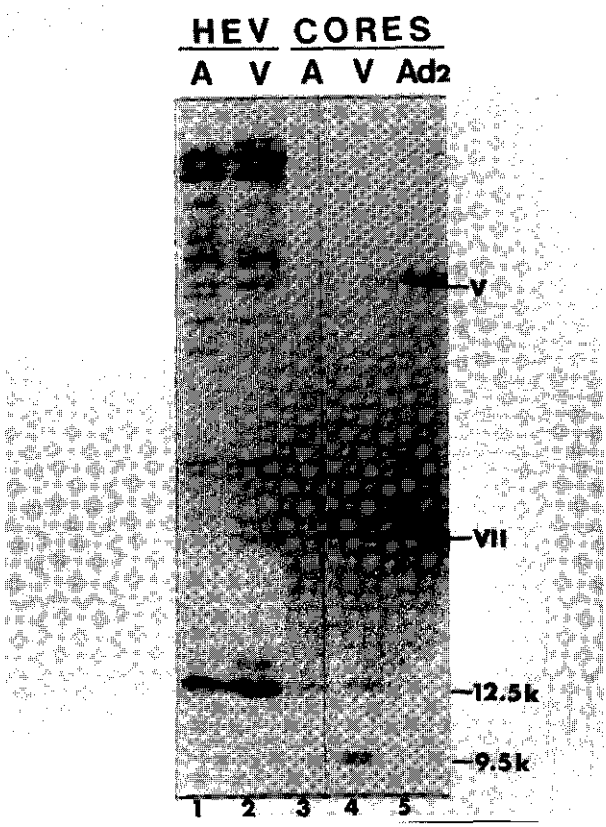

Fig. 8. Analysis of the core proteins of HEV-A (lane 3), HEV-V (lane 4), and Ad2 (lane 5) present in purified nucleoprotein cores separated by SDS-PAGE in 13% gels and stained with Coomassie brilliant blue R-250. The profiles of the HEV-A and HEV-V polypeptides are shown in lane 1 and 2, respectively. The positions of the HEV and the Ad2 (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 and 44k vs 43k, of HEV-A and HEV-V, respectively. The electrophoretic migration pattern of the native HEV soluble proteins was different from the pattern of Ad2 proteins. The migration order of the penton, penton base, and IIIa proteins of HEV and Ad2 was similar, but it was inversed in the case of the hexon, and fiber proteins (2,26). 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 Ad2 fiber and penton base proteins relative to Ad2 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 (31). In addition, it is an important neutralizing antigen (J.V. van den Hurk and S. van Drunen Littel-van den Hurk, Can J Vet Res 1988, manuscript accepted). No differences were observed in electrophoretic mobility between the hexons of HEV-A and HEV-V in native or denatured conditions. Furthermore, 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 preparation).

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 EDS76V (15,23) has pentons with single fibers, and this is in contrast to the double fibers present on penton bases of fowl adenoviruses (15,25,27). 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 observed difference in migration on polyacrylamide gels of HEV-A and HEV-V penton bases under non-denaturing and denaturing conditions probably underlies a difference in the primary structure of these proteins.

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 (17nm). The molecular weight of 29k was lower than that found for the long fibers of Ad2 (62k), FAV-1 (65k), and EDS76V (67k) but resembled more closely those found for the shorter fibers of Ad3 (34.8k), and FAV-1 (44.5k) (27,31,32,34). The observation of relatively short fibers with a low molecular weight is in agreement with the suggested relationship between the length of the native fiber protein and the size of the polypeptide (32). 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 preparation). 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 (15,27,37).

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 Ad2. Similar results for HEV-A, HEV-V, and Ad2 were obtained by FA staining, ELISA of soluble proteins and purified virus, and Western blotting. Moreover, the recognition of the IIIa protein of HEV and Ad2 is the first evidence of a shared antigenic determinant found on a human and an avian adenovirus. 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 virus-dissociation.

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 Ad2 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 EDS76V might also be analogous (34).

The 12.5k and 9.5k proteins were identified as core proteins of HEV. They are smaller than those of Ad2 (48k and 18.5k) and more closely ressemble those of FAV-1 (20k, 12k, and 9.5k) (28,31). 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 Ad2 is that the smallest core protein (9.5k, 9.5k, and 18.5k respectively) is more tightly attached to viral DNA than the larger one(s) (28,31).

This report strengthens the arguments for the classification of HEV in the family of the 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 Ad2. 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).

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CHAPTER 5

In Vitro Replication of Group II Avian Adenoviruses

Propagation of Group II Avian Adenoviruses in Turkey and Chicken Leukocytes

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SUMMARY. An avirulent hemorrhagic enteritis virus isolate (HEV-A) as well as a virulent HEV isolate (HEV-V), both belonging to the group II avian adenoviruses, were successfully propagated in turkey blood leukocyte cell cultures. HEV antigens were detected as early as 12 hr after infection of the cells, using HEV-specific monoclonal antibodies in a fluorescent antibody test, and virus particles were observed in the nuclei of infected cells at 18 to 24 hr after infection. Light microscopy as well as electron microscopy revealed the presence of HEV in the nuclei of non-adherent as well as in adherent cells. The non-adherent infected cells had the characteristics of immature mononuclear leukocytes while the adherent cells had monocyte-macrophage characteristics. HEV produced in turkey leukocytes was mostly cell-associated, particularly with the non-adherent cells. HEV-A could be serially passed in turkey blood leukocyte cultures at least 7 times. Various methods employed to culture virus indicated that cells grown in spinner cultures were superior to stationary cultures. In contrast to the successful infection of HEV in turkey leukocytes, the infection of chicken leukocytes with either HEV or splenomegaly virus (SV) of chickens, or turkey leukocytes with SV, was poor.

INTRODUCTION

Hemorrhagic enteritis (HE) is an acute disease of turkeys caused by hemorrhagic enteritis virus (HEV). HE is characterized by depression, bloody droppings and death, and usually occurs in commercial turkey flocks in 6 - 12 week old birds (4,5). HEV of turkeys, marble spleen disease virus (MSDV) of pheasants and splenomegaly virus (SV) of chickens are serologically closely related viruses which are tentatively classified as members of the group or type II avian adenoviruses (4,5,7,8,15).

HE in turkeys can be and is prevented effectively by administration in the drinking water of a live virus vaccine propagated in turkeys (6,9,26). This vaccine is obtained by making a crude extract from spleens of turkeys orally infected with an avirulent HEV (HEV-A) isolate. This crude spleen vaccine is used locally by turkey producers because until recently no vaccine of higher quality and safety was commercially available.

In contrast to most mammalian and fowl adenoviruses which can be propagated easily in epithelial cell cultures of their homologous hosts,

difficulties were encountered with the in vitro replication of HEV because this virus did not replicate in a simlar system (5,21). The first successful propagation of HEV in a cell line for vaccine production was reported by Nazerian and Fadly (21,22). They propagated HEV in Marek's disease virus (MDV)-transformed B lymphoblastoid cell lines obtained from turkey tumors (20). Although the virus replicates in these transformed cells, the use of such a vaccine is disputed and its safety features must be carefully evaluated because MDV is present in these cells (20).

The goal of this investigation was to develop and test a cell culture system in which HEV can be propagated efficiently, and ultimately to use HEV-A produced in this cell culture system as a vaccine for HE in turkeys. Recently, I reported on the propagation of HEV in turkey leukocytes (30). In this paper the replication of both the HEV-A and the virulent HEV (HEV-V) isolates in cell culture is described.

MATERIALS AND METHODS

Turkeys. Day-old small white diamond hybrid poults (Chinook Belt Hatcheries, Calgary, Alberta, Canada) raised in isolation were used in all experiments. Poults were bled at regular intervals to determine passive antibody titers against HEV using an antibody-ELISA (31).

Viruses. The HEV-A isolate (pheasant origin), the HEV-V isolate (turkey origin), and the SV isolate (chicken origin) were obtained as lyophilized crude spleen extracts from C.H. Domermuth, Virginia Polytechnic Institute, Blacksburg, Virginia. Both HEV isolates were passed in turkey poults by oral inoculation (31), first by crude spleen extract and then as CsCl-purified virus. Crude or purified virus preparations from spleens of the fifth or higher passage level in turkey poults were used for inoculation of the cell cultures. Crude spleen extract of SV passed once in chickens was used for inoculation of the cell cultures. All HEV preparations in PBS containing 15% glycerol were filter-sterilized through a 0.45 μ filter and stored at -70 C.

Cell culture. Turkey leukocytes were prepared from heparinized blood (50 units/ml) collected from 8 to 20 week old turkeys which were sero-negative for HEV (ELISA titers <10). The leukocytes were isolated by two methods. (i)

Ficoll-Paque method: Whole blood was centrifuged through Ficoll-Paque (Pharmacia, Montreal, Quebec, Canada) for 15 to 20 min at 800 g at room temperature. The mononuclear leukocytes, at the Ficoll-Paque interface, were collected for further processing and culturing virus. (ii) "Slow-spin" method (14): Blood was first centrifuged for 3 min at 150 g followed by a further 10 min at 35 g at 4 C. Plasma and buffy coat cells were collected for further processing. Cells, obtained by both methods, were washed twice in RPMI 1640 and resuspended at a concentration of 10^7 cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), Hepes buffer (25 mM), and gentamycin (50 mg/l) (Gibco, Grand Island, New York). The leukocytes were grown either in stationary culture or in spinner cultures (Techne, Cambridge, UK) at 41 C in an atmosphere of 95% relative humidity and 5% CO_2. In general, leukocyte cultures were infected 1 to 3 hr after seeding with crude spleen extract, purified spleen extract or cell culture produced HEV at a concentration of 5-20 tissue culture infectious doses 50 ($TCID_{50}$)/ml culture medium. $TCID_{50}$'s were determined by titration of virus preparatins in stationary turkey leukocyte cultures (10^7 cells/ml). The percentage of infected cells in smears made three days post-infection, was determined by the fluorescent antibody (FA) test and the 50% value was calculated (10). Cell cultures were harvested 2 to 3 days after infection and stored at -70 C. Chicken leukocytes were obtained from chicken blood isolated by the Ficoll-paque method and grown in stationary cultures as described for turkey leukocytes. Differential cell counts of cytocentrifuge cell preparations were determined after staining with a Diff-Quick set stain (American Scientific Products, McGaw, Illinois) (19).

Immunofluorescence. Cell smears of the non-adherent cell population of HEV-infected and control cells were made with a cytocentrifuge on microscope slides. HEV-infected and control adherent cells were grown in Lab-Tek tissue culture chambers (Miles, Naperville, Illinois). The cells were fixed in methanol for 5 min. Subsequently, they were incubated with a monoclonal antibody (MAb) cocktail consisting of hybridoma supernatants of MAb's 11B6, 14E1, and 15G4, all at a final concentration of 1:10 for 1 hr at 41 C. These MAb's react specifically with a wide range of group II avian adenovirus isolates (32). Thereafter, the cells were incubated with affinity-purified fluorescein-labelled goat-anti mouse IgG (Boehringer Mannheim, Dorval, Quebec) for 1 hr at 41 C. The cells were mounted with PBS-glycerine (1:1, v/v). The

presence of HEV antigen in the cells was observed with a Zeiss IM35 microscope equipped with epifluorescence illumination. The number of infected cells was estimated by counting fluorescent and total cells in several microscope fields (usually 500 to 1,000 cells). Only cells in which the nuclei exhibiting fluorescence were used to calculate the percentage of infected cells while the adherent cells showing fluorescent specks in the cytoplasm were not counted. Pictures were taken with a neofluar x 40 objective.

Phagocytosis. Leukocytes were cultured in Lab-Tek culture dishes and infected with HEV. Twenty-four hours post-infection, phagocytosis using carbon particles (29) or latex beads (25) was determined. After incubation with the particles for 30 min at 41 C, cells were fixed, stained for HEV antigen by the indirect fluorescence antibody technique and examined with the Zeiss microscope for fluorescence and phagocytosis.

Electron microscopy. Non-adherent cells taken from the culture dishes and adherent cells removed from the culture dishes by gentle scraping were collected separately and centrifuged. Cells were resuspended in PBS and washed twice. The cells were fixed in 3% glutaraldehyde in sodium cacodylate buffer for 2 hr at 0 C, washed in sodium cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated through graded ethanol and propylene oxide, and embedded in epoxy resin (Epon). Ultrathin sections were cut with a diamond knife, post-stained with 2% uranyl acetate and lead citrate, and examined with a Philips 410LS electron microscope.

Virus distribution. Turkey leukocytes isolated from blood using the Ficoll-Paque method, were grown in stationary cultures. In experiment 1 the cells were infected with 10 $TCID_{50}$ of HEV-A from crude spleen extracts and in experiment 2 with 5 $TCID_{50}$ of purified HEV-A. Three days post-infection non-adherent cells and culture medium were removed from the flasks and separated by centrifugation. Adherent cells were scraped from the flasks. $TCID_{50}$ were determined to evaluate the distribution of infectious virus in the two cell populations, after HEV was released from the cells by two freeze-thaw cycles and sonication, and in the cell culture medium.

Passage and testing of HEV-A produced in turkey leukocytes.

Experiment 1. Leukocytes, isolated from blood by Ficoll-Paque centrifugation and cultivated in stationary cultures at 10^7 cells/ml, were infected with 20 $TCID_{50}$ of HEV-A from crude spleen extract. After three days in culture, cells and culture medium were collected and virus was released from the cells by two freeze-thaw cycles and sonication (passage one). This procedure was repeated six times using virus produced in the previous passage to infect the next cell culture. At each step 10% of the previous cell culture suspension was used to infect the leukocytes of the next culture. Culture conditions were the same as described before.

Experiment 2. Leukocytes, isolated from blood by a "slow-spin" centrifugation and propagated in spinner culture at 10^7 cells/ml, were infected with 10 $TCID_{50}$ of CsCl-purified HEV-A. Virus was released after 3 days in culture as described for experiment 1 (passage one) and used for infection of new leukocytes. Virus was passed four times in cultures using 2% of the previous cell culture suspension for infection of the next culture. $TCID_{50}$ were determined to evaluate virus replication in both experiments.

RESULTS

Analysis of HEV-infected cells. Initially experiments were carried out using spleen cells because a relatively high number of these cells contain virus particles after infection of turkeys with HEV. After leukocytes were found to be the target cells in which HEV replicates, blood leukocytes were used since it was easier to process and isolate large quantities of leukocytes from blood than from spleens, and birds can be bled repeatedly.

After purification by the Ficoll-Paque method, the isolated cell suspensions contained in addition to lymphocytes (30-40%), monocytes (3-9%), granulocytes (0-1%), and a high quantity of thrombocytes (50-65%). The cells were cultured in RPMI 1640 and 10% FBS, and then infected with HEV-A or HEV-V. After 18 to 24 hr smooth shiny cells were observed in leukocyte cultures infected with HEV-A (Fig. 1) or HEV-V (data not shown). These cells were larger than the average lymphocyte and were loosely attached to the plastic surface of the culture dish or flask, in which they were growing. Their numbers increased in time and they were often found in pairs or clusters after several days in culture. Similar cells were also observed in control cultures but they were present in much smaller numbers (Fig. 1). The presence of

infected cells was analysed by a FA screening test using monoclonal antibodies

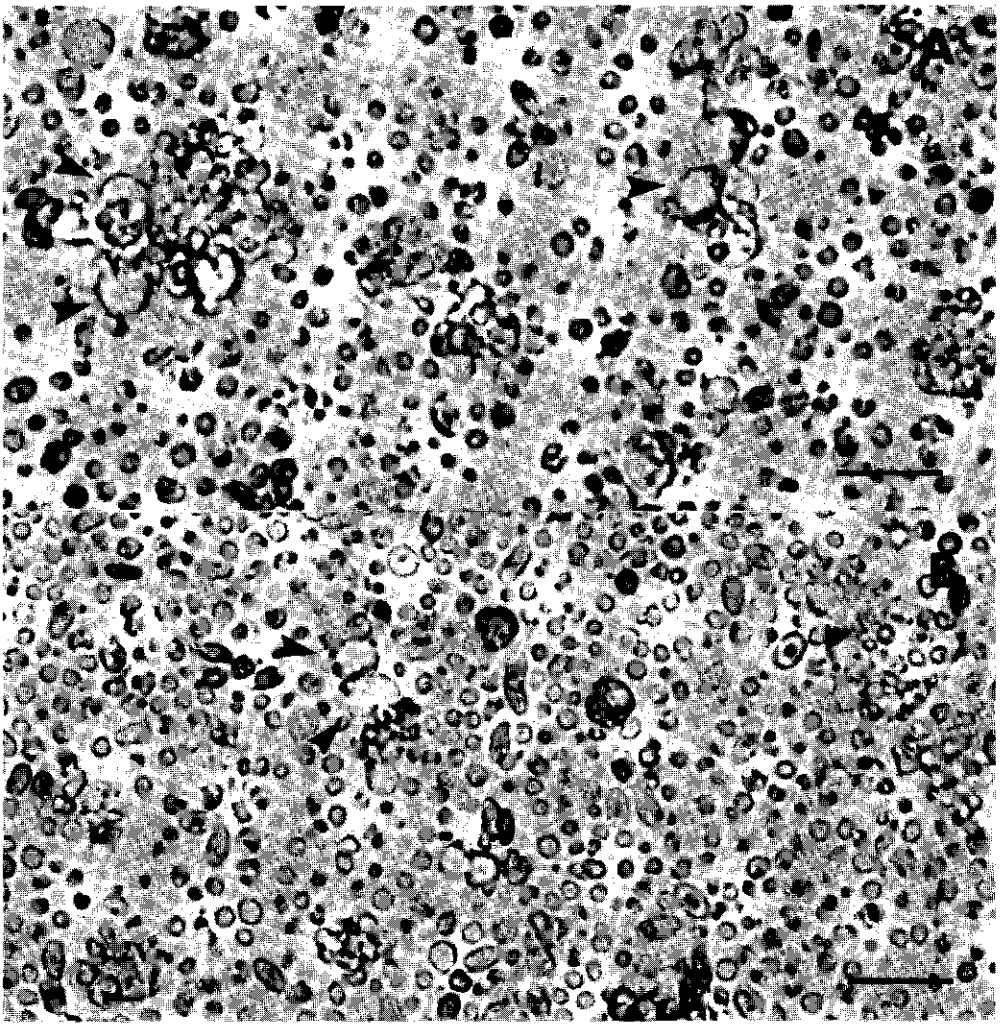


Fig. 1. Photographs of HEV-A infected (panel A) and control leukocytes (panel B) growing in cell culture. Note the large shiny cells (arrowheads) which are present in both panels. Photographs were taken 48 hr post infection. Bar represents 50 μm.

which react specifically with the hexon protein of the group II avian adenoviruses. Two to three days after infection a low percentage (1-5%) of the cells became infected with HEV (Fig. 2). Infected cells were mostly enlarged and 60 to 85% of the larger cells showed the presence of HEV when analysed by the FA test. The percentage of infected cells did not differ whether crude spleen extract or purified HEV was used as inoculum. The virus strain used did

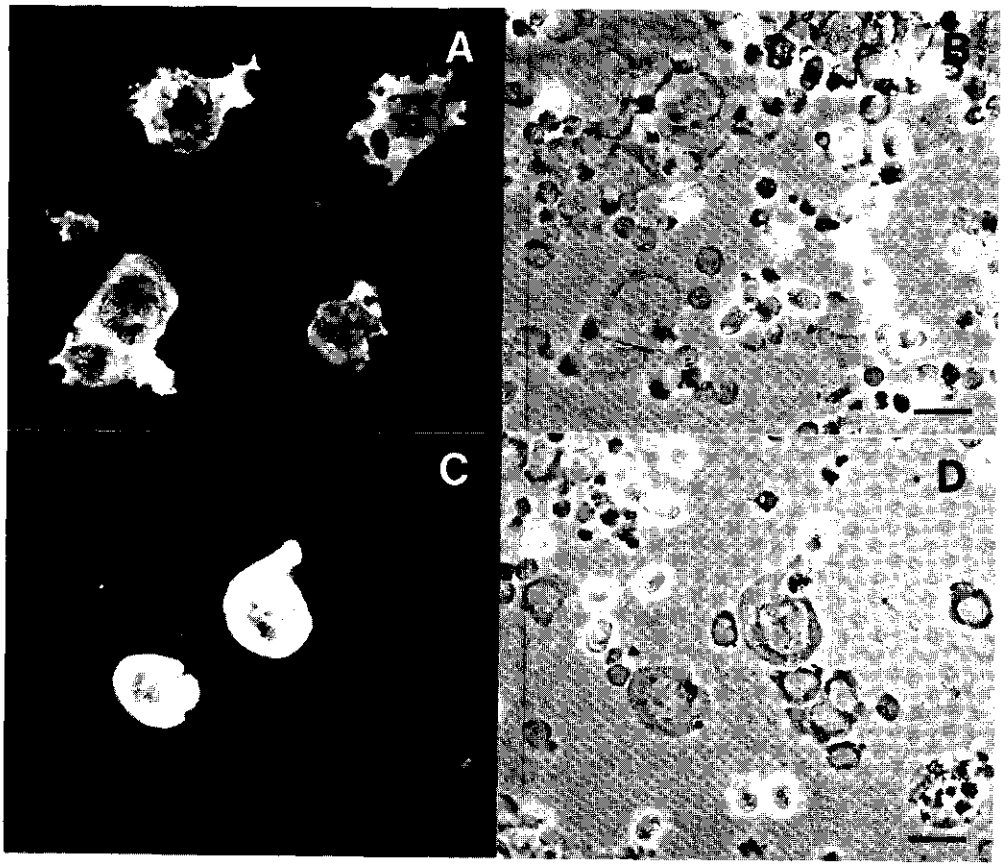


Fig. 2. Location of HEV-A (panels A and B) or HEV-V (panels C and D) infected turkey leukocytes visualized by indirect immunofluorescent staining of the hexon protein using a cocktail of MAb's. Cell smears were made 48 hr post infection and were fixed in methanol. Panels A and C, fluorescence visualized by uv microscopy; panels B and D, cell morphology visualized by phase contrast. The bars in the panels represent 20 μm.

not affect this result either. No antigen was detected in uninfected control cells.

When the leukocytes in the cultures were divided in to non-adherent and adherent cells, HEV-infected cells were detected in both groups (Fig. 3).

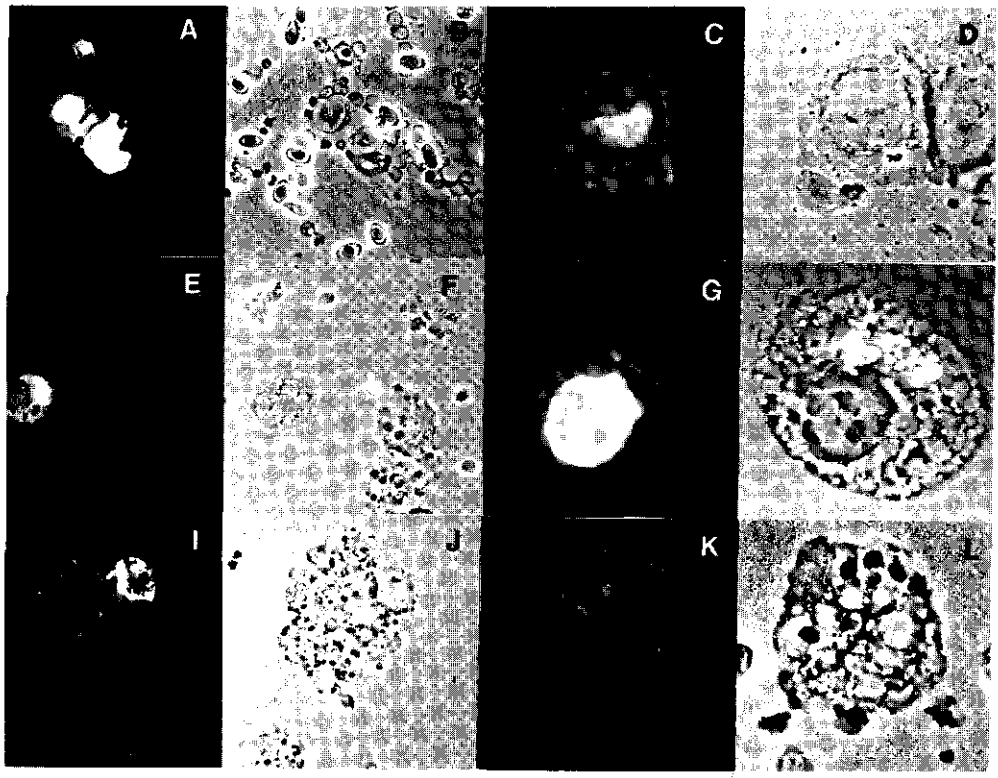
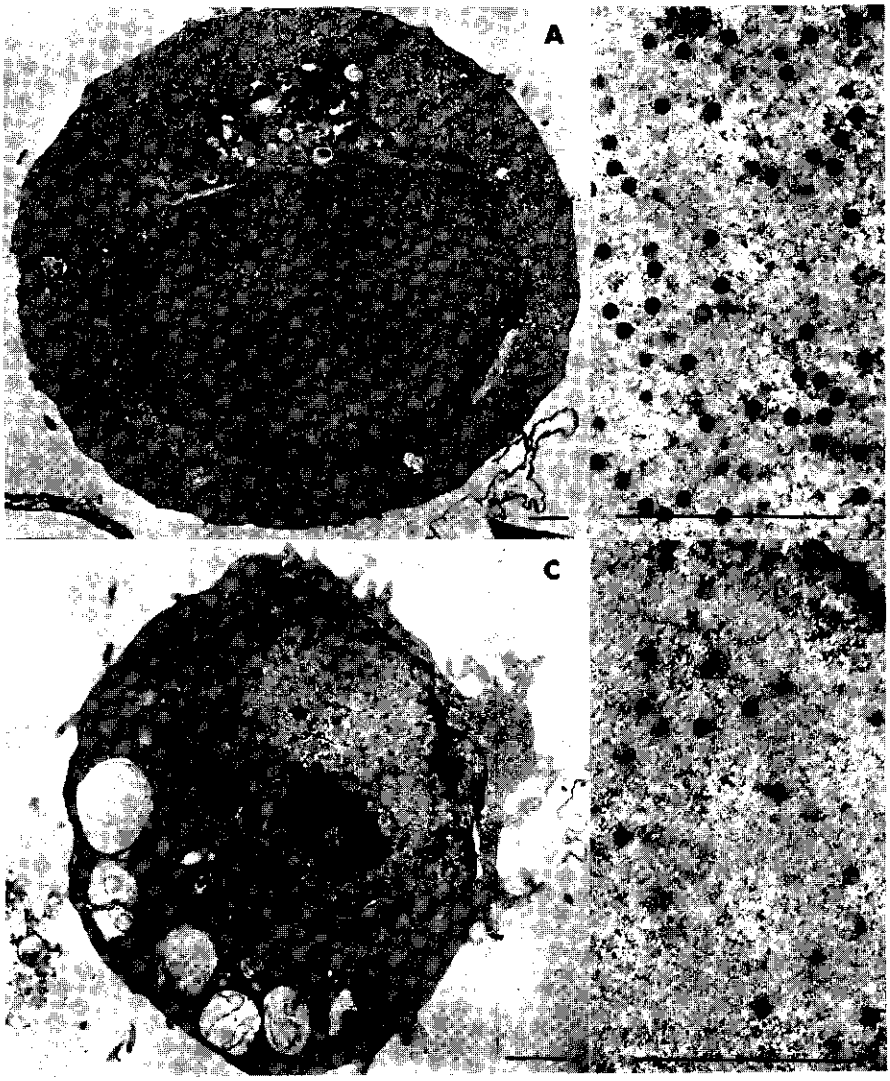


Fig. 3. Detection of HEV-A infected cells in non-adherent (panels A through D) and adherent cell populations (panels E through L) visualized by indirect staining of the hexon protein using a cocktail of MAb's. Cell smears were made 48 hr post infection. Panels A,C,E,G,I, and K are photographs under uv illumination of the same fields shown in panels B,D,F,H,J, and L, respectively, taken under phase contrast. Note the typical smooth infected cells with small vacuoles in panels A,B,C and D, the granular infected cells with many large vacuoles in panels E,F,G, and H, and the cells with fluorescent speckles in panels I,J,K, and L (arrow heads).

The non-adherent infected cells were large smooth-looking immature cells when observed with the light and electron microscope. HEV particles were present in the nuclei of the infected non-adherent cells (Fig. 4). The HEV-infected cells in the adherent group resembled monocyte-macrophage cells. Sections of infected adherent cells showed HEV particles in the nuclei of these cells as



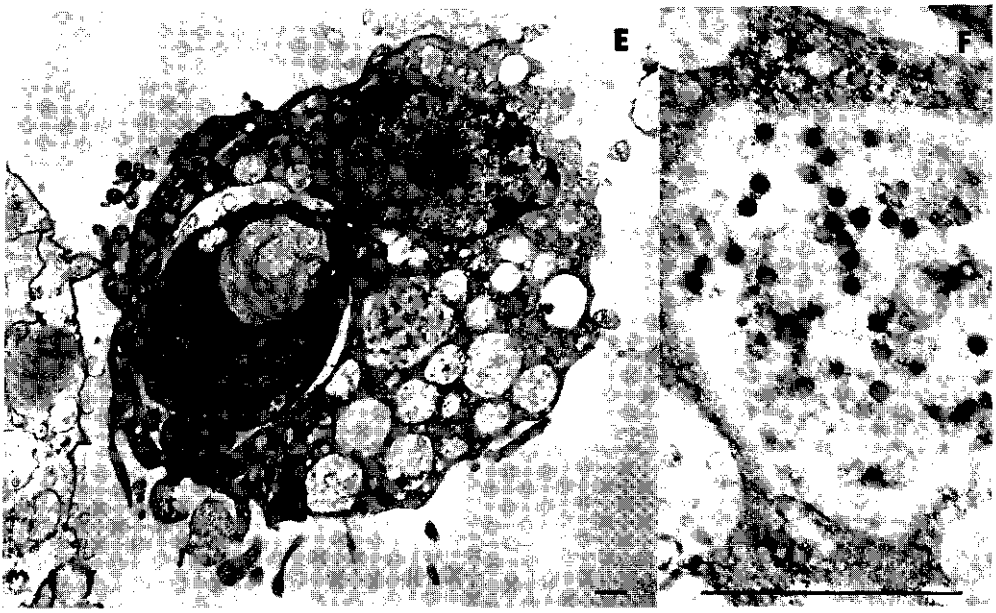


Fig. 4. Electron micrographs of HEV-A infected leukocytes 48 hr post infection. Panel A shows a typical smooth non-adherent cell with HEV in its nucleus, panel C shows a typical adherent cell with HEV in its nucleus, and panel E shows a cell with HEV particles in its cytoplasm. Panels B, D, and F are details of A, C, and E, respectively, at a higher magnification, clearly showing HEV particles. The bars in the panels represent 1 μm.

well (Fig. 4). In addition, ingested virus particles were observed in the cytoplasm of some monocyte-macrophage cells (Fig. 4).

The HEV target cell population was further characterized by investigating their phagocytic activity. Most of the infected adherent cells did ingest latex or carbon particles (Table 1, Fig. 5) and also had high non-specific esterase activity (data not shown). In contrast, only a low percentage of the infected non-adherent cells had ingested carbon or latex particles or stained for non-specific esterase activity. These data confirm that the adherent infected cells are probably monocyte-macrophage cells, but they are not conclusive about the nature of the non-adherent cells.

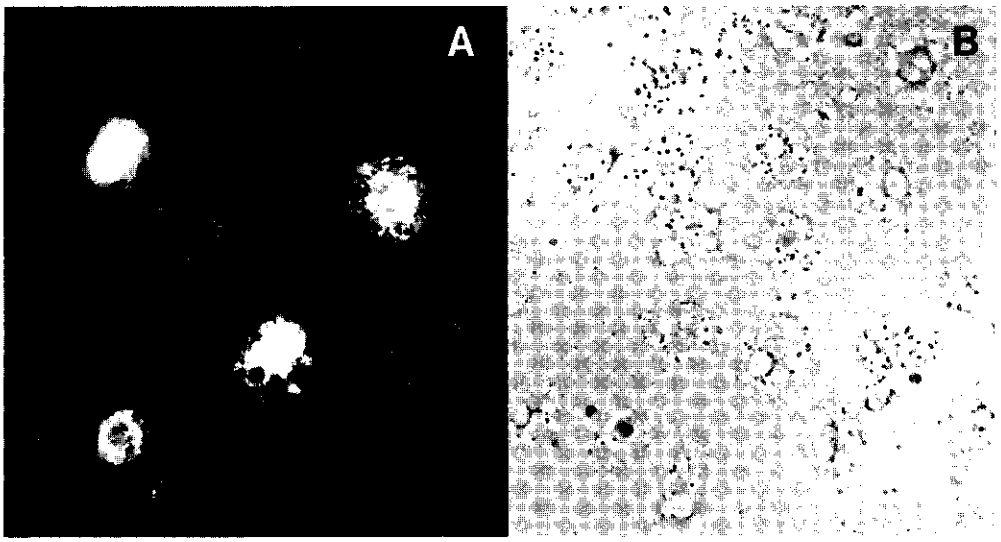


Fig. 5. Phagocytosis of latex particles by adherent cells infected with HEV-A. Panel A shows fluorescence visualized by uv microscopy and panel B shows cell morphology of the same field visualized by phase contrast.

Table 1. Phagocytic activity of turkey blood leukocytes infected with HEV-A.

Cell Population	Infected Cells[A]	Infected phagocytic cells[B]	
		Carbon	Latex
Adherent cells	1.3	89.0	94.0
Non-adherent cells	2.5	2.0	1.0

[A] Percentage infected cells determined by FA test.

[B] Percentage infected cells with phagocytic activity.

The various subfractions were also analyzed to determine whether infectious virus was mostly cell-associated or free in the medium, and whether it was associated with adherent or non-adherent cells. Table 2 demonstrates that most of the virus was cell-associated and predominantly present in the non-adherent cell population even though the infectivity of infected adherent and non-adherent cells was the same on a per cell basis. From this information it is evident that HEV-A is present in all subfractions and they should be kept together to obtain optimum virus yields.

Table 2. Distribution of cell-associated and cell-free HEV-A produced in turkey leukocyte cultures.

	$TCID_{50}$	
	Exp.1[A]	Exp.2[A]
Adherent cells	8	2
Non-adherent cells	32	8
Cell culture supernatant	3	1

[A]Two independant experiments; see Materials an Methods for details.

In order to determine the kinetics of infection a time course experiment was conducted. Antigen could be detected within 12 hr post-infection in the non-adherent cells while in the adherent cells, antigen was not observed until 20 hr post-infection (Fig. 6). Virions were observed in the nuclei of infected non-adherent cells at 18 to 24 hr after infection (not shown). Accumulated data from 30 experiments in which leukocytes were infected with HEV-A showed that the highest percentage of infected cells was found 2 to 3 days post-infection with a range of 1 to 14% and an average of 4.3% (SD$\pm$3.2%). The large variation in percentage of infected cells was caused by a number of

factors including variation in susceptibility between cells of different birds, age of the birds, and different cell culture conditions. The peak of infectious HEV-A production was also found 2 to 3 days post-infection (data not shown). When cells were kept in culture for more than four to five days, the number of non-adherent cells decreased gradually and after 10 days virtually none were observed at all. The only surviving cells in culture were large adherent phagocytic cells which were sometimes multinuclear. HEV-A could still be detected in the cytoplasm of some of these cells by FA

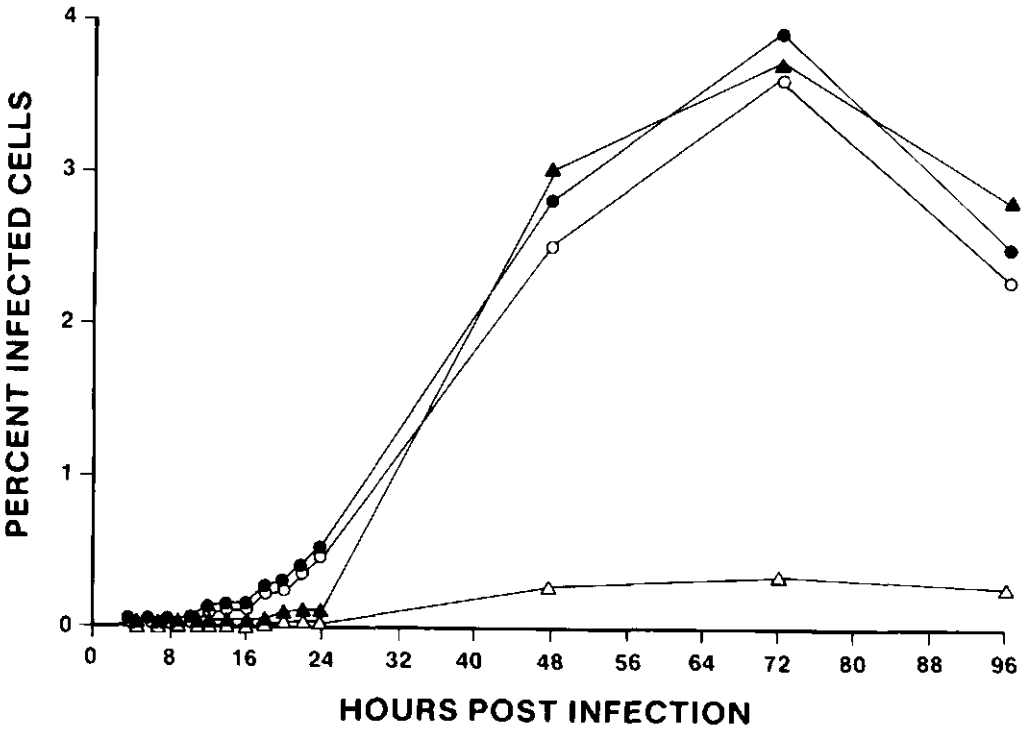


Fig. 6. Time course experiment of HEV infection in turkey leukocytes. Infection of the cells with crude spleen HEV-A was determined using the FA test. Symbols: percentage infected non-adherent cells/total non-adherent cells, ● ; percentage infected non-adherent cells/total cells, ○ ; percentage infected adherent cells/total adherent cells, ▲ ; percentage infected adherent cells/total cells, △.

screening ten days post-infection but gradually all cells became negative for HEV-A antigen after three weeks (data not shown). A similar infection pattern was observed when turkey leukocytes were infected with HEV-V (data not shown). Attempts to establish cell lines from primary turkey leukocytes for HEV propagation were all unsuccessful.

Virus passage in leukocytes. In order to investigate whether HEV-A was actually replicating in cell culture, resulting in the production of infectious virus, two experiments were carried out in which virus was passed seven or four times, respectively. The data in Table 3 demonstrate that infectious virus was produced in cell culture, because it could be passed and diluted and retained its infectivity. In addition, virus produced after several passages was even more infectious than after one passage (Table 3).

Table 3. HEV-A production after several passages in turkey leukocyte cultures.

Experiment 1[A]		Experiment 2	
Passage level	$TCID_{50}$	Passage level	$TCID_{50}$
1	5	1	47
7	20	4	87

[A] Two independent experiments; see Materials and Methods for details.

HEV-A production under various conditions. The effect of two variables on virus production, method of leukocyte isolation and culture condition, was investigated with the purpose to improve virus yield. The cell suspensions purified by the "slow-spin" method, contained in addition to lymphocytes (85-95%), monocytes (5-10%), granulocytes (1-2%), and thrombocytes (1-2%). The

number of thrombocytes was much lower following the "slow spin" method than after the Ficoll-Paque method (50-60%). In general, higher and more consistent virus production was observed when the leukocytes were obtained with the "slow-spin" method than with the Ficoll-Paque method. Moreover, more infectious virus was generated when the cells were maintained in a spinner culture than in a stationary culture (Table 4). Another advantage of the spinner culture was that larger cell quantities (1 to 10l) could be processed for HEV production requiring less work in handling of the cells than when cells were grown in flasks.

Table 4. Comparison of HEV-A production by two different leukocyte isolation methods and culture conditions.

Culture condition	Leukocyte isolation method	
	Ficoll-Paque	Slow spin
Stationary culture	11(4-20)[A,B]	28(27-29)[C]
Spinner culture	27(23-31)[C]	73(23-135)[B]

[A] Average $TCID_{50}$ and range in brackets
[B] Data from 4 experiments
[C] Data from 2 experiments

Infection of chicken and turkey leukocytes with HEV-A and SV. In order to determine whether higher virus yields could be obtained using a different cell culture system, the potential of HEV and SV propagation in chicken leukocytes was investigated. The ability of SV to replicate in turkey cells was also analyzed. Table 5 shows that leukocytes of eight chickens were only infected at a low rate with both HEV-A and SV. In a comparable experiment conducted with turkey leukocytes a normal infection was obtained with HEV-A,

whereas a poor infection was found with SV (Table 6). Fluorescent cells, detected only in infected and not in control cultures, were enlarged, had intranuclear inclusions, and belonged mostly to the non-adherent cell population in both experiments.

Table 5. Infection of leukocytes from 8 different chickens with HEV-A or SV at 3 and 4 days post-infection.

Leukocytes from chicken number	3 days post-infection		4 days post-infection	
	HEV-A	SV	HEV-A	SV
152	±[A]	±	±	±
156	±	±	±	-
158	±	-	±	-
159	±	-	-	-
163	-	-	±	±
164	±	-	±	-
169	±	-	-	-
170	±	-	-	±

[A] Percentage of cells stained in the FA tests ranging from ++ to -:
++, ≥ 1.0%; +, 1 - 0.1%; ±, 0.1% - 0.01%; -, < 0.01%

DISCUSSION

This report describes the propagation of HEV in a primary cell culture. After infection of turkey leukocytes in cell culture it was observed that viral antigen increased as detected by FA testing, HEV particles occurred in the nucleus, and the amount of infectious virus increased after inoculation. Moreover, HEV could be serially passed at least seven times in

turkey leukocytes. Previously, Perrin et al (24) inoculated spleen cells with HEV but they did not show that the recovered virus was other than the inoculum, by conducting titration experiments or passing HEV in cell culture. Fasina and Fabricant (12) were able to infect spleen lymphocytes with HEV as detected by immunofluorescence; however, all attempts to passage the virus in their cell cultures failed. The only successful continuous replication of HEV was reported by Nazerian and Fadly (21) using lymphoblastoid B cells derived from a Marek's disease tumor (20).

TABLE 6. Infection of turkey leukocytes from 6 different turkeys with HEV-A or SV at 3 and 4 days post-infection.

Leukocytes from turkey number	3 days post-infection		4 days post-infection	
	HEV-A	SV	HEV-A	SV
101	++[A]	-	++	-
102	++	±	++	±
103	++	-	++	-
104	++	-	++	-
107	++	-	++	-
108	++	±	++	±

[A] Percentage of cells stained in the FA test ranging, from ++ to -: ++, ≥ 1.0%; +, 1 - 0.1%; ±, 0.1 - 0.01%; -, < 0.01%.

After infection, virus particles were found in three groups of cells, which probably belong to the mononuclear cell lineage at different stages of maturation. The largest group of infected cells consisted of apparently immature cells but they were difficult to identify. These cells were non-adherent, smooth, non-phagocytic cells containing many virus particles in their nuclei. They might be immature cells, which are normally present in avian blood (19), or they might look immature due to changes induced by virus

infection. Immature infected cells, resembling these non-adherent blood leukocytes, have been observed in the spleens and other organs of turkeys infected with HEV (16). However, it can not be ruled out that these cells are immature lymphocytes. Lymphocytes (12) and more specifically B lymphocytes (11,21) were thought to be the cells in which HEV could replicate. Identification of the cells susceptible to HEV will be possible when MAb's which will specifically recognize leukocyte subpopulations are available. The second group consisted of cells with the characteristics of monocyte-macrophage cells grown in vitro (1). These characteristics include rapid attachment to the surface, aggregation of the cells, sometimes resulting in the formation of large clumps and multinuclear cells, an increase in size, development of many phase-dense granules and phase-lucent vacuoles, and phagocytic features. HEV particles were found in the nuclei of these infected cells. These cells resembled the reticular cells with macrophage characteristics found in spleens of HEV-infected turkeys (16). The third group of cells had the same characteristics as the cells in the second group but virus particles were only present in the cytoplasm and not in the nuclei of the cells. Apparently, after internalization of HEV in these cells, the virus is not released from the receptosomes in the cytoplasm and transferred to the nucleus which is required for an adenovirus replication (23). This process is probably followed by transfer of the virions from the receptosomes into phagolysosomes in which they are degraded, because no HEV antigen was detected in these cells three weeks post-infection. Phagocyting cells with virus particles in cytoplasmic vacuoles were also observed in organs of HEV-infected turkeys (16,21).

Most of the infectivity was cell-associated which could be expected of virus that replicates in the nucleus (3). However, after cell death and degeneration the amount of virus in the medium did not increase markedly. An explanation might be that virus either was not released from the cells or was ingested by macrophages and thus removed from the medium in the cell cultures. This last assumption is in agreement with the phagocytosis of HEV observed in macrophages by the FA test (fluorescent speckles in the cytoplasm) and by electron microscopy (HEV particles in the cytoplasm). Uptake of virus by phagocytes followed by inactivation might also provide an explanation for the higher virus yield obtained when cells were grown in spinner instead of stationary culture, because constant movement in the spinner culture may

decrease the ingestion of virus.

Only a low percentage of the leukocytes was found to be infected with HEV. One reason for this observation might be that HEV infection and replication is restricted to a subpopulation of the cells which is susceptible to the virus. Another explanation might be retardation of the first infection steps as described for human adenovirus type 2 (Ad2) infection of human peripheral blood leukocytes (2,13,18). Although this virus attached to 26% of the lymphocytes, virus uncoating and virus DNA synthesis were slower, and the percentage of infected cells and infectious virus production were much lower than in susceptable HEp-2 cells (13). It was suggested that virus production in lymphoid cells required cells actively engaged in DNA and protein synthesis because higher virus yields were obtained in lymphocytes after stimulation with phytohemagglutinin and in several lymphoblastoid cell lines (13,18). A third possibility might be that after an initial infection and replication in some susceptible cells, spreading to and replication in other cells was inhibited by interferon. Although human adenoviruses are relatively resistant to interferon, mutants lacking the gene coding for virus-associated RNAI are known to be sensitive to interferon (17). Furthermore, Ad12, 13, and 31 are potent inducers of interferon in non-permissive chicken embryo fibroblast cells (27,28).

The potential of HEV-A and SV propagation in chicken leukocytes, as an alternative for virus propagation in turkey leukocytes was investigated. However, chicken leukocytes were not very susceptible to either HEV or SV. This might be a feature of the chicken cells. In addition, infection of turkey leukocytes by SV was poor compared with a HEV-A infection. Although HEV and SV are serologically related (5) and are able to infect both chickens and turkeys causing spleen enlargement and serological response in these animals (6, and J.V. van den Hurk, unpublished data), this does not exclude the possibility that there might be differences between these viruses. Genotypic differences might be responsible for the difference in infectivity.

In conclusion, this report shows that HEV replicates in turkey mononuclear blood leukocytes, predominantly in immature non-adherent cells, but also in adherent monocyte-macrophage type cells. The practical implications of this study for the development of an efficacious and safe vaccine for HEV will be discussed in the accompanying communication (J.V. van den Hurk, submitted for publication).

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CHAPTER 6

Cell Culture Vaccine for HE in Turkeys

Efficacy of Avirulent Hemorrhagic Enteritis Virus Propagated in Turkey Leukocyte Cultures for Vaccination against Hemorrhagic Enteritis in Turkeys.

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SUMMARY. Avirulent hemorrhagic enteritis virus (HEV-A) propagated in turkey leukocyte cell culture was tested as a vaccine to prevent hemorrhagic enteritis (HE) in turkeys in experimental and field trials. Immunization of turkeys with live HEV-A resulted in protection against a challenge with virulent HEV (HEV-V) as measured by the serological response and the absence of clinical disease and HEV antigen in spleens. In field trials, nineteen out of twenty flocks seroconverted within 21 days after vaccination with live HEV-A distributed in the drinking water. The overall immune response of the turkeys in these flocks was 96%. Most importantly, neither clinical HE nor other adverse effects caused by HEV-A vaccination were observed in any of the vaccinated flocks. Since maternal antibodies can interfere with the immune response to the vaccine, the optimum time for vaccination was determined. Using an established half life value of 4.25 days, and knowing the ELISA titer of the maternal antibodies and age of the turkey, the time of vaccination could be calculated taking into account that maternal antibody titers should be lower than 40 to vaccinate the turkeys successfully and induce protection. In vivo tests with HEV-A preparations confirmed the replication of the virus in turkey leukocyte cultures and the potential to pass it in culture without loss of potency. Furthermore, the results of the in vivo analysis of virus obtained from non-adherent cells, adherent cells, and cell-free medium was similar to those obtained in the in vitro analysis. Both assays showed that most infectious virus was associated with preparations of the non-adherent cell population. The potency of HEV-A preparations was dependent on the production method and varied from an average 570 to 8,135 doses per ml.

INTRODUCTION

Hemorrhagic enteritis (HE) is an infectious disease of turkeys which is characterized by depression, intestinal bleeding, and death (5). This disease is caused by hemorrhagic enteritis virus (HEV) which is tentatively classified as a group II or type II avian adenovirus (3,5). HE usually occurs between 6 and 11 weeks of age and is most common in 7 to 9 week-old birds (5). Younger birds are usually refractory to the disease as a result of the presence of maternal antibodies (3,10,13,20).

Two vaccines have been used to prevent HE. The first is a crude spleen extract prepared from turkeys infected with avirulent HEV (HEV-A) (6,7,19).

The second vaccine contains HEV-A grown in a lymphoblastoid cell line (RP19) derived from a Marek's disease virus (MDV)-induced tumor (8,9,14,15,16). Although both vaccines elicit protective immunity in turkeys, the safety features of these vaccines are disputed and have to be carefully evaluated.

The overall goal of this study was to develop and test an efficacious vaccine for HE in turkeys, using HEV-A produced in turkey leukocytes as previously described (J.V. van den Hurk, manuscript submitted). Potency and safety testing was carried out and the vaccine was tested under experimental and field conditions. In addition, since high levels of maternal antibodies to HEV can interfere with vaccination, the rate of decline of these antibodies was studied so that the optimum age for vaccination could be determined.

MATERIALS AND METHODS

Virus and HEV-A preparations. The origin, characteristics and propagation of HEV-A and virulent HEV (HEV-V) have been described previously (20). The details of the propagation of HEV-A in turkey leukocytes have been described elsewhere (J.V. van den Hurk, manuscript submitted).

Enzyme-linked immunosorbent assays (ELISA's). HEV antibodies were determined in an indirect ELISA using CsCl-purified HEV for coating and rabbit anti-turkey IgG-peroxidase conjugate for detection of the antibodies (20). HEV antigen was determined in an indirect sandwich ELISA using turkey antibodies for capture, and a cocktail of monoclonal antibodies (MAb's 11B6, 14E1, and 15G4), followed by goat anti-mouse IgG-peroxidase conjugate for detection of the antigen (17,20,21).

Potency testing of HEV-A preparations. An assay was developed to measure the protection of poults against HE challenge after immunization with cell culture-produced HEV-A. The potency of HEV-A preparations was established in dose-challenge experiments in which the minimum dose necessary to protect 6 week-old poults against a challenge with 100 effective doses 95 (ED_{95}) of the HEV-V isolate was determined. The ED_{95} was based upon the presence or absence of HEV antigen in the spleens five days post-infection, and was defined as the dose that produced antigen in the spleens in 95% of 6 week-old birds. An infection with 100 ED_{95} of HEV-V caused intestinal bleeding in an average of

60% (range 32-88%), death in 28% (range 12-50%), and HEV antigen in the spleens in 100% of unvaccinated turkeys.

In the potency tests, six to seven week-old turkeys, which had been raised in isolation were placed in separate isolation rooms (8 turkeys per group). Poults were vaccinated orally with 1 ml of serial 10-fold dilutions of each batch of cell culture-produced HEV-A. Fourteen days after vaccination the turkeys were challenged with 100 ED_{95} of HEV-V, and 5 days post-challenge the survivors were killed and examined. Two criteria were used to establish the protective titers in turkeys five days following challenge. First, the HEV antigen titers were determined in the spleen extract of each poult. ELISA antigen titers $\leq$ 100 indicated protection. Second, spleen enlargement, a characteristic of a HEV infection, was evaluated by calculating the spleen/body weight ratios, which were multiplied by 10^3 for reporting purposes. A ratio x $10^3 \leq 1.40$ indicated protection except in birds that were bleeding intestinally. The Chi square (X^2)test was performed to evaluate protection: i) dependency of antibody and antigen titers, and ii) dependency of antibody titer and spleen/bird weight ratio. The number of doses per ml cell culture harvest (potency) was calculated from the ED_{90} based upon protective antibody levels using the method of Reed and Muench.

Potency of HEV-A propagated under different conditions. The potency of HEV-A propagated under different conditions was determined in dose-response challenge trials. First, the potency of HEV-A propagated in turkey leukocytes at passage levels one and seven (experiment 1), or at passage levels one and four (experiment 2), was determined. Second, turkey leukocytes were infected with either HEV-A from crude spleen extracts (experiment 1) or with purified HEV-A (experiment 2). Three days post-infection the cell cultures were harvested and separated into non-adherent cells, adherent cells, and culture medium fractions, and the potency of each of the three fractions was determined. Third, the potency of HEV-A produced in leukocytes, isolated by the Ficoll-Paque or "slow-spin" method (12) and grown in stationary or spinner cultures, was determined. Details of the cell culture conditions, infection, and harvest of HEV-A preparations are described elsewhere (J.V. van den Hurk, manuscript submitted).

Maternal antibodies. In a first experiment the presence and decline of maternal antibody titers were determined in 42 groups of turkeys, all from different hatches with an average of 75 poults. All groups were housed at VIDO. Antibody titers were determined by ELISA in sera, obtained from five randomly selected poults that were bled when they were one day old in all groups and at weekly intervals in 16 groups thereafter. In the second experiment the decline of maternal antibodies was investigated in blood samples, collected from three groups of ten poults (three different hatches) when they were one day old and at 8 weekly intervals thereafter. Slopes and half life values were calculated from graphs in which the maternal antibody titers of individual birds ($\log_{10}$ ELISA titers) were plotted against the age of the birds.

Safety screening of HEV-A preparations. All HEV-A preparations were screened before inoculation into poults for the presence of bacteria including mycoplasma, fungi, and extraneous viruses. Only when all tests were negative were potency trials conducted in the birds. Moreover, HEV-A preparations used in the field trials were tested for side-effects by intramuscular and oral inoculation of 10 poults with a 100 to 1,000 times higher dose than was necessary for induction of a protective immune response.

Field trials. Field trials were conducted using two different HEV-A preparations. The leukocytes of both cultures were isolated by the Ficoll-Paque method and the cells were grown in stationary cultures. The first preparation (A) was obtained from turkey leukocytes infected with HEV-A from a crude spleen extract and harvested two days post-infection and it had a potency of 1260 doses/ml. The second preparation (B) had a potency of 165 doses/ml. It was obtained from a second passage of HEV-A in turkey leukocytes initially infected with CsCl-purified HEV-A, and it was harvested three days post-infection. In the first field trial two flocks were vaccinated with preparation A. The birds of flock 1 were 4 weeks old and those of flock 2 were 6 weeks old at the time of vaccination.The second field trial was conducted in 20 flocks with an average of 6,000 birds using both preparations A and B. The birds in these flocks were four to seven weeks old. The selected flocks had been raised under confinement on premises where no HE vaccine had been used previously. After stimulation of thirst by withholding water for 2

hr, the first half of the vaccine solution, diluted in clean fresh water supplemented with powdered milk (250g/80l) as stabilizer, was distributed evenly over the empty waterers at a concentration of 5,000 doses/80l. As soon as the waterers were empty the second half of the required vaccine was prepared and the procedure repeated. Birds were encouraged to move around when the vaccine was distributed to increase the number of birds vaccinated as a result of drinking. Blood was collected at random from 25 birds at the time of vaccination and three weeks after vaccination from all flocks, and at slaughter from 4 flocks for serological testing.

RESULTS

Calculation of the protective antibody titer. The protective antibody titer was determined after vaccination of turkeys with HEV-A propagated in turkey leukocytes. A highly significant association ($p < 0.001$) was found between an antibody titer ≥ 20 and protection as defined by the absence of antigen (titer ≤ 100) in spleen extracts (Table 1). Under these conditions 280/297 (94%) of the birds with an antibody titer ≥ 20 were protected (antigen titer ≤ 100), whereas only 47/241 (20%) of the birds with an antibody titer <20 were protected. A similar highly significant association ($p < 0.001$) was calculated between an antibody titer ≥ 20 and protection based upon spleen/body weight ratio ≤ 1.40 (Table 2). In this case 213/233 (92%) of the birds with an antibody titer ≥ 20 were protected, whereas only 32/192 (16%) of the birds with an antibody titer <20 were protected. In addition, following vaccination with HEV-A and challenge with HEV-V no intestinal bleeding or death occurred in any of the birds with a HEV antigen titer <100 or a serum antibody titer ≥ 20. Based on these results an induction of a protective antibody titer ≥ 20 in turkeys following immunization with HEV-A was used to calculate the potency (no. doses/ml) of HEV-A preparations in dose-response experiments. Moreover, an induction of a protective antibody titer ≥ 20 determined experimentally was used for the evaluation of a serological response in turkeys following vaccination with HEV-A in the field trials.

Immunization of turkeys with cell culture-propagated HEV-A at various passage levels. HEV-A preparations at various passage levels were tested for their capacity to induce protection against HE in turkeys. Table 3

Table 1. Association between HEV antigen titer in spleen extracts and serum antibody titer of turkeys following vaccination with HEV-A and challenge with HEV-V.

Antibody titer	Spleen antigen titer ≤100	>100	Total poults
<20	47	194	241
≥20	280	17	297

X^2 test. The association between an antibody titer ≥20 and protection as defined by the absence of antigen (titers ≤ 100) in spleen extracts was highly significant ($p < 0.001$).

Table 2. Association between spleen/body weight ratio and serum antibody titer of turkeys following vaccination with HEV-A and challenge with HEV-V.

Antibody titer	Spleen/body weight ratio ≤1.4	>1.4	Total poults
<20	32	160	192
≥20	213	20	233

X^2 test. The association between an antibody titer ≥20 and protection as defined by a bird/weight spleen ratio $x10^3$ ≤1.4 was highly significant ($p < 0.001$).

illustrates that immunization with HEV-A preparations, which were once or seven times subcultured, resulted in an antibody response and protection against challenge with HEV-V in a dose-dependent manner. The potency of the HEV-A preparations varied from 282 to > 10,000 doses/ml. The results of these in vivo experiments confirmed that HEV-A replicated in cell cultures, and that after repeated passage HEV-A could still be used to induce protection in turkeys. In addition, a high association was found between the presence of antibodies (titers $\geq$ 20), the absence of HEV antigen in the spleen (titers $\leq$ 100) and spleen/bird weight ratios ($\leq$1.40) in birds that were not bleeding intestinally.

Comparison of HEV-A potency in cell culture sub-fractions. Previously, HEV-A propagated in turkey leukocytes has been found to be present in adherent cells, non-adherent cells, and culture medium (J.V. van den Hurk, manuscript submitted for publication). These culture fractions were analyzed for their capacity to induce protection in turkeys. The potency of these preparations was tested in dose-response challenge trials and expressed in ED_{90}/ml. Table 4 illustrates that in two independent experiments the highest potency was associated with the non-adherent cell fraction. Since HEV-A was present in each subfraction cells and culture media were kept together for maximalization of the vaccine production.

Table 4. Potency of cell-associated and cell-free HEV-A propagated in turkey leukocyte cultures.

Cell culture fraction	Potency[A] (%)[B]	
	Experiment 1[C]	Experiment 2[D]
Adherent cells	251 (7%)	116 (8%)
Non-adherent cells	3,170 (89%)	1,260 (88%)
Cell-free medium	145 (4%)	63 (4%)

[A] ED_{90}/ml

[B] Potency of fraction/total potency

[C] Leukocytes were infected with crude spleen HEV-A

[D] Leukoyctes were infected with purified HEV-A

Table 3. Potency testing of HE at various passage levels in turkey leukocytes.

Experiment	Passage level	Vaccine dilution	Clinical disease[A]	Antibody titer[B]	No. with Antibody titer >20[B]	Spleen/BW ratio[C]	No. with spleen/BW ratio<1.4[C]	HEV titer in spleen[D]	No. with spleen titer<100[D]	No. protected	Potency[E] (doses/ml)
1[F]	1	Controls	2/7	<10	0/7	1.75	1/7	37,234	0/7	0/7	282
		10^{-4}	3/7	28	1/7	1.75	1/7	17,083	1/7	1/7	
		10^{-3}	1/8	589	6/8	1.16	5/8	559	6/8	6/8	
		10^{-2}	0/8	2,407	8/8	0.97	8/8	<100	8/8	8/8	
		10^{-1}	0/8	1,761	8/8	0.75	8/8	<100	8/8	8/8	
	7	Controls	4/8	<10	0/8	1.62	3/8	22,371	0/8	0/8	1,445
		10^{-4}	3/8	105	3/8	1.52	3/8	1,733	3/8	3/8	
		10^{-3}	0/8	5,865	8/8	0.73	8/8	<100	8/8	8/8	
		10^{-2}	0/8	6,368	8/8	0.65	8/8	<100	8/8	8/8	
		10^{-1}	0/8	9,486	8/8	0.61	8/8	<100	8/8	8/8	
2[G]	1	Controls	4/8	<10	0/8	1.48	4/8	1,663	0/8	0/8	>10,000
		10^{-4}	0/8	6,226	8/8	0.73	8/8	<100	8/8	8/8	
		10^{-3}	0/8	11,401	8/8	0.77	8/8	<100	8/8	8/8	
		10^{-2}	0/8	6,034	8/8	0.57	8/8	<100	8/8	8/8	
	4	Controls	5/7	<10	0/7	1.87	3/7	10,261	0/7	0/7	6,295
		2×10^{-5}	4/8	<10	0/8	1.55	3/8	8,857	0/8	0/8	
		2×10^{-4}	0/8	822	8/8	1.07	7/8	<100	8/8	8/8	

A Clinical disease = occurrence of disease, intestinal bleeding or death within 5 days post-challenge.
B Geometric mean ELISA antibody titer 5 days post-challenge.
C Spleen/body weight ratio.
D Geometric mean ELISA antigen titer of HEV in spleen.
E Calculated by the method of Reed and Muench.
F Leukocytes were isolated by ficoll-paque method, grown in stationary culture, and initially infected with crude spleen HEV-A.
G Leukocytes were isolated by "slow-spin" method, grown in spinner culture, and initially infected with purified HEV-A.

Potency analysis of HEV-A propagated in turkey leukocytes, isolated and cultured under various conditions. In an attempt to obtain optimum virus yields, a number of methods to propagate the virus were investigated. The highest yields were obtained with the combination of the "slow spin" method for isolation and spinner culture for maintenance of the cells (Table 5).

Table 5. Comparison of HEV-A production in turkey leukocytes obtained by various isolation methods and cultured under various conditions.

Culture Condition	Leukocyte isolation method	
	Ficoll-Paque	Slow Spin
Stationary culture	570 (60-1,445)[A,B]	2,035 (1,160-2,910)[C]
Spinner culture	2,850 (2,000-3,700)[C]	8,135 (3,700-12,560)[B]

[A] Average potency (ED_{90}/ml) and range in brackets

[B] Data from 4 HEV-A preparations

[C] Data from 2 HEV-A preparations

Maternal antibodies versus immunization. Most turkeys have maternal antibodies against HEV when they hatch (5,10,13). This information agrees well with the observation that only one out of forty-two turkey groups (all from different hatches) used at VIDO lacked maternal antibodies. The antibody response after immunization was thought to be dependent on the level of maternal antibodies of the turkeys.

Interference by maternal antibody with the serological response to immunization with HEV-A in a field trial is clearly shown in Table 6. When vaccination was performed at 4 weeks of age (flock 1) the mean pre-vaccination maternal antibody titer was 68 and only 11/25 (44%) poults seroconverted as defined by titers $\geq$ 20 at 21 days after vaccination. In contrast, 24/25 (96%) poults seroconverted in the flock vaccinated at six weeks (flock 2) when maternal antibody titers had declined to a mean of 9. This difference in protective antibody response could also be expected from the number of birds with maternal antibody titers $\geq$ 40 at the time of vaccination, which was 10/25 in flock 1 and 0/25 in flock 2.

Table 6. Effect of maternal antibody (MA) level on the serological response after vaccination with HEV-A in turkey flocks at 4 and 6 weeks.

Flock	Age at vaccination	HEV Antibody Titer: Before vaccination[A]	After vaccination[B]	No. with MA titers ≥40/ No. tested[C]	No. responded/ No. tested[D]	Response (%)
1	4 weeks	68	41	10/25	11/25	44
2	6 weeks	9	360	0/25	24/25	96

[A] Geometric mean ELISA antibody titers at the day of vaccination.

[B] Geometric mean ELISA antibody titers three weeks post-vaccination.

[C] Tested at the day of vaccination.

[D] Ratio between the no. of turkeys which responded with an antibody titer ≥20 and the no. of turkeys tested.

In order to determine when a flock should be vaccinated, the decline of maternal antibodies in poults was studied. The rate of decline of maternal antibody levels of the ten individual poults in the three groups was nearly the same as is illustrated in Fig. 1 for the poults of group 1. Furthermore, an average value of 4.25 days for the half life and an average value of -0.072 for the slope were determined for the maternal antibodies of these groups (Table 7). Using the half life value of 4.25 days, and knowing the ELISA titers at day one of the three groups of ten turkeys, it was found that calculated and actual maternal antibody titers corresponded very well (Table 7).

The decline of the maternal antibody titers was further investigated in 16 groups of turkeys. Table 8 shows that the mean of the maternal antibody titers of these groups declined with age at a similar rate as that determined for the 3 groups of 10 birds with slopes of -0.074 and -0.072 respectively. Furthermore, the level of the maternal antibodies declined to below 40 in the majority of the birds by 5 to 5 weeks of age. These results suggest that 75 to 94 percent of these turkeys were expected to react with a protective antibody response after vaccination with HEV-A at 5 to 6 weeks using maternal antibody

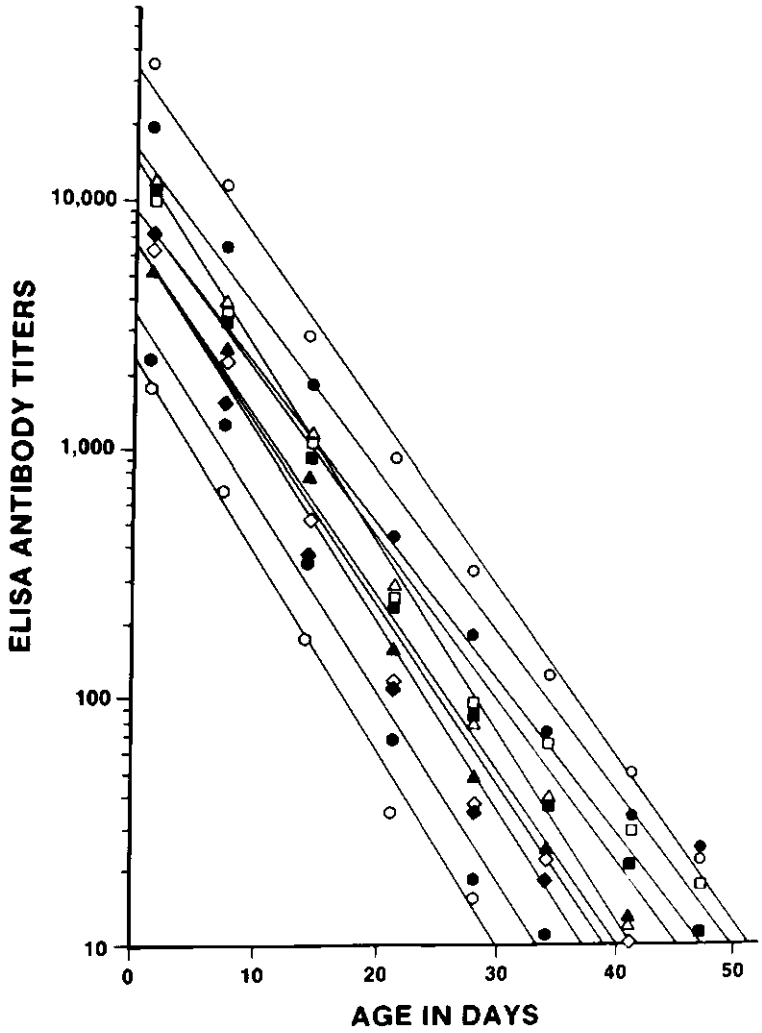

Fig. 1. Decline of HEV maternal antibody titers in the poults of group 1. The $\log_{10}$ of HEV maternal antibody titers, determined by ELISA when turkeys were 1, 7, 14, 21, 28, 34, 41, and 47 days old, was plotted against age. A slope of -0.071 ± 0.006 (Mean $\pm$ SD) and a half life of 4.25 ± 0.34 days (mean $\pm$ SD) were calculated. Sera from turkey 2108: ○ , 2109: ● , 2112: □ , 2107: ■ , 2104: △ , 2105: ▲ , 2106: ◇ , 2103: ◆ , 2111: ✹ , 2110: ✪ .

titers below 40 as a requirement for such a response. The wide range of maternal antibody titers within poults of the same age should be also noted.

Under experimental conditions a highly significant association ($p<0.0001$) was found between maternal antibody titers <40 and a response (antibody titer ≥ 20) after immunization with HEV-A (Table 9). Under these conditions 141/150 (94%) of the turkeys with a maternal antibody titer <40 responded whereas only 3/15 (20%) of the birds with a $40 \leq$ maternal antibody titers ≤ 80 showed an immune response.

Table 7. Decline of maternal antibodies against HEV: determination of slopes, half lives, and correlation between measured and calculated maternal antibodies in three groups of ten turkeys.

Group	Slope[A]	Half Life (days)[A]	Correlation[B] coefficient(r)
1	-0.071 ± 0.006	4.25 ± 0.34	0.95
2	-0.071 ± 0.003	4.26 ± 0.22	0.97
3	-0.073 ± 0.006	4.23 ± 0.38	0.95
Mean	-0.072	4.25	

[A] Arithmic mean $\pm$ SD of 10 turkeys.

[B] The correlation between the number of days for maternal antibody titers to decline to 40 was determined as measured by ELISA and calculated from the ELISA titers at day 1 using a half life value of 4.25 days for the antibodies. Highly significant values were determined for the correlation coefficients ($P<0.001$).

Table 8. Decline of maternal antibodies against HEV in 16 turkey groups.

Age	Maternal antibody titer[A] (range)	No. with MA titers <40/ No tested	% with MA titers <40[B]
1 day	5,274 (280 - >100,000)	0/80	0%
1 week	2,022 (120 - 23,000)	0/80	0%
2 weeks	345 (<10 - 800)	5/80	6.3%
3 weeks	116 (<10 - 3,400)	14/80	17.5%
4 weeks	45 (<10 - 350)	37/80	46.3%
5 weeks	17 (<10 - 190)	60/80	75.0%
6 weeks	<10 (<10 - 45)	75/80	93.8%
7 weeks and older	<10 (<10 - 29)	80/80	100%

[A]Geometric mean maternal antibody (MA) titers of 16 different turkey groups determined by ELISA. A slope of -0.074 was calculated from the $\log_{10}$ of the maternal antibodies and the age of the birds.

[B]Percentage of the birds which is expected to respond with a protective antibody titer after vaccination with HEV-A using maternal antibody titers <40 as a requirement for such a response.

Table 9. Effect of the occurrence of maternal antibodies on the serum antibody response after vaccination of turkeys with HEV-A.

Maternal antibody titer	Antibody titer after vaccination <20	Antibody titer after vaccination ≥20	Total poults
<40	9	141	150
≥40 and ≤80	12	3	15

X^2 test. The association between maternal antibody titer <40 and a protective antibody response (antibody titer ≥20) after immunization with HEV-A was highly significant ($p < 0.001$).

Field trials. Field trials were conducted in 20 flocks in Canada using two HEV-A preparations. No adverse effects in any of the birds were observed when these HEV-A preparations were tested by intramuscular or oral inoculation with a 100 to 1,000 times higher dose than was required for induction of a protective immune response. A very good serological response was obtained after vaccination with HEV-A in nineteen out of twenty flocks with an average response of 96% (Table 10). Only one flock (no 9) showed a lower response of 46%. No significant difference in response was found between the flocks vaccinated with preparation A or B. In addition, no clinical HE or other adverse effects caused by vaccination were observed in any of the 20 flocks. All birds tested in the four flocks at slaughter had protective antibody titers against HEV. The ELISA titers (geometric mean titers) between these flocks varied from 6,823 to 11,274. In the 12 flocks that were vaccinated when they were 5 weeks old 254 out of 309 birds (82%) had maternal antibody titers <40 (data not shown). This figure is in good agreement with the observation that 275 out of 293 birds (94%) responded to the vaccination, resulting in a protective antibody titer 3 weeks post-vaccination.

DISCUSSION

A good correlation was found between an antibody titer $\geq$ 20 against HEV and protection as defined by absence of HEV antigen in spleens and spleen/body weight ratios x 10^3 $\leq$ 1.40. One reason that such low antibody titers were protective might be that during an early response IgM antibodies were measured which are known to be particularly effective against invading microorganisms (2,11,17). Antibodies are known to play a role in protection against HE, because administration of HEV antiserum to turkeys prevented occurrence of clinical disease upon HEV-V infection of these birds (4,5). Another possibility might be protection elicited by cell-mediated immune (CMI) reactions. CMI protection might also elucidate the appearance of a group of turkeys that showed no detectable serological response after vaccination and challenge, but was protected based on the absence of viral antigen in the spleens. The importance of CMI response for control and resolution of human adenovirus infections has been described (18).

The successful propagation of HEV-A in turkey leukocytes has been reported

Table 10. Serology data of turkey flocks vaccinated with HEV-A propagated in turkey leukocytes (Field trials)

Flock	HEV-A preparation	Prevaccination Age[A]	Prevaccination Titer[B]	Post-vaccination Age	Post-vaccination Titer[B]	Response No. positive/total	Response %[C]
1	B	4 1/2	41	7 1/2	4,607	25/25	100
2	B	4 1/2	45	7 1/2	2,575	25/25	100
3	A	4	26	7	1,023	22/22	100
4	B	6	<10	9	3,256	25/25	100
5	A	5	19	8	1,773	20/21	95
6	A	4 1/2	52	7 1/2	908	23/23	100
7	A	5	17	8	1,153	21/21	100
8	B	5	32	8	834	25/25	100
9	B	5	28	8	87	11/24	46
10	A	5	36	8	2,726	26/26	100
11	A	5	11	8	1,260	25/25	100
12	B	5	13	8	1,823	25/25	100
13	B	5	30	8	706	22/25	88
14	B	5	16	8	1,114	26/27	96
15	A	5	17	8	1,878	27/27	100
16	A	5	18	8	9,131	25/25	100
17	A	NA[D]	NA	NA	5,274	24/24	100
18	A	6 1/2	<10	9 1/2	6,742	25/25	100
19	A	5	19	8	1,581	22/22	100
20	A	5 1/2	34	9	1,494	25/25	100
Average	-	5.0	25	8.0	2,497	469/487	96

[A] Age in weeks.

[B] The geometric mean titers as measured by ELISA.

[C] Percentage response = number of turkeys with ELISA titer $\geq$ 20 three weeks after vaccination.

[D] Not available.

previously (J.V. van den Hurk, manuscript submitted). In this study, the usefulness of cell-culture produced HEV-A for the immunization of turkeys against HE was investigated. In the dose response challenge experiments it was found that HEV-A produced in cell culture induced protective immunity in turkeys. These in vivo experiments confirmed the replication of HEV-A in turkey leukocytes, the possibility to pass HEV-A in culture without loss of infectivity, and the distribution of HEV-A over adherent and non-adherent cells, and cell-free culture medium. Both in vitro and in vivo analyses showed that HEV was mostly cell-associated which is a common feature of adenoviruses (1). In addition, the highest potency in turkeys and the highest virus yield in cell culture were obtained when turkey leukocytes were isolated by the "slow spin" method and cultured in spinner flasks (J.V. van den Hurk, manuscript submitted).

In general, there was a good correlation between the infectivity of HEV-A propagated in leukocytes determined in cell culture ($TCID_{50}$) (J.V. van den Hurk, manuscript submitted) and in turkeys (ED_{90}). However, the in vitro test was about 100 times less sensitive. These results are in contrast with those found for HEV produced in RP19 cells where the in vitro test was more sensitive (16). Possible reasons for the lower sensitivity of the in vitro HEV-infectivity test might be phagocytosis of HEV-A by phagocyting cells in which the virus does not replicate, and a possible production of interferon which might affect virus production in mixed turkey leukocyte cell cultures.

The serological response in the field trials of the turkeys, that were immunized with HEV-A propagated in turkey leukocytes, was very good in 19 out of 20 flocks. The overall immune response of the turkeys in these 20 flocks was 96%. Such a good response probably resulted from replication and transfer of virus from one bird to another rather than from all 96% of the birds being vaccinated directly by drinking the HEV-A preparation. Horizontal transmission of HEV between birds after vaccination has also been observed by other investigators (8,19). The reason for the poor response (46%) in one flock is not known, but based on the consistency of seroconversion in the other flocks and in experimental trials, it was suspected that the vaccine was not properly administered to this flock.

Seroconversion in the field trials was considered to be caused by vaccination rather than by infection with field strains on the basis of the following observations: i) no clinical disease was observed in any of the 20

flocks, while on some of the farms previous flocks had HE; and ii) an antibody response was detectable in 96% of the birds within 3 weeks after vaccination when they were 7.5 to 9.5 (average of 8) weeks old. This antibody response generally occurred earlier than that observed after an HEV infection in the field which is usually apparent when birds are 8 to 12 weeks old (based upon seroconversion 7 days after the peak of disease incidence when birds are 7 to 11 weeks old; 5,20, J.V. van den Hurk, unpublished data).

Most young turkeys have maternal antibodies against HEV which might interfere with a successful vaccination. Therefore, the decline of these maternal antibodies and the level at which they did not interfere with an HEV-A immunization were investigated. In general, based upon maternal antibody levels against HEV in poults housed at VIDO and maternal antibody levels and protective antibody response in poults in the field trials, an HE vaccination at five weeks of age is recommended. From the maternal antibody titers of a random sample of young turkeys tested at a known age, the appropriate time for immunization can now be determined using a half life value of 4.25 days and a maternal antibody titer <40. However, the timing of the vaccination might still be problematic when there is a wide variation in maternal antibody titer between individual birds.

This HE vaccine shares the advantages of many live avirulent vaccines which usually require only one vaccination to induce a good and long-lasting immunity. Furthermore, the high yield in cell culture, ease of preparation, and oral administration in the drinking water made it economically attractive as a vaccine. Disadvantages of this vaccine are that it is propagated in a primary cell culture system involving the isolation of cells for the production of each new virus batch. In addition, since it is a live virus vaccine stress and immunosuppression may occur. An alternative would be a killed or subunit vaccine which would not have these disadvantages. However, such a vaccine would not be cost-effective, because the production and administration costs would be much higher than those of a live HEV-A based vaccine given in the drinking water.

HEV-A produced in turkey leukocytes or RP19 cells can be successfully used as a vaccine for HE in turkeys. In the case of the RP19 cells HEV-A is propagated in turkey lymphoblastoid cells, which are derived from a tumor induced by MDV (14), while in this report HEV-A is propagated in primary turkey leukocytes, a mixed population of normal cells. HEV-A vaccine preparations

produced in RP19 cells were reported to be free of MDV, as long as they contain no cellular material (8,15). However, there is always the possibility that MDV is released simultaneously with HEV-A in culture medium from degenerating HEV-A-infected cells.

In conclusion, immunization of turkeys with live avirulent HEV propagated in turkey leukocytes has been effective in experimental and field trials. Furthermore, HEV-A propagated in turkey leukocytes can probably be used for the immunization of pheasants against marble spleen disease caused by marble spleen disease virus, which is serologically related to HEV (5,6). The HE vaccine propagated in turkey leukocytes is licensed and commercially available since March 1986.

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CHAPTER 7

GENERAL DISCUSSION

In cells infected with a human adenovirus about 40 virus-specific polypeptides are synthesized during a lytic infection (6). At least nine of these polypeptides form the structural units of which an adenovirus virion is built up. In addition to being important for the structure of the virion, the major outer capsid proteins, hexon, fiber, and penton base, play an important role in recognition, attachment and penetration of the virus into the host cell. They also elicit an immune response in the host species.

Until recently, neither the HEV proteins had been studied and characterized, nor a suitable cell culture system for the propagation of HEV had been identified. HEV had to be purified from the spleens of infected turkeys, which was not the best source for the generation of pure and adequate virus preparations. Therefore, specific antibodies were required to identify the HEV polypeptides. Consequently, monoclonal, monospecific, and polyclonal antibodies were generated and used for i) characterization and identification of HEV polypeptides; ii) purification, characterization, and identification of HEV soluble proteins; iii) recognition of HEV target cells; iv) quantification of HEV antigen; v) in vitro quantification of HEV infectivity; and vi) investigation of protective antibody levels in turkeys after vaccination.

After PAGE and Western blotting of CsCl-purified HEV preparations, eleven polypeptides were detected, which were specifically recognized by antibodies in convalescent turkey serum. The apparent 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, and those for the HEV-V polypeptides 96k, 57k, 51k, 43k, 37k, 34k, 29k, 24k, 21k, 12.5k, an 9.5k. Six of these polypeptides were further characterized by PAGE, Western blotting, ELISA, sucrose gradient centrifugation and electron microscopy. The identified HEV polypeptides were: 96k as hexon polypeptide, 57k as IIIa polypeptide, 51/52k as penton base polypeptide, 29k as fiber polypeptide, and 12.5k and 9.5k as core polypeptides.

The molecular weights of the major polypeptides of HEV-A, HEV-V, FAV-1, EDS76V, and Ad2 are compared in Table 1. This table shows similar molecular weights for the hexon and IIIa polypeptides, a difference in molecular weight of the

penton base and fiber, and similarity between the core polypeptides of HEV and the lower molecular weight core polypeptides of FAV-1.

Table 1. Molecular weights of the major polypeptides of HEV, FAV-1, EDS76V, and Ad2.

Polypeptide	HEV-A	HEV- V	FAV-1[B]	EDS76V[C]	Ad2[D]
Hexon	96[A]	96	100	126	109
Penton base	52	51	92	65	85
Fiber	29	29	65 and 44.5	67	62
IIIa	57	57	62[E]	-[F]	66
Core proteins	12.5 and 9.5	12.5 and 9.5	20, 12 and 9.5	20[G]	48 and 18.5

[A] Apparent molecular weights x 10^{-3} determined by PAGE

[B] Li et al (17)

[C] Tod and McNulty (29)

[D] Philipson (23)

[E] Similar molecular weight, not identified as IIIa

[F] Not known

[G] Similar molecular weight, not identified as a core protein

The ten monoclonal antibodies that recognize the hexon protein of HEV probably all react with conformational epitopes because the antigenic sites were not recognized anymore after dissociation and denaturation of hexons in polypeptides. These monoclonal antibodies reacted with complete virions and eight of them neutralized the infectivity of HEV in cell culture. Based upon these results and taken into account that the hexon base is less accessible to antibodies, the monoconal antibodies probably react with the trimeric top part of the hexon. In addition, the monoclonal antibodies reacted with more than one site on each hexon suggesting that the HEV hexon probably exists of three identical polypeptides each of which has one identical antigenic site. In addition to recognizing the hexon in the virion, the monoclonal antibodies reacted with the free hexon (ELISA of purified hexons and detection by immunoblotting) and they might react with the nascent hexon polypeptide (cytoplasmic FA staining of HEV-infected cells).

An interesting result was the presence of a common epitope on the IIIa protein of HEV and Ad2. This is the first observation of a common antigenic site on a structural protein in avian and mammalian adenoviruses. This conserved site might have a function in the adenovirus architexture. Hypothetically conservation of this site might be important for linkage of the penton or peripentonal hexons to the core, since this function might be filled by the IIIa protein (31). The epitope shared by these viruses was not present on the surface of the capsid, but it was concealed in the virion which could be expected of a common epitope shared by viruses with such phylogenetically remote hosts.

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 the 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 adenoviruses. 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 than HEV-A penton base, and 3) a combination of 1 and 2. The presence of free pentons in peripheral blood of several fatal cases of adenovirus pneumonia in young children might be supportive for this hypothesis (15).

The classification of HEV as an adenovirus has been based upon size, morphology, presence in the nucleus of infected cells, ether resistence, and density in CsCl of the virions (3,5,14,30). This classification is endorsed by: 1) common properties of the structural proteins of HEV and other adenoviruses, including the identification of hexon, penton, penton base, and fiber by electron microscopy, polypeptide profile of the structural proteins after PAGE, and sedimentation coefficient values of the major capsid proteins; and 2) homology of the IIIa protein of HEV and Ad2.

HEV lacks the group-specific antigen shared by the fowl adenoviruses, and EDS76V (5). In addition, HEV and EDS76V carry single fibers at their vertices, in contrast to the fowl adenoviruses which carry double fibers. Finally, ten monoclonal antibodies reacted with nine isolates of the group II avian adenoviruses, whereas none of the five serotypes of the fowl adenoviruses were recognized. These data are in support for a subdivision of the avian adenoviruses in either subgroups (subgenera) or groups (genera).

After infection of turkey leukocytes with HEV, two types of infected mononucleated cells were detected by immunofluorescence and electron microscopy. The first type of infected cells consisted of immature cells, either of monoblast or lymphoblast parentage, which resembled immature HEV-infected cells in turkeys (14). Various investigators have suggested that lymphocytes are the target cells for HEV infection (7,8,22). However, the first group of investigators has not determined the true identity of the infected cells, which were observed in peripheral blood of HEV-infected turkeys, and assumed that these cells were lymphocytes (8). The second group has demonstrated that the RP19 cells which they used for HEV infection experiments has B cell rather than T cell characteristics, but these RP19 cells are MDV-transformed cells and transformation might have changed their original properties (21,24). The second type of infected cells were monocyte-macrophage cells, which clearly had the characteristics of mononuclear phagocytes, and which resembled reticuloendothelial cells observed by electron microscopy in organs of HEV-infected turkeys. In addition to phagocytes in which HEV particles were observed in the nucleus, there were also cells in which virions only occurred in the cytoplasm. The conclusion can be drawn from the data available thus far is that the HEV target cells are monocytes. However, the possibility that lymphocytes also function as target cells can not be excluded.

We demonstrated that HEV can infect turkey leukocytes and production of infectious virus in these cells was demonstrated by: i) appearance of nuclear HEV antigen; ii) appearance of HEV particles in the nucleus; and iii) retention of infectivity in vitro and in vivo after serial passage in cell culture. Thus, in conclusion, HEV replicates in vitro in lymphoblast or monoblast cells and monocyte-macrophage cells, and infectious virus is generated in these cells.

In contrast to the lytic infections of epithelial cells caused by mammalian and group I avian adenoviruses, only a low percentage of the total cell population of both adherent and non-adherent leukocytes became infected with HEV. This is not exceptional because limited infections of lymphocytes with human adenoviruses in vivo and in vitro have been reported (2,12,13,16,26). The limitation of HEV infection to a low percentage of cells might have been caused by various factors of which the most relevant ones are discussed . First, the number of susceptable cells may be limited to cells with HEV-receptors which might form a small portion of the total cell population. Second, a higher percentage of the cell population may initially become infected, but the infection may be abortive in most of them. This might happen in

the adherent cell population where virus replication takes place in some cells but not in others where virus transfer to the nucleus is "blocked" in the cytoplasm. HEV might be trapped in the receptosomes without being released into the cytoplasm, which is not unlike the fate of glutaraldehyde-fixed Ad2 virions, observed in cytoplasmic vesicles in HeLa cells (27). Alternatively, virions might be transferred from the receptosomes into phagolysozomes in which they are degraded, instead of released in the cytoplasm which is required for adenovirus replication. Third, HEV might only undergo a full replication cycle in actively metabolizing cells as suggested for human adenoviruses (12,16). After an HEV infection blast cells appear in cell culture of which many are infected with HEV. However, it is not clear whether these appear blast-like due to virus infection or whether they became first blast cells and were subsequently infected by HEV.

HEV resembles the fastidious human adenovirus types 40 and 41 which cause intestinal infections in man (1,4,9,28). Ad40 and Ad41 replicate in intestinal cells and up to 10^{11} virions are present per ml stool (9,25). Like HEV these viruses do not replicate in epithelial or epitheloid cell cultures commonly used for mammalian and group I avian adenoviruses. However, they can replicate in permissive 293 cells which are transformed by Ad5 and which contain the E1 gene of Ad5 (10,28). This leads to the question whether HEV and the fastidious adenoviruses may be replicating in different cells than the other adenoviruses. However, these cells have not been identified yet. In addition, when the reticuloendothelial cells of many organs in which HEV replicates are not the primary cells during an HEV-infection in the birds, the identity of the cells that are initially infected remains to be determined. Knowledge of these primary target cells might eventually lead to an improved cell culture system for HEV propagation.

Pathogenic strains of HEV are able to infect young turkeys and cause disease, loss in production, and immunosuppression which may predispose the birds to other diseases (5,18-20). For the prevention of HE, the efficacy of a potential vaccine consisting of apathogenic HEV propagated in turkey leukocytes was tested. The immunization conditions were established experimentally at VIDO, and then trials were conducted to test the vaccine in turkeys under field conditions.

First, since most turkeys possess maternal antibodies to HEV, it was determined that for the induction of a protective antibody response after immunization with HEV-A, maternal antibody titers in turkeys had to be <40. This level was reached in most turkeys when they were 5 to 6 weeks old. Furthermore, the time of vaccination

could be determined more accurately from the established half life value of 4.25 days and a maternal antibody titer <40 which does not interfere with a protective antibody response. Second, a potency test was developed to determine the number of doses in HEV-A preparations. In this test, the time of challenge after immunization and the time for antigen analysis in spleen extracts after challenge were based upon data described in Chapter 2. The potency was determined in dose-response challenge experiments. In addition, induction of an antibody titer ≥20 after immunization with HEV-A was shown to be protective. Third, the safety of all HEV-A preparations was determined by screening in vitro for the presence of contaminants before they were used in turkeys. The preparations used in the field trials were also tested for adverse effects in vivo.

Table 2. Comparison of the properties of HE and human adenovirus vaccines.

	Vaccine preparations			
	HEV-A from turkey leukocytes	HEV-A from RP19 cells	Hexon from turkey leukocytes	Ad4+7 from HEK cells
Propagation	Primary cell culture	Cell line	Primary cell culture	Cell line
Stability	+++[A]	+++	++++	+++
Adjuvants	Not required	Not required	Required	Not required
Vaccine type	Live apathogenic virus	Live apathogenic virus	Subunit	Live attenuated virus
Inoculation	Oral	Oral	Injection	Oral
Vaccination(s)	Once	Once	More than once	Once
Costs	+	+	+++	++
Safety	+++	++	++++	+++
Response	++++	+++	+++	+++

[A] Range from low to very high: + low; ++ medium; +++ high; ++++ very high.

After immunization of turkeys with live HEV-A propagated in turkey leukocytes had been shown to be safe and efficacious under experimental conditions, field trials were conducted. Nineteen out of twenty flocks seroconverted within 21 days of vaccination with live HEV-A distributed in the drinking water. The overall immune response of the turkeys in the 20 flocks was 96%. In addition, neither clinical HE nor other adverse effects caused by HEV-A vaccination were observed in any of the vaccinated flocks.

In Table 2 the properties of the HE vaccine produced in turkey leukocytes, the HE vaccine produced in RP19 cells, a subunit HE vaccine (J.V. van den Hurk, manuscript in preparation) consisting of the hexon protein, and the human adenovirus vaccine used to immunize recrutes for acute respiratory disease are shown. The propagation of HEV-A in primary turkey leukocytes instead of in an established cell line is a disadvantage of this vaccine. The RP19 cell line is not ideal for vaccine production either because it contains MDV and only cells at passage levels between 10 and 20 are recommended for virus propagation (21,22). HEV-A itself, however, has a number of attractive qualities for use as a live virus vaccine because: i) it does not revert to a more pathogenic form; ii) it appears to be antigenically stable; and iii) it is able to induce long-lasting protection against wild-type virus (5,22, this dissertation). In contrast to the many positive points of the live adenovirus vaccines, safety will be a continuous concern. Factors of concern are: i) contaminating microorganisms in the vaccine preparations which might cause disease; ii) effect of incorporation of adenovirus gene(s) in host DNA as was observerd after infection of humans with adenoviruses (11); and iii) spreading of the virus from vaccinates to non-vaccinates which might be a concern when the non-vaccinates are immunocompromised and therefore not able to react normally. Although a hexon subunit vaccine will be safer and more stable, the major disadvantage of such a vaccine is that it is expensive to produce and administer to turkeys. After the efficacy and safety of the HE vaccine propagated in turkey leukocytes had been demonstrated, this vaccine was licensed in April 1986 and it is now widely used. The HE vaccine propagated in RP19 cells is licensed since August, 1987.

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SUMMARY

Hemorrhagic enteritis (HE) in turkeys is an acute infectious disease characterized by depression, intestinal bleeding, and death. HE occurs worldwide affecting 6 to 12 week-old turkeys and lasting 4 to 6 days. This economically important disease is caused by hemorrhagic enteritis virus (HEV), a turkey adenovirus which is tentatively classified as a member of the group II avian adenoviruses. Serologically related HEV strains with marked differences in pathogenicity for turkeys have been described. Until recently, only 2 vaccines were available for the prevention of HE in turkeys. Both are live virus vaccines containing avirulent HEV (HEV-A) and both elicit protective immunity in turkeys. However, since the first vaccine is a crude extract prepared from spleens of turkeys infected with HEV-A, and the second vaccine is propagated in a transformed cell line contaminated with Marek's disease virus, their safety features are questionable.

HEV is unique among the adenoviruses because it is not antigenically related with the mammalian or group I avian adenoviruses. Its classification as an adenovirus is based upon common physical, chemical, morphological and structural properties. An adenovirus is composed of 240 hexons and 12 pentons, outer capsid proteins which give the virus its characteristic icosahedral shape, capsid associated proteins, and core proteins associated with the double-stranded linear DNA genome with a molecular weight of 17 - 30 x 10^6. Until recently, HEV and its structural proteins had been poorly characterized due to the lack of a suitable *in vitro* system for virus propagation. In summary, there was a need for an improved vaccine for HE in turkeys, and the development of a such a vaccine would be facilitated by the discovery of a cell type suitable for HEV replication and by a more basic knowledge of the virus itself.

The major goal of the research described in this dissertation was the development and testing of a safe and efficient vaccine for HE in turkeys. In order to achieve this goal, a cell culture system for virus propagation as well as methods to measure virus replication *in vitro* and protection in

immunized birds had to be developed. In addition, the knowledge of virus and viral components had to be expanded.

The development and application of sensitive and specific enzyme-linked immunosorbent assays (ELISAs) for the quantitation of HEV antibodies in turkey sera and HEV antigen in tissue extracts is described in Chapter 2. The presence and decline of maternal antibody titers in sera of poults and seroconversion and induction of protective antibody titers in turkeys following immunization with HEV-A were determined by ELISA (Chapters 2 and 6). The ELISA for the titration of antigen was used to monitor protection in turkeys following immunization with HEV-A and challenge with virulent HEV (HEV-V) (Chapter 6). A strong antigenic relationship between HEV-A and HEV-V was measured with both ELISAs.

The characterization of both HEV-A and HEV-V and their structural proteins, purified from spleens of infected turkeys is described in the Chapters 3 and 4. The electron microscopic data on the size (72nm) and structure of the virion and its density in CsCl (ρ = 1.34 g/cm^3), as well as the profile of the viral polypeptides in polyacrylamide gels showing molecular weights ranging from 96,000 to 9,500, justified the classification of HEV as an adenovirus. The major structural proteins were identified as hexon, penton, penton base, fiber, IIIa, and core proteins based on their structure observed by electron microscopy and/or recognition by specific antibodies. Free hexon and penton proteins, purified by immunoaffinity chromatography using monoclonal antibodies, had identical properties as their counterparts in the virus. The hexon was an important neutralizing antigen. The penton of HEV consisted of a single fiber attached to its penton base, a feature shared with the mammalian adenoviruses and the avian egg drop syndrome 1976 virus, but not with the fowl adenoviruses which have double fibers. In contrast to the many common properties of HEV-A and HEV-V, serological differences between the fibers of and differences in electrophoretic migration between the penton bases of both strains were observed. The IIIa proteins of HEV and human adenovirus type 2 shared a common epitope. This is the first antigenic relationship detected between avian and mammalian adenoviruses.

The propagation of HEV-A and HEV-V in turkey blood leukocyte cells is

described in Chapter 5. The presence of HEV in the nuclei of non-adherent as well as in adherent cells was revealed by electron microscopy and by light microscopy, using a fluorescent antibody test. The non-adherent infected cells had the characteristics of immature mononuclear leukocytes while the adherent cells had monocyte-macrophage characteristics. HEV-A could be serially passed in turkey leukokcytes at least seven times. Optimum conditions for virus propagation in turkey leukocyte cultures and harvest times were determined. HEV could not be produced in chicken leukocytes.

HEV-A, propagated in turkey leukocyte cell cultures, was tested as a vaccine to prevent HE in turkeys in experimental and field trials (Chapter 6). Immunization of turkeys with live HEV-A resulted in protection against a challenge with HEV-V as measured by the serological response and the absence of clinical disease and HEV antigen in spleens. In the field trials, 19 out of 20 flocks seroconverted within 21 days after vaccination with live HEV-A distributed in the drinking water. The overall immune response of the turkeys in these flocks was 96%. Most importantly, neither clinical nor other adverse effects caused by HEV-A vaccination were observed in any of the vaccinated turkeys in the experimental and field trials. The optimum time of the vaccination of poults was determined in relation to interference with maternal antibodies.

SAMENVATTING

Hemorrhagic enteritis (HE) is een akute infektieuze ziekte in kalkoenen die gekenmerkt wordt door depressie, darmbloeding en mortaliteit. HE komt voor in de hele wereld, tast 6 tot 12 weken oude kalkoenen aan en duurt 4 tot 6 dagen. Deze ekonomisch belangrijke ziekte wordt veroorzaakt door hemorrhagic enteritis virus (HEV), een kalkoene-adenovirus dat voorlopig geklassificeerd is als een group II avian adenovirus. Serologisch verwante HEV stammen met duidelijke verschillen in pathogeniteit zijn beschreven voor kalkoenen. Tot voor kort waren er slechts 2 vaccins beschikbaar ter voorkoming van HE in kalkoenen. Beide zijn levend virus vaccins die verzwakt HEV (HEV-A) bevatten en beide induceren immuniteit in kalkoenen. Aangezien het eerste vaccin echter bestaat uit een ongezuiverd extrakt, verkregen uit de milt van HEV-A-geinfekteerde kalkoenen, en het tweede wordt vermeerderd in een getransformeerde celkultuur die gekontamineerd is met Marekvirus, laat de kwaliteit van deze vaccins te wensen over.

HEV is een uniek adenovirus omdat het geen antigene verwantschap vertoont met de mammalian of de group I avian adenoviruses. De klassifikatie van HEV is gebaseerd op het bezit van gemeeschappelijke fysische, chemische, morfologische en strukturele eigenschappen. Een adenovirus is opgebouwd uit 240 hexons en 12 pentons, capsid eiwitten die de karakteristieke vorm aan het virus geven, eiwitten geassocieerd met de capsid, en core eiwitten geassocieerd met het dubbelstrengig lineair DNA genoom dat een molekuulgewicht heeft van 17 tot 30 x 10^6. Sinds kort waren HE virus en strukturele eiwitten bijna niet gekarakterizeerd door het gebrek aan een passend in vitro systeem voor virus vermeerdering. Kortom, er was een behoefte aan een verbeterd vaccin voor HE in kalkoenen en de ontwikkeling van zo'n vaccin zou vergemakkelijkt worden door de ontdekking van een geschikte cel voor HEV vermeerdering en door een meer uitgebreide basiskennis van het virus.

Het belangrijkste doel van het onderzoek beschreven in dit proefschrift was de ontwikkeling en analyse van een veilig en doelmatig vaccin tegen HE in kalkoenen. Om dit doel te bereiken moesten behalve een celkultuur voor virus vermeerdering bovendien methoden ter bepaling van de virus replikatie in vitro en bescherming van geimmuniseerde vogels ontwikkeld worden.

De ontwikkeling en toepassing van gevoelige en betrouwbare enzyme-linked immunosorbent assays (ELISAs) voor het meten van HEV antilichamen in

kalkoene-sera en HEV antigeen in weefselextrakten wordt beschreven in Hoofdstuk 2. De aanwezigheid en afname van maternale antilichaamtiters in sera van jonge kalkoenen, en serumconversie en induktie van beschermende antilichaamtiters in kalkoenen na immunisering met HEV-A werden bepaald met de ELISA (Hoofdstukken 2 en 6). De ELISA voor de titratie van antigeen werd gebruikt om bescherming te verifieren in kalkoenen na immunisatie met HEV-A en challenge met virulent HEV (HEV-V) (Hoofdstuk 6). Een sterke antigene verwantschap tussen HEV-A en HEV-V werd gemeten met beide ELISAs.

De karakterisering van HEV-A, HEV-V en hun strukturele eiwitten, gezuiverd uit de milt van geinfekteerde kalkoenen, wordt beschreven in de Hoofdstukken 3 en 4. De elektronenmikroskopische resultaten van de grootte (72 nm) en struktuur van het virus, de dichtheid van het virus in CsCl (ρ=1.34 g/cm^3), zowel als het elektroforese patroon van de virale polypeptiden in polyacrylamide gels met molekuulgewichten varierend van 96.000 tot 9.500 bevestigde de juistheid van de klassifikatie van HEV als een adenovirus. De identiteit van de voornaamste strukturele eiwitten kon worden vastgesteld als hexon, penton, penton base, fiber, IIIa, en core eiwitten op de basis van hun struktuur waargenomen met de elektronenmikroskoop en/of herkenning met behulp van specifieke antilichamen. Vrije hexon en penton eiwitten, die verkregen werden na zuivering met immunoaffiniteitschromatografie waarbij monoklonale antilichamen werden gebruikt, vertoonden dezelfde eigenschappen als de overeenkomstige eiwitten in het virus. De hexon was een belangrijk neutraliserend antigeen. De penton van HEV bestond uit een enkele fiber gehecht aan de penton base, een kenmerk dat HEV gemeen heeft met zoogdier-adenovirussen en het vogel-egg drop syndrome 1976 virus, maar niet met de kippe-adenovirussen die dubbele fibers bezitten. In tegenstelling tot de vele eigenschappen die HEV-A en HEV-V gemeen hebben staan de serologische verschillen tussen de fibers en de verschillen in mobiliteit van de penton bases van beide stammen. De IIIa eiwitten van HEV en menselijk adenovirus type 2 bezitten een gemeenschappelijk epitoop. Dit is de eerste antigene verwantschap die gevonden is tussen vogel- en zoogdier-adenovirussen.

De vermeerdering van HEV-A en HEV-V in witte bloedcellen van kalkoenen wordt beschreven in Hoofdstuk 5. Met behulp van elektronenmikroskopisch en lichtmikroscopisch onderzoek met een antilichaam-fluorescentietest werd de aanwezigheid van HEV zowel in de kernen van cellen die groeien in suspensie

als in cellen die groeien in een monolaag aangetoond. De geinfekteerde cellen die groeiden in suspensie hadden eigenschappen van onvolledig gedifferentieerde mononucleaire witte bloedcellen, terwijl de geinfekteerde cellen die in een monolaag groeiden kenmerken ten toon spreidden van monocyten en makrofagen. HEV-A kon minstens 7 keer in celkultuur gepasseerd worden. Optimale kondities voor virus vermeerdering in witte bloedcelkulturen van kalkoenen en oogsttijd werden bepaald. HEV kon niet worden vermeerderd in witte bloedcellen van kippen.

HEV-A, vermeerderd in witte bloedcelkulturen van kalkoenen, werd getest als een vaccin ter voorkoming van HE in kalkoenen in experimentele en veldproeven. Immunisatie van kalkoenen met levend HEV-A resulteerde in bescherming tegen een challenge met HEV-V, wat geverifieerd werd met een stijging in antilichaamtiters alsook met de afwezigheid van klinische infektie en HEV antigeen in de milt. In de veldproeven vertoonden 19 van de 20 groepen kalkoenen seroconversie binnen 21 dagen na vaccinatie met levend HEV-A toegevoegd aan het drinkwater. De gemiddelde immuunresponse van de kalkoenen in de groepen was 96%. Van groot belang was ook dat geen klinische infekties en geen andere schadelijke bijverschijnselen werden aangetroffen in de HEV-A-gevaccineerde kalkoenen tijdens de experimentele en veldproeven. De beste tijd om kalkoene-kuikens te immuniseren werd bepaald met inachtneming van mogelijke interferentie van maternale antilichamen.

Curriculum Vitae

Jan Vincent Joseph Maria van den Hurk werd op 28 maart 1944 geboren te 's Hertogenbosch. Hij bezocht het St. Janslyceum te 's Hertogenbosch waar hij in 1964 het diploma HBS-b behaalde. In hetzelfde jaar werd begonnen met een studie aan de Landbouwuniversiteit te Wageningen waar het kandidaatsexamen werd afgelegd in 1973. Gedurende de jaren 1965 en 1966 werd de studie onderbroken en was de auteur werkzaam als laboratorium assistent bij de AKZO te Arnhem. De praktijktijd werd doorgebracht bij de afdeling microbiologie en immunologie van de universiteit van Montreal, in Montreal, Canada, van september 1972 tot september 1973. In januari 1975 werd het doctoraal diploma behaald met virologie als hoofdvak, organische chemie als verzwaard bijvak, en biochemie als bijvak. Vanaf 1975 tot 1980 was de auteur verbonden als wetenschappelijk medewerker bij de afdeling microbiologie en immunobiologie van de universiteit van Montreal, in Montreal, Canada. Sinds 1980 is hij werkzaam als wetenschappelijk medewerker bij de Veterinary Infectious Disease Organization (VIDO), in Saskatoon, Canada, waar het onderzoek heeft plaatsgevonden dat geleid heeft tot dit proefschrift.