Bovine Adenovirus Type 10 Identified in Fatal Cases of Adenovirus-Associated Enteric Disease in Cattle by In Situ Hybridization

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A severe, naturally occurring enteric disease of cattle in which adenovirus inclusions are present in the intestinal vascular endothelium has been recognized in several countries; three different adenovirus serotypes have been isolated from affected animals. An in situ hybridization technique for the detection of bovine adenoviral DNA was developed and applied to tissue from 13 cattle in Northern Ireland diagnosed to have the disease. Bovine adenovirus serotype 10 (BAV-10) was identified in the vascular inclusions of all cattle, providing strong evidence that adenoviral enteric vascular disease in cattle is associated with this serotype. The existence of BAV-10 has only recently been recognized. The first molecular biology-based technique for the diagnosis of BAV-10 infection is described. The animals in the present study are the first in which BAV-10 has had a confirmed role in a pathologic process.

There have been reports from several countries of a severe, naturally occurring enteric disease of cattle aged from 6 days old to adults in which adenovirus-induced nuclear inclusions were found in the vascular endothelium of the intestine (8, 9, 12, 13, 15, 17). No other enteric pathogens were identified in most of these animals. This, in conjunction with the fact that the enteric lesions found in these animals can be explained by the vascular damage caused by virus replication, provides strong circumstantial evidence that the enteric disease is caused by the adenovirus. At present this type of adenoviral involvement in enteric disease can only be diagnosed by identification of the inclusions and/or by immunocytochemical examination of affected tissues. Thus, diagnosis is normally only possible in fatal cases and only if appropriate tissues are examined. This, together with the fact that there are likely to be nonfatal forms of the disease, means that such adenoviral involvement in enteric disease is probably underdiagnosed.

To improve our ability to diagnose vascular adenoviral infection in animals with enteric disease, and, hence, to determine its incidence, it is first important to identify the specific cause. To date, three different serotypes of bovine adenovirus (BAV) have been isolated from affected animals (2, 9, 10, 13), and there is serological evidence for the involvement of a fourth serotype in an additional animal (17). These findings raise the possibility that several serotypes of BAV are capable of replicating in the vascular endothelium, leading to enteric disease. Alternatively, the disease may be caused by one serotype, with the reported isolations of other serotypes representing coincidental simultaneous infections. Direct examination of the viral inclusions in affected animals by in situ hybridization (ISH) with serotype-specific probes allows for the specific determination of the involvement of these serotypes in the disease and would overcome the interpretive difficulties associated with virus isolation. This report describes the use of a

DNA probe which hybridizes specifically to BAV serotype 10 (BAV-10) and probes made from BAV-4 DNA (specific for BAV-4, -5, -6, -7, and -8) and BAV-1 DNA (specific for BAV-1, -2, -3 and -9) to examine the vascular inclusions in archival tissue samples from 13 cattle in Northern Ireland that died of adenovirus-associated enteric disease. In view of the results obtained, the key clinical and pathologic features of these animals are presented.

Clinical histories. The 13 affected cattle (the principals) were from different farms and ranged in age from 5 to 12 months. They were clinically affected in either April-May or September through to November. Nine animals were at pasture. The environments of the other four animals are not known. The affected animals were of mixed breeds and were mainly beef type. Most animals presented with a history of either sudden death or a short illness lasting 12 to 24 h. One animal was ill for 2 days, and one animal had a history of unthriftiness but died unexpectedly. With the exception of the latter animal, sick animals were profoundly depressed and were recumbent. Subnormal temperatures were reported in four animals. Most animals developed severe diarrhea or dysentery before death. Two of these animals were presented to the laboratory with a provisional clinical diagnosis of abdominal catastrophe.

Pathologic findings. Macroscopically, all principal cattle had enteritis. In 11 animals, the ileal mucosa in particular was hemorrhagic with a fibrinous exudate on the surface. In 2 of these 11 animals, there was marked necrosis over Peyer's patches. Colitis was present in some animals, and a few superficial abomasal ulcers and/or abomasal edema were present in some animals. One animal had pulmonary edema. Lung lesions were not observed in any of the other animals.

Histopathologically, large basophilic or amphophilic intranuclear inclusions were found in the vascular endothelium of the lamina propria and submucosa of the small intestine (Fig. 1) in 11 animals (the intestines of the remaining two animals were not examined). The affected nuclei were enlarged, often markedly so, and had marginated chromatin. Fibrinoid degeneration of arterioles and mucosal hemorrhage

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FIG. 1. Submucosal blood vessel, small intestine. There are numerous enlarged endothelial nuclei with marginated chromatin (arrows). Large amphophilic inclusions are present in these nuclei. Hematoxylin and eosin stains were used. Bar, 17 μ m.

were common. In one animal, in which mucosal autolysis was minimal, fibrin microthrombi were visible in some superficial capillaries. Nuclear inclusions were also present in the vascular endothelium of the renal cortices of all nine animals in which it was examined (Table 1) and sometimes in the vascular endothelium of small blood vessels in the colon, abomasum, mesenteric lymph node, kidney, and liver (portal and occasionally central veins) (Table 1). Of the two animals whose intestines were not collected for histopathologic examination, typical inclusions were found in the renal vascular endothelium. Apart from the adenoviral associated lesions, no evidence of any other infectious or toxic cause of enteritis was found in any of the animals.

ISH. The tissues examined by ISH in the present study included samples of small intestine from 11 of the principal cattle and kidney tissue from the remaining 2 principal cattle. These tissues had been fixed in 10% neutral buffered formalin and were embedded in paraffin. Formalin-fixed paraffin-embedded sections from seven animals with other adenoviral diseases (see controls) and glutaraldehyde-fixed paraffin-embedded preparations of separate BAV-1-, BAV-3-, and BAV-10-infected cell cultures were also examined for control purposes.

Preparation of probes. Cloned BAV-10-specific DNA (6) was prepared as follows. A Northern Ireland isolate of BAV-10 (2) was propagated in low-passage-number bovine testicle cells. Virus particles were purified and DNA was extracted as described previously (3). *Pst*I-cleaved fragments of the DNA were randomly cloned into the pMOB plasmid by using a TN1000 kit (GOLD Biotechnology Inc., St. Louis, Mo.) according to the manufacturer's instructions. The DH5 α strain of *Escherichia coli* was transformed, and after alkaline mini plasmid purification, a recombinant plasmid containing the *Pst*I-D fragment (approximately 2,050 bp [6]) was selected on the basis of size. A sample of uncleaved plasmid with the *Pst*I-D insert and a second sample containing purified *Pst*I-D fragment excised from the plasmid were labelled with digoxigenin (DIG) by using a commercial kit (DIG DNA Labelling and Detection Kit; Boehringer Mannheim UK, Lewes, England) according to the manufacturer's instructions. The 5' and 3' ends of the cloned *Pst*I-D fragment of BAV-10 have been sequenced.

Two additional probes made of cloned DNA from BAV-1 (the *Eco*RI-B fragment in pUC18 [4]) and BAV-4 (pBAV401, a 15-kb long *Cl*AI-*Sal*I fragment cloned in the pKH47 vector [5]) were labelled with DIG as described above.

Development of ISH method for BAV-10. Autoclaved distilled water was used to prepare all solutions. Unless otherwise specified, all washes consisted of two 10-min treatments in 5 mM Tris-buffered saline (TBS [pH 7.6]). The hybridization mixture was as described by Brigati et al. (7), except that it contained 1 $\times$ Denhardt's solution (Sigma Chemicals, Poole, England) and 200 μ g of carrier DNA per ml.

Two 5- μ m sections of paraffin-embedded tissue from all 13 principal cattle were placed on separate glass slides which had been coated with 3-amino propylethoxysilane and deparaffinized by immersion in histosol (Shandon Scientific Ltd., Astmoor, England) for 5 min and then immersion in 99% methylated spirits for 3 min. Endogenous peroxidase was blocked by using 0.5% hydrogen peroxide in methanol for 20 min. Sections were treated in 0.02 N HCl in water for 10 min and washed in TBS (pretreatment 1); this was followed by the addition of 0.1% Triton X-100 (Sigma Chemical Company) in TBS for 1.5 min (pretreatment 2). Sections were then digested with proteinase K (Sigma Chemical Company) at a concentration of 0.5 mg/ml in 0.05 M Tris HCl (pH 7.6)–5 mM EDTA at 37°C for 20 min. The proteinase K reaction was stopped by immersing the sections in 1% glycine in TBS for 30 s, and the sections were washed. The sections were next fixed in 4%

TABLE 1. Relative numbers of and organ distribution of vascular endothelial adenoviral inclusions found in each of the 13 principal cattle by histopathologic examination

Tissue	Relative no. and distribution in animal no. ^a :												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Small intestine	+++	+++	++	ne	+++	+++	++	++	+++	ne	+++	+++	++
Colon	+++	++	ne	ne	ne	ne	ne	ne	++	ne	ne	+++	ne
Mesenteric lymph node	ne	+	ne	ne	ne	ne	ne	++	ne	ne	ne	ne	ne
Abomasum	ne	+++	ne	ne	ne	ne	ne	ne	++	ne	ne	ne	ne
Kidney	ne	+++	ne	++	+++	++	+	+++	ne	+++	+++	+++	ne
Liver	—	+	ne	+	+	+	ne	+	ne	ne	—	—	ne
Spleen	ne	—	ne	ne	ne	ne	ne	—	ne	ne	ne	ne	ne
Lung	ne	ne	ne	+	ne	ne	ne	ne	ne	—	—	—	ne
Heart	ne	ne	ne	ne	ne	ne	—	ne	ne	ne	—	ne	ne
Brain	ne	ne	ne	—	ne	ne	—	ne	ne	ne	ne	ne	ne
Esophagus	ne	ne	—	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne

^a +++ , inclusions in most $\times 250$ magnification fields; ++ , inclusions in many $\times 250$ magnification fields; + , inclusions found only after searching multiple fields; — , no intranuclear inclusions; ne, organ not collected for histopathologic examination.

TABLE 2. ISH controls and specificity testing

Sample tested		Result with the following probe:		
Origin of sample	Infecting adenovirus	BAV-10	BAV-4 ^a	BAV-1
Infected cell culture	BAV-1	—	—	+
Infected cell culture	BAV-3	—	—	+
Infected cell culture	BAV-10	+	—	—
Natural infection	Virus in 13 principal cattle	+	—	— ^b
Experimental infection	Fowl adenovirus type 8	—	NT ^c	NT
Experimental infection	Egg drop syndrome virus	—	NT	NT
Natural infection	Canine adenovirus type 1	—	NT	NT
Natural infection	Renal adenovirus in a pig	—	NT	NT
Natural infection	Renal adenovirus, ovine ^d	—	NT	NT
Natural infection	Enteric adenovirus, ovine ^e	—	NT	NT
Experimental infection	Guinea pig adenovirus	—	NT	NT

^a See section on specificity testing for details on the positive control.^b The result is for 6 of the 13 principal cattle tested.^c NT, not tested.^d See reference 16.^e See reference 14.

paraformaldehyde in TBS for 5 min and were washed in TBS (pretreatment 3). Then, 30 μ l of hybridization mixture containing labelled plasmid with viral insert (concentration 200 ng/100 μ l) was added to one section from each animal, while 30 μ l of hybridization mixture containing the labelled BAV-10 DNA excised from the plasmid (concentration 50 ng/100 μ l) was added to the second section from each animal. Coverslips were placed on top, and the sections were denatured at 90°C for 10 min, immediately cooled on ice, and allowed to hybridize for 18 h at 42°C. The sections were washed consecutively for 5 min at 37°C in the following: 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl containing 0.015 M sodium citrate [pH 7.0]) (two washes), 0.2 $\times$ SSC and 0.1 $\times$ SSC, and then 0.1 $\times$ SSC at room temperature. After washing in TBS, the slides were incubated in 1% blocking reagent (Boehringer Mannheim UK) in 0.1 M Tris-HCl (pH 7.5)–0.15 M NaCl (Tris-HCl–NaCl) for 20 min at 37°C and were washed twice in 0.1 M Tris-HCl–NaCl for 10 min. The sections were then incubated in 0.5% peroxidase-conjugated anti-DIG antibody (anti-DIG POD; Boehringer Mannheim UK) for 45 min, washed in Tris-HCl–NaCl for 20 min, and finally, incubated in a freshly prepared solution of 0.05% diaminobenzidine tetrahydrochloride in 0.2 M Tris-HCl (pH 7.6) containing 0.01% hydrogen peroxide for 7 to 10 min. The sections were then washed in TBS and were counterstained lightly with hematoxylin.

Effect of pretreatments. Serial sections from six animals were subsequently used to test the effect of the omission of each of the pretreatments and then the omission of all of the pretreatments on the intensity of labelling.

Controls and specificity testing. The sensitivity and specificity of the uncleaved plasmid containing BAV-10 DNA probe had initially been tested by Southern and dot blot hybridization experiments (6) with purified viral DNA and normal stringency conditions, which showed that the prototype BAV-10 strain (10) and the four Northern Ireland isolates of BAV-10 (2) gave a positive reaction, while the other known BAV serotypes and a range of ovine and porcine adenoviruses remained negative. The specificity of this probe was further tested by ISH in the present study, first, by comparison with the results obtained when the excised DIG-labelled cloned BAV-10 DNA fragment was used for hybridization and, second, by comparison with the results for an extensive array of controls detailed below and summarized in Table 2.

The BAV-10 probe was hybridized with 5- μ m sections of

BAV-1, BAV-3, and BAV-10 cell culture preparations by the ISH protocol described above but excluding pretreatments 1 to 3. The BAV-1 probe (specific for BAV-1, -2, -3, and -9 [4]) (concentration, 100 ng/100 μ l) and BAV-4 probe (specific for BAV-4, -5, -6, -7, and -8 [5]) (concentration, 100 ng/100 μ l) were similarly hybridized with separate sections of the BAV-10-infected cell culture preparation. The efficacy of the ISH protocol for BAV-4 was confirmed by the inclusion of sections from a calf experimentally infected with BAV-4. These sections were kindly supplied by E. Scanziani, Istituto di Patologia Veterinaria e Patologia Aviaria, University of Milan, Milan, Italy. For heterologous hybridizations, the stringency of the washes was reduced by carrying out only two washes in 2 $\times$ SSC to increase the likelihood of nonspecific hybridization occurring.

Formalin-fixed paraffin-embedded sections (containing adenovirus inclusions) from the following animals were reacted with the BAV-10 probe: animals with two ovine adenoviral diseases (14, 16), chickens experimentally infected with fowl adenovirus serotype 8 or egg drop syndrome virus, animals with naturally occurring porcine adenovirus and canine adenovirus type 1 infection, and a guinea pig with experimental adenovirus infection. Pretreatments 1 to 3 were included in the ISH protocol for these animals.

As additional negative controls, sections from all of the principal cattle were subjected to the full ISH protocol, except that either no probe or BAV-4 probe was added to the hybridization mixture. Tissues from six principal cattle were also hybridized with the BAV-1 probe. For the BAV-4 and BAV-1 probes, the stringency of washing was reduced by carrying out only two washes in 2 $\times$ SSC.

Results of ISH. The vascular inclusions in the 13 principal cattle positively hybridized with BAV-10, giving a strong reaction end product (Fig. 2; Table 2) in all cases except one, in which the hybridization signal was noticeably weaker. The test tissue in that case was very autolytic. There was no obvious difference in the numbers of labelled nuclei or in the intensity of labelling when the intact plasmid with the viral DNA insert was used as the probe or when the excised purified viral DNA fragment was used. Omitting pretreatments 1 to 3 either singly or totally resulted in minor inconsistent variations in the intensity of the hybridization signal. These were not regarded as significant.

The inclusions were unlabelled in sections from the principal

FIG. 2. Submucosal blood vessel, small intestine. Several enlarged endothelial nuclei are strongly labelled (arrows) by ISH with a DIG-labelled BAV-10-specific probe (probe 2). Hematoxylin counterstain was used. Bar, 14 µm.

cattle to which either no probe, the BAV-4 probe, or the BAV-1 probe was added, despite less stringent washes following probe hybridizations. The inclusions in all of the other adenovirus-infected cattle did not become labelled with the BAV-10 probe.

The nuclei of cells in the BAV-10-infected cell culture preparation hybridized with the BAV-10 probe, but not with either the BAV-4 or the BAV-1 probe, even when less stringent washes were used with the latter two probes. The nuclei of cells in the BAV-1 and BAV-3 cell culture preparations did not hybridize with the BAV-10 probe, again even when less stringent washes were used.

Discussion. Severe enteric disease in cattle associated with adenoviral inclusions in the intestinal vascular endothelium has been reported in several countries. BAV-4 (one isolate), BAV-7 (one isolate), and BAV-10 (five isolates) have been recovered from affected animals (2, 9, 10, 11, 13). Rising antibody titers to BAV-3 have been reported in animals in contact with an additional affected animal (17). The recovery of three serotypes from affected animals and the circumstantial evidence for the involvement of a fourth serotype raise the possibility that several serotypes of adenovirus are capable of replicating in the vascular endothelium to produce this form of disease or, alternatively, that two, or indeed all, of the isolates were from animals with coincidental simultaneous infections. It is also possible, particularly if the adenovirus isolates were from animals with coincidental infections, that the serotypes recovered reflect the susceptibilities of the different cell culture systems used for attempted virus isolation, since, for example, BAV-10 viruses are difficult to isolate (2, 6), growing best in bovine testis cells. These interpretive problems could be overcome by direct examination of the DNA in the inclusions to determine the serotype.

We have developed and tested an ISH method which enables the specific, accurate, and rapid detection of BAV-10 DNA in tissue sections. Using this, we have shown the presence of BAV-10-specific DNA in the vascular inclusions in all 13 animals with adenovirus-associated enteric disease. This provides strong evidence that BAV-10 is the specific serotype replicating in the vascular endothelium in animals with this form of disease. While our test animals were all from within

Northern Ireland, it is of interest that the first isolate of BAV-10 was recovered from a similarly diseased animal in New Zealand (10, 11). Nonetheless, it would be of interest to test additional animals with this disease from other countries.

The existence of BAV-10 was first confirmed in 1989 (10), and to date, only five isolates have been obtained (2). The 13 animals in the present series are the first in which BAV-10 has had a confirmed role in a disease process, i.e., enteric vascular disease. Therefore, the main clinicopathologic findings in the affected animals have been presented. The clinical history in most of our animals was rather characteristic, usually of a sudden-onset severe depressive disease with a short clinical course, and can resemble an abdominal catastrophe. In some cases, in-contact animals had diarrhea, but in other cases they did not. The pathologic findings in the animals can be explained by the primary vascular damage caused by the adenovirus. No other known enteric pathogens were identified. The age of the affected animals means that the death of even one animal represents a significant loss to the farmer.

At present, adenoviral replication in the vascular endothelium of cattle with enteric disease is probably underdiagnosed, first, because there is no test suitable for diagnosis of the disease in live animals and, second, because diagnosis requires histopathologic or immunocytochemical examination of the intestine. The latter examinations are often not carried out because intestine decomposes rapidly after death. We have found that the kidney is useful for histopathologic diagnosis because it was affected in all of the nine animals whose kidneys were examined and it does not undergo autolysis as quickly as the intestine.

It seems probable that there will be nonfatal forms of disease associated with the replication of adenovirus in the vascular endothelium. A diagnostic test is needed to enable recognition of the disease in live affected animals, and thus to establish its overall importance as a cause of bovine diarrhea. If BAV-10 is found to be the only serotype involved, then it may be possible to make a diagnosis in live affected animals by examination of acute- and convalescent-phase sera for rising antibody titers. However, serum neutralization tests would be required, since the different bovine adenovirus serotypes share some antigens (1, 2, 15). Such tests are labor-intensive and would be particularly so in this case because BAV-10 grows poorly in cell culture and monoclonal antibodies to BAV-10 are not available. Since our work has confirmed the specificity of the *Pst*I-D fragment for BAV-10 and the sequences of the 5' and 3' ends are known, it should be possible to develop a PCR test for the detection of viral DNA. A PCR test for examination of blood could prove to be useful for the diagnosis of BAV-10 in clinically affected animals.

Nucleotide sequence accession numbers. The 5' and 3' ends of the cloned *Pst*I-D fragment of BAV-10 have been submitted to GenBank under accession numbers U25262 and U25263, respectively.

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