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Tissue Tropism and Target Cells of Bluetongue Virus in the Chicken Embryo

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In situ cytohybridization was used to determine the tissue tropism and target cells for replication of bluetongue virus (BTV) in the developing chicken embryo. Hybridization with a biotinylated probe specific for segment 3 of BTV serotype 17 detected viral replication in embryos inoculated with U.S. serotypes 2, 10, 11, 13, and 17 or sheep blood containing a BTV field strain. At the final stages of infection, when the embryos were hemorrhagic, viral infection could consistently be detected in the brain, kidney, spinal cord, heart, lung, and liver, with the brain and kidney most severely affected. Other tissues, such as the retina, skin, tongue, and intestinal villi, also supported viral replication in some embryos. Greater concentrations of virus tended to be localized within epithelial cells, such as those lining the kidney tubules and tertiary bronchi of the lungs. Kinetics studies with BTV serotypes 11 and 17 and a field strain indicated that within 24 h after inoculation, viral replication occurred initially in the brain and kidney. By 48 h, viral replication was also detected in the lungs, heart, and spinal cord, with the liver being severely infected by 72 h. Low levels of hybridization could be detected in embryos infected with epizootic hemorrhagic disease virus, which is antigenically related to BTV.

Bluetongue virus (BTV), which has a 10-segment double-stranded RNA genome, is the prototype of the orbivirus genus in the Reoviridae family. Bluetongue, which is transmitted by *Culicoides* spp., is an infectious, noncontagious, and, under certain circumstances, hemorrhagic disease in ruminants. Infection during gestation may result in congenital malformations or death of the developing fetus (16, 19) or lead to persistently infected carriers (13, 15). Studies defining the tissue tropism of BTV have depended primarily on virus recovery techniques. Virus may be isolated from lymphocytes, neutrophils, or erythrocytes, as well as from semen, after insect-mediated infection with BTV (2, 12). The involvement of these cells may indicate that they are sites of viral replication, but could also reflect the strong affinity that BTV apparently has for cellular membranes (24). In deer, BTV has been isolated from a number of tissues, including the lung, liver, kidney, spleen, and tongue, after inoculation of egg-adapted virus (23). However, the frequency of isolation from each organ ranged from 55 to 70%, and no specific tissue could be recommended for optimal BTV recovery. Likewise, virus has been reisolated from a variety of organs of in utero-infected fetuses (A. J. Luedke, personal communication). The fetal brain has been found to be a predominant target for viral replication and pathogenesis, as indicated by the development of hydranencephaly and porencephaly (16, 19).

Although Osburn et al. (18) reported that BTV could be detected in fetal tissue by immunofluorescent techniques, the preferred method for confirmation of BTV infection generally involves intravascular (i.v.) inoculation of embryonating chicken eggs (ECE) followed by several blind passages of embryo homogenates on cultured cells (3, 5). Because of difficulties encountered in isolating virus and, thus, in identifying carriers, regulatory agencies throughout the world impose restrictions on the movement of cattle and importation of otherwise desirable germ plasm and embryos. A sensitive and economical assay for detection of carriers would be valuable in the certification of BTV-free animals. Nucleic acid hybridization techniques have proven valuable in demonstrating target organs for viral replication, as well as providing sensitive, rapid alternatives for diagnosis of viral and genetic diseases (1, 4, 7). These techniques are less dependent on genomic expression, making it possible to detect defective or nonexpressed genomes. Target tissues and viral infection of individual cells, even when relatively few cells are infected, can be readily detected (7).

In this report, in situ hybridization techniques were used to identify the specific target cells and organs for viral replication in longitudinal sections of ECE infected with several BTV serotypes indigenous to the United States. Since all the major organs could be viewed simultaneously in each section, this model proved to be convenient for studying tissue tropism of BTV, as well as the effects of dose and the kinetics of infection in a developing animal.

MATERIALS AND METHODS

Viruses. The serotype 10, strain 8, collected in California; serotype 11, Station strain, Texas; serotype 13, strain 67-41B, Idaho; serotype 17, strain 62-45S, Wyoming; and epizootic hemorrhagic disease viruses (EHDVs), U.S. serotype 1, New Jersey, and serotype 2, Alberta, laboratory prototype strains used were obtained from the Arthropod-Borne Animal Diseases Research Laboratory, Laramie, Wyo. All prototype strains were adapted to BHK-21 cells. Blood from a field-infected sheep from western Texas was used as the source of BTV in one study. BTV had previously been isolated from this blood by i.v. inoculation of ECE (5). The avian infectious bronchitis virus (IBV) Arkansas and Newcastle disease virus (NDV) GB used for this study were egg-adapted viruses.

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Tissue preparation. Eleven-day-old ECE were infected i.v. with each viral strain, and at various intervals of time after inoculation, the embryos were harvested. After harvesting, the wings and legs were removed from the embryos, and the tissue was embedded in OCT (Miles Scientific) at −70°C. Longitudinal sections (8 μm) of the specimens were cut with a Lipshaw cryotome at −20°C, transferred to glass slides treated as described by Haase et al. (7), and coated with Histostik (Accurate Chemical and Scientific Co., Westbury, N.Y.). The sections were then air dried, dipped in phosphate-buffered saline, and fixed in freshly prepared 4% paraformaldehyde for 15 min (22). After fixation, sections were rinsed in phosphate-buffered saline, dehydrated in increasing concentrations of ethanol, and stored at 4°C (7).

Probe preparation. The biotinylated probe was prepared by nick translation of a BTV cDNA for serotype 17 segment 3, which was previously shown to be specific for all the U.S. serotypes (20, 21). The BTV cDNA insert was excised from the plasmid with *Hin*dIII, separated from the plasmid on an agarose gel, and electroeluted as described in Maniatis et al. (17). The insert was labeled by nick translation in the presence of biotinylated dUTP (instruction manual of Enzo Bio-Chem, New York, N.Y.). The efficiency of nick translation was determined by monitoring the incorporation of [^{3}H]dATP. Optimal labeling of the probe was achieved by lowering the concentration of DNA (<0.5 μg/50 μl) and increasing the concentration of DNase (>1 ng/50 μl).

Hybridization assays. The in situ hybridization procedure used in this study was a modification of the methods described by Singer et al. (22) and Haase et al. (7). Prehybridization was found to be unnecessary. Sections were overlaid with 30 to 40 μl of hybridization solution (50% formamide, 2× SSC [0.15 M sodium chloride, 0.015 M sodium citrate], 5× Denhardt solution, 10% dextran sulfate, 100 ng of salmon sperm DNA per ml, 1 mg of whole chicken embryo RNA per ml, and 20 to 50 ng of biotinylated cDNA, per slide). Silicon-treated cover slips were placed over the sections covered with hybridization solution and sealed with rubber cement. The slides were then heated for 10 min at 80°C, and hybridization was allowed to proceed overnight at 37°C. Posthybridization, washing and visualization were performed as described for the Enzo kit. Positive hybridization was detected by the reaction of streptavidin-horseradish peroxidase, diaminobenzidine tetrahydrochloride, and hydrogen peroxide. The nuclei were counterstained with a 0.25% solution of methyl green in 0.03 M sodium acetate, pH 4.8. Positive hybridization was indicated by areas with dark red-brown grains surrounding blue-green nuclei counterstained with methyl green.

Dot blot hybridization was done with RNA extracted with phenol-sodium acetate, pH 5, buffer from uninfected BHK-21 cells or from cells infected with BTV 11 or EHDV 1. A modification of the procedures described by Kafatos et al. (11) and Schleicher & Schuell, Inc., Keene, N.H., was used. Briefly, the RNA was pretreated with formaldehyde, formamide, and phosphate-SSC buffer. After heating to 65°C for 10 min, the solution with 1 μg of RNA or fourfold serial dilutions was blotted onto nitrocellulose pretreated with 20× SSC (17) with a Bio-Dot Apparatus (Bio-Rad Laboratories) manifold. After drying, the samples were prehybridized for 4 h at 37°C in 50% formamide–5× Denhardt solution–0.1% sodium dodecyl sulfate–150 μg of calf thymus DNA per ml–5× SSC (17) and hybridized for 24 h at 37°C in a solution of 50% formamide–2× Denhardt solution–150 μg of calf thymus DNA per ml–5× SSC and 10^6 cpm of probe labeled by nick translation with [α-^{32}P]dATP (17) per ml.

RESULTS

Tissue tropism of BTV and probe specificity. In situ cytohybridization with a recombinant cDNA probe specific for segment 3 of BTV 17 was used to detect viral replication in ECE individually infected with laboratory strains of the U.S. serotypes of BTV: 2, 10, 11, 13, and 17. At least three embryos were infected per virus. Hemorrhagic embryos were harvested after they had died, which was between 3 and 5 days after inoculation of BTV. Thin (8 μm) longitudinal sections of the OCT-embedded embryos were used for these in situ studies. Figure 1 shows such a whole embryo section stained with hematoxylin and eosin. Various organs including brain, eye, kidneys, liver, spinal cord, heart, intestines, and lungs can be readily identified in sections. In addition, the spleen could also be seen in occasional sections.

Although some tissue organization can be discerned only at the lower magnifications, higher-power (about 1,000×) objectives were necessary to accurately score BTV infectivity in the cytoplasm. Figures 2a and b illustrate a control section of brain from an uninfected 15-day-old embryo at 250× and 1,000× magnification. This control is representative of the assay with uninfected cells from other tissues, since only nuclei were stained, and thus all the tissues were very similar at the higher magnifications. The viral RNA-probe hybrids in the following black and white figures are seen as dark granules in the cytoplasm surrounding lighter gray nuclei. In embryos infected with any of the five U.S. serotypes, positive hybridization could consistently be detected throughout the brain, kidneys, lungs, heart, liver, and spinal cord. However, greater concentrations of virus were demonstrated in the external granular layer of the cerebellum of the brain (Fig. 2c and d), in renal tubule epithelial cells (Fig. 3a, b, and c), and cells lining the tertiary bronchi of the lungs (Fig. 3d and e). In addition to the hybridization found in these organs, viral replication could be demonstrated occasionally in other tissues of embryos, such as the retina (Fig. 3f and g), skin, tongue, and breast muscle.

Of the six tissues in which virus was consistently demonstrated, more viral RNA was found in the brains and kidneys, with less in the spinal cord and liver, whereas the amount of BTV RNA found in the hearts and lungs was intermediate relative to the other tissues (Table 1). Although the embryos were severely hemorrhagic, the positive signals in BTV-infected sections were specific and not a result of

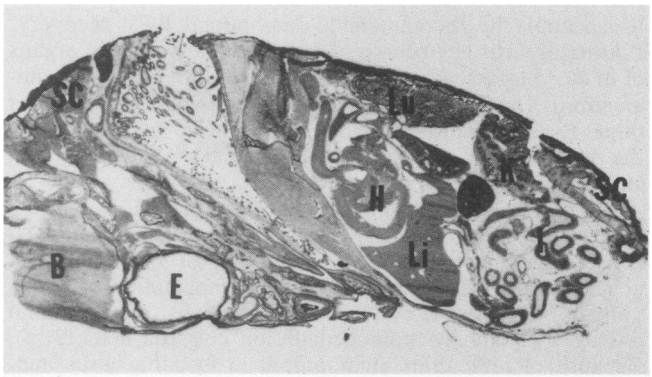

FIG. 1. Hematoxylin-and-eosin-stained longitudinal section of a 15-day-old chicken embryo. Individual organs: B, brain; E, eye; H, heart; Li, liver; Lu, lung; I, intestine; S, spleen; SC, spinal cord; K, kidney.

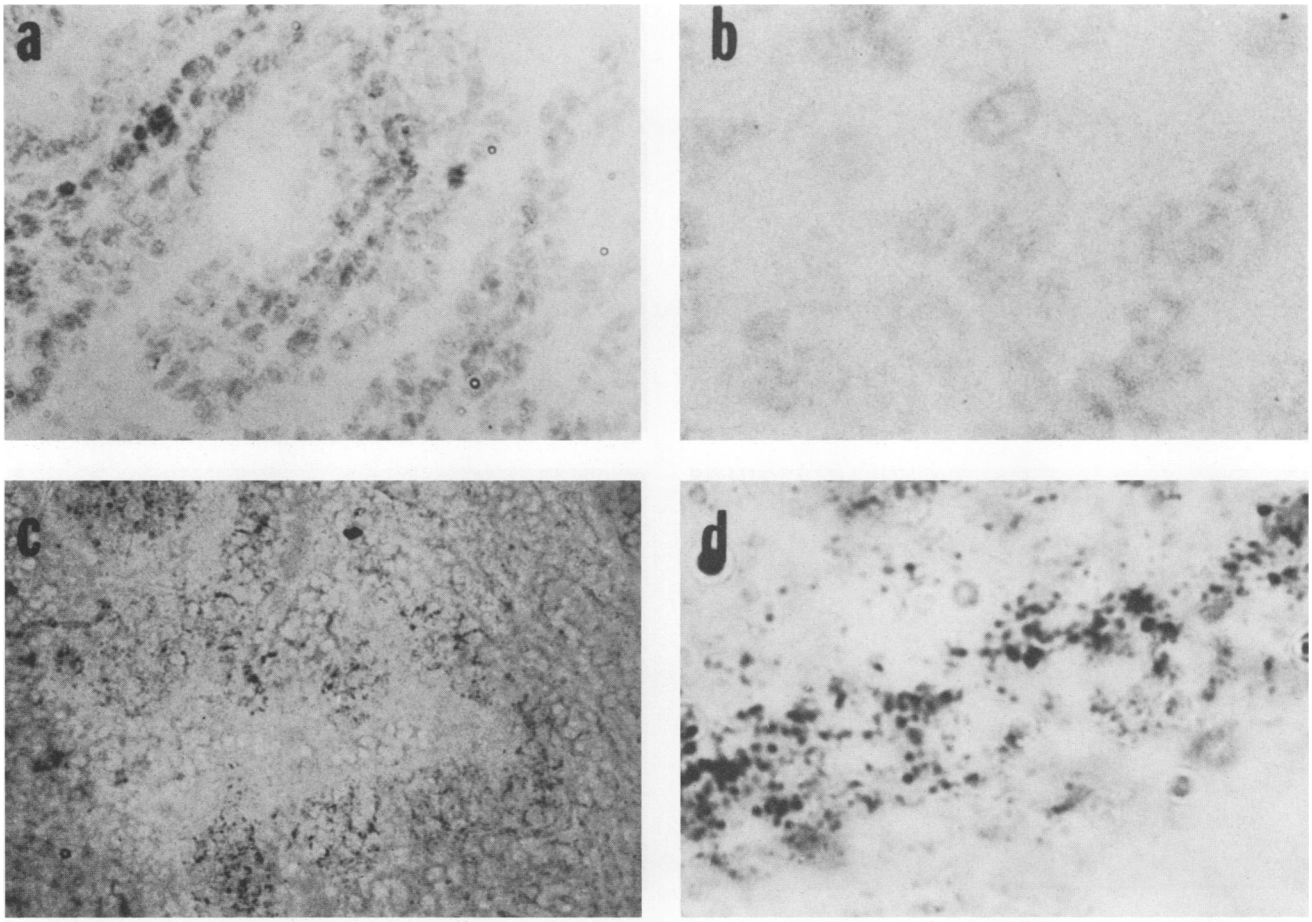

FIG. 2. In situ hybridization of sections of the brain from an uninfected 15-day-old embryo (a, ×250; b, ×1,000) and a BTV 11-infected 15-day-old embryo (c, ×250; d, ×1,000) harvested 4 days after infection.

extensive lesions in blood vessels, because hybridization was not seen in the embryos infected with NDV, which, like BTV, causes hemorrhages in embryos. Embryo sections infected with IBV, another unrelated virus which served as an additional control, were also negative.

Hybridization, however, was found in both the brains and kidneys of embryos harvested 5 days after infection with either serotype of EHDV (Table 1), although there was no evidence of gross pathology in these ECE and these strains did not kill embryos. The relative amount of hybridization was less in the brain than the kidneys of EHDV-infected embryos, and the amount in both these tissues was considerably less than that seen in the brain and kidneys of BTV-infected embryos. Since it had been reported that the segment 3 BTV 17 probe was not specific for EHDV 1 RNA in transfer blot assays (20), a dot blot assay with extracted RNA was used to compare EHDV, BTV serotype 11, and BHK-21 RNA (Fig. 4). Under the conditions used, the probe did have specificity for EHDV as well as BTV RNA. However, the amount of hybridization to BTV was considerably greater than that to EHDV RNA. Attempts to eliminate the in situ hybridization to EHDV by increasing the hybridization temperature to 42°C resulted in less hybridization in both the EHDV- and BTV-infected tissues. At 45°C, hybridization was not found in tissues infected with either virus; however, this may be the result of cell disintegration found at this temperature.

Kinetics and dose-response studies. BTV infection was examined by in situ hybridization of ECE at various intervals of time after infection with various concentrations of virus. Tenfold dilutions ranging in concentration from $10^{2.5}$ to $10^{5.5}$ 50% egg-lethal doses (ELD_{50}s) per ml of BTV serotype 11 were used, and the embryos were harvested at 4, 8, 16, 24, 48, 72, 96, and 120 h after infection or when the embryos died (from 72 h on). The earliest that replication of either virus could be detected was 24 h after viral inoculation with the $10^{4.5}$ and $10^{5.5}$ ELD_{50} doses (Table 2), and the effect of decreasing concentrations of virus tended to extend the time at which viral RNA could be detected, so that hybrid-

TABLE 1. Viral specificity of BTV 17 segment 3 cDNA in in situ hybridization assays

Virus	Virus detected[a]					
	Brain	Kidney	Heart	Lung	Spinal cord	Liver
BTV[b]	++++	++++	+++	++	++	+++
EHDV[c]	+	++	−	−	−	−
IBV	−	−	−	−	−	−
NDV	−	−	−	−	−	−

[a] Concentrations from high (++++) to undetectable (−).
[b] BTV serotypes 2, 10, 11, 13, and 17.
[c] EHDV serotypes 1 and 2.

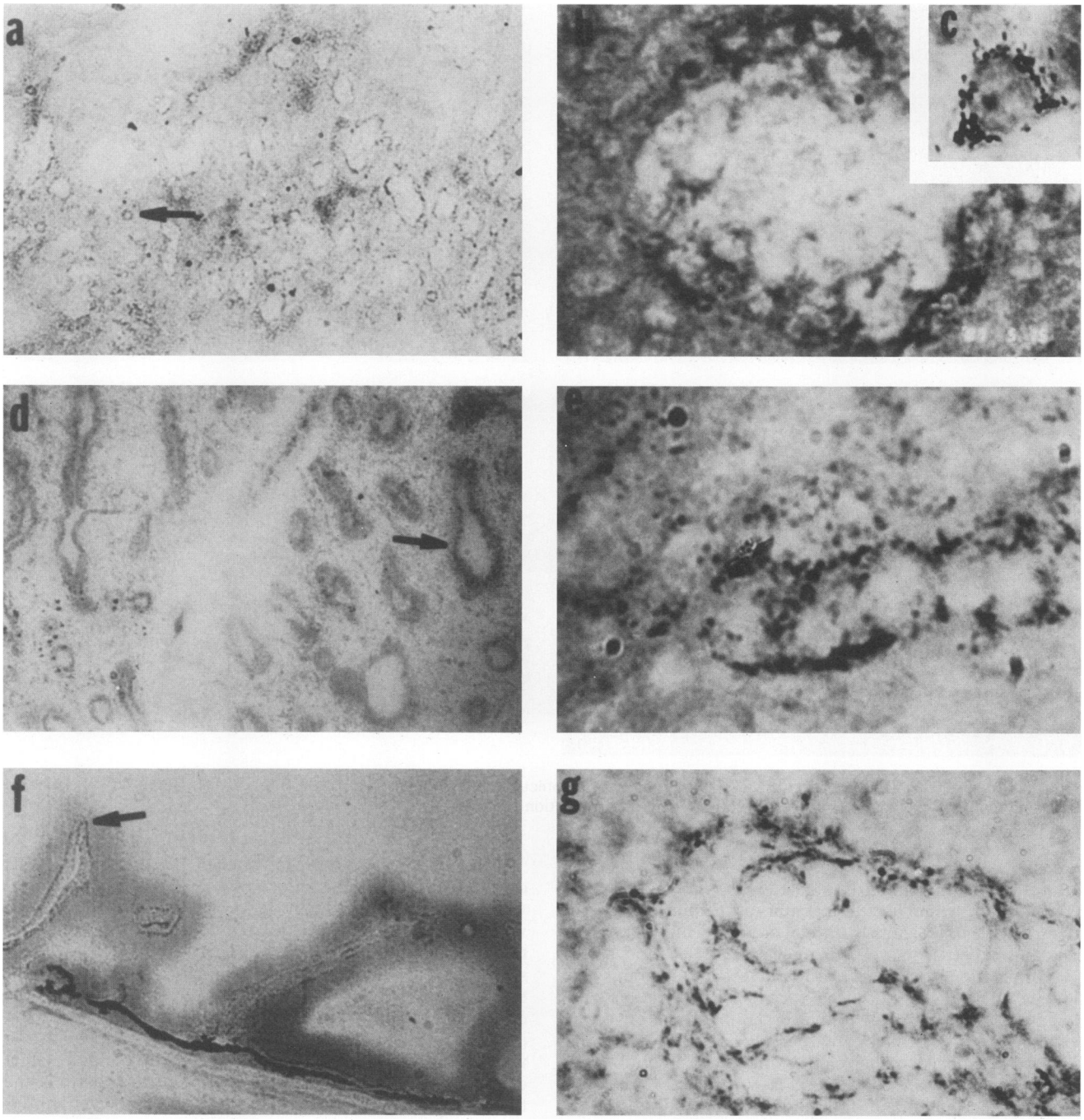

FIG. 3. In situ hybridization of sections of BTV 11-infected kidney (a, b, and c, a single cell supporting viral replication), lung (d and e), and retina of the eye (f and g) of a 15-day-old embryo. Magnification: a, d, and f, 40×; b, e, and g, 1,000×; c, 2,000×. Arrows indicate portions of the 40× magnification which are enlarged in panels d and f.

ization could not be detected until 72 h after infection with the $10^{2.5}$ and $10^{3.5}$ ELD_{50}s.

At 24 h, when there were no visible signs of virus-induced lesions, infection of kidney and brain tissues was evident with the higher concentrations of inoculum (Table 3). By 48 h, BTV could also be detected in the cells surrounding the spinal cord and in the lung and heart. By 72 h, virus was readily detectable throughout the embryo, when the hybridization was similar to that found in the previously described hemorrhagic embryos. Parallel experiments with BTV 17 gave similar results. Because of the low background occasionally seen in the liver, it was sometimes more difficult to identify positive hybridization at the lower concentrations of virus, and consequently, there may have been more BTV replication present in this organ than we have reported at 24 and 48 h.

Detection of BTV from a field specimen. The sensitivity of this assay in detecting viral RNA early in infection, that is, before signs of disease, suggested that it had potential as a diagnostic procedure to confirm BTV in field samples. Iso-

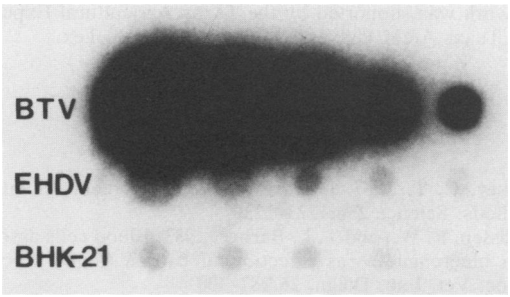

FIG. 4. Dot blot hybridizations of [α-^{32}P]dATP-labeled BTV 17 segment 3 cDNA with RNA from uninfected and BTV- and EHDV-infected chicken embryos. Fourfold dilutions of the RNA were added to each well, starting with 1 μg of RNA in the first well on the left.

lation of a strain of BTV had been made in our laboratory from a blood specimen of a sheep during a 1985 bluetongue outbreak in western Texas. Embryos infected with sheep blood (about 3.7 ELD_{50}s) from which the isolation had been made were examined at 24, 48, and 72 h after inoculation or at the time of death (Table 3). Five embryos were examined each day. After 24 h, viral replication was detected throughout the brain and kidneys and in a few cells of the spinal cord. As with the laboratory strains used above, after the second day, replication in this unmodified strain could be detected in a number of cells in the heart and lungs, but also in the liver. Also at 48 h, positive replication could be seen in other tissues throughout the embryos, including the epithelial cells of the intestinal mucosa (Fig. 5a and b), which had not been found to support viral replication in embryos infected with the laboratory strains; the retina; breast muscle; tongue; and skin. Figure 5c is a 1,000× magnification of an uninfected intestinal mucosa. This Texas field strain appeared to be more virulent, since it affected more tissues and affected tissues earlier than the BTV 11 and 17 laboratory strains.

TABLE 2. Effects of time and viral concentration on the detection of BTV infectivity

Dose (log_{10} ELD_{50})	Hybridization[a] at time postinoculation:					
	16 h	24 h	48 h	72 h	96 h	120 h
2.5	–	–	–	+	+	+
3.5	–	–	–	+	+	+
4.5	–	+	+	+	+	+
5.5	–	+	ND	ND	ND	ND

[a] After i.v. inoculation of 11-day-old chicken embryos with BTV 11. Symbols: –, hybridization was negative in the longitudinal embryo sections; +, positive hybridization was found in the embryo sections; ND, not done.

TABLE 3. Time that RNA was detectable after infection with BTV serotype 11 or 17 or the western Texas field strain

Tissue	Time of detection (h postinoculation)	
	11 or 17[a]	Field strain[b]
Brain	24	24
Kidney	24	24
Spinal cord	48	24
Heart	48	48
Lung	48	48
Liver	72	48

[a] Virus titer, 4.5 ELD_{50}.
[b] Virus titer, 3.7 ELD_{50}.

DISCUSSION

In situ cytohybridization was used to detect BTV infection in developing chicken embryos. Since all the major organs of

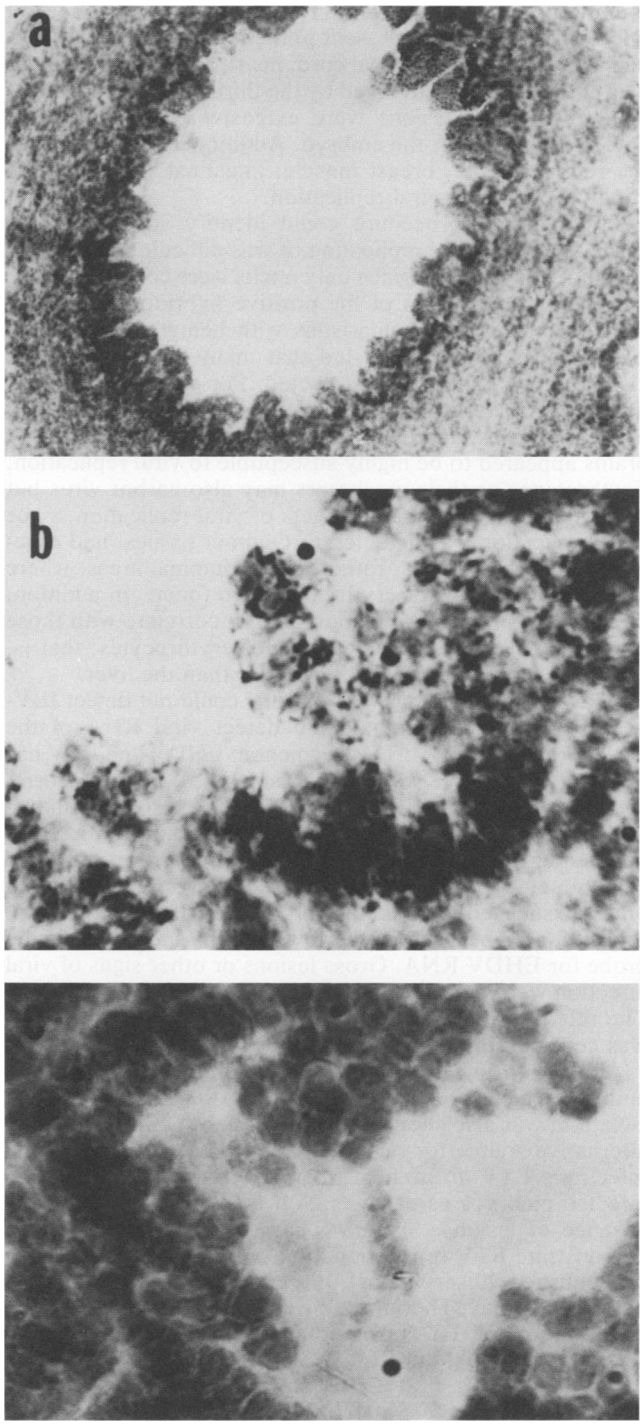

FIG. 5. In situ hybridization of sections of BTV field strain-infected intestines at (a) 40× and (b) 1,000× and of uninfected intestine at 1,000× (c).

a longitudinal section of the embryo can be viewed simultaneously on a single slide, the chicken embryo was a convenient model for examining the pathogenesis of BTV infection, which is known to cause congenital malformations in the developing fetus (16, 19). The initial target organs where replication could consistently be detected as early as 24 h after inoculation of BTV were the brain and kidneys. Similarly, congenital malformations in in utero-infected fetal calves and sheep were also reported to be targeted to the brain (16, 19). At 48 h after BTV inoculation of ECE, viral infection was not only present in the brain and kidneys, but had also spread to the spinal cord, heart, and lungs. The liver was unquestionably involved by the third day. At the time of death, when the lesions were extensive, viral RNA was detected throughout the embryo. Additional tissues such as the retina, tongue, breast muscle, intestinal villi, and skin could also support viral replication.

Although our procedure could identify specific organs associated with viral replication, it was difficult to define the specific cells involved since only nuclei were counterstained. However, the location of the positive hybridization within tissue sections and comparisons with hematoxylin-and-eosin-stained sections suggested that many cell types, especially epithelial cells, were infected. For example, the cells lining tubules in the kidney, the retina, the epithelial mucosa of the intestines, and external layer of the cerebellum of the brains appeared to be highly susceptible to viral replication. Erythrocytes or their precursors may also harbor virus but did not appear to be major targets of viral replication, since viral replication was detected throughout tissues, and especially in epithelial cells, rather than in luminal areas, where high concentrations of erythrocytes are found. In addition, target organs for viral infection did not correlate with those organs having high concentrations of erythrocytes; that is, the brain supported replication earlier than the liver.

Whereas the segment 3 BTV probe could not detect IBV- or NDV-infected cells, it could detect viral RNA of the closely related EHDV. This segment in EHDV and BTV has been shown to have sequence homology (6), and the corresponding proteins of EHDV and BTV have also been shown to share antigenic epitopes (8). Viral replication in EHDV-infected embryos, however, was only detected in the brain and kidneys. Hybridization may not have been found in other tissues either because of the low level of EHDV replication or because of the low level of specificity of the probe for EHDV RNA. Gross lesions or other signs of viral infection were not detected in the EHDV-infected embryos. The relative specificity of the probe used for EHDV RNA was considerably less than for BTV.

Along with in situ hybridization techniques, the chicken embryo, which is the preferred medium for unmodified BTV isolation, was also shown in these studies to offer a potential alternative source for rapid and sensitive diagnosis of BTV infection. BTV from field samples can be confirmed in infected embryos as early as 24 h postinfection and in the absence of lesions. Since we have recently been able to demonstrate BTV replication after only 4 h of hybridization (unpublished observations), the procedure can be done within an 8-h day. Therefore, the amount of time required for confirmation of BTV can conceivably be reduced from several weeks to 2 days.

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