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Pathogenesis of Type II Avian Adenovirus Infection in Turkeys: In Vivo Immune Cell Tropism and Tissue Distribution of the Virus

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Hemorrhagic enteritis virus (HEV), a type II avian adenovirus, causes intestinal hemorrhages and immunosuppression in turkeys. In this study, we exposed turkeys to virulent HEV and examined fractionated spleen cells for the presence of viral DNA by in situ hybridization and amplification of DNA extracted from virus-infected cells by PCR. HEV replication was detected only in the immunoglobulin M-bearing B lymphocytes and macrophage-like cells but not in the $CD4^+$ or $CD8^+$ T lymphocytes. The inability to infect T cells distinguishes type II avian adenoviruses from lymphotropic mammalian adenoviruses which infect and replicate in T cells. Furthermore, these data suggested that HEV-induced immunosuppression in turkeys may be due to the effect of the virus on B lymphocytes and macrophages. We also examined tissue tropism of HEV by in situ hybridization conducted on sections of lymphoid and nonlymphoid tissues. Large numbers of HEV-positive cells were detected in spleen and cecal tonsils. Diminutive viral activity was present in the intestines, the principal site of HEV-induced pathology. Thus, intestinal pathology was not associated with local cytopathic viral replication. This result and our previous observation that cyclosporin A abrogated intestinal hemorrhaging in HEV-infected turkeys strongly suggested that intestinal lesion induced by this virus may be immune system mediated.

Hemorrhagic enteritis (HE) is an acute disease of turkeys caused by hemorrhagic enteritis virus (HEV), a type II avian adenovirus. The disease is characterized by depression, splenomegaly, intestinal hemorrhages, and immunosuppression (3). Although HE has been recognized since 1937 (17), the pathogenesis of the disease is not well understood. Precise identity of the cells that support HEV replication and the mechanism(s) involved in the generation of intestinal lesions remain obscure. Adenoviruses along with rotaviruses have been considered the most common causes of pediatric viral gastroenteritis in humans (2). However, the pathogenic mechanisms of enteric adenoviruses of humans remain unknown because a suitable animal model is lacking. HE in turkeys is highly reproducible after oral inoculation with adenovirus. The possibility of using HE in turkeys as an animal model to study the pathogenesis of human enteric adenoviruses has not been examined.

Mammalian adenoviruses have been shown to replicate in T cells, B cells, and other lymphoid cells (30). Previous studies have suggested that HEV may replicate in the mononuclear phagocytic cells in the turkey (3, 10). Although to date there is no direct evidence that avian adenoviruses replicate in vivo in lymphocytes, we and others have recently obtained data that strongly suggest that lymphoid cells may be susceptible to infection by HEV (24, 28). We have shown that B lymphocytes may be important for viral replication because the levels of viral antigen in the spleen were profoundly reduced in turkeys depleted of B lymphocytes (24). We have also noted that HEV causes a reduction in the relative proportion of immunoglobulin M-bearing (IgM^+) B lymphocytes in the spleen and peripheral blood of infected turkeys (24). Furthermore, HEV has been propagated in vitro in peripheral blood leukocytes (28) and a herpesvirus-transformed B-lymphoblastoid cell line of turkeys (12).

Oral infection of susceptible turkeys with HEV results in well-pronounced splenic enlargement and intestinal hemorrhages in 4 to 6 days (3). The spread of HEV in the body following oral infection is not well understood. Interestingly, HEV replication has not been demonstrated by immunological methods in the intestinal epithelial cells at the site of hemorrhage, although epithelial cell degeneration and sloughing was very much evident (3, 6, 9). Detection of adenovirus replication in rat pneumocytes required the use of in situ hybridization technique when immunological methods failed (22).

Our objectives were (i) to examine the tropism of HEV for various subpopulations of immune cells and (ii) to monitor the spread of HEV to various organs, particularly lymphoid organs. We provide the first direct in vivo evidence that an avian adenovirus infects B lymphocytes and macrophage-like cells but not the $CD4^+$ and $CD8^+$ T lymphocytes. Tissue tropism studies by in situ hybridization revealed that HEV replicated to high levels in the spleens and cecal tonsils of infected turkeys but not in the intestines, thus providing evidence that HEV-induced intestinal hemorrhaging was not due to viral replication in the intestinal epithelial cells. These findings coupled with our earlier observations that experimentally induced immunodeficiency modulated disease outcome (24) have important implications on the involvement of immune mechanisms in viral pathogenesis.

MATERIALS AND METHODS

Turkeys. White Nicholas turkey poults were obtained on the day of hatch from a commercial hatchery (Willmar Poultry Company, Willmar, Minn.) and held in isolation for the duration of the study. Specific-pathogen-free poults lacking anti-HEV antibodies were obtained from the National Animal Disease Center, Ames, Iowa. All experimental groups were reared separately for the duration of study in Horsfall-Bauer-type isolation units supplied with biologically filtered air. Turkeys were provided with food and water ad libidum.

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TABLE 1. Effects of HEV on lymphocyte subpopulations in the spleens of infected turkeys (day 4 p.i.)

Group (n = 3)	Mean % of positive cells[a] ± SD		
	$CD4^+$	$CD8^+$	IgM^+
Controls	22.5 ± 0.7	22.0 ± 1.4	39.0 ± 2.8
HEV-infected turkeys	37.0 ± 1.4[b]	23.0 ± 2.8	23.0 ± 2.0[b]

[a] All viable splenic mononuclear cells were analyzed.
[b] Significantly different from control values ($P < 0.05$).

Viruses. Cell culture-propagated virulent HEV (12) and marble spleen disease virus (12) were kindly provided by K. Nazerian (Avian Disease and Oncology Laboratory, East Lansing, Mich.). Herpesvirus of turkeys was obtained from Solvay Animal Health, Mendota Heights, Minn. Each turkey was inoculated per os with 1,000 50% turkey infectious doses of HEV (21). Chicken embryo lethal orphan virus (32) and inclusion body hepatitis virus (4) were obtained from the American Type Culture Collection (Rockville, Md.).

Enrichment for adherent macrophages. Adherent macrophages were obtained by published protocols (31). Briefly, splenic mononuclear cells were obtained by layering single-cell suspensions of splenocytes on a Histopaque 1083 (Sigma, St. Louis, Mo.) gradient. The mononuclear cells were washed thrice in phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium (Sigma) containing 20% fetal bovine serum (Sigma) at a concentration of 10^7 cells per ml. The cell suspension was incubated in plastic tissue culture plates at 39 C for 2 h. Nonadherent cells were removed by washing the plates five times with PBS. The adherent cells were harvested by scraping with a cell scraper.

Monoclonal antibodies and flow cytometric sorting of lymphocyte subpopulations. Mouse monoclonal antibodies CT4 and CT8 that recognize the chicken homologs of mammalian CD4 and CD8, respectively, were purchased from Southern Biotechnology Laboratories, Birmingham, Ala. A monoclonal antibody to the μ chain of the chicken IgM molecule was a gift from F. Davison (Compton, England). The cross-reactivity of anti-chicken CD4, CD8, and μ antibodies with turkey lymphocytes and staining procedures have been described in detail elsewhere (25). Briefly, spleens were aseptically harvested from turkeys and crushed to make a single-cell suspension of splenocytes. The splenocyte suspension was layered on Histopaque 1083 (Sigma), and mononuclear cells were harvested after centrifugation at 3,000 × *g* for 10 min. The mononuclear cells were stained with fluorescein isothiocyanate-conjugated anti-chicken CD4 or phycoerythrin-labeled anti-chicken CD8 by direct immunofluorescence. B lymphocytes were stained by indirect immunofluorescence, using anti-chicken μ as a primary antibody and fluorescein isothiocyanate-conjugated goat- anti-mouse IgG (Sigma) as a secondary antibody. Purified populations of $CD4^+$, $CD8^+$, and IgM^+ lymphocytes (10×10^6 cells of each phenotype) were obtained by fluorescence-activated cell sorting, using a FACStar Plus (Becton Dickinson, Mountain View, Calif.). A postsort analysis was performed to assess the purity of the sorted lymphocytes. The sorted lymphocytes were >98% pure.

Development of a DNA probe to detect HEV. Viral DNA extracted (20) from purified HEV (obtained from spleens of infected turkeys [16]) was digested with *Eco*RI (GIBCO BRL, Gaithersburg, Md.), and the sizes of restriction fragments were compared with previously published data (33). The *Eco*RI fragments of HEV DNA were cloned into plasmid pGem 3Z (Promega, Madison, Wis.) by standard protocols (18), and the recombinant plasmids were used to transform competent *Escherichia coli* JM109 cells. Recombinant plasmids digested with *Eco*RI were fractionated by electrophoresis on low-melting-point agarose (FMC, Rockland, Maine), and the inserts were purified from the gel by using a DNA purification kit (GIBCO BRL). The gel-purified inserts were labeled with digoxigenin by using a DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.). The labeled inserts were screened for reactivities against DNA from heterologous viruses, namely, type I avian adenoviruses (chicken embryo lethal orphan virus and inclusion body hepatitis virus) and herpesvirus of turkeys, by the Southern blot hybridization assay (1). On the basis of nonreactivity to heterologous viruses, a fragment of the HEV genome, 2,200 bp in size, was selected as the probe for HEV. The size of the insert in the plasmid carrying the 2,200-bp HEV fragment was reduced to 564 bp by further digestion with *Hin*dIII, ligation, and transformation into competent *E. coli* JM109 cells. This 564-bp probe was used for in situ hybridization studies.

In situ hybridization for HEV. In situ hybridization was done on unsorted splenic mononuclear cells, adherent cells, purified subpopulations of lymphocytes, and 6-μm-thick paraffin-embedded tissue sections fixed in paraformaldehyde-lysine-periodate buffer (11) as described earlier (7, 8). Deparaffinized tissue sections or cells deposited on slides were permeabilized by exposure to 0.2 N HCl followed by 2× standard saline citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 70°C, and proteinase K (Sigma) at 37°C. Proteinase K-treated slides were treated with 2× SSC containing 100 μg of DNase-free RNase (Boehringer Mannheim) per ml at 37°C for 30 min and exposed to 20% (vol/vol) glacial acetic acid for 20 s at 4°C. Slides were postfixed in 4% paraformaldehyde in PBS for 1 h at room temperature, and DNA was denatured by being incubated in a solution of 95% formamide (U.S. Biochemical, Cleveland, Ohio) in 0.1× SSC at 65°C for 15 min and immediately dipped in ice-cold 0.1× SSC for 2 min. Hybridization was performed with 500 ng of digoxigenin-labeled probes (HEV probe or a 500-bp *Hin*dIII-digested lambda DNA fragment as a control probe) per ml at 42°C under Parafilm for 18 h. After posthybridization washes, the hybrids were visualized by the colorimetric detection method, using alkaline phosphatase-conjugated antidigoxigenin antibodies (8). Slides were counterstained with neutral red or eosin, mounted under a coverslip with Permount, and examined under a microscope. All sections from control and HEV-infected turkeys were evaluated semiquantitatively. Tissues were classified as no signal (0), low (1), mild (2), moderate (3), or high (4) numbers of positive cells. Signal prevalence scores of 1, 2, 3 and 4 were defined as 1 to 5 positive cells, 6 to 10 positive cells, 11 to 50 positive cells, and >100 positive cells, respectively, per 10 high-power microscopic fields (magnification of ×40).

PCR. DNA sequencing of the 564-bp *Eco*RI-*Hin*dIII fragment of HEV DNA was performed by using a *Taq* DyeDeoxy Terminator Sequencing Kit (Applied Biosystems, Foster City, Calif.). Vector-derived SP6 and T7 sequences were used as primers for the ends of the cloned DNA fragment. The sequences were analyzed by the Genetics Computer Group (Madison, Wis.) sequence analysis software package. Comparison of the HEV sequences with those in the GenBank revealed high degree of homology to the penton base protein gene of various adenoviruses. The sequence information was used to design a set of primers, each 22 bases long, to amplify a 234-bp fragment. The sequences of the sense and the antisense primers were 5′-CCAGCTACAATCGACATTGACA-3′ and 5′-TGTATCTCGAACAGGAGGCAAG-3′, respectively. The PCR was performed with sample DNA from 10^6 cells. The conditions for PCR amplification were optimized (0.1 μM each primer, 1.5 mM $MgCl_2$, and 2.0 U of *Taq* polymerase per 100-μl reaction mixture) by using a PCR core kit (Boehringer Mannheim). A total of 30 cycles were carried out in a thermocycler (Perkin-Elmer Cetus), each cycle consisting of denaturation for 1 min at 94°C, annealing for 30 s at 65°C, and extension for 1 min at 72°C. To ensure complete synthesis, the last cycle was extended at 72°C for 10 min. Aliquots (25 μl) of amplified products were separated by electrophoresis on 2% agarose gels, and bands were visualized by ethidium bromide staining.

Experimental plan. To study the immune cell tropism of HEV, two groups of 4-week-old birds (n = 3), obtained from a commercial hatchery at 1 day of age, were used. One group was inoculated with HEV (1,000 50% turkey infectious doses per bird) orally (21). The other group of birds was mock infected and served as a virus-free control. Because the highest level of viral antigen is reached in the spleen on day 4 postinfection (p.i.) (5), we harvested spleens from the experimental animals on day 4 p.i. The spleens were processed to obtain adherent cells, and flow cytometric analysis of subpopulations of lymphocytes was performed as described above. Analysis of relative proportions of lymphocyte subsets in the spleen was performed on all infected and virus-free birds. However, purified subsets of lymphocytes were obtained by flow cytometric sorting of splenic mononuclear cells from two turkeys each from HEV-infected and virus-free groups.

To study the viral replication kinetics, two groups of 4-week-old specific-pathogen-free birds (n = 18) were used as described above. At 24-h intervals, three birds from each group were euthanized, and tissue samples from intestines, bursas of Fabricius, cecal tonsils, thymuses, and spleens were collected in paraformaldehyde-lysine-periodate buffer for analysis by in situ hybridization.

RESULTS

Detection of HEV in the immune cells of the spleen by in situ hybridization. All turkeys infected with HEV showed typical splenomegaly on day 4 p.i. indicative of successful infection. Spleens were harvested to analyze and purify subpopulations of immune cells.

The results of flow cytometric analysis of lymphocyte subpopulations are shown in Table 1. In confirmation of our earlier findings (24), infection of turkeys with HEV caused a

TABLE 2. Analysis of splenic immune cells of turkeys for HEV DNA by in situ hybridization

Cell type	Mean % of cells positive for HEV[a]	
	Turkey 1	Turkey 2
Unsorted mononuclear	1.0	1.2
Adherent	2.6	3.3
IgM^+	2.0	2.7

[a] Five hundred cells were examined at a magnification of ×40. No HEV was detected in the immune cells from mock-infected virus-free turkeys. HEV DNA was not detectable in $CD4^+$ or $CD8^+$ lymphocytes in either turkey 1 or turkey 2.

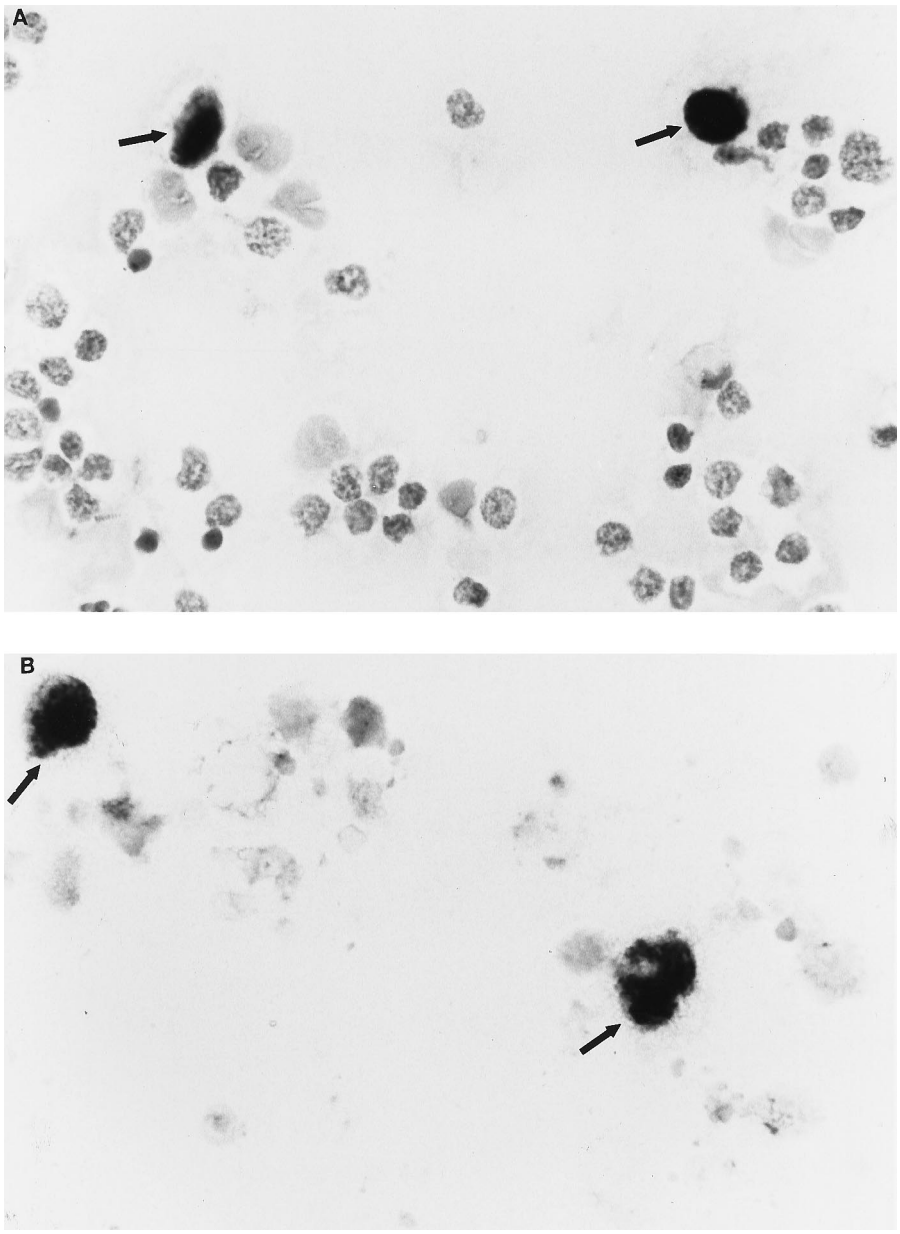

FIG. 1. Detection of HEV DNA in splenic mononuclear cells by in situ hybridization (magnification, ×100). (A) Unsorted mononuclear cells; (B) adherent cells; (C) IgM^+ B lymphocytes; (D) T lymphocytes ($CD4^+$ and $CD8^+$ cells in equal proportion). Arrows point to HEV-positive cells.

reduction in the relative proportion of IgM^+ cells and an elevation in the relative proportion of $CD4^+$ cells in the spleen. HEV caused no detectable changes in the relative proportions of $CD8^+$ cells.

Unsorted splenic mononuclear cells, adherent cells, and purified subsets of lymphocytes obtained from HEV-infected and virus-free turkeys were examined for the presence of HEV DNA by in situ hybridization (Table 2 and Fig. 1). Mononuclear cells from mock-infected turkeys were negative. Also, no positivity was detected when mononuclear cells from infected turkeys were hybridized with a negative control probe (digoxigenin-labeled *Hin*dIII-digested lambda DNA fragment), thus establishing the specificity of HEV detection. HEV DNA was detected in the nuclei of unsorted mononuclear cells from HEV-infected birds (Fig. 1A). Approximately 1% of the unsorted mononuclear cells were HEV positive (Table 2).

Examination of splenic adherent cells for HEV DNA revealed that the mean percentage of virus-positive cells was 2.6 to 3.3 (Table 2). The morphology of the positive cells was consistent with that of macrophages (Fig. 1B). Analysis of purified subpopulations of splenic lymphocytes for HEV DNA revealed that viral DNA was detectable only in the IgM^+ B lymphocytes (Fig. 1C), not in the $CD4^+$ or $CD8^+$ lymphocytes (Fig. 1D). The HEV-positive IgM^+ B lymphocytes appeared enlarged with scant cytoplasm, and the viral DNA was localized in the nucleus (Fig. 1C). The mean percentage of HEV-positive IgM^+ cells was 2.0 to 2.7 (Table 2). The proportions of macrophages and IgM^+ cells in the splenic mononuclear cells

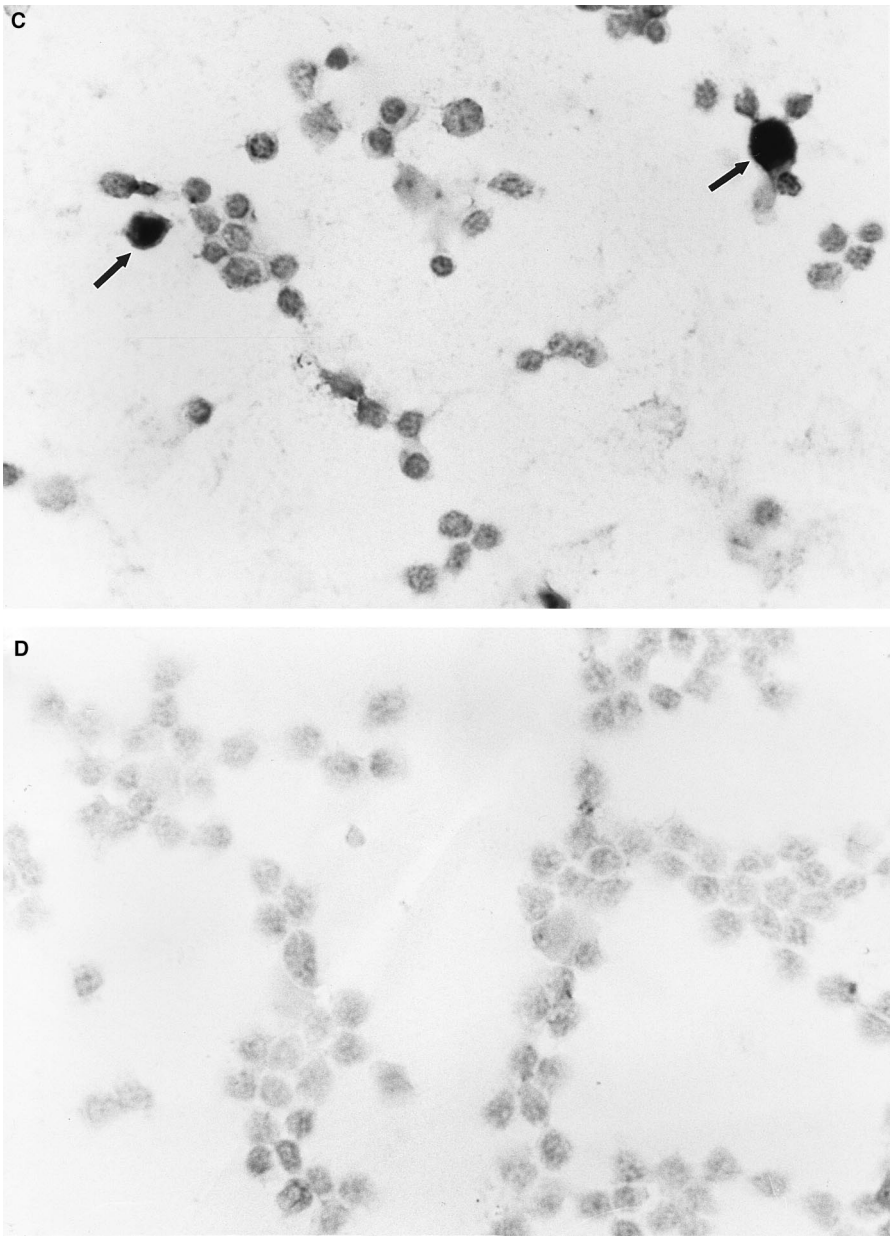

FIG. 1—*Continued.*

of turkeys were estimated to be 5 to 8% (23a) and ~30% (25), respectively. Thus, ~2.9% of adherent cells and 2.3% of IgM^+ cells infected with HEV translates to a total of ~1.0% of HEV-infected mononuclear cells. Taken together, these data suggest that macrophage-like cells and IgM^+ B lymphocytes and not the T lymphocytes were the in vivo targets for HEV replication in the spleen.

Detection of HEV DNA in the splenic mononuclear cells by PCR. To confirm the results of in situ hybridization analyses, PCR was used to detect HEV DNA in the lymphocyte subpopulations. The PCR was used to increase the degree of sensitivity of detection, especially to ascertain that HEV does not infect T cells because a very low copy number of target HEV DNA in the T cells may have escaped detection by nonradioactive in situ hybridization. A total of 10^6 lysed mononuclear cells or purified lymphocyte subsets were analyzed with PCR primers amplifying a 234-bp fragment of the HEV genome. As illustrated in Fig. 2, HEV DNA was detectable in the unsorted mononuclear cells and IgM^+ cells. Viral DNA was not detectable in the $CD4^+$ and $CD8^+$ lymphocytes. These data confirmed the results obtained by in situ hybridization.

Spread of HEV to various lymphoid organs after oral inoculation. The replication kinetics of HEV in various lymphoid organs and intestines following infection by the oral route was studied by in situ hybridization. The tissue distribution and the extent of HEV replication in various organs on different days p.i. are shown in Table 3. On day 1 p.i., virus was first detected at very low levels in the lamina propria of the duodenum, cecal tonsils, and the bursa of Fabricius, suggesting that HEV may enter the bloodstream after initial replication in these organs. Although the virus-positive cells in these organs appeared to belong to the lymphoid lineage, the precise identity or the

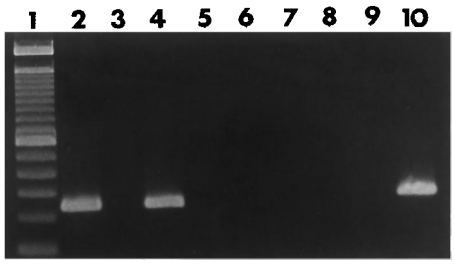

FIG. 2. Detection of HEV DNA in subpopulations of splenic mononuclear cells by PCR. Lanes: 1, 100-bp marker; 2, mononuclear cells from an HEV-infected bird; 3, mononuclear cells from a virus-free control bird; 4, IgM^+ B cells from an HEV-infected bird; 5, IgM^+ B cells from a virus-free control bird; 6, $CD4^+$ T cells from an HEV-infected bird; 7, $CD4^+$ T cells from a virus-free control bird; 8, $CD8^+$ T cells from an HEV-infected bird; 9, $CD8^+$ T cells from a virus-free control bird; 10, positive control plasmid containing an insert from HEV genome from which the primer sequences were designed.

phenotype of these cells was not determined. HEV was detected in the spleen as early as 2 days p.i. Very high levels of viral replication were detected in the spleen and the cecal tonsils on day 3 p.i. The highest level of HEV was detected in the spleen (Fig. 3A) on the day 4 p.i., which coincided with the appearance of intestinal hemorrhages, extensive lymphoid depletion, and hyperplasia of the mononuclear phagocytes in the spleen. The cells that replicated HEV in the spleen (Fig. 3A) and cecal tonsils (Fig. 3B) were distributed evenly in the tissues and were either lymphocytes or the cells of the mononuclear-phagocytic system. The thymic medulla contained macrophage-like cells that were positive for HEV on day 4 p.i. (Fig. 3C). On day 4 p.i., although there was extensive hemorrhage, necrosis, and sloughing off of the duodenal mucosal cells, low viral activity was detected at the site of hemorrhage. Only a few HEV-positive cells were present in the lamina propria; the mucosal epithelium was devoid of detectable virus-infected cells (Fig. 3D). These data confirmed our previous speculation that HEV-induced intestinal lesions are not due to the extensive local viral replication in the intestine. HEV was detectable in the bursa of Fabricius at low levels throughout the course of infection (Fig. 3E). These data indicated that the spleen and cecal tonsils were the principal target organs for HEV replication.

DISCUSSION

In this study, we used in situ hybridization and DNA amplification by PCR to examine the presence of the genome of a type II avian adenovirus (HEV) in subpopulations of immune cells in turkeys. Our findings provide first direct evidence that in vivo, HEV may infect and replicate in the IgM^+ B lymphocytes and macrophages. Susceptibility of these cell types to HEV in vitro was noted in earlier studies (12, 28). The fact that all viruses cytopathic for the immune cells cause various levels of immunosuppression has been known for many years (29, 34). Infection of mononuclear cells by HEV may explain one of the mechanisms of immunosuppression associated with HE in turkeys (3, 23).

We had earlier reported that HEV induced a reduction in the proportion of IgM^+ cells in the spleen and peripheral blood of turkeys (24). Now we show that IgM^+ B lymphocytes were infected with HEV, implying that HEV-induced IgM^+ B-cell depletion may be partly due to lytic infection of B lymphocytes. Direct viral cytopathic effect alone did not account for the reduction in the relative proportions of IgM^+ B lymphocytes (16% reduction) in the spleens of infected turkeys, because the percentage of HEV-positive IgM^+-cells as determined by in situ hybridization was only ~2.3. These findings suggested two possibilities: (i) cytopathic viral replication may not be the only mechanism involved in the depletion of IgM^+ B lymphocytes, or (ii) we have underestimated the HEV-infected IgM^+ cells. We have recently obtained data that the spleens of turkeys infected with HEV contained a large number of IgM^+ apoptotic cells (unpublished data). Thus, we speculate that HEV-induced apoptosis may be one of the alternate mechanisms involved in the depletion of IgM^+ B lymphocytes in addition to cytopathic viral replication. The possibility that we may have underestimated the number of HEV-positive IgM^+ cells cannot be excluded, since virus-infected dying or dead cells may have been included in the pellet when viable mononuclear cells were purified on a Histopaque gradient for sorting by flow cytometry.

We studied by in situ hybridization the tropism and spread of HEV to various lymphoid organs and the intestine following oral inoculation. HEV was detected in the intestine, bursa of Fabricius, and cecal tonsils at day 1 p.i., and the cellular morphology of the positive cells was consistent with that of lymphocytes. These data implied that HEV may replicate in these organs in the lymphocytes initially before entering the systemic circulation and eventually reaching the spleen. The precise identity of the HEV-infected lymphocytes in the gut-associated lymphoid tissue needs to be determined. Consistent with earlier reports (5, 9), the virus reached peak levels in the spleen on day 4 p.i., coinciding with the occurrence of hemorrhagic lesions in the intestine.

In HE, intestinal hemorrhages are more prominent in the duodenum than in other parts of the intestines (3). Interestingly, very low levels of virus-specific signals detectable by in situ hybridization were observed in the duodenum on days 3 and 4 p.i., irrespective of the presence or absence of intestinal lesions. The small numbers of virus-positive cells detected in the intestine were always located in the lamina propria of the duodenum; no virus-positive cells were detected in the mucosal epithelial cells. These data indicated that degeneration and sloughing of the intestinal epithelial cells associated with HEV infection may not be sequelae to cytopathic viral infection. These findings support previous data which strongly suggested that intestinal hemorrhage in HE may be due to an immune system-mediated phenomenon (14, 15, 24). Immunosuppression induced by treatment of turkeys with cyclosporin A protected against HEV-induced intestinal hemorrhage without affecting viral replication in the spleen (24), providing evidence that activated T lymphocytes may play a pivotal role in the

TABLE 3. Prevalence of virus-specific signals in the tissues of turkeys inoculated orally with HEV as determined by in situ hybridization

Days post-infection	No. of HEV-positive birds/no. examined (mean score of virus-positive cells)[a]				
	Bursa of Fabricius	Cecal tonsils	Duodenum	Spleen	Thymus
1	2/3 (0.6)	3/3 (1.0)	2/3 (0.6)	0/3 (0)	0/3 (0)
2	3/3 (1.0)	3/3 (1.0)	0/3 (0)	2/3 (0.6)	0/3 (0)
3	3/3 (1.0)	3/3 (3.0)	2/3 (0.6)	3/3 (3.0)	3/3 (1.0)
4	3/3 (1.3)	3/3 (3.3)	3/3 (1.0)[b]	3/3 (4.0)	3/3 (1.6)

[a] The HEV^+ cells in the sections were quantitated by counting 10 high-power microscopic fields (magnification of ×40) per tissue. On each sampling day, three birds were sampled from each group, and signal prevalence was scored as described in Materials and Methods.

[b] Pronounced intestinal hemorrhages were detected in all HEV-infected birds.

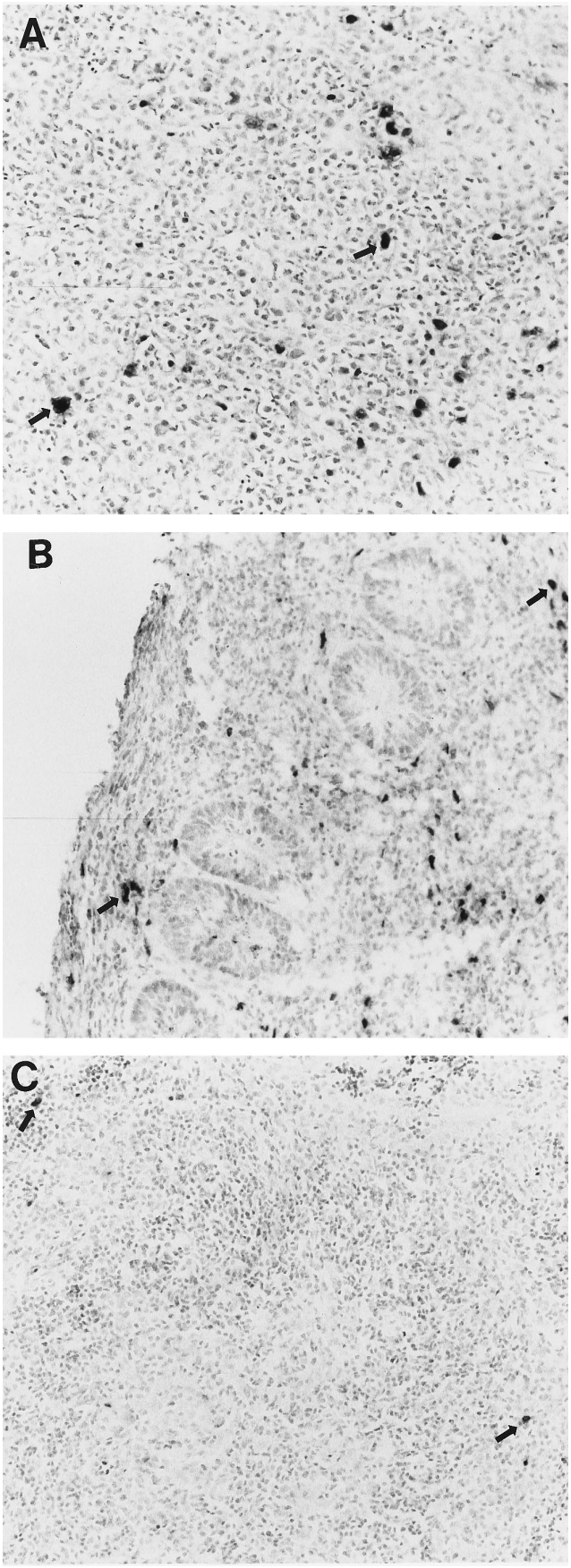

FIG. 3. Tissue distribution of HEV on day 4 p.i. as detected by in situ hybridization (magnification, ×18.6). (A) Spleen; (B) cecal tonsils; (C) thymus; (D) duodenum; (E) bursa of Fabricius. Arrows indicate representative HEV-infected cells.

genesis of HEV-induced intestinal lesions. In this and a previous study (24), we noted an increase in the proportion of $CD4^+$ T lymphocytes in the spleens of HEV-infected turkeys. It was reported recently that the proportion of a subpopulation of $CD4^+$ T lymphocytes ($CD4^+$ $Leu8^-$) was increased in patients with inflammatory bowel disease (13). Whether the modulation of T lymphocytes by infection with HEV in turkeys has relevance to the pathogenesis of HE is not known. Hemorrhagic intestinal lesions similar to HE have been reproduced in experimental animals treated with either endotoxin or tumor necrosis factor alpha (TNF-α) (26, 27). Preliminary studies indicated that thalidomide, a specific antagonist of TNF-α (19), inhibited the development of HEV-induced intestinal hemorrhages in turkeys (23a). If intestinal bleeding in the turkeys infected with HEV is an immune system-mediated phenomenon, the roles of proinflammatory cytokines, particularly TNF-α, need to be examined.

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