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Functional Diversity in Vascular Endothelial Cells: Role in Coxsackievirus Tropism

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Six plaque-purified virus isolates were obtained from liver and heart tissues of a DBA/2 mouse infected 7 days earlier with 10^4 PFU of coxsackievirus group B type 3. Each virus isolate was assayed in vitro for infectivity to vascular endothelial cells (VEC) of the liver, lungs, and heart. Both the percentage of VEC infected and the mean progeny PFU produced per infected VEC were determined. Virus isolates from the heart showed greater infectivity and replication in heart VEC than in VEC derived from either the liver or lungs. Similarly, virus isolated from the liver preferentially infected liver VEC. Virus receptor expression varied between VEC populations, as demonstrated by binding studies with a [^{35}S]methionine-radiolabeled heart virus and by enzyme-linked immunoadsorption assay studies with a monoclonal antibody to the coxsackievirus group B type 3 receptor on heart tissue. Finally, the heart and liver virus isolates were injected (10^4 PFU) intraperitoneally into BALB/c mice. After 7 days, the animals were sacrificed, and the hearts, livers, and lungs were evaluated for tissue injury and virus concentrations. Viruses originally isolated from the heart preferentially infected the heart when reinjected into animals and caused severe myocarditis. Viruses originally derived from the liver most consistently reinfected the liver, although significant virus concentrations were also detected in the heart. The liver virus isolates, however, were incapable of causing myocarditis. Thus, selective tropism of viruses for particular organs in vivo corresponds to the ability of these isolates to infect VEC in vitro.

Vascular endothelial cells (VEC) act as an important interface between the vascular space and the parenchyma of an organ. VEC in different organs show distinctive surface characteristics which can influence biological functions (1). Such functions include preferential homing of circulating cells, nutrients, or hormones to selected tissues through receptors expressed at the cell surface. Thus, VEC are not homogeneous throughout the body but have unique properties depending upon the organ and species (2, 9, 15, 17, 20). These differences probably affect normal tissue physiology. The VEC could function in disease pathogenesis by determining the location of tumor metastases or organ tropism of an infectious agent. Auerbach et al. found that tumor cells adhere best in vitro to VEC derived from certain tissues and that the in vitro adhesion profile often resembles metastasis formation in vivo (1). Other investigators found that VEC from human umbilical veins and bovine thoracic aortas show distinct infectivity patterns with herpes simplex virus, polio virus, and coxsackie virus group B type 4 (7). However, it was not resolved whether differences reflected either the species or the tissue source of the VEC.

Generally, picornavirus infections either are asymptomatic or are restricted to mild febrile illnesses with coldlike symptoms (11). Occasionally, infections lead to far more serious manifestations including, but not restricted to, polydermatomyositis (8), orchitis (4), insulin-dependent diabetes mellitus (5), myocarditis (10), meningitis, and encephalitis (19). Clearly, tropism of a particular virus for selected tissues represents one factor influencing disease incidence and severity. A virus which is incapable of infecting insulin-producing beta cells is less likely to cause diabetes than a virus which can infect such cells. Similar considerations of virus tropism undoubtedly affect the development of other virus-associated diseases, including myocarditis. This report addresses the role of VEC as mediators of tissue tropism for coxsackievirus group B type 3-induced myocarditis as observed by using a murine model.

MATERIALS AND METHODS

Animals. BALB/c mice were originally purchased from Cumberland Farms, Clinton, Tenn. Adult male (7 to 9 weeks) and neonatal (0 to 3 days) animals were obtained from colonies maintained at the University of Vermont for these experiments. An adult DBA/2 male mouse (9 weeks) was obtained from Jackson Laboratory, Bar Harbor, Maine.

Virus. Coxsackievirus B3 (Nancy strain) (CVB3) was originally obtained from J. F. Woodruff. Tissue virus isolates were produced by inoculating a DBA/2 mouse intraperitoneally with 10^4 PFU of the stock CVB3 preparation. The animal was sacrificed 7 days after infection, and the heart and liver were aseptically removed, weighed, and homogenized in Dulbecco minimal essential medium (DMEM) (GIBCO Laboratories, Grand Island, N.Y.) containing 2% fetal bovine serum (FBS) (GIBCO) and antibiotics (100 U of penicillin and 100 μg of streptomycin per ml) (GIBCO). Cellular debris was removed by centrifugation at 300 × *g* for 10 min. Supernatants were serially diluted, and titers on HeLa cell monolayers were determined in the plaque-forming assay. Plaques were identified with an inverted microscope, and the agar overlying the individual plaques was carefully removed. Fresh DMEM–2% FBS was added to the agar from each plaque in separate tubes, and the mixtures were vortexed to release maximal virus. The agar was removed by centrifugation at 300 × *g* for 10 min, and the supernatant was subjected to a second round of plaque purification. The virus isolates were added to separate 25-cm^2 tissue culture flasks (Corning Glass Works, Corning, N.Y.) containing confluent HeLa cell monolayers. After overnight incubation, the monolayers showed greater than

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90% cytopathic effect. The virus preparations were centrifuged to remove cellular debris, divided into aliquots, and frozen at −70°C until use. Titers were determined for samples of each virus isolate.

Preparation of VEC. Livers, hearts, and lungs of neonatal BALB/c mice were removed, minced, and rinsed with two to three changes of phosphate-buffered saline (PBS). The minced tissue was digested at 37°C for 45 min with a mixture of 0.2% collagenase and 0.1% DNase in DMEM containing 5% FBS. The digested tissue was disrupted by repeated pipetting, and the resulting cell suspension was centrifuged at 160 × *g* to pellet the cells. The cell pellet was suspended in 55% isotonic Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.), and a gradient was formed by layering 37% Percoll, 29% Percoll, and saline on top. The gradient was centrifuged at 400 × *g* for 15 min. Dead cells were found mostly in the top band of the gradient, while viable endothelial cells were found at the interface between the 37% and 55% layers. The endothelial cells were removed, washed, and cultured in 25-cm^2 tissue culture flasks in DME/F12 (GIBCO) containing 5% FBS, 20 U of heparin per ml, 10 μg of endothelial cell growth supplement (Sigma Chemical Co., St. Louis, Mo.) per ml, 5 μg of insulin (Sigma) per ml, 5 μg of transferrin (Sigma) per ml, 5 ng of selenium (Sigma) per ml, and 200 μg of Endo-Gro (VEC TEC, Inc., Schenectady, N.Y.) per ml. Endothelial cells were subcultured by brief (2-min) treatment with 0.01% trypsin at 37°C. This further enriched the culture with endothelial cells, because they detached from the surface first and fibroblasts preferentially remained attached. Preparations were judged to contain 90% or more endothelial cells, as reported previously (C. E. Haisch, P. A. Lodge, S. A. Huber, and F. T. Thomas, Transplantation, in press).

Infectious center assay. The infectious-center assay was used to determine the percentage of VEC infected. Monolayers containing approximately 5 × 10^4 VEC in 6-mm-diameter tissue culture wells (flat-bottom polystyrene tissue culture 96-well plates; Corning Glass Works) were incubated with 5 × 10^6 PFU of virus for 1 h, washed, and cultured with DMEM–5% FBS for 19 h. The monolayers were washed and treated with 0.25% trypsin (GIBCO) to produce single-cell suspensions. The cells were incubated with 100 μg of CVB3 virus-neutralizing antibody (clone 8A6) per ml for 30 min at room temperature and counted, and 100 cells were added to HeLa cell monolayers in 200 μl of DMEM–5% FBS. The cultures were incubated for 45 min at 37°C and then overlaid with 0.6% agar in DMEM–5% FBS. After 2 days, 10% buffered Formalin was added to inactivate the virus, the agar was removed, the monolayers were stained with 2% crystal violet, and the plaques were counted. Each plaque was considered to represent a single infected cell.

Monoclonal antibodies MAb. The hybridoma clones producing an immunoglobulin M (IgM) CVB3-neutralizing antibody (8A6) and an IgM antibody to a heart autoantigen (10A1) have been described in detail elsewhere (21). Hybridoma clones producing antibodies to class I (K^dD^d, clone 34-7-23S) and class II (IA^d, clone MK-D6) major histocompatibility complex antigens were obtained from the American Type Culture Collection (Rockville, Md.). Hybridoma cells were grown in ascites form in BALB/c mice treated with 0.5 ml of 2,6,10,14-tetramethylpentadecane (pristane; Sigma) and 500 R of irradiation. Immunoglobulin was purified from ascites fluid by precipitation with 40% ammonium sulfate and by Sephadex G-10 chromatography (Pharmacia).

Infection of mice. Mice were infected intraperitoneally with 10^4 PFU of virus in 0.5 ml of PBS. Mice were sacrificed by sodium pentobarbital overdose.

Organ virus titer. Organs were removed aseptically, weighed, and homogenized in DMEM–2% FBS. Cellular debris was removed by centrifugation at 300 × *g* for 10 min, and the titers of virus in the supernatants were determined by using the plaque-forming assay (21).

Histology. Tissue sections were fixed in 10% buffered Formalin and stained with hematoxylin and eosin. Inflammation was scored on a scale of 0 to 4 by S.A.H., with 0 representing no inflammation and 4 representing confluent lesions throughout the tissue section.

Enzyme-linked immunosorbent assay. VEC in tissue culture plates (6-mm-diameter wells) were fixed with 0.3% glutaraldehyde for 30 s, washed six times with PBS containing 0.05% Tween 20, incubated with 10 μg of primary MAb for 30 min at room temperature, washed six times, and incubated with a 1:100 dilution of urease-conjugated goat anti-mouse IgM/IgG or goat anti-rat IgG antibody (Sigma) for 30 min. The secondary antibody was removed by a washing with PBS-Tween 20, followed by a washing with double-distilled water, and 50 μl of the urease substrate (0.008 g of bromcresol purple, 0.1 g of urea, and 0.074 g of EDTA in 100 ml of distilled water; pH 4.8) for 60 min at 37°C. Optical density was read at 599 nm. Specific absorbance was determined by subtracting optical density values obtained in wells without the primary antibody from values of samples with primary antibody (21).

Radiolabeled-virus binding studies. Confluent monolayers of HeLa cells in 75-cm^2 flasks were washed with methionine-free medium (DMEM-M) and incubated in DMEM-M for 4 h. The medium was removed, and 10^7 PFU of a plaque-purified heart variant (H3) were added in 0.5 ml of DMEM-M containing 2% dialyzed FBS. The cultures were incubated for 90 min at 37°C, the monolayers were washed with DMEM-M, and 10 ml of DMEM-M containing 0.5% dialyzed FBS was added. After 2 h at 37°C, 1 mCi of [^{35}S]methionine was added, and the incubation was continued overnight until cytopathic effect was 90% complete. The cells and supernatant were removed and alternately frozen and thawed three times. The cellular debris was removed by centrifugation at 1,000 × *g* for 10 min. Triton X-100 (Sigma) was added to the supernatant to a final concentration of 1%, and the supernatant was layered on 30% sucrose–1% Triton X-100. The samples were centrifuged at 100,000 × *g* for 4 h. The pellet was suspended in and dialyzed against PBS. The titers and radioactivity were determined for the resulting virus preparation, which was then divided into equal portions, and frozen at −70°C. The virus contained approximately 2,050 cpm/10^4 PFU. For binding studies, 10^4 VEC were cultured with 10^4 to 10^7 PFU of radiolabeled virus alone or with a 10^3-fold excess of unlabeled virus to control for nonspecific binding. After incubation at 4°C for 15 to 30 min, the cells were washed to remove unattached virus and trypsinized, and radioactivity in the cells and supernatant was measured in Ecolite scintillation cocktail (Dupont, NEN Research Products, Boston, Mass.) with a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Specific binding represents the counts per minute bound with radiolabeled virus alone minus the counts per minute bound in the presence of cold virus (21).

Statistics. Statistical evaluations were performed by using either the Wilcoxon ranked score or the Student *t* test.

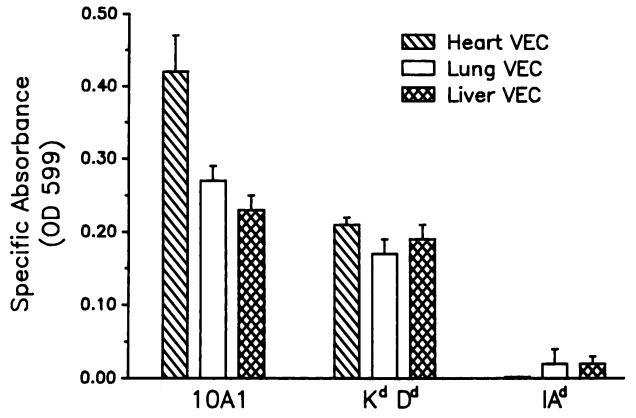

FIG. 1. Antigen expression on VEC. VEC preparations derived from different organs were incubated with 3 μg of MAb to the CVB3 receptor (10A1) per ml and class II (IA^d) and class I (K^dD^d) major histocompatibility complex antigens in a urease enzyme-linked immunosorbent assay. Results represent mean absorbance ± standard error of the mean of four replicates in one of two duplicate experiments. OD 599, Optical density at 599 nm.

RESULTS

Relative reactivity of various organ-derived VEC to MAb binding. The initial experiments evaluated VEC derived from heart, liver, and lung tissues of BALB/c mice for reactivity to three MAb by using the enzyme-linked immunosorbent assay. MAb directed to class I (K^dD^d) and class II (IA^d) major histocompatibility complex antigens and to an antigen on cardiac tissues involved in autoimmunity (10A1) were used. The different VEC preparations were indistinguishable on the basis of reactivity to class I and class II major histocompatibility complex antigens, being equivalently positive for the former and equivalently negative for the latter (Fig. 1). However, the cell populations showed distinctive expression patterns for the MAb to the cardiac antigen. Heart VEC consistently demonstrated approximately twice as much reactivity to MAb 10A1 as did either of the other VEC preparations. These results suggest that heart VEC express more virus receptors and autoantigens recognized by pathogenic effectors per cell than VEC derived from other areas do.

Comparison of virus binding to different VEC. Plaque-purified virus variants were isolated from either liver or heart tissue of a CVB3-infected mouse. One virus variant isolated from the heart (H3) was used to produce [^{35}S]methionine-

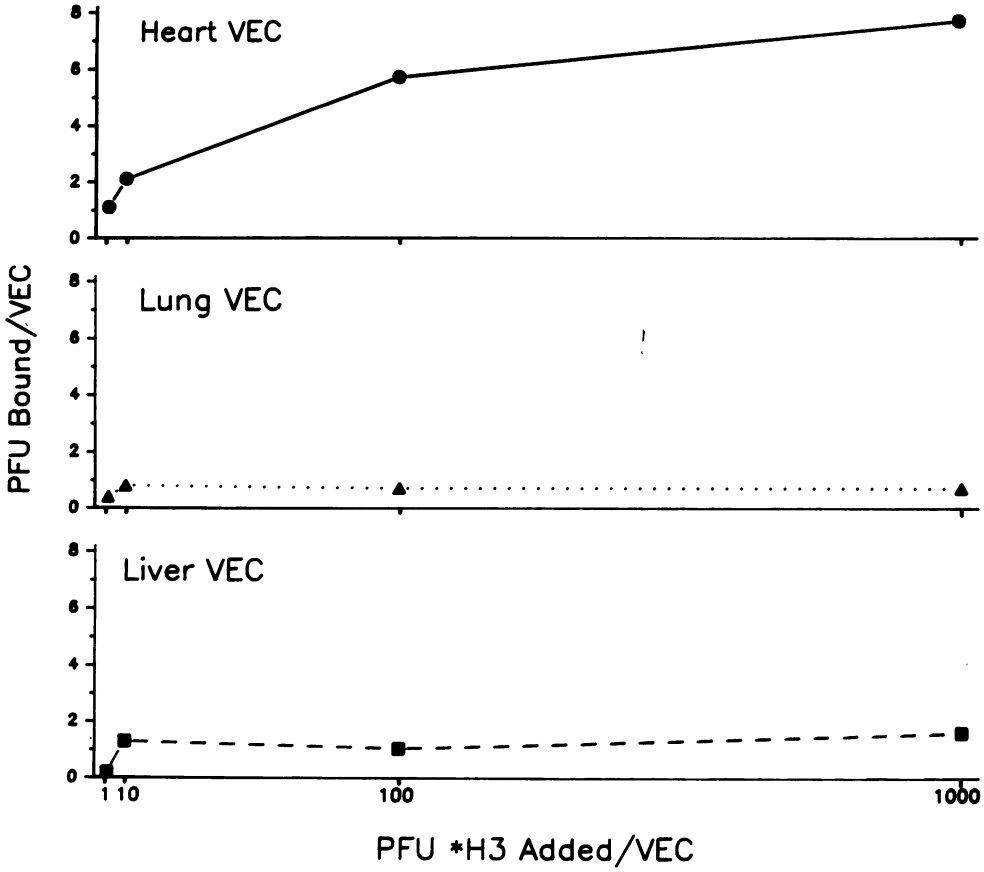

FIG. 2. Virus binding to VEC. A plaque-purified heart isolate (H3) of CVB3 was radiolabeled (*H3) and added at different concentrations (in PFU per VEC) to cultures of endothelial cells, either alone or with a 10^3-fold excess of unlabeled H3 virus. After incubation at 4°C for 15 min, the cells were washed and trypsinized, and radioisotope in the cells and supernatant was measured. Specific virus binding to the cells was determined by subtracting the counts per minute bound in cultures in the presence of unlabeled virus from the counts per minute in cultures containing labeled virus alone. The number of PFU bound per VEC was determined by dividing the average counts per minute bound per VEC in four replicate cultures by the calculated counts per minute per PFU of virus.

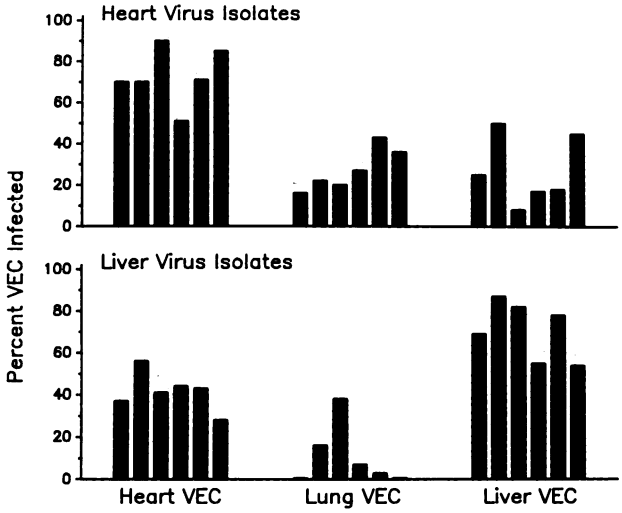

FIG. 3. Relative susceptibility of VEC from different tissues to virus infection. Six plaque-purified isolates of CVB3 were isolated from the heart and liver of an infected mouse. Each isolate was incubated on VEC derived from different tissues at multiplicity of infection of 10 PFU per cell for 20 h at 37°C. The VEC were thoroughly washed, treated with virus-neutralizing antibody to eliminate extracellular virions, counted, and evaluated by infectious-center assay for the percentage of cells infected. Each bar represents the mean percent infection of three replicate samples for each of six different plaque-purified virus isolates from either the liver or the heart, as indicated. Percent infection of heart VEC with heart isolates was significantly greater than percent infection of either lung or liver VEC at $P < 0.05$. Percent infection with liver isolates was greater in liver VEC than in heart VEC ($P < 0.05$) and lung VEC ($P < 0.01$). Percent infection in heart VEC was greater than in lung VEC ($P < 0.05$). *H3, [^{35}S]methionine-labeled H3.

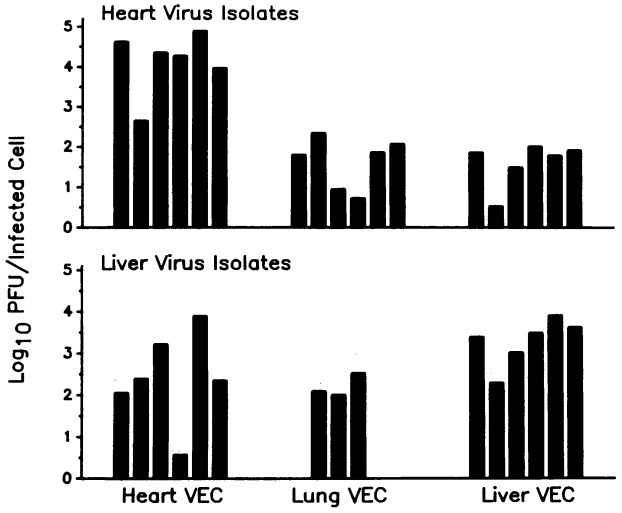

FIG. 4. Virus replication in infected cells. Replicate samples containing 100 of the VEC infected with virus isolates in Fig. 3 were lysed, and the titer of released intracellular virus was determined. The PFU were divided by the number of infected cells, as determined in the infectious center assay. Each bar represents the calculated average $\log_{10}$ PFU per infected cell for individual heart and liver virus isolates.

labeled virus. This virus was added to monolayers of murine heart, lung, and liver VEC at concentrations ranging from 1 to 10^3 PFU per cell. After incubation at 4°C, the unattached virus was removed. The average PFU of radiolabeled virus binding to each type of VEC was determined by dividing the counts per minute bound to each VEC by the counts per minute in each PFU of the radiolabeled virus (Fig. 2). Heart-derived VEC bound approximately 6 to 8 PFU of the radiolabeled virus isolated from the heart, whereas only 1 to 2 PFU were taken up by each of the other two VEC types.

Comparison of virus infectivities of different VEC preparations. The above results confirm the enzyme-linked immunosorbent assay studies and suggest that VEC might differ markedly in susceptibility to infection. To test this possibility, six plaque-purified isolates from the heart and six from the liver were incubated with VEC monolayers in vitro. After 20 h of incubation, the cells were trypsinized to form single-cell suspensions, counted, and divided into six aliquots containing 100 viable cells each. Three aliquots were saved for titer determinations, as described below. The remaining three aliquots for each virus isolate were used to determine the number of infected cells by the infectious-center assay (Fig. 3). The six heart isolates infected between 50 and 90% of the heart VEC, which was significantly greater ($P < 0.05$) than the percentage of either lung (18 to 45% of cells) or liver (5 to 50% of cells) VEC infected by the same isolates. In comparison, plaque-purified isolates from the liver were significantly more infectious for liver VEC (approximately 50 to 80% of the cells were infected) than for lung (0 to 40% infection, $P < 0.01$) and heart (28 to 58% infection, $P < 0.05$) cells. Not only did VEC preparations from various organs differ with regard to the percentage of cells infected with each viral isolate, but the average amount of virus produced per infected cell also differed (Fig. 4). The remaining three aliquots of cells that were saved from the experiment described above were alternatively frozen and thawed to release intracellular virus. The titers of the supernatants were determined by serial dilution. The mean PFU produced per infected cell was calculated by dividing the virus titer in each aliquot by the mean percentage of VEC infected, as determined by the infectious-center assay. The differences were most apparent with virus from the heart. These isolates reproduced significantly better in heart VEC than in either of the other cell preparations ($P < 0.01$). Liver virus isolates showed no significant differences in PFU produced per infected cell among the three VEC types. Thus, heart virus isolates demonstrated both preferential infection and replication in heart-derived VEC. However, liver virus isolates showed differences only in infectivity of VEC. All VEC types supported liver virus isolate replication once the virus entered the cell.

Correlation of in vitro and in vivo viral tropism. The following experiments investigated the abilities of the virus isolates from the different organs to infect and cause specific tissue injury in vivo. Three BALB/c mice were inoculated intraperitoneally with 10^4 PFU of each virus isolate. Seven days later, the animals were killed. Hearts, livers, and lungs were evaluated histologically (Fig. 5 and 6) and virologically (Fig. 7). Inflammatory lesions were rarely observed in either the lung or the liver but were quite severe in the heart. The six heart virus isolates consistently induced significant myocarditis (Fig. 5). With the exception of one isolate, virus derived from the liver was significantly less pathogenic ($P < 0.01$). Virus titers in the three organs generally resembled the results obtained by infecting VEC in vitro. Infection of mice with the isolates from the heart clearly produced greater infectivity of the heart than of the lungs or the liver per 100 mg (wet weight) of tissue. Virus isolated from livers infected

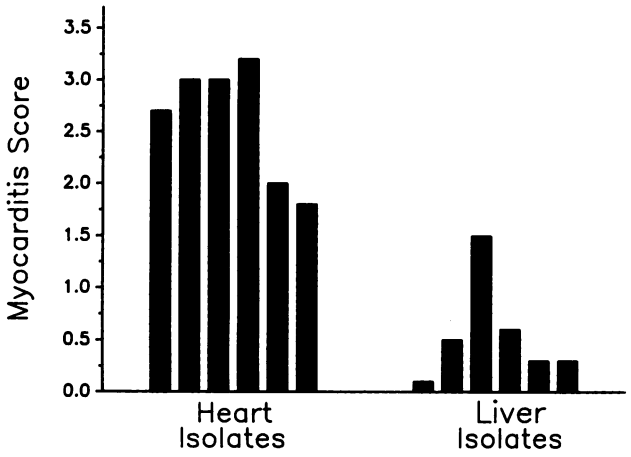

FIG. 5. Myocardial inflammation in mice infected with heart and liver virus isolates. BALB/c mice were inoculated with 10^4 PFU of the heart and liver virus isolates and sacrificed 7 days later. Hearts were removed and evaluated histologically for inflammation by using a scoring system of 0 (no myocarditis) to 4 (confluent lesions throughout the myocardium). Each bar represents the mean score for three animals inoculated with each virus isolate. Myocarditis produced by heart virus isolates exceeded that produced by liver virus isolates at $P < 0.01$.

the liver better than the lungs ($P \leq 0.05$), as determined by Wilcoxon ranked score analysis. No statistical differences were noted between the liver virus isolates infecting the liver and the heart.

DISCUSSION

The virus receptor has long been viewed as the premier factor determining viral tropism. Organs composed of cells devoid of specific viral receptors should largely escape both infection and tissue injury. However, the parenchymal cells of an organ are rarely in direct contact with the circulatory system but are separated from the elements of the blood by the VEC. For a virus to infect an organ, it must move from the circulatory system into the underlying tissue. Thus, the virus must either circumvent or infect the VEC. In capillaries, transport of small particles and nutrients from the circulatory system to the organ parenchyma occurs through transcytotic vesicles in the VEC (3). Materials from the lumen of the vessel are taken up by endocytosis into the vesicle and exit into the tissue by exocytosis. This method might allow virus penetration from the circulatory system into the organ without requiring direct interaction with receptor molecules on the VEC. Thus, the VEC would not act as a barrier to the infection and would presumably play a minimal role in tropism.

An alternative mechanism of infection of an organ involves specific interactions of the virions with the endothelial cell. VEC lacking receptors for certain viruses would act

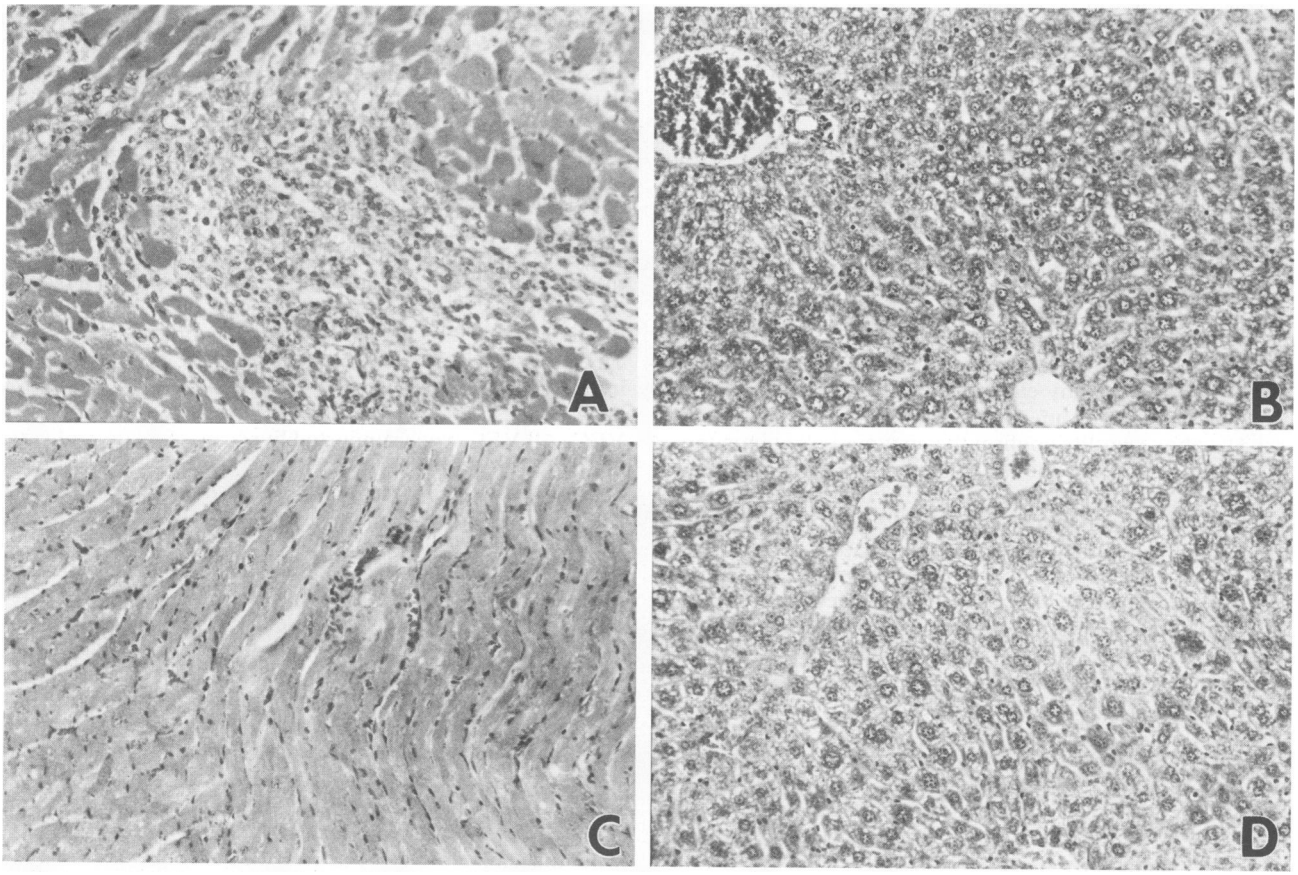

FIG. 6. Representative histological sections of hearts from animals inoculated with heart (A and B) and liver (C and D) virus isolates. The sections were stained with hematoxylin and eosin. Magnifications, ×25 (A and C) and ×50 (B and D).

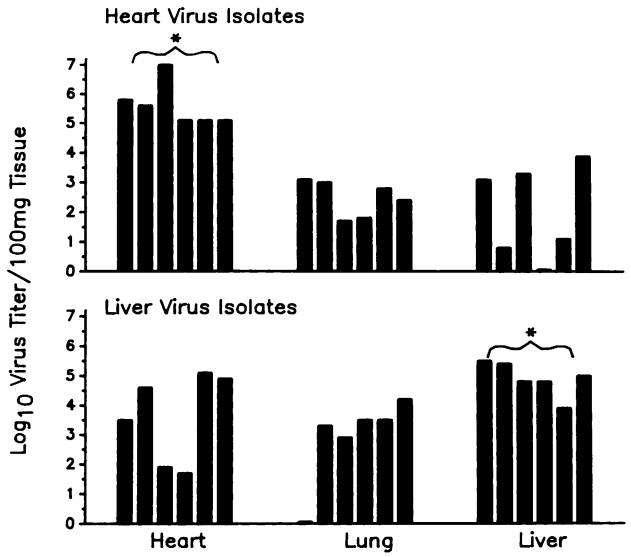

FIG. 7. Virus concentrations in organs from mice inoculated with heart and liver virus isolates. BALB/c mice were inoculated intraperitoneally with 10^4 PFU of the heart and liver virus isolates and were sacrificed 7 days later. Hearts, lungs, and livers were removed, and the titers of virus were determined. Each bar represents the mean virus titer from three animals infected with each virus isolate. *, Titers exceed values for other tissues at $P < 0.05$.

as effective barriers to infection. However, where the endothelial cell expresses appropriate receptors, virions bind to the VEC membrane and either undergo preferential transport through the transcytotic vesicles or infect the endothelial cell proper. Infection of the VEC with virus replication and subsequent cell lysis could release high titers of the infectious agent onto the underlying cells. The VEC might act as a seeding mechanism of viral entry into an organ. VEC producing the most progeny virions per infected cell would provide optimal opportunities for parenchymal-cell infection.

Both VEC-dependent and independent mechanisms of virus tropism probably occur in vivo. In the present system, VEC from different organs clearly showed distinct susceptibilities to CVB3 infections. Heart virus isolates which preferentially react to heart-derived VEC in vitro also showed greater infectivity for the heart in vivo, suggesting that the VEC from which virus is isolated determines tissue tropism. The results obtained with the liver virus isolates are somewhat different. Here, viruses derived from liver, while showing relative tropism for liver VEC in vitro, retained significant infectivity for heart tissue in vivo. One of two explanations for the observed differences seems likely. First, viruses isolated from heart tissue show consistently greater efficiency for binding to and replicating in heart VEC than in liver VEC. In contrast, viruses isolated from the liver bind poorly to heart VEC in vitro, but once infected, heart VEC appear nearly as capable of supporting virus replication as the liver-derived cells. Thus, circulating liver virus isolates may show poor tropism for heart VEC in vivo, but even reduced numbers of infected heart cells could still produce high virus concentrations in the organ over a 7-day period. A second explanation is that liver virus isolates use receptors to infect heart cells in vivo different from those detected in vitro. Work from several laboratories indicates that picornaviruses can use more than one cell receptor (6, 13, 21), and receptor usage may be dependent on cell type (18). Studies of the cellular receptor for poliovirus revealed that nonpermissive tissues express receptor mRNA, suggesting that posttranslational modifications may affect virus binding (16). Therefore, virus variants which can bind more than one receptor have an extended host cell range. Furthermore, receptor selection is crucial in pathogenicity (21). Interactions with other receptor molecules, even though they may result in efficient infection of the heart, preferentially induce suppressor T cells and no cardiac injury (unpublished data). Clearly, liver VEC are poor expressers of the cardiac virus receptor, as demonstrated in both radiolabeled virus-binding studies and ELISA, using the 10A1 MAb. Thus, liver virus isolates which successfully infect heart tissue may select receptors which fail to initiate autoimmunity. Evidence for this concept rests primarily on the minimal myocarditis present in mice given liver virus isolates.

Although VEC preparations differ in virus receptor expression, they appear quite similar in other respects. In the present instance, all VEC populations expressed equivalent amounts of class I major histocompatibility complex molecules yet were negative for class II molecules. Therefore, while all VEC undoubtedly share many cell surface characteristics, other characteristics may be organ specific and may reflect differences in function. This report certainly supports such a concept. Although the virus strain is an important determinant of infectivity, the VEC may also play a major role both in viral tropism and in disease pathogenesis.

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