A Murine Model for Coxsackievirus B3-Induced Acute Myocardial Necrosis for Study of Cellular Receptors as Determinants of Viral Tropism

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Receptor Hypotheses

A number of studies have contributed to the working hypothesis that specific cellular receptors, if present in sufficient quantity, serve as major determinants of both the host range and tissue tropism in the pathogenesis of human and animal picornavirus infections [14, 21, 28, 36, 42]. Though picornaviruses contain relatively small genomes, they produce a bewildering array of diseases. It is likely that one of the several factors contributing to disease diversity is due to the specific requirement of individual viruses for host cell receptors. For example, we have postulated [12a] that genetic changes in the virion attachment site could occur during virus replication in infected individuals. These changes could influence the course of the disease [9, 23, 51] by altering the virus receptor specificity. This would be of special importance if different receptors occurred on different tissues.

We also have hypothesized [12a] that receptors may be different (polymorphic) in genetically dissimilar individuals. If so, these differences may result in the selection and amplification of variant viruses and thereby cause a more or less severe disease than that produced by the parental virus in other individuals. Furthermore, we view cellular receptors as having a dual function, i.e., to bind virus and to facilitate disassembly of the capsid to release the viral genome [17]. In some cases virus may attach to specific receptors, but infection does not occur because of some limitation in the capacity of the receptors to eclipse virus infectivity [52]. Finally, recent results from our laboratory reveal that more than one type of specific receptor protein may exist on cell surfaces for binding a given virus and influence virus tropism [19, 30, 48]. Collectively, these several observations and hypotheses (Table 1) illustrate the complexity which can be encountered in investigation of cellular receptors for picornaviruses and the role of receptors as determinants of virus tropism.

Nevertheless, results of studies of other virus-host cell systems support the concept that receptors influence pathogenesis. For example, a strain of mice (SJL/J) resistant to mouse hepatitis virus (a coronavirus) is homozygous for an autosomal recessive mutant receptor gene and cannot attach virus, whereas, a normal mouse strain (BALB/c) which produces a specific receptor is susceptible to this virus infection [3]. Another example of receptor-mediated pathogenesis can be found for

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Table 1. Hypotheses about cellular receptors for picornaviruses

1. Determinants of viral tropism in the pathogenesis of infection.
2. Receptor variation among individuals may account for selection of virus variants which may be more (or less) virulent than parental virus.
3. Receptors have dual functions: to attach virus and to initiate virus disassembly.
4. More than one type of specific receptor may serve to initiate virus infection.

reoviruses in a mouse model [54]. Reovirus T1 has a distinct tropism for ependymal cells of the brain, whereas reovirus T3 seeks out neurons. However, since these viruses share a receptor on mouse L cells [37], undoubtedly more than one receptor specificity accounts for the specific histotropisms of reoviruses in mice [8].

Despite continued progress in learning about cellular receptors for viruses in general [16] and picornaviruses in particular, little is known about their molecular structure, cellular function or role in viral pathogenesis. This chapter outlines some of our knowledge about receptors on cultured cells and presents some preliminary experimental results together with strategies for testing our several hypotheses about receptors for coxsackieviruses (CV) of group B (CVB) in a murine model.

Receptors on Cultured Cells for CVB

Receptor specificity for the different species of picornaviruses was first demonstrated by the binding competition between two virus serotypes which share the same receptor [11, 12, 18, 39, 47]. On HeLa cells, specific receptors were discovered for polioviruses, CVB, and for two separate families of human rhinoviruses, respectively. Studies from our laboratory have focused attention on the characterization of the HeLa cell receptor for the CVB. Our current knowledge of the properties of the receptor for the prototype CVB3 (Nancy strain) is summarized in Table 2. A receptor protein of approximately 50 kilodalton (kd) has been identified and purified from HeLa cells [40]. This protein is currently being sequenced (N-terminal amino acids), and oligonucleotide probes are being prepared to permit screening of a HeLa cell cDNA library for isolation of a receptor gene (collaboration with R. Colonno and J. Tomasinni, Merck, Sharp, and Dohme Research Laboratories, West Point, PA). Whether this glycoprotein [35] occurs in pentameric units to comprise a receptor site as suggested previously [13] remains to be determined. Additional studies are also needed to determine whether purified receptors reconstituted in liposomes possess the postulated dual functions of virus attachment and virus disassembly.

A second cellular receptor for a host range variant of CVB3, referred to as "CVB3-RD" following selection by serial passage in human rhabdomyosarcoma cells (RD), has been identified [48]. This receptor has been designated as "human receptor 2" (HR2) to distinguish it from the 50-kd receptor (HR1) described

Table 2. Properties of receptors from HeLa cells for CVB3 (Nancy)

	Reference
1. Chymotrypsin sensitive; trypsin resistant	61
2. Inactivated by periodate, B glucosidases, and mannosidase	35
3. Regeneration requires mRNA and protein synthesis	37 a
4. Under genetic control of cell	41
5. Integral plasma membrane protein approximately 50-kd monomere	40
6. Approximately 275-kd (Sepharose 4B) multimere	35
7. Approximately 10^5 sites/cell	12
8. Density on sucrose 1.06	35
9. Virus species specificity	18
10. Stable at pH 1, 2 °C, 10 min; 60 °C, 30 min	62
11. Monoclonal antibodies to receptor block virus attachment	20 30

above. HR2 has been found on RD cells, which lack HR1, whereas HeLa cells possess both HR1 and HR2. The Buffalo green monkey kidney cell line possesses simian receptor one (SR1) which appears to be closely related to HR1 by assays with a receptor-specific monoclonal antibody, designated "RmcB" [30]. Another receptor-specific monoclonal antibody, designated "RmcA", has specificity for HR2 [20, 30]. These and other receptors for CVB which are found on cells of different species (mouse, rat) are listed in Table 3. Human erythrocytes also have a receptor (HR3) to which CVB3-RD and RmcA attach. Thus, more than one receptor for CVB may serve to initiate infection and each receptor will need to be characterized.

Table 3. Nomenclature for CVB cellular receptors defined by cells and probes[a]

Receptor	Origin		Probe		
Designation	Species	Cell	Virus	Rmc[d]	Other
HR1	Human	HeLa	CVB1–6	B	Ad2F[e]
HR2	Human	RD[b,c]	CVB3-RD	A	–
HR3	Human	Erythrocyte	CVB3-RD	A	–
MR1	Mouse	YAC-1	CVB1–6	–	Ad2F
RR1	Rat	L8	CVB3	–	–
SR1	Monkey	BGM	CVB1–6	B	Ad2F

[a] It is anticipated that additional receptors will be found that can be distinguished by use of specific probes
[b] Rhabdomyosarcoma cells
[c] HR2 are also found on HeLa cells
[d] Receptor-specific monoclonal antibody
[e] Adenovirus type 2 fiber protein (studies with K. Lonberg-Holm)

Table 4. Comparative number of binding sites of different cells for CVB3 and the monoclonal antibodies RmcA and RmcB. (From [30])

Ligand	Binding sites		
	HeLa (n)	RD (n)	BGM (n)
CVB3	1.8×10^4	–[a]	2.7×10^3
CVB3-RD	5.6×10^5	3.7×10^3	3.3×10^3
RmcA	2.5×10^6	2.7×10^4	–
RmcB	5.0×10^4	–	3.6×10^4

[a] Less than 100 per cell as limit of detection, considered to be absent

Monoclonal antibodies have also been prepared against cellular receptors for polioviruses [44, 46] and rhinoviruses [10]. These reagents, which are very useful for probing the different cellular receptors, were first obtained by Campbell and Cords [6] for a receptor epitope from HeLa cells for some of the CVB. A different monoclonal antibody against another HeLa cell receptor epitope for CVB2, CVB4, and CVB6, but not reactive for CVB1, CVB3, and CVB5 has recently been described [26]. It is predicted that many additional epitope specificities, recognized by other monoclonal antibodies, will be identified if the search is continued.

The number of receptors per susceptible cell for most picornaviruses is relatively low, even though there may be as many as 10^5 receptor molecules per HeLa cell. This value is undoubtedly high for most normal cells in the intact animal. We have estimated the number of receptors for CVB3 and CVB3-RD on HeLa cells, RD cells, and BGM cells using radiolabeled virions and monoclonal antibodies (Table 4) [30]. The approximate number of specific binding sites per cell at ligand saturation was determined by extrapolation of the slope of the nonspecific binding to intercept the ordinate at zero input. The results of this type of assay are represented in Fig. 1, which depicts the titration of receptors for CVB3-RD on RD cells. It will be a challenge to conduct quantitative studies of receptors on cells in situ which have a low receptor density.

The relationship between patterns of pathogenesis and the quantitative distribution of specific virus receptors, however, must be examined in the whole animal since cultured cells may synthesize receptors which are not expressed in vivo [29]. In searching for receptors for CVB in the experimental mouse model, the effects of age and genetic strain of the animals will also be of critical interest. Molecular biological methods, in addition to the use of labeled receptor ligands, may permit more sensitive assays of receptors in mouse tissues. For example, receptor mRNA could be measured by hybridization with labeled DNA probes prepared from receptor genes. We soon hope to be able to apply some of the tools derived from molecular studies of the CV receptors on cultured cells to the ultimate question of how a virus causes a specific pattern of disease.

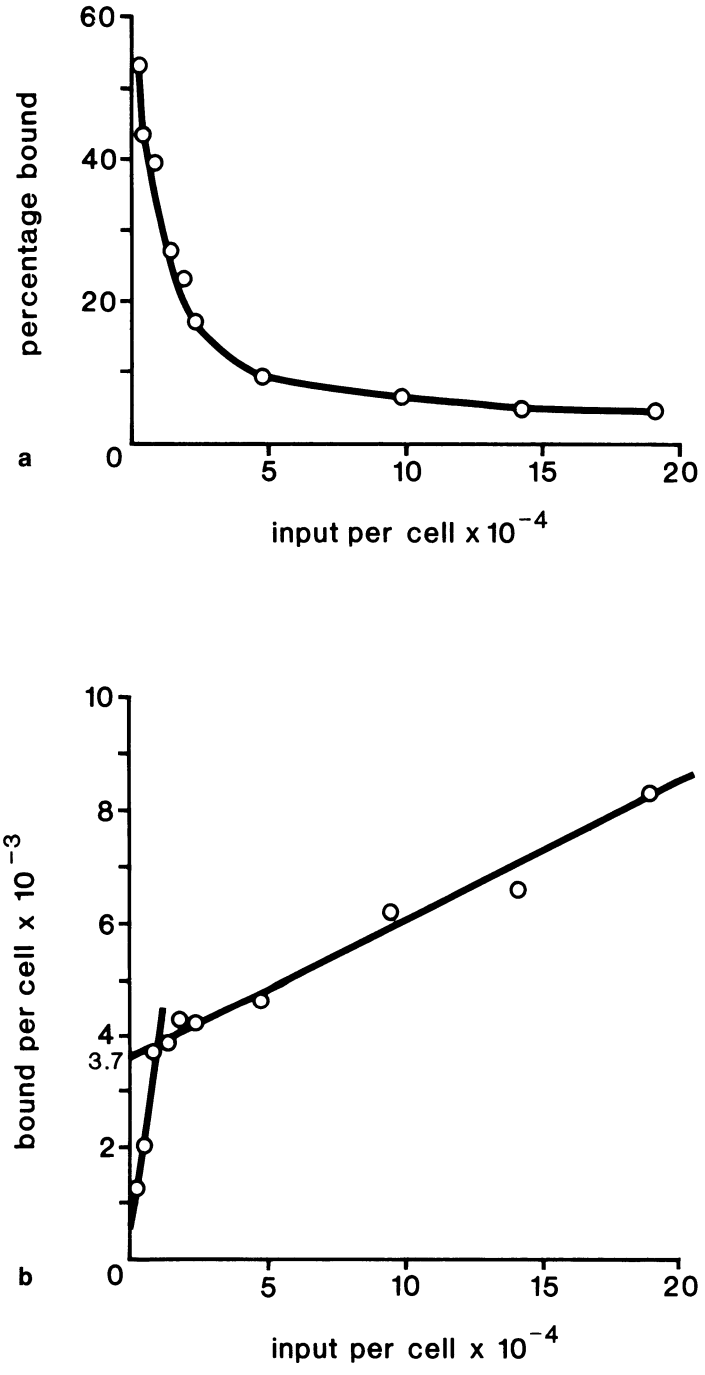


Fig. 1 a, b. Titration of receptors for CVB3-RD on RD cells. Dilutions of ^{35}S-CVB3-RD were incubated 90 min with 10^7 RD cells per milliliter at 24°C. The cells were washed twice and counted for radioactivity. **a)** The relationship of input virus multiplicity to percentage of cell-bound labeled virions. **b)** The results in **a** transformed to particles bound per cell input virus multiplicity. Approximately 3.7 x 10^3 virions were estimated to bind specifically per cell

CVB3-Induced Histopathology in Different Strains of Inbred Mice

We have compared the temporal sequence of tissue damage following the intraperitoneal inoculation of a myocarditic strain of CVB3 (Woodruff strain; CVB3-W) into 8-week-old mice of three inbred strains, BALB/C, SJL/J and C3H/HeJ. A strain of BALB/c mice has been shown to produce cytotoxic T lymphocytes which resulted in myocardial injury following CVB3 infection [27], although this reponse has not been uniformly detected [49]. The SJL/J mouse has a tendency to develop a diabetes-like disease following infection with CB4 or encephalomyocarditis virus [60], whereas the C3H/HeJ mouse has a partial defect in cell-mediated immunity with diminished macrophage stimulation to lipopolysacharide [58].

Although our initial objectives were to study the development of virus-induced heart disease in BALB/c mice, we found that these animals died following inoculation of only 5×10^4 plaque-forming units (PFU) of CVB3-W (Table 5). This observation prompted a histopathologic study of multiple tissues at intervals following CVB3-W infection. In general, three phases of disease were identified (Table 6). Phase I (pancreatic disease) occurred 2–3 days postinfection (p.i.) and was characterized by subtotal or total pancreatic acinar cell necrosis with sparing of islets. Phase II (hepatic disease) occurred 4–5 days p.i. and was marked by hepatocyte swelling and vacuolization leading to coagulative necrosis. We concluded that the BALB/c mice, inoculated with 5×10^4 PFU of virus, died from massive hepatocellular necrosis. If a lower amount of inoculum was used, the mice developed reversible changes in the liver and went on to Phase III (heart disease) which developed 7–10 days p.i. The heart disease was characterized by multifocal myocardial necrosis with calcification as shown in Fig. 2. Resolution of lesions occurred over a 28-day period. It is significant that there was no evidence of an inflammatory infiltrate in the early phase in the pancreas, the liver, or the heart; and the pathologic changes were associated with increasing titers of virus. Although peak virus titers preceded peak tissue damage by 1–3 days, only a mild inflammatory cell infiltrate composed of scattered lymphocytes and macrophages was observed secondary to coagulative necrosis. In each case inflammation was associated with removal of necrotic cellular debris caused by virus infection and not to immunopathologic mechanisms [25].

Table 5. Comparative susceptibility of BALB/c and C3H/HeJ mice to CVB3-W. (Modified from [19])

Mouse strain[a]	Virus inoculum	%Surviving at 14 days (%)
BALB/c	5×10^2	40
	5×10^4	0
C3H/HeJ	5×10^6	100

[a] Eight week-old males

Table 6. General pattern of disease following i. p. inoculation of CVB3-W into BALB/c mice

Disease phase	Primary target organ	Days of peak change	Description of pathology
I	Pancreas	2–3	Total acinar cell necrosis followed by cell drop-out Inflammation minimal after coagulative necrosis
II	Liver	4–5	Hepatocyte swelling, vacuolization and fatty change leading to coagulative necrosis Begins pericentrally
III	Heart	7–10	Multifocal myocardial necrosis with calcification Lesions resolve over time Inflammation follows viral-induced necrosis

[a] Leads to death if inoculum contains 5 x 10^4 PFU, whereas animals resolve liver disease and progress to heart disease with 5 x 10^2 PFU

The C3H/HeJ mice, infected with as much as 5 x 10^6 PFU of CVB3-W, neither died (Table 5), nor developed histologic evidence of liver disease (Table 7). However, these animals developed prominent multifocal myocardial necrosis with onset on day 7 p.i.. SJL/J mice developed only subliminal liver disease and some myocardial necrosis. However, a chronic inflammatory skeletal muscle disease was produced in these mice following infection with CVB3-WRD [53]. Thus, CVB infections of inbred mice provide a system in which different patterns of pathogenesis can be compared with possible differences in cellular receptors between strains of mice and between different organs.

Table 7. Comparative occurrence of pathologic changes in selected organs of mouse strains infected with CVB3–W

Mouse strain[a]	Inoculum (PFU)	Occurrence of pathologic changes[b]			
		Pancreas	Liver	Heart	Skeletal muscle
C3H/HeJ	5 x 10^6	+	–	+	–
SJL/J	5 x 10^6	+	+/–	+	+
BALB/c	5 x 10^2	+	+	+	–

[a] Eight-week-old males
[b] Fourteen days p. i.

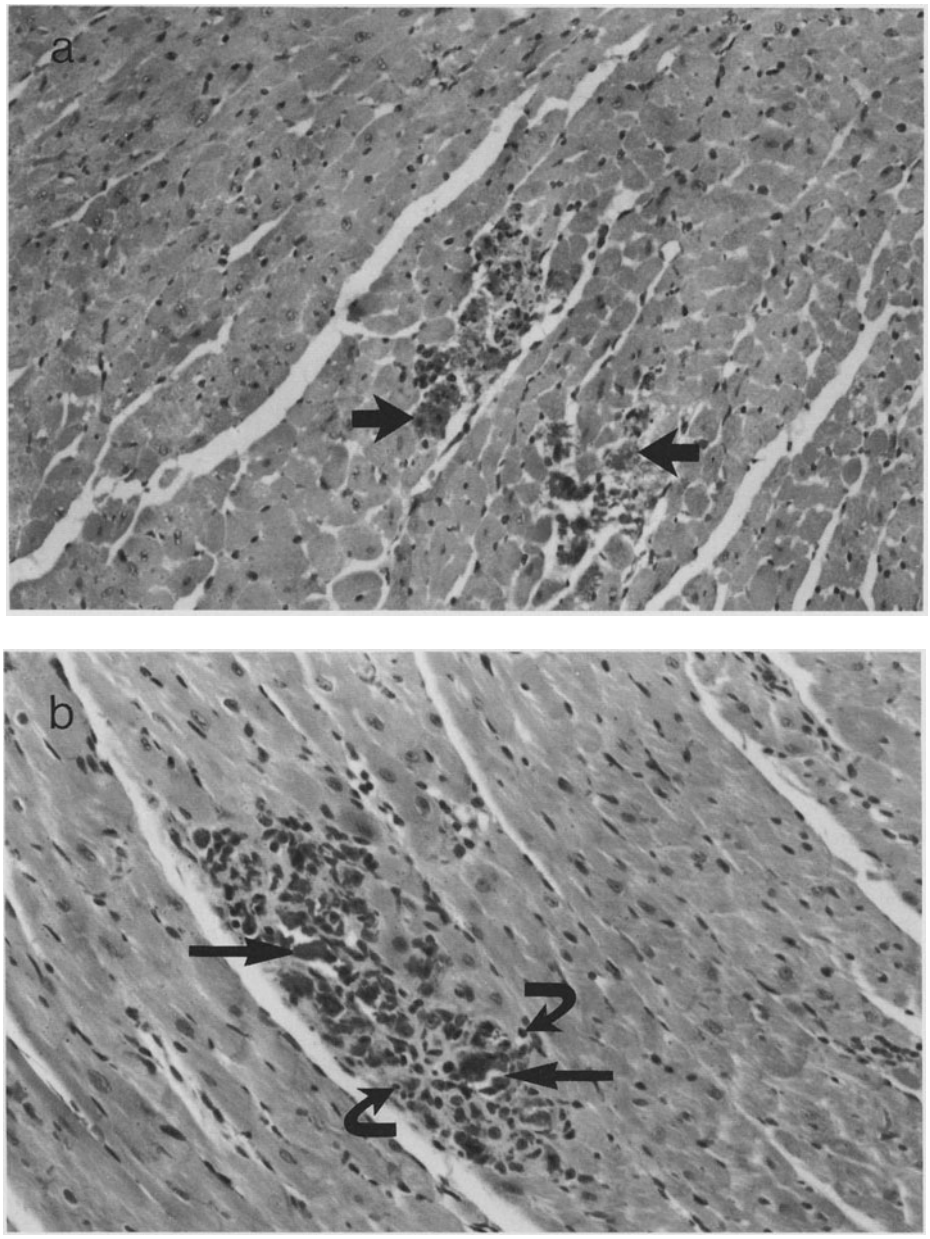
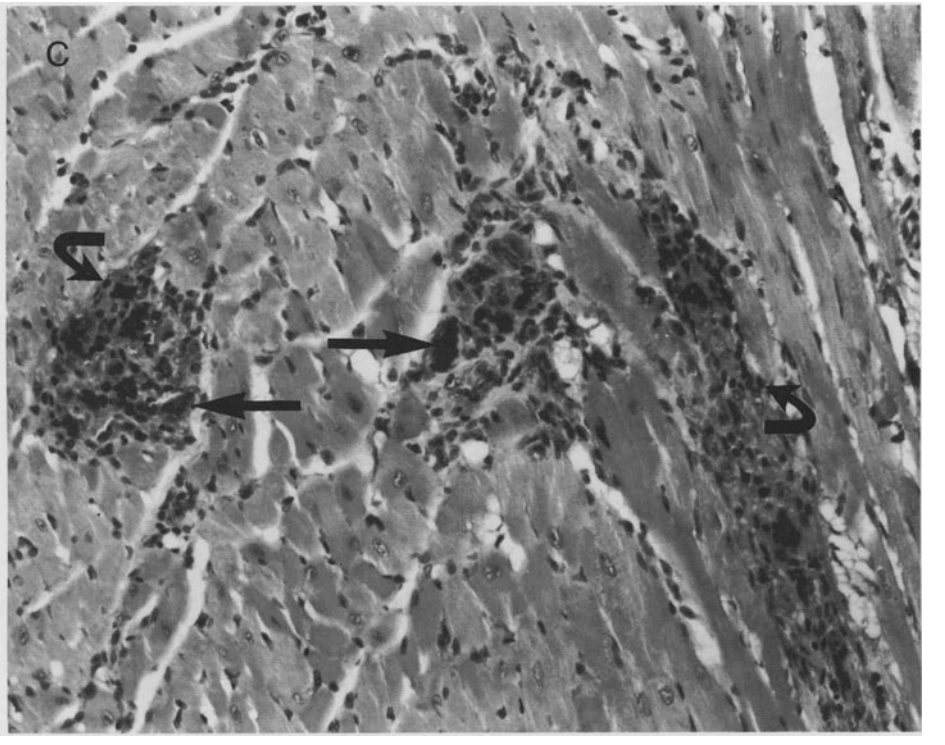


Fig. 2 a-d. Male C3H/HeJ mice 6–8 weeks old were infected i.p. with 5 x 10^6 CVB3-W. **a)** At day 7 p.i., heart shows focal areas of myocardial cell necrosis *(straight arrows)*. Remainder of heart is normal. **b)** At day 10 p.i., foci of necrotic myocardial cells show calcification *(straight arrows)*. Scattered lymphocytes have entered the lesion *(curved arrows)*. **c)** At day 14 p.i., lesions have attained maximal size and contain areas of dense calcification *(straight arrow)*. A mild to moderate infiltrate is seen *(curved arrows)*. Note the continual focal nature of the heart lesions which spare the remainder of the myocardium. **d)** At day 28 p.i., areas of focal necrosis with calcification *(straight arrows)* are undergoing resorption. Note reduced size of lesion. From [25] H & E, X 100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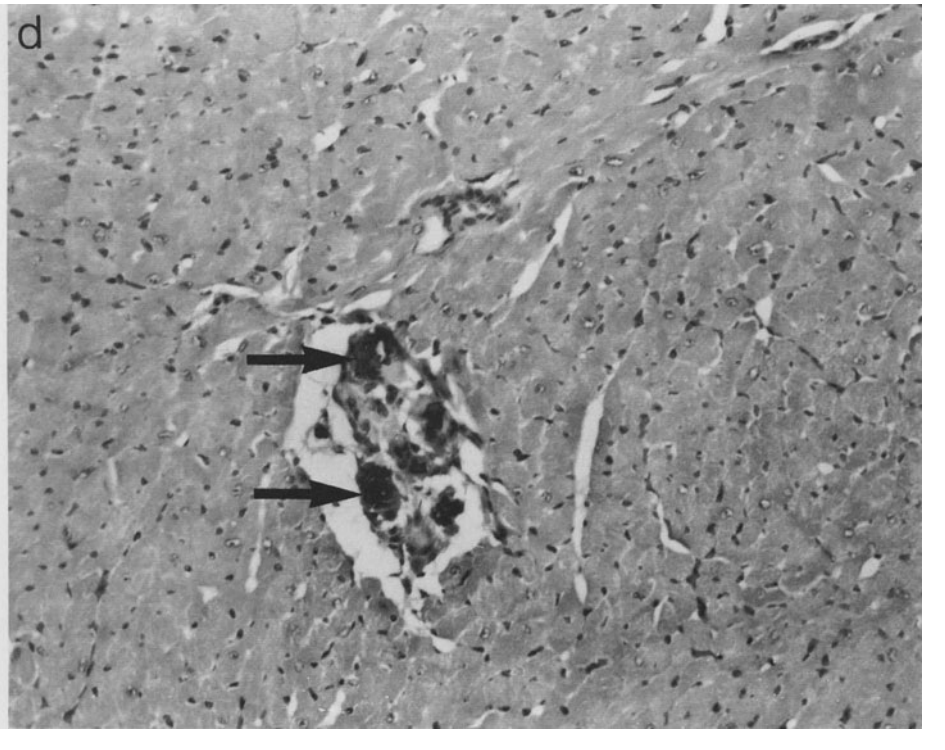
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Strategies for Localization of Receptors, Receptor mRNA, and Virus in Mouse Tissues

It has been axiomatic to us that receptors contribute to the pattern of pathogenesis of the CVB. Yet there are presently only imprecise methods for measurement of the distribution of receptors in different organs and in different individuals or strains of experimental animals [36]. This deficiency is attributable to the low number of receptor molecules on the cell surface and to the fact that neither picornavirus virions, nor any virion substructure or polypeptide can be used to probe for the presence of receptors in situ [15, 50]. Earlier studies which attempted to quantitate receptors in tissue homogenates have been aided by use of methods to solubilize the receptor proteins prior to assay [34]. Currently we are developing molecular and monoclonal antibody probes that are better suited to determining if there is a correlation between receptor concentration and susceptibility to CVB infection with accompanying pathologic change.

Localization of Receptors

Receptors in detergent-solubilized extracts of different tissues can be measured by quantitative immunoblot assay using monoclonal and, as available, high titer polyclonal antibodies. Recently, we have also found that radiolabeled virions can be employed in dotblots and in viroblot assays (comparable to Western blot) [3, 45]. Solubilized receptor proteins can be adsorbed to nitrocellulose and detected with either radiolabeled antibody or virions. Bound radioactive probe is detected by radioautography and can be quantitated by densitometry [56] or by counting radioactivity. In addition, immunogold labeling techniques can be used to determine receptor density and distribution on cells by electron microscopy [55]. We seek to compare the presence and amount of receptors for CVB on different organs from BALB/c, C3H/HeJ, and SJL/J strains of mice which show different sensitivities to infection and differences in the ability of virus to produce liver necrosis (Table 7).

Localization of Receptor mRNA

There are two reasons to examine receptor mRNA as well as receptor protein for identifying the distribution of receptors in human and animal tissues. In some situations (e.g., when there is rapid turnover or down-regulation in infected animals or low numbers of receptor molecules) it may not be possible to demonstrate functional receptors by direct assay. More significantly, it may be possible to determine the distribution of structurally different but antigenically homologous receptor proteins by using specific probes for their mRNA [38].

Initially, we plan to use labeled copies of receptor specific cDNA for detection and quantitation of receptor mRNA. If it is found that there are different species of related mRNAs, probes with different specificities can be prepared from restriction fragments of their cDNA.

Localization of Virus

Viruses have been detected in infected tissues by plaque titration or by immunohistologic identification of viral antigen in sections. Viral RNA can also be detected by in situ hybridization [4, 5]. To evaluate fully the persistent state of infection, we will probe for both plus and minus strands of viral RNA [7]. Detection of viral RNA permits identification of latently or defectively infected cells which may not contain infectious particles [2, 22, 32]. We intend to quantitate viral nucleic acids in organs of infected mice by dot hybridization [31] and then to examine sections of major organs by both immunoperoxidase staining for viral antigens and by in situ hybridization using ^{35}S-labeled probes or peroxidase-labeled nucleic acid probes to identify viral RNA.

Summary

We hypothesize that specific cellular receptors serve as major determinants of CVB tropism in the pathogenesis of human and animal diseases. The CVB cause a wide spectrum of human diseases including: pleurodynia, meningitis, pancreatitis, myositis, hepatitis, pericarditis, and heart muscle disease [43]. They also produce a similar spectrum of diseases in inbred mice which provide useful animal models for laboratory investigation [1, 19, 24, 30a, 33, 57, 59]. An increasing amount of information about the nature of cellular receptors for the CVB has been obtained from studies of cultured cells; however, cultured cells may express receptors which are not expressed in vivo. The challenge remains to apply this information to determine receptor distribution, receptor abundance, and receptor specificities by in situ methods in human tissues and in animal models. Several strategies for the detection of receptors by monoclonal antibodies and molecular probes have been presented. The finding of more than one receptor species for prototype and variant viruses on different target cell types will make these determinations more complicated than previously anticipated.

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