THE NATURAL HISTORY OF ENCEPHALOMYOCARDITIS VIRUS-INDUCED MYOSITIS AND MYOCARDITIS IN MICE

Viral Persistence Demonstrated by In Situ Hybridization

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Some viral infections can initiate a chronic inflammation that seems to become self-sustaining. Early in the infection, the initiating virus can be cultured from the tissues, and then in chronically inflamed tissue it no longer can be. Antiviral humoral and cellular immunity develops, and even viral antigens disappear. Sometimes an autoimmune reaction can be demonstrated during the chronic phase. If viruses or other microbes do play a role in initiating autoimmune diseases, as is often proposed, it is likely that the clinical illness represents the stage of the disease when the inciting microbe has ceased to be culturable. Sporadic attempts to grow putative inciting agents from patients or animals with autoimmune disorders have been largely disappointing. Understanding the transition, therefore, from the initiating viral event to the self-sustaining chronic inflammatory phase with an autoimmune mechanism is central to understanding whether and how microbial agents might cause autoimmune diseases.

Among the most intensively studied viral-initiated sustained inflammatory processes are those caused by picornavirses. These small RNA viruses are generally lytic to the cells they infect. They do not integrate into the host cell genome, and they have been thought not usually to persist in a latent intracellular phase. They usually cease to be culturable from tissue soon after infection. Rarely, culturable virus, viral antigens, or viral nucleic acid may be demonstrated months or years later (1–5). There is, however, evidence that they may persist in tissue culture in altered form (6, 7).

To study the transition from acute viral infection to chronic inflammatory phase in more detail, we have followed presence of viral genetic material in mice infected with a myotropic variant of encephalomyocarditis virus (EMCV),[1] EMC-221A, by in situ hybridization as well as by slot blot hybridization, virus culture, and standard histologic techniques. We have chosen this virus because it causes a myositis and myocarditis resembling those found in the human autoimmune disease polymyositis, for which there is substantial but still indirect evidence of picornaviruses as the inciting agents (8).

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[1] *Abbreviations used in this paper:* ECMV, encephalomyocarditis virus; PFU, plaque-forming units.

Materials and Methods

Virus Infection of Animals. 9-wk-old female BALB/cAnN female mice (National Cancer Institute) were inoculated intraperitoneally with 10^4 or 10^6 plaque-forming units (PFU) of a myotropic variant of EMCV, EMC 221A (8), and were killed by carbon dioxide anesthesia and autopsied at weeks 1, 2, 3, 4, 6, 8, and 12. Tissue samples from each animal were quickly frozen on dry ice and stored at −80°C until use. The remaining tissues were fixed in 10% buffered formalin for 8–24 h and then were paraffin embedded. Slides were coded before examination, and tissue sections were evaluated in a blinded manner. Mononuclear cell infiltrates were graded as follows: 0 = no infiltrate, 1 = sparse, 2 = mild, 3 = moderate, and 4 = severe mononuclear cell infiltration (8).

Virus and Antiviral Antibody Titration. Portions of the frozen tissues from two animals per time point were rapidly weighed together and then homogenized with a Polytron homogenizer. Appropriate dilutions were tested for virus on confluent monolayers of L-929 cells (American Type Culture Collection, Rockville, MD). Blood specimens for antiviral antibodies were obtained just before mice were killed. All titrations were carried out on the same day by end-point neutralization on heat-inactivated sera.

Probes. A cDNA designated A8, representing the 3′-terminal 1.4 kb of EMC 221A, was prepared from poly(A) RNA from a concentrated stock of EMC-221A obtained by standard techniques from the supernatant of L-929 cells infected with the virus (9, 10). The cDNA, E9-21, representing bases 389–844 of EMC, was the gift of Ann Palmenberg, University of Wisconsin, Madison, WI (11). The cDNA for the mouse β-actin coding sequence was the gift of Bruce Patterson, National Institutes of Health and Margaret Buckingham, Institute Pasteur, Paris, France. To make single-stranded RNA probes, cDNA was transferred to plasmid pΓ7/T3α-18 (Bethesda Research Laboratories, Gaithersburg, MD).

Single-stranded probes were synthesized with uridine 5′-[^{35}S]thiotriphosphate (Amersham Corp., Arlington Heights, IL) for in situ hybridization or with uridine 5′-[^{35}P]triphosphate (Amersham Corp.) for slot blot hybridization using T7 Polymerase (Promega Biotec, Madison, WI). When used for in situ hybridization, the labeled RNA was partially digested by alkaline hydrolysis in carbonate buffer.

An RNA standard for quantitative slot blot hybridization was obtained by synthesizing complementary unlabeled RNA from the appropriate plasmid and determining the concentration of nucleic acid by absorbance at 260 nm. Size standards for single-stranded RNA were purchased from Bethesda Research Laboratories.

RNA Extraction. RNA for slot blot hybridization was prepared from an aliquot of homogenized frozen tissue by the hot phenol method (12). Hybridization on Nytran was carried out at 55°C in 50% formamide, 1X Denhardt's solution, 0.02 M sodium phosphate, 1.25 M sodium chlorida, 0.5 M Tris Cl, pH 8.0, 0.1 M EDTA, 10% dextran sulfate, sheared DNA 0.1 mg/ml, and ^{32}P-labeled probe, usually at 10^6 cpm/ml. After ~16 h the blot was washed as recommended by the manufacturer at 68°C. The density of each slot was read at three locations with an Ultrascan laser densitometer with a recording integrator (LKB Instruments, Bromma, Sweden). To control for the recovery of RNA during the isolation procedure, a separate blot was probed for β-actin mRNA. Because the amount of actin message varied from tissue to tissue and at different stages of the illness, we used the information only to determine whether or not RNA recovery during the extraction was adequate. In these experiments, the limit of sensitivity was ~10^7 molecules of viral nucleic acid per slot.

For Northern blot analysis of the RNA, a sample of frozen tissue was homogenized in ice cold guanidine isothiocyanate solution, and the RNA was prepared immediately (9). A Nytran blot was hybridized at 55°C for 16 h and washed three times at 65°C for 15 min in 1X SSPE, 0.5% SDS, and at 60°C for 15 min in 0.1X SSPE, 0.5% SDS.

All autoradiographs were exposed to Kodak XAR-2 film at −80°C with a single Cronex intensifying screen (Eastman Kodak Co., Rochester, NY).

In Situ Hybridization. In situ hybridization was then carried out by a modification of the method of Moench et al. (13). Formalin-fixed, paraffin-embedded tissues were sectioned onto silanated activated slides (14, 15) and were deparaffinized, hydrated by standard methods, and then incubated in 0.2 N HCl for 20 min at room temperature, digested with 2.5 mg/ml proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 15 min at 37°C,

acetylated in 0.25% (vol/vol) acetic anhydride (Sigma Chemical Co., St. Louis, MO) in 0.1 M triethanolamine HCl, pH 8.0, for 10 min at room temperature. The slides were washed in distilled water, dehydrated in graded ethanols, and air dried. All water was treated with diethylpyrocarbonate (Calbiochem-Behring Corp., San Diego, CA). The slides were then prehybridized in 2× SSC, 50% deionized formamide, 10 mg/ml *Escherichia coli* tRNA (BMB), 0.02% (wt/vol) Ficoll, 0.02% (wt/vol) polyvinyl pyrrolidone, 0.02% BSA, and 0.3 mg/ml sheared salmon sperm DNA for 2 h at 45°C. The sections were then hybridized overnight at 45°C in probe solution (50% formamide, 0.3 M NaCl, 0.01 M Tris, pH 7.4, 0.5 mM EDTA, 1 mg/ml BSA, 0.2% Ficoll, 0.02% PVP, 1 mg/ml tRNA, 20 mM DTT, 0.02% Triton X-100) containing 10^5 cpm/μl of probe. The next day the slides were washed with the 2× SSC for 10 min at room temperature twice, 2× SSC, 0.1% Triton X-100, 1 mM EDTA, 5 mM DTT for 15 min at 60°C, and 2× SSC at 60°C for 10 min six times, then dehydrated in graded ethanols plus 0.3 M ammonium acetate, air dried, and were then dipped in autoradiographic emulsion, NTB-3 (Eastman Kodak Co.) diluted 1:1 in 0.6 M ammonium acetate. Exposure was for 7 d at 4°C. The slides were developed in D19, fixed in 30% sodium thiosulfate, and stained with hematoxylin and eosin.

All tissues were hybridized to EMC probes, A8 and E9-21, as well as to a λ phage probe (Promega Biotec) as a control for specificity. The probe to mouse β-actin was used to evaluate efficacy of the technique; mouse myoblasts cultured on slides and infected with EMC 221A served as positive controls. Sections were considered positive if there was focal silver grain accumulation over cells and not present over the same area in the control tissue nor in the background (see Discussion).

Results

Clinical Illness. As previously described, EMC-221A induced roughened fur and a waddling gait in >90% of the infected animals, beginning 3–4 d after inoculation, peaking 6–10 d after inoculation, and slowly resolving over the next several weeks (8). Animals inoculated with a higher viral dose (10^6 PFU/animal) appeared to develop more acute and more severe symptoms than those given a lower viral dose (10^4 PFU/animal). Control mice appeared unchanged, and none died spontaneously during the course of the experiment; 1/35 low dose animals and 2/45 high dose animals died spontaneously. Both low and high dose animals rapidly developed antibody responses that were near maximal by 14 d and remained at that high titer throughout the 12 wk of observation. Uninfected animals remained negative.

Viral Persistence and Inflammation. At the time of autopsy, portions of brain, heart, skeletal muscle, liver, and spleen from each mouse were rapidly frozen for later viral culture and extraction of RNA. Other portions were fixed in formalin for routine histologic examination and for in situ hybridization. By using a group of parallel analyses of the same tissue, we hoped to determine: (*a*) how long living virus persisted in each organ, (*b*) how long viral nucleic acid sequences could be detected by techniques that do not require a complete infectious viral particle to be present; and (*c*) the relationship between viral persistence and inflammation. In all these studies the results of observations in animals injected with 10^4 PFU and with 10^6 PFU differed little and are pooled where noted in presentation of the data.

Skeletal Muscle. Skeletal muscle inflammation was moderately severe at 7 d with severe myocyte degeneration and mononuclear cellular infiltration. By 2 wk, it had diminished substantially, and by 3 wk, it had virtually disappeared. Culturable virus was present in high titer (260–1,100 PFU/mg) at 7 d, but only a rare PFU was present at 2 wk and none thereafter (Table I).

At 1 wk, many myocytes were infected with virus, as judged by in situ hybridiza-

TABLE I
Summary of Viral Detection Assays

Week	Tissue	Culture (PFU/mg tissue)		Slot blot*		In situ hybridization‡	
		LD	HD	LD	HD	LD	HD
1	Muscle	1,100	260	0	+ +	2/2	3/3
	Heart	380	240	?	+	2/2	3/3
	Brain	47	17	+ +	+ +	2/2	2/2
2	Muscle	0	0.014	0	?	3/3	2/3
	Heart	0.17	0	+ +	+ +	3/3	2/3
	Brain	0.019	0.012	0	?	2/3	3/3
3	Muscle	0	0	0	?	3/4	2/4
	Heart	0	0	0	0	2/4	1/4
	Brain	0	0.01	+	0	3/3	1/2
4	Muscle	0	0	0	+ +	0/4	0/5
	Heart	0	0	0	0	0/4	0/5
	Brain	0.084	0	+ +	0	2/4	0/3

Methods as described under Materials and Methods; all assays were negative at 6-, 8-, and 12-wk time points.

* + + is $>10^7$, + is 10^6–10^7, 0 is $< 10^6$ molecules/mg tissue; (?) uncertain data because of low β-actin messenger yield.

‡ Data expressed as number of animals positive per total number of animals tested in each group.

tion (Fig. 1). In situ hybridization proved more sensitive at tracking the presence of viral nucleic acid than either culture or slot blot (except for an unexplained strong slot blot signal in high dose animals at week 4). There was a positive signal up to 3 wk and rare positive cells were seen as late as 4 wk (Table I and Fig. 1 *C*).

Of great interest was the relationship between virus presence by in situ hybridization and inflammation since they could be correlated on the same slide. Virus was sometimes present only in a limited segment of a myofiber, the remainder appearing normal (Fig. 1 *D*). Although concordance of the two was common, virus was sometimes present in an area without apparent inflammation, and inflammation was often intense in areas where virus was sparse or absent (Fig. 1, *A* and *D*).

Heart. Acute viral myocarditis was followed by chronic myocarditis in all the infected animals we examined. At late time points, marked fibrosis was noted, and sometimes there was calcification. Culturable virus was present in high titer (2.4–3.8 $\times$ 10^2 PFU/mg) at 7 d, in low titer at 2 wk in the low dose group, and absent thereafter. As in skeletal muscle, a strong in situ hybridization signal remained at 2 wk when culturable virus was almost gone and was still present at 3 wk (Fig. 2 *A* and *C*). Slot blot signal was again less reliable.

As in the skeletal muscle, viral presence by in situ hybridization was not strongly correlated with inflammation, and of course, late inflammation did not at all correlate with the presence of virus nucleic acid.

Brain. By routine histological techniques, most of the animals had subacute encephalitis as evidenced by focal scattered microglial nodules, and this persisted throughout the 12 wk of observation. Virus was present by culture in high titer at 7 d and in low titer as late as 4 wk (Table I). By in situ hybridization, viral nucleic acids from both 5′ and 3′ ends of the EMC genome were also present in almost all

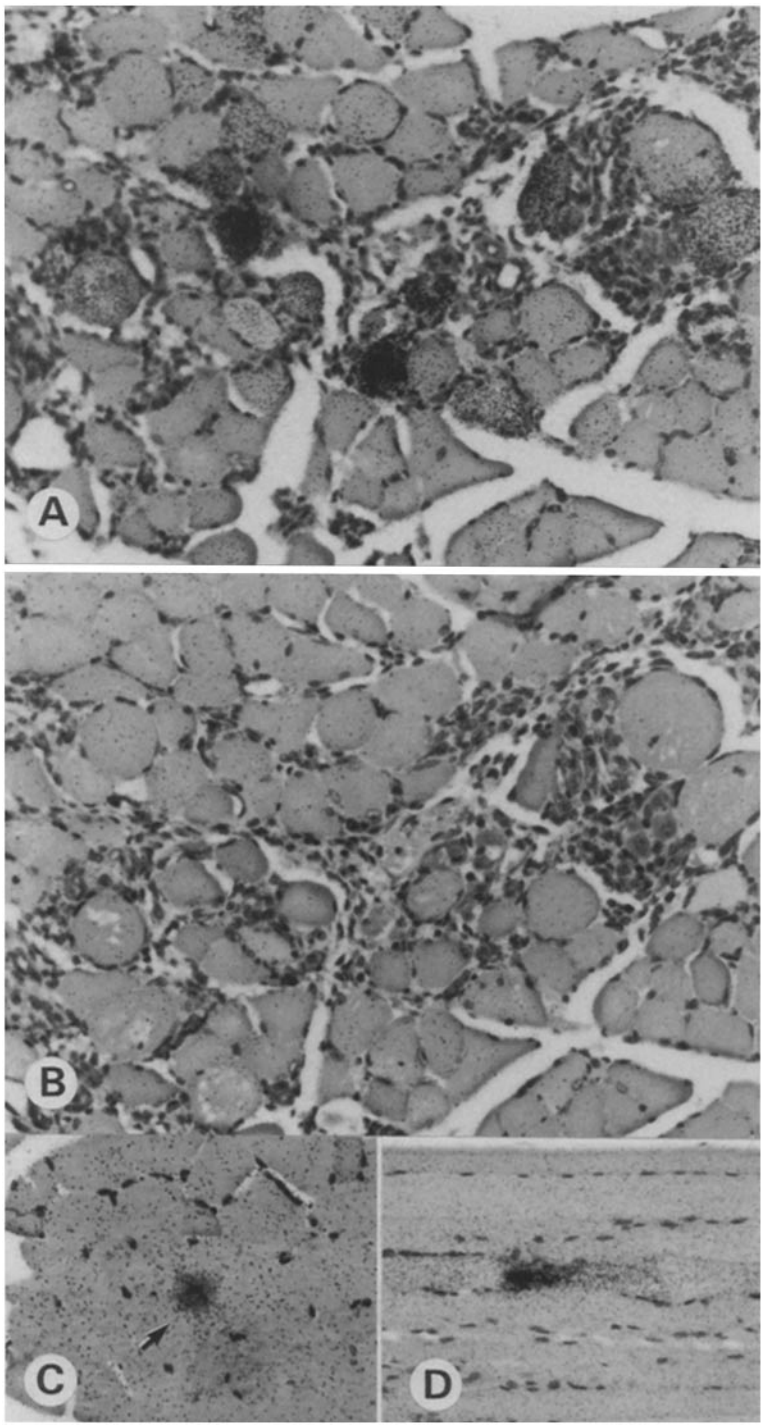

FIGURE 1. Relationship of skeletal muscle inflammation and viral presence by *in situ* hybridization. (*A*) Cross-section of skeletal muscle from a high dose animal 1 wk after EMCV inoculation showing severe mononuclear cell infiltration in endomysial and epimysial locations with variation in myofiber size and numerous areas of virus localization indicated by focal silver grain accumulations. (*B*) A serial section of the same area, which has been hybridized with a control probe. (*C*) Cross-section of skeletal muscle in a 3-wk animal showing a single positive focus (*arrow*). (*D*) Longitudinal section of skeletal muscle showing virus localization within a segment of a single myofiber from an animal 1 wk after infection. *A, B,* and *C*: ×215; *D*, ×108.

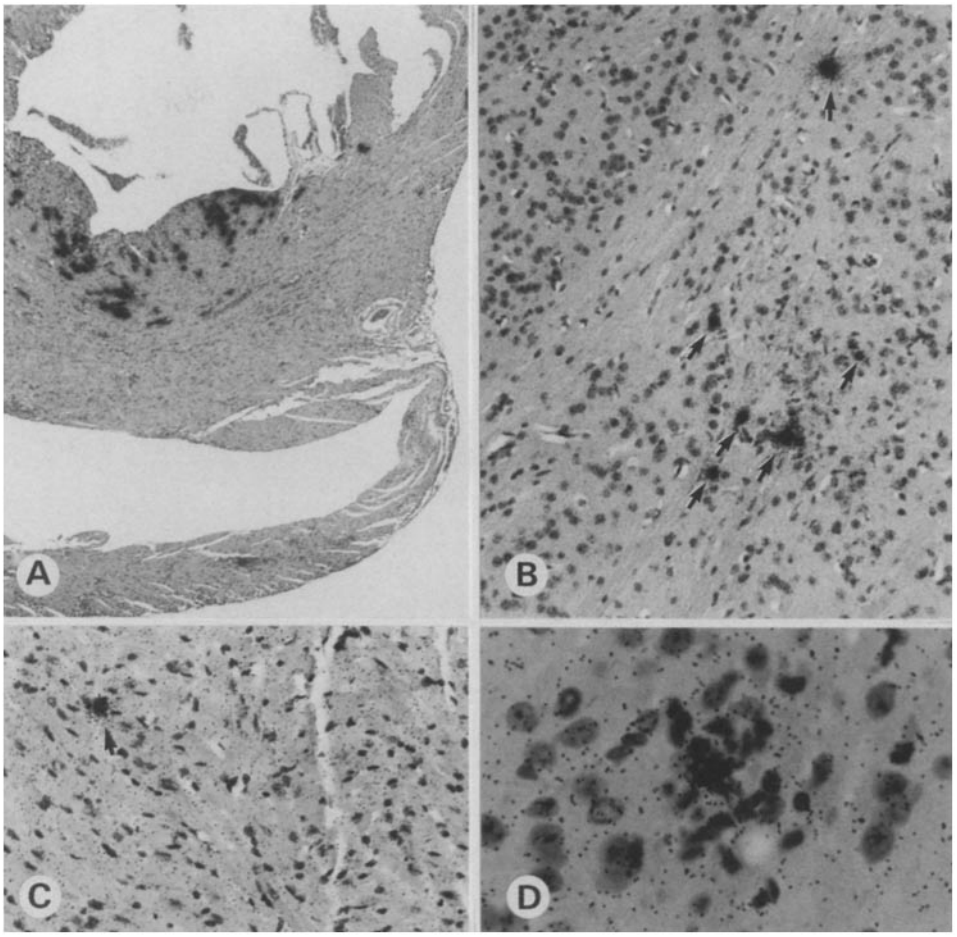

FIGURE 2. Viral persistence in the heart and brain by in situ hybridization. (*A*) Low power view of the heart of a low dose animal at 1 wk showing numerous positive signals in the septal area of the left ventricle. (*B*) Low power view of the brain of a 1-wk animal showing multiple areas of positive signal (*arrows*). (*C*) Higher power view of heart at 3 wk showing a single strong focus of positivity (*arrow*). (*D*) High power view of a microglial nodule from the brain of a low dose 4-wk animal. *A*, ×89; *B* and *C*, ×178; *D*, ×355.

animals up to 3 wk, in some animals at 4 wk, but not at 6 wk or thereafter (Fig. 2, *B* and *D*). Using an estimate of the in situ signal, taking into account both the frequency of infected cells and the amount of signal per cell, as much viral nucleic acid was present at 2 wk as at 1 wk, despite an almost 1,000-fold difference in culturable virus. Slot blot intensity paralleled the in situ results, but was less sensitive at most points (Table I).

Early in infection, virus was present widely throughout the brain by in situ hybridization, but later viral involvement was most prominent in the area of the hippocampus. Of note was that cells in this area and occasionally elsewhere in the brain sometimes showed a marked autoradiographic signal in uninfected animals or with a control λ phage probe. The mechanism of this nonspecific signal is unknown.

Spleen and Liver. Many of the infected animals at weeks 1 and 2 showed focal periportal chronic inflammatory infiltrates that were not seen in the livers of infected animals at later time points. This was in contrast to the follicular hyperplasia seen in the spleens of most of these animals that was noted throughout the 12-wk observation period.

Northern Blot Analyses. Northern blots of RNA extracted from frozen tissues were probed with E9-21, a probe for the 5′ end of the virus. A number of bands were detected: one at ~8.2 kb presumbably represented whole virus, and there were strong bands at 3.5 kb and 1.0 kb with faint bands at 2.0, 1.3, and 0.55 kb. When the same blots were probed with A8, a probe for the 3′ end of the virus, whole virus genome was not discernible, but bands at 1.6 kb and 0.8 kb were present.

When infected myoblast cultures were extracted with ice-cold guanidine isothiocyanate solution as described to minimize degradation of RNA (9) and the RNA was immediately separated by ultracentrifugation, Northern blots probed with E9-21 showed a higher proportion of full-length RNA but also some material at ~2.9 kb, 1.3 kb, and 0.9 kb, and those probed with A8 showed bands at 3.3 kb and 1.3 kb. Both uninfected and infected cells showed faint bands at 3.3 kb (E9-21) or 1.5 kb (A8). In both myoblast and tissue extracts, an actin probe revealed a strong band (1.73 kb) and a faint band (1.25 kb). Thus the material detected by slot blot and in situ hybridization of RNA extracted from tissue may represent not only complete viral genomes but also incomplete chains as would be present in cells actively synthesizing virus, partially digested RNA, and a small amount of crosshybridizing RNA from host cells. It was not possible to quantitate the RNA on the Northern blots, but all the bands described were present in brain, heart, skeletal muscle, and spleen at weeks 1 and 2 but only in skeletal muscle at week 3.

Discussion

In animals, some viruses clearly cause apparently self-sustaining inflammation that long outlasts the presence of culturable virus. Picornaviruses are among the best-studied examples. In Coxsackievirus B3-induced murine myocarditis, both autoantibodies directed at cardiac myosin and lymphocytes reactive to autologous muscle have been described as arising in the wake of infection in susceptible mouse strains (16, 17). Virus can be cultured only for the first week or two, and a prompt, long-lasting humoral immunity to the virus develops (18). In Theiler's virus-induced demyelinating disease of the central nervous system, an immune response to virus-infected cells develops (1, 3). The virus can be cultured from spinal cord tissue for at least 5 mo (1), virus antigen is detectable 2.5 yr after infection despite negative cultures (2), virus and inflammation are closely associated in the chronic phase of the illness (19), and a cellular immune response to virus-infected cells may be responsible for the inflammation (20).

A full understanding of the role of the initiating virus in diseases like these depends in part upon knowing exactly how long, where, and in what form the virus persists in the host. The technique of culturing virus in a standard cell line will detect only complete virus particles that have retained the ability to bind, penetrate, multiply in, and destroy the indicator cell line. In fact, within a picornavirus-infected cell, or the culture supernatant of an infected cell culture, a variety of other forms exist. There are particles that appear morphologically complete but have lost the ability to replicate in target tissue, unassembled coat proteins, other viral proteins,

RNA-negative strand intermediates, complete viral genomes not yet incorporated into particles, and incompletely synthesized viral genomes. Such incomplete forms outnumber infectious particles by several orders of magnitude for picornaviruses (7, 21, and our unpublished observations).

To understand more completely the relationship between EMCV infection and the myositis and myocarditis it initiates in mice, we have attempted to detect noninfectious virus by nucleic acid hybridization with probes for both the 5′ and 3′ ends of the viral genome. The illness in these animals resembles human polymyositis and myocarditis both biochemically and histologically (8). Since there is considerable circumstantial and now even direct evidence (22) for a role of Coxsackievirus in these illnesses, a more complete understanding of the model illness may illuminate the human counterparts.

In this illness, culturable virus persists in the brain for at least 4 wk, but is virtually gone from heart and skeletal muscle by 2 wk. By contrast, in situ hybridization, confirmed by Northern blot hybridization, showed the presence of viral genome for a week or two longer in skeletal muscle and heart despite a culture technique that could easily detect a single plaque of complete infectious virus in 100 mg of tissue, a substantial fraction of the weight of the organ.

Inflammation, however, has a very different history in these organs. In skeletal muscle it lasted as long as virus could be detected by in situ hybridization, and was succeeded by fibrosis. By contrast, in the heart the virus disappeared as rapidly, but inflammation was evident throughout the 12 wk of observation. Furthermore, even when both inflammation and virus were present by histology and in situ hybridization early in the infection, they were not always present in the same place, as noted by Kandolf et al. (23). It is interesting that in human biopsies from patients with myositis, it is similarly common to see isolated necrotic cells without adjacent inflammation and inflammation in the perimysium. Subacute inflammation within the brain with occasional microglial nodules long outlasted viral-induced cell destruction.

With the more sensitive technique of viral nucleic acid hybridization (see below), especially in situ on tissue sections, it is apparent that viral material persists beyond the ability to detect it by culture. It is likely, furthermore, that it persists in this model for at least some time longer. For a virus such as EMCV (and many related picornaviruses), which usually multiplies rapidly to a large number of progeny per cells, in situ hybridization is doubtless the best technique for locating such cells, even if they are present only rarely. By scanning an entire 4 × 4 mm section of muscle cells containing up to 3×10^4 cells in cross-section, a single infected cell can be detected. A conservative estimate of the sensitivity of probe E9-21 is that a cell with $\sim 10^2$ to 10^3 molecules of E9-21 can be found in this section (i.e., in 1 of 3×10^4 cells) (our unpublished observations). By contrast, for a virus that exists at a very low level per cell, or even only one integrated copy in occasional cells, in situ hybridization will fail, and extraction methods from a larger volume of tissue are required.

With the techniques we used, we are not now able to detect EMC viral genome beyond 4 wk, but we would miss cells with <100 viruses/cell at a density of $<1/10^5$ or a much lower concentration of virus unless it is present in most cells. We are currently engaged in trying to increase the sensitivity of detection. Since viral proteins may persist in the absence of viral reprodution, a similar program to enhance detection of viral proteins will be necessary.

Summary

Picornaviruses can initiate chronic inflammation that persists after the virus can no longer be cultured from inflamed tissues. In an attempt to understand this transition we have sought evidence for viral persistence by methods that detect viral genome independent of whether or not whole competent virus is present. In mice infected with a myotropic variant of encephalomyocarditis virus, EMC-221A, virus can be cultured in high yield at 1 wk and in low yield at 2 wk from skeletal muscle, heart, and brain; a small number of plaque-forming units could be cultured from brain at 4 wk. By contrast, in situ hybridization detected viral nucleic acid at least a week or two thereafter, often in single cells. In the skeletal muscle, inflammation disappeared by 3 wk, but in heart it remained for the full 12 wk of observation. In the brain, microglial nodules, sometimes with associated viral nucleic acid, were present for a long period. Application of this technique allows a more accurate assessment of the role of viral persistence in the pathogenesis of virus-initiated but apparently autoimmune inflammation.

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