Differences in the Two-Dimension-Gel Electrophoresis Protein Patterns of the Lethal K and Benign B Variants of Encephalomyocarditis Virus (43321)

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Abstract. **Variants of encephalomyocarditis virus (EMCV) are indistinguishable by hyperimmune serum. In spite of their antigenic similarity, they produce different disease syndromes in susceptible strains of mice. To understand the basis for the diversity in pathogenicity, studies have been initiated to characterize each of the virus variants. In this study, two-dimensional gel electrophoresis was used to compare the proteins produced by the benign EMCV-B with those produced by lethal EMCV-K. The data show that (i) the replication cycle of each of the virus variants is characteristic of picornaviruses, (ii) the VP1 of EMCV-K is more basic than that of EMCV-B, and (iii) three proteins, one a major component of VP1, the other two with molecular weight of about 12,000, are present in EMCV-K but not in EMCV-B.** [P.S.E.B.M. 1991, Vol 198]

Encephalomyocarditis virus (EMCV) is a cardiovirus in the family Picornaviridae (1). It is composed of several variants that are indistinguishable by hyperimmune antiserum cross-protection (2) and complement-fixation tests (3), but are not antigenically identical by hemagglutination inhibition tests (4, 5). These viruses are ubiquitous in nature and have been isolated from insects, birds, and mammals including humans (6, 7). They are highly infectious for a variety of small laboratory animals where, in spite of their close serologic relationship, the variants target different tissues (8), causing pathogenesis which mimics important human diseases such as insulin-dependent diabetes mellitus (9), vasculitis (10), myocarditis (11), and polymyositis (12).

In order to understand the basis for the diversity in pathogenicity, we have initiated studies to characterize each of the virus variants. In this communication we report differences in the isoelectric points (pI) of the viral proteins produced by the lethal EMCV-K and the nonpathogenic EMCV-B variants.

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Materials and Methods

Viruses. The nonpathogenic B variant (EMCV-B) was obtained from J. W. Yoon, NIH, Bethesda, MD. The K variant (EMCV-K) recently isolated by us from the EMCV-B stock (8) is lethal at 1 plaque-forming unit (PFU) per mouse, but unlike EMCV-B does not induce detectable levels of interferon either *in vivo* or in cell culture (unpublished observation). Both viruses were propagated as described previously (13).

Replication Cycles. Culture plates (60 mm) were seeded with 4×10^6 L929 cells 24 hr before use. One hour before infection, the confluent cell cultures were placed at 5°C. Either EMCV-K or EMCV-B at a multiplicity of infection of 10 PFU/cell was then adsorbed to each of the cell cultures for 1 hr at 5°C. After the adsorption period, the cultures were washed twice with ice-cold Hanks' balanced salt solution, refed with warm (37°C) culture medium, and incubated at 37°C. At the times indicated in Figure 1, duplicate samples were removed and the cells were scraped with a rubber policeman into the culture medium. The cell suspensions from duplicate plates were pooled and cell-associated virus was released by sonication (Heat Systems

model W-220F; 20 KHz vibrations, 30-W power output) for 10 sec in an ice bath. After removal of cell debris by centrifugation (2000 *g*, 10 min), the supernatant fluids were collected and stored at −70°C until assayed for PFU content.

Immunoprecipitation. Immunoprecipitations were done using mouse antisera directed against EMCV total proteins. At 6-hr postinfection (10 PFU/cell), mouse L929 cells (ATCC) infected with either EMCV-K or EMCV-B were labeled for 4 hr with "Tran ^{35}S label" (ICN, Irvine, CA) at 0.5 mCi/ml. After the labeling period, the cell monolayers were lysed by adding 1 ml of hot (65°C) 1% sodium dodecyl sulfate (SDS). The cell lysate was diluted 1/10 with 9 ml of phosphate-buffered saline-Tween 20, and the cellular debris was removed by centrifugation (2000 *g*, 10 min, 4°C). Polyclonal mouse antisera (200 μl; 15,000 viral PFU neutralizing units), raised against CsCl-purified EMCV-D particles and absorbed with lysates from uninfected L929 cells, was then added to the supernatant fluids and incubated at 4°C for 10 hr. The antigen-antibody complexes were sequestered by the addition of 200 μl of protein A-Sepharose GMB (Sigma) and incubated with shaking for 1 hr at room temperature. The bead pellet was collected by centrifugation (2000 *g*, 4°C, 10 min) and washed with phosphate-buffered saline-Tween 20. The pellet was washed twice more as described above and analyzed as described below.

Two-Dimensional Analysis of Immunoprecipitated Viral Proteins. *First dimension: isoelectric focusing.* Isoelectric focusing was done with the method of O'Farrell (14) using Isolytes 4-8 (Isolabs, Akron, OH). The gels were cast in glass tubes (180 mm o.d., 1.5 mm i.d.; Bio-Rad) and allowed to polymerize overnight. The upper chamber of a standard tube gel apparatus was filled with 0.1 *N* NaOH and the bottom chamber with 0.06% H_3PO_4. The gels were pre-run according to the following schedule: (i) 200 V, 15 min; (ii) 300 V, 30 min; and (iii) 400 V, 1 hr. The radiolabeled, immunoprecipitated viral proteins were dissolved in lysis buffer (9.8 *M* in urea, 2% NP-40, 2% ampholytes 7–9, and 100 m*M* dithiothreitol) and loaded onto the gels. The samples were run at 400 V for 15 to 18 hr, followed by an additional 2 hr at 800 V to sharpen the bands. The tube gels were then extruded into 5 ml of equilibration buffer A (0.5 *M* Tris, 5% basal medium Eagle, pH 6.8) for 15 min, followed by a second equilibration in 10 ml of buffer B (0.06 *M* Tris base [pH 6.8]; 2% SDS, 5% BME, 10% glycerol) for another 15 min. At this point the gels were frozen at −70°C or loaded onto the second dimension gel.

Second dimension. SDS-polyacrylamide electrophoresis. The second dimension gels (SDS-10% polyacrylamide electrophoresis) were used to determine the molecular weights of the polypeptides separated in the first dimension. The IEF tube gel was loaded on top of the SDS-polyacrylamide gel and anchored with 2 to 3 ml of melted 1% agarose in buffer (equilibration buffer B with 0.002% bromophenol blue). The gels were run at constant current (9 to 11 mA) until the bromophenol blue reached the bottom of the gel. After electrophoresis the gel was fixed and stained for 2 hr with Coomassie blue (40% methanol, 10% acetic acid, and 2.5 g/liter Coomassie blue (23) to visualize the molecular weight markers and internal standards. After visualization of the markers and standards, the gels were soaked in EN^{3}Hance (New England Nuclear, Boston, MA) for 1 hr, dried, and fluorographed at −70°C for 4 to 7 days.

Results

Replication Cycles. The one-step replication cycles of the viruses were determined in L929 cells as described in Materials and Methods and are shown in Figure 1. From these data it is evident that the replication kinetics of the two virus variants are similar, consistent with those of picornaviruses, and that the production of mature virions is complete by 10-hr postinfection.

Two-Dimensional Electrophoresis of Immunoprecipitated EMCV-K and EMCV-B Viral Proteins. ^{35}S-Radiolabeled EMCV proteins synthesized in infected L929 cells were immunoprecipitated with polyclonal antiserum as described in Materials and Methods. The antiserum was produced in mice against CsCl-purified viral particles and was preabsorbed with lysates from L929 cells. Two-dimensional SDS-10% polyacrylamide gel electrophoreses of these immunoprecipitated proteins were done several times, using different prep-

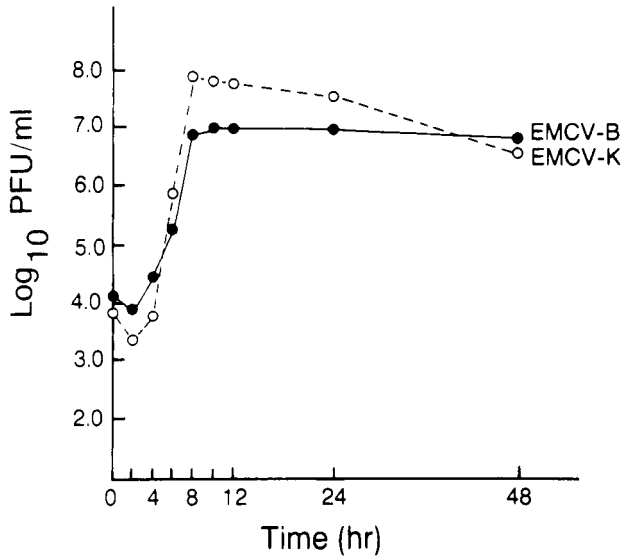

Figure 1. One-step replication cycles. Confluent monolayer cultures of L929 cells were infected with either EMCV-K or EMCV-B as described in Materials and Methods. Samples were collected at the times indicated and the total number of viral PFU determined. The data are the average of two separate experiments containing pooled duplicate plates at each sample time point.

arations. With very minor variations (relative size and position of some of the spots), the results were essentially identical each time the experiment was done. Representitive data are shown in Figure 2. The IEF was run as the first dimension, with pI determinations made using linear plots of pI markers: β-lactoglobulin A, pI 5.13; carbonic anhydrase B, pI 5.85; and myoglobin, pI 6.76 and 7.16. These internal markers were also included in each viral sample. The second dimension was on SDS-10% polyacrylamide electrophoresis, with protein standards (Promega, WI) serving as molecular weight markers. The two-dimensional electrophoresis detected more than 20 proteins for each virus. The dotted lines in the schematics connect the major spots and show the position of proteins of similar molecular weight. These data suggest that the viral proteins exist as heterogeneous complexes or "pI isomers" (15, 16). The two-dimensional analysis also shows that there are some protein spots absent (indicated by arrows) or have shifted in the EMCV-B pattern when compared with that of EMCV-K. These data are consistent with the notion that most of the proteins of EMCV exist as a heterogeneous pI complex. Uninfected control cell cultures treated in a similar manner were negative (no protein spots) and are not shown.

Based on molecular weight comparisons with known standards, the proteins in line 1 are consistent with polymerase E (51 kDa); line 2, ϵ (36 kDa); line 3,

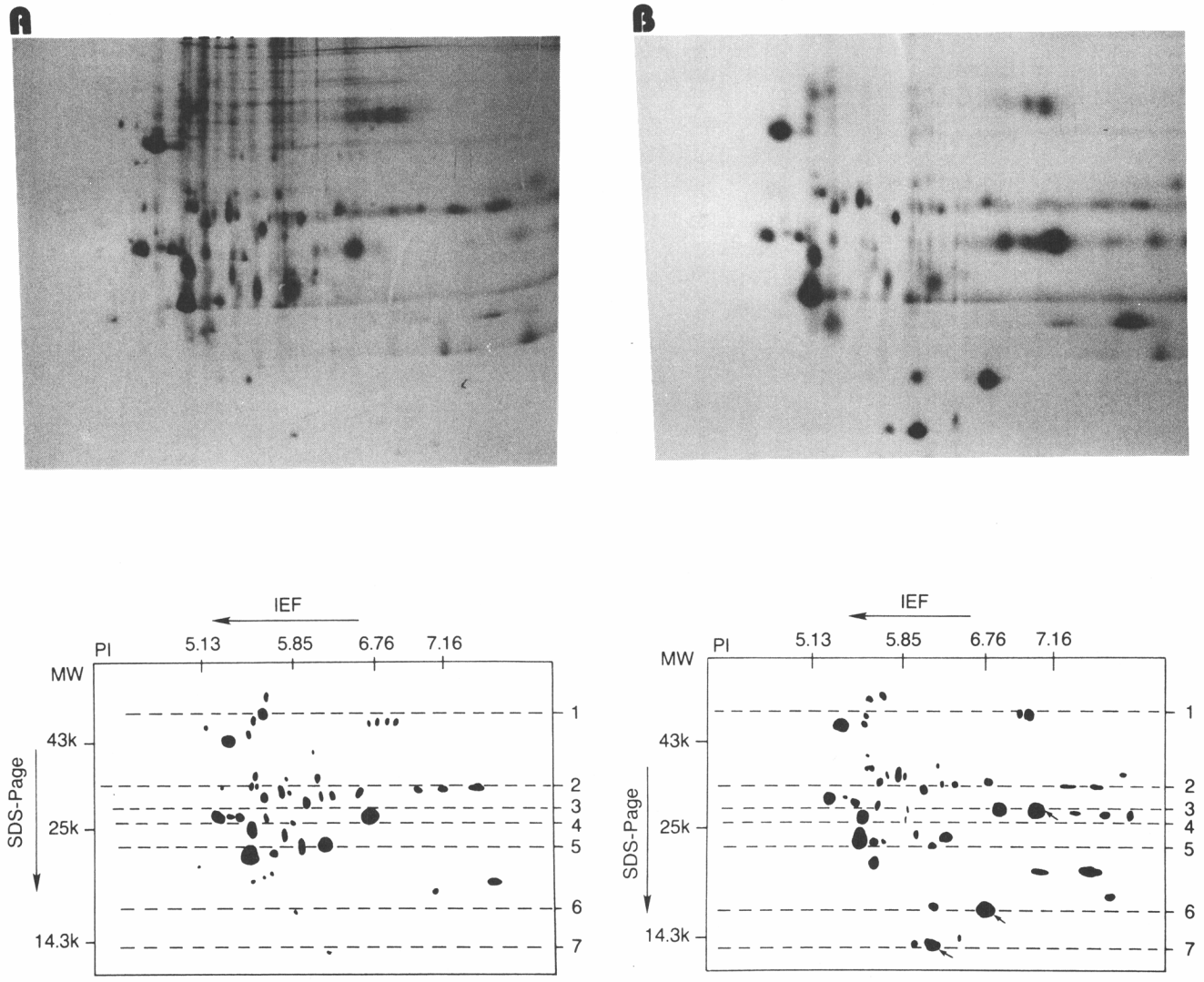

Figure 2. Two-dimensional gel electrophoresis of immunoprecipitated ^{35}S-labeled EMCV-K and EMCV-B virus proteins. (A) EMCV-B. (B) EMCV-K. Cultures of L929 cells were infected with either EMCV-B or EMCV-K at a multiplicity of infection of 10 PFU/cell. At 6-hr postinfection, the cultures were labeled with "Tran ^{35}S-label" for 4 hr and then lysed. The cell extracts were immunoprecipitated with antiserum to EMCV and analyzed by two-dimensional gel electrophoresis (IEF followed by 10% SDS-polyacrylamide electrophoresis). Samples with the same amount of radioactivity (100,000 cpm) were run on IEF gels as the first dimension. The first dimension tube gels were then layered on the second dimension SDS-polyacrylamide electrophoresis gels. The radiolabeled protein spots were detected by fluorography. pI determinations on the first dimension were made using pI markers: pI 5.13, pI 5.85, pI 6.76, and pI 7.16. Size determinations in the second dimension SDS-PAGE polyacrylami gels were made using protein molecular weight markers.

α (32 kDa; VP1); line 4, β (29 kDa; VP2); line 5, γ (25 kDa; VP3); line 6, protein G, (17 kDa); and line 7, protein H, a virus protein (VP) g-containing precursor (12 kDa). The small capsid protein δ (7 kDa) was not detected in these preparations, possibly due to elution during equilibration of the first dimension gel or because its pI may be greater than 8 (17).

The data show that α (VP1) consists of at least three species, whereas β (VP2) and γ (VP3) have only one major component. They also suggest that the α components of EMCV-K are more basic in nature than those of EMCV-B.

Discussion

In previous studies we showed that the B and K variants of EMCV differ in tissue tropism and in the disease syndrome produced in ICR Swiss mice (8). These observations suggest that there might be subtle differences in the capsids of these serologically identical viruses which are recognized by receptors on the host cells. In this study, the charge characteristics of the viral proteins produced by each of these virus variants were determined using two-dimensional gel electrophoresis. The advantage of this technique is that it provides a "panoramic view" of the entire coding capacity of the viral genome, identifying differences which can then be studied more specifically with other techniques.

One-step replication cycles were done to ensure that the replication of each virus variant was the same in the cells used for these experiments. The results in Figure 1 show that the replication kinetics of the viruses are the same and are consistent with those of picornaviruses. The lower yield of EMCV-B reflects the ability of this virus to induce the production of relatively large amounts of interferon in L929 cells (18). This conclusion is consistent with previous studies showing that interferon produced by L929 cells in response to infection by EMCV-MM interferes with the replication of the virus (19). In contrast, when EMCV variants replicate in either baby hamster kidney (BHK21) or HeLa cells, the yields are essentially identical (data not shown).

The data in Figure 2 show that multiple charge isomers (pI isomers) exist for the EMCV viral polypeptides. The charge heterogeneity among individual viral polypeptides can be explained by cleavage of amide groups, involvement of sulfhydryl-disulfide transitions, and phosphorylation or loss of terminal amino acids. These have been established to be some of the causes of microheterogeneity, particularly in the poliovirus system (20). Another explanation is that, since errors in amino acid composition (substitutions) occur with RNA genomes, substitutions at the site(s) of cleavage could reduce the protease specificity for that site so that cleavage would occur preferentially at an alternate site.

There appears to be two main differences between the proteins of the virus variants. First, in the α (VP1) proteins (line 3 in the schematics), the isoelectric point of the major component of EMCV-K is more basic (about 7.0) than that of EMCV-B (about 6.6), and a major spot seen in EMCV-K (arrow) is missing in EMCV-B. Second, at a molecular weight of about 12,000, two spots, presumably corresponding to ppH (VPg-containing precursors), are evident in EMCV-K, but are missing in EMCV-B. The experiment has been repeated on four different occasions, and the differences reported above have been evident each time.

Since VP1 has been shown to be important in the pathogenicity of other picornaviruses (21, 22), the more basic nature of the major VP1 component in EMCV-K may be related to its ability to infect the central nervous system of mice when administered by the intraperitoneal route of inoculation. The missing spots may reflect deletions or point mutations. This notion is supported by Eun et al. (23) who recently reported that the nucleotide sequences of the VP1 genes of EMCV-B and EMCV-D differed by five amino acids which were due to point mutations. These mutations were shown to increase the hydropathy profile of VP1 for the EMCV-D as compared with EMCV-B. Two of these mutations may be expressed on the surface of the virion, thus mediating receptor attachment differences which may account for the diabetogenic properties of EMCV-D and nondiabetogenicity of the B variant. This hypothesis is further supported by recent evidence from our laboratory (24) which shows that EMCV-D binds to a greater extent to pancreatic β cells than does EMCV-B.

Our results are consistent with those of other investigators who have reported differences between variants of EMCV which could help explain their pathogenic behavior. For example, Ray et al. (25) found that an oligonucleotide is missing in EMCV-B which is present in EMCV-D. Yoon et al. (26) reported that the B and D variants differ by a single point mutation in the oligonucleotide, A for EMCV-D and G for EMCV-B. This change rendered the EMCV-B RNA digestible by RNase T1 at that site, producing fragments smaller than the resolution limit of 14 bases. Jordan et al. (27) reported that the 103-nucleotide sequence from the 5′ noncoding region of EMCV-D and EMCV-B was identical and that the restriction maps of the entire open reading frame of both variants did not reveal any difference. However, the poly(C) tract of EMCV-B was estimated to be three bases shorter than that of EMCV-D. The biologic significance of this change is not known. Other studies provide evidence that the noncoding regions of the viral genome may have important functions such as controlling the efficiency of certain steps in replication (28, 29).

Differences in nonstructural proteins (polypeptides H and G) may also be of significance in infection.

Support for this notion comes from studies by Yin and Lomax (30) who reported host range mutants of human rhinovirus in which nonstructural proteins are altered. Similar mutations in nonstructural genes have been reported for poliovirus (31, 32) which result in a virus which does not selectively inhibit host cell protein synthesis; this may help to determine the pathogenicity of the virus. Minor et al. (33) report novel strains of poliovirus altered in both structural and nonstructural proteins; the two may serve to complement each other in controlling some functions in the viral life cycle which are not yet completely understood.

Our data show that there are differences in the proteins generated by EMCV-K and EMCV-B during replication in L929 cells. These differences may confer upon the viruses properties which allow one virus to preferentially interact and infect one host cell type over another.

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