Two amino acids, Phe 16 and Ala 776, on the polyprotein are most likely to be responsible for the diabetogenicity of encephalomyocarditis virus

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The diabetogenic D variant of encephalomyocarditis virus (EMC-D) was previously shown to differ from the non-diabetogenic B variant (EMC-B) by 14 nucleotides out of 7829 bases. Similar approaches with a new non-diabetogenic variant, EMC-DV1, obtained by plaque purification of the EMC-D variant stock pool, enabled us to narrow down further the possible genomic area responsible for the diabetogenicity of EMC virus. EMC-DV1 does not induce interferon in vitro, differing from the highly interferon-inducing EMC-B. The complete nucleotide sequence of EMC-DV1 was determined by RNA-dependent DNA sequencing and cDNA sequencing. The genomic size and organization of EMC-DV1 are similar to those of EMC-D and EMC-B, with a long open reading frame encoding a polyprotein of 2292 amino acids. Comparative analyses of sequence information as well as biological activities of EMC-DV1 with EMC-D and EMC-B suggest that (i) the diabetogenicity is apparently distinct from the ability to induce interferon, which is probably due to the single U base insertion at position 765 in EMC-B, and (ii) the diabetogenicity of EMC virus is most probably controlled by one or both of two amino acids, Phe 16 (on the leader peptide) and Ala 776 (152nd amino acid on the VP1) on the polyprotein.

Introduction

Encephalomyocarditis (EMC) virus, a member of the picornaviruses, the icosahedral structure of which contains 60 copies of four different capsid proteins, VP1 to VP4, and encloses a plus-sense, single-stranded RNA genome, has now evolved to several known variants. The D variant of EMC virus (EMC-D) induces diabetes in susceptible mice by infecting and destroying the insulin-producing pancreatic beta cells (Yoon et al., 1977; Yoon & Notkins, 1983). In contrast, the B variant (EMC-B), which was isolated together with EMC-D from an M variant stock pool (Craighead & McLane, 1968), is completely non-diabetogenic, but induces a substantial amount of interferon (Yoon et al., 1980). In spite of these remarkable biological differences, EMC-D and EMC-B are almost indistinguishable in several physicochemical and immunological properties. These observations have led to a hypothesis that the diabetogenicity of EMC-D and non-diabetogenicity of EMC-B are at least partially attributable to their ability to induce interferon (Yoon et al., 1980, 1983; Cohen et al., 1983; Jordan & Cohen, 1987).

We have recently shown by comparing the nucleotide sequences of EMC-D and EMC-B that the differences in biological activities of the two variants are due to a maximum of 14 nucleotide changes out of 7829 bases. The changes consist of one base insertion, two part deletions and eight point mutations (Bae et al., 1989). This investigation was initiated to narrow down further the number of nucleotides possibly responsible for the diabetogenic activity and/or interferon inducibility of EMC virus, from the 14 nucleotides identified previously. We have isolated a new variant, designated EMC-DV1, by plaque purification from the EMC-D variant stock pool. The newly isolated EMC-DV1 variant is neither diabetogenic nor interferon-inducing. Together with these biological characteristics of EMC-DV1, determination of the complete nucleotide sequence and its comparison with those of EMC-D and EMC-B provide us with further understanding of the diabetogenicity and interferon inducibility of EMC virus at the
molecular level. Our present data suggest that the diabetogenicity of EMC virus is not contingent on its ability to induce interferon, and that one or two amino acids, one on the leader peptide and the other on the capsid protein VP1, are most likely to be responsible for the diabetogenicity of EMC virus.

Methods

Materials. All enzymes used in this experiment were purchased from Bethesda Research Laboratories (BRL), Pharmacia and Boehringer Mannheim Biochemicals (BMB). Cell culture media and serum were obtained from Gibco/BRL. Nucleotide sequences were assembled and analysed using Pustell DNA sequence analysis software from International Biotechnologies.

Isolation of EMC-DV variant. Stock pools of EMC-D virus (Yoon et al., 1980) were passaged five times by inoculating a mouse fibroblast cell line (L929 cells) at an m.o.i, of 30 (Yoon et al., 1977). The virus was assayed on L929 cell monolayers and the titre was expressed as p.f.u. Plaque purification was carried out essentially as described elsewhere (Yoon et al., 1980). Briefly, EMC-D virus was diluted to a concentration of less than 1 p.f.u./ml and 0.5 ml aliquots were adsorbed to L929 cell monolayers in 60 mm culture dishes. After adsorption for 1 h at 37 °C, cultures were overlaid with 2% methylcellulose in Eagle's minimal essential medium (EMEM) containing 5% foetal calf serum (FCS). Monolayers were stained 2 days later with neutral red at a 1:20 000 dilution. After 24 h eight single plaques were selected on the basis of plaque size and shape and were amplified in L929 cell cultures. These eight clones were tested for their ability to produce diabetes in mice and to induce interferon in L929 cell cultures. Of these clones one was found to be neither diabetogenic nor capable of inducing interferon and was designated EMC-DV1.

Animals and glucose assay. SJL/J male mice (5 to 6 weeks old) were obtained from Jackson Laboratory. Ten mice per group were inoculated i.p. with plaque-purified viruses (3 × 10^5 p.f.u. per mouse). Glucose levels were measured in blood from the retro- orbital venous plexus by use of a glucose oxidase assay with o-dianisidine dihydrochloride as indicator dye (Yoon et al., 1980). Blood for glucose tolerance tests (GTTs) was obtained 60 min after i.p. injection of 2 mg of glucose per g weight. Non-fasting (NF) glucose levels were measured 7 and 14 days after infection, and GTTs were performed and stored at - 70 °C for over 4 days to inactivate the viruses.

Induction and measurement of interferon. Confluent monolayers of L929 cells in 60 mm Petri dishes were exposed to 1, 10 and 100 m.o.i, of virus. After 1 h the unadsorbed virus was removed and the exposed cells were refed with 6 ml EMEM containing 2% FCS. Two days later, the culture supernatant was harvested and cleared by centrifugation. The supernatant was acidified to a pH below 2 by the addition of 1 M-HCl, and stored at 4 °C for 4 days to inactivate the viruses.

Interferon in the supernatant fluids of cell cultures was assayed as described previously (Lewis, 1987). In brief, the acidified interferon solution was neutralized by adding 1 M-NaOH. Monolayers of L929 cells in microplates were incubated for 24 h at 37 °C with 100 μl of threefold serially diluted samples. Then 25 μl of vesicular stomatitis virus (Indiana strain, American Type Culture Collection) was added to each well at a concentration of 4 × 10^5 p.f.u./ml. After 48 h the interferon titre of each sample was calculated as endpoint protection units (IRU) by comparison with reference interferon (mouse a and β fibroblast interferon; Sigma).

cDNA cloning and sequencing. Virus was grown in L929 cells, purified by CsCl and sucrose gradient centrifugations and used to prepare viral RNA by phenol–chloroform extraction (Yoon et al., 1988). The RNA was precipitated twice with ethanol, dissolved in diethylypyrocarbonate-treated water and stored at - 70 °C. Purified viral RNA (0.5 to 1 μg) was denatured by heating at 95 °C for 3 min and annealed with PstI-digested, oligo(dT)-tailed pT718R plasmid by slow cooling. The cloning was performed by a method modified from that of Okayama & Berg (1982). pT718R (Pharmacia) was digested with PstI and T-tailed at both 3' ends in 100 μl of reaction mixture containing Co2+ buffer (BRL), 0.1 mM-dTTP and 30 units of terminal deoxynucleotidyl transferase at 37 °C for 30 min. One T-tailed end of this vector was removed by HindIII digestion. After annealing of the EMC-DV RNA with the T-tailed vector, first and second strand cDNA were synthesized using avian myeloblastosis virus (AMV) reverse transcriptase (BMB), RNase H (BRL), and Escherichia coli DNA polymerase 1 (BMB) according to the method of Gubler & Hoffman (1983). After T4 DNA polymerase (BMB) treatment to remove any 3' overhangs at both ends of the cDNA and the vector, the cDNA-vector was recircularized by blunt-end ligation using T4 DNA ligase. The recombinant clones were transfected into E. coli DH5αF' competent cells (BRL) and screened by LacZ- phenotype. A clone containing a 2600 bp cDNA insert was selected and used for DNA sequencing as described previously (Bae et al., 1989).

Amplified cDNA was restriction-mapped, subcloned into appropriate enzyme site(s) of the pT718R (or pT719R) vector and sequenced by the dideoxyribonucleotide chain termination method (Sanger et al., 1980) using [α-32P]dATP [sp. act. 500 Ci/mmol (18.5 TBq/mmol) NEN/ DuPont], M13 reverse primer, and Sequenase (US Biochemicals). Overlapping restriction fragments were subcloned and sequenced bidirectionally to eliminate any ambiguity.

RNA sequencing. The first 5' 5300 genomic sequences of EMC-DV1 except for the poly(C) tract were determined by DNA sequencing. The first primer, complementary to bases 5530 to 5549, was synthesized by use of the previous cDNA sequence information. After 32P-labelling with T4 polynucleotide kinase and [α-32P]dATP [sp. act. 6000 Ci/mmol (222 TBq/mmol) NEN/DuPont], 5 ng of the 32P-labelled primer was annealed with 1 to 2 μg of purified DV1 viral RNA in a 12 μl annealing buffer containing 250 mM-KCl and 10 mM-Tris-HCl pH 8.3, by heating at 95 °C for 2 min and then incubating sequentially at 70 °C for 5 min, at 65 °C for 6 min and at 42 °C for more than 1 h. To this annealing mixture, 15 μl of reverse transcription buffer [24 mM-Tris-HCl pH 8.3, 16 mM-MgCl2, 8 mM-dithiothreitol, 0.4 mM-dATP, 0.4 mM-dCTP, 0.8 mM-dGTP, 0.4 mM-dTTP, 100 μg/ml actinomycin D, 30 units of RNasin (Pharmacia), 10 μCi (0.37 TBq) [α-32P]dATP and 20 units of AMV reverse transcriptase] was added and 6 μl reaction mixture was distributed into each reaction tube containing 1 μl of termination mixture of 0.1 mM-ddATP, 0.1 mM-ddCTP, 0.1 mM-ddGTP and 0.2 mM-ddTTP, respectively. The samples were incubated at 42 °C (or 50 °C) for 30 min and were applied to an 8%polyacrylamide sequencing gel containing 7 M-urea. The following sequences were determined by the primer extension RNA-directed DNA sequencing method. Seven diabetogenic clones (EMC-D) of eight plaque isolates were partially sequenced around the area covering the nucleotide differences (shown in Table 2) by the same method.

For the determination of the length of the 5' poly(C) tract, 2 μg of EMC-DV1 RNA was digested with 100 units of RNases T1 and U2 (BRL) for 45 min at 55 °C in 20 μl of 25 mM-sodium citrate buffer pH 5.0 containing 1 mM-EDTA. The fragments were labelled at the 5'-end using [γ-32P]dATP and T4 kinase, and then applied to an 8% polyacrylamide gel. The poly(C) tract, actually poly(C)-A, was
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EMC-DV1 RNA

Fig. 1. Genomic organization of EMC-DV1 and its sequencing strategy. Large arrow on the right-hand side (3DV-1/8) shows cloned cDNA starting from the 3' end, and short arrows indicate subcloning span and cDNA sequencing direction. Circle-attached small arrows indicate RNA-directed DNA sequencing starting from each synthetic primer (open circle). The asterisk (*) denotes the poly(C) tract, the length of which was determined by enzymic RNA sequencing.

extracted from the gel by incubation overnight at room temperature with an extraction buffer composed of 0.5 M-ammonium acetate, 10 mM-EDTA, 10 mM-magnesium acetate, 10 mM-vanadyl ribonucleoside complex and 40 μl/ml carrier yeast tRNA. The extracted RNA sample was sequenced with RNase CL3 (BRL) for 5 min at 37 °C in 10 mM-sodium phosphate buffer (pH 6.5) containing 20 mM-KCl and 10 μM-EDTA, and with RNase PhyM (BRL) for 15 min at 55°C in a reaction mixture containing 25 mM-sodium acetate (pH 5.0), 7 mM-urea and 1 mM-EDTA.

Results

Isolation of EMC-DV1 variant and its biological activities

To select a mutated non-diabetogenic variant from an EMC-D stock, individual plaques were amplified, and inoculated into SJL/J male mice. Only one out of eight plaque-isolated clones did not produce diabetes and was designated EMC-DV1. EMC-DV1 showed a multiplication efficiency on L929 cell cultures of 5 × 10⁹ to 10¹⁰ p.f.u./ml, similar to that of EMC-D. When mice were inoculated with EMC-DV1, the mean glucose level was 148 ± 22 mg/dl. None of the 30 infected mice became diabetic. In contrast, over 90% of the mice inoculated with the other clones became diabetic as expected for EMC-D. The mean blood glucose level of these groups ranged from 395 to 486 mg/dl. As controls, original stocks of both EMC-D and EMC-B were inoculated into mice. The mean glucose level of EMC-D-inoculated mice was 420 mg/dl and 95% of the mice became diabetic. In contrast, the mean glucose level of EMC-B-inoculated mice was 132 mg/dl and none of the animals became diabetic (Table 1).

To see whether the non-diabetogenic EMC-DV1 variant could induce interferon, L929 cells were infected at three different m.o.i. (1, 10 and 100), and the maximum titers are shown in IRU based on reference interferon purchased from Sigma.

Table 1. Biological activities of EMC-DV1, EMC-D and EMC-B

<table>
<thead>
<tr>
<th>Virus</th>
<th>Number of mice</th>
<th>Serum glucose* (mg/dl)</th>
<th>Number of mice diabetic/total</th>
<th>Induction of interferon in L929 cell culture†</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMC-DV1</td>
<td>30</td>
<td>148 ± 22</td>
<td>0/30</td>
<td>ND‡</td>
</tr>
<tr>
<td>EMC-D</td>
<td>20</td>
<td>420 ± 35</td>
<td>19/20</td>
<td>ND</td>
</tr>
<tr>
<td>EMC-B</td>
<td>20</td>
<td>132 ± 19</td>
<td>0/20</td>
<td>8 × 10⁴ IRU/ml</td>
</tr>
<tr>
<td>Control§</td>
<td>10</td>
<td>126 ± 16</td>
<td>0/10</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Expressed as glucose index: [4 × (NF day 14) + 2 × (NF day 7) + 2 × (GTT day 17) + 1 × (GTT day 10)] / 10.
† Interferon was induced at three different m.o.i. (1, 10 and 100), and the maximum titers are shown in IRU based on reference interferon purchased from Sigma.
‡ ND, Not detectable.
§ Mock infection with medium.
Table 2. Nucleotide and deduced amino acid differences among the genomes of EMC-DV1, EMC-D and EMC-B

<table>
<thead>
<tr>
<th>Nucleotide (amino acid) position*</th>
<th>Nucleotide difference</th>
<th>Amino acid difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EMC-DV1</td>
<td>EMC-D</td>
</tr>
<tr>
<td>Length</td>
<td>Poly(C) tract</td>
<td>129</td>
</tr>
<tr>
<td>Point mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>875 (16)</td>
<td></td>
<td>CUU</td>
</tr>
<tr>
<td>1930 (366)</td>
<td></td>
<td>ACC</td>
</tr>
<tr>
<td>2840 (671)</td>
<td></td>
<td>ACU</td>
</tr>
<tr>
<td>2873 (682)</td>
<td></td>
<td>GCA</td>
</tr>
<tr>
<td>2888 (687)</td>
<td></td>
<td>GAA</td>
</tr>
<tr>
<td>3155 (776)</td>
<td></td>
<td>ACC</td>
</tr>
<tr>
<td>3239 (804)</td>
<td></td>
<td>GUC</td>
</tr>
<tr>
<td>3242 (805)</td>
<td></td>
<td>UCG</td>
</tr>
<tr>
<td>4396 (1188)</td>
<td></td>
<td>UCG</td>
</tr>
<tr>
<td>5836 (1668)</td>
<td></td>
<td>GCU</td>
</tr>
<tr>
<td>Insertion§</td>
<td>765</td>
<td></td>
</tr>
<tr>
<td>Deletion§</td>
<td>7829</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>7830</td>
<td>G</td>
</tr>
</tbody>
</table>

* Nucleotides and deduced amino acids are numbered on the basis of the consensus sequence of EMC-D, EMC-B and EMC-DV1.
† NA, Not applicable.
‡ ND, No difference.
§ Deletion and insertion relative to the EMC-D sequence.

**cDNA clone and genomic sequence of EMC-DV1**

From the several cDNA clones covering the 3' end region of the viral genome, clone 3-DV1/8 was selected and used for sequencing the 3' end 2600 bases. The restriction map, subcloning and sequencing strategies are shown in Fig. 1. The first 5300 base sequences were determined by primer extension RNA-directed DNA sequencing and enzymic RNA sequencing using 25 synthetic primers as described in Methods (Fig. 1). We were able to read 250 to 300 bases per primer-dependent RNA sequence, and used four more primers (p-2938, p-3127, p-3259 and p-3515) to confirm the VP1 area.

**Comparison of EMC-DV1 genome with those of EMC-D and EMC-B**

The EMC-DV1 genome (except the 5' VPg and 3' poly(A)) consists of 7828 bases, between those of EMC-D (7829 bases) and EMC-B (7824 bases) in size, and contains a long open reading frame of 6876 nucleotides starting at position 828, which encodes a polyprotein of 2292 amino acids. The number of coding amino acids and the L434 genomic organization are the same as those of EMC-D and -B and are very similar to those of other cardioviruses including EMC-RRR (Palmenberg et al., 1984).

The sequence differences and deduced amino acid changes among EMC-D, EMC-B and EMC-DV1 are summarized in Table 2. EMC-DV1 has seven different nucleotides in the polyprotein coding region in comparison with that of EMC-D. Four of them direct amino acid substitutions due to first base changes of each codon (from DV1 to D): position 875 (C-U; Leu-Phe) on the L gene, and positions 2873 (G-A; Ala-Thr), 2888 (G-C; Glu-Gln), 3239 (G-A; Val-Ile) and 3242 (U-A; Ser-Thr) on the ID (VP1) gene. Meanwhile, two of the seven plaque-isolated diabetic clones (EMC-D) were found to have nucleotide A (Thr) instead of G (Ala) at position 2840 on the VP1 (Fig. 2). The remaining three nucleotide differences at positions 1930 (C-U), 4396 (G-A) and 5836 (U-C) do not have any influence at the amino acid level. EMC-DV1 has 129 Cs on the poly(C) tract, and is one C shorter than that of EMC-D (130 Cs) and two Cs longer than that of EMC-B (127 C).

Compared with EMC-B, EMC-DV1 shows 10 nucleotide differences in the coding and non-coding regions. Among the five mutations in the coding region, four are located on the ID gene and result in amino acid substitutions (from DV1 to B): positions 2873 (G-A; Ala-Thr), 2888 (G-C; Glu-Gln), 3239 (G-A; Val-Ile) and 3242 (U-A; Ser-Thr). One point mutation at 5836 on the 3C (P22) gene is silent. The other five nucleotide differences in the 5' and 3' non-coding areas are deletions and an insertion. EMC-DV1 has neither the U base insertion nor two base (AG) deletion found in EMC-B at position 765 and the 3' end poly(A) junction area, respectively. The remaining two nucleotide differences occur at the poly(C) tract.
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Fig. 2. Amino acid sequence comparisons among several cardiovirus VP1s. The sequence of VP1 was numbered from the N terminus to the C terminus. Dash indicates that the amino acid at that site is identical to the residue in the same position in the EMC-D sequence. Positive and negative symbols denote the diabetogenicity of each strain. Thr 47, indicated by arrow, was found in place of Ala 47 in two of the seven plaque-isolated diabetogenic viruses. Asterisks (*) show the residues involved in the 'pit' area in Mengovirus (Luo et al., 1987). The hydrophilic patch containing three proline residues is underlined, and the 152nd amino acids on the VP1 in several cardioviruses are enclosed in the box.

Discussion

The separation from EMC-M virus stock of diabetogenic EMC-D and non-diabetogenic EMC-B made it possible to define the genomic area responsible for the diabetogenicity as closely as 14 nucleotide differences (Bae et al., 1989). The present isolation of another variant, EMC-DV1, which is non-diabetogenic and different from EMC-D and also different from EMC-B in interferon inducibility, enabled us to analyse the diabetogenic sites more closely. The comparative study on the biological activities and full sequence information of the three different EMC variants (EMC-D, EMC-B and EMC-DV1) led us to narrow down the potential diabetogenic sites to two, from the previously identified 14 nucleotides.

Sequence comparison (Table 2) shows that EMC-B, the unique interferon-inducing variant, has the following specificities in the genomic sequence: U base insertion at 765, 2873-A, 2888-C, 3242-A and two part deletions of three Cs at the poly(C) tract and AG at the 3' end. Among these sequence specificities, our structural analysis with the FOLD program (Zuker & Stiegler, 1981) revealed that the sequence from 720 to 830 around the 765-U insertion was predicted to possess a strong hairpin structure with the folding energy of -8.3 kJ/mol. Nucleotide 765 is located at a stem–loop junction of the sequence which is highly conserved among several cardioviruses except the 765-U insertion in EMC-B (Bae et al., 1989). The U base insertion at this site causes a one base pair longer stem and an apparent subtle conformational change on the secondary structure in EMC-B. The snap-back structures of vesicular stomatitis virus (Marcus & Garcione, 1989) or hairpin structures of the reovirus S1 mRNA transcript (Bischoff & Samuel, 1989) have been reported to be important in mediating the induction of interferon. The predicted strong secondary structure of EMC virus may thus be associated with the interferon-inducing potential, and the U base insertion in this region may have changed the EMC-B genome to make it a potent interferon inducer.

The mechanism by which EMC-D, but not EMC-B or other variants, destroys the pancreatic beta cells is not well understood. Interferon inducibility of EMC-B was thought to be related to the non-diabetogenicity of EMC-B. However, the isolation of a new variant (EMC-DV1),
which does not induce interferon and is non-diabetogenic even though its RNA genome is almost the same as that of EMC-D, enables us to exclude the possibility of the involvement of interferon in the non-diabetogenicity of EMC virus.

Another possibility is that the U base insertion in the highly conserved hairpin structure at the 5' non-coding region may affect the capacity of EMC-B virus to replicate for several reasons, as discussed previously (Bae et al., 1989), resulting in 10- to 100-fold lower titres in cell culture. Nevertheless, our data show that both variants, either having U base insertion (EMC-B) or not (EMC-DV), are non-diabetogenic. This suggests that a U base insertion or deletion at position 765 does not affect the diabetogenicity of EMC virus regardless of whether it affects the induction of interferon or influences the replication capacity.

Combining the biological data (Table 1) and the sequence analysis (Table 2), the deletion or insertion of 3'-AG and the four point mutations causing amino acid substitutions on the ID gene [in the order DV1-D-B, at positions 2873 (GCC-GCC-ACC; Ala-Ala-Thr), 2888 (GAA-GAA-CAA; Glu-Glu-Gln), 3239 (GUC-AUC-ΔUC; Val-Ile-Ile) and 3242 (UCG-UCA-ACG; U-U-A; Ser-Ser-Thr)] are unlikely to affect the diabetogenicity of EMC virus, because either or both of non-diabetogenic EMC-B and EMC-DV have the same nucleotides or amino acids as diabetogenic EMC-D at the corresponding sites (Table 2). The three silent mutations at positions 1930 (C-U-C), 4396 (G-A-G) and 5836 (U-C-C) may affect genomic stability, but we could not find any secondary structure around these areas which might be involved in the genomic stability. The variation of one or two nucleotides in the length of the 3' poly(C) tract may not affect the infectivity of EMC virus to beta cells. Meanwhile, two of the seven plaque-isolated diabetogenic clones (EMC-D) have nucleotide A (Thr) instead of G (Ala) at position 2840 on the VP1 (Fig. 2), indicating that the coding change mutation at position 2840 (ACU-GCU; Thr-Ala; amino acid 47 on the VP1) probably does not affect the diabetogenicity of EMC virus.

These comparative data analyses lead us to conclude that the two coding change mutations in EMC-D, at positions 875 (U; Phe) on the L gene and 3155 (G; Ala) on the ID gene, are the most likely candidates for controlling the diabetogenicity of EMC virus. Thus one or both of Phe 16 (L) and Ala 776 (residue 152 on the VP1) on the polyprotein may have a critical role in the diabetogenicity of EMC-D virus. Of the two amino acids, Phe 16 is located on the leader peptide for which no biological role is known. However, the L gene, which is the first part of the 5' coding region, is relatively free of secondary structure. This means that the L gene change is unlikely to affect the induction of interferon. In addition, direct amino acid comparison among several cardiovirus strains shows that some non-diabetogenic variants (Mengo-M and EMC-RRR) also have Phe at position 16 on their leader peptides, suggesting that Phe-16 may not play a significant role in the diabetogenicity of EMC-D virus.

On the other hand, Ala 776 (amino acid 152 on the VP1) seems to be involved in viral attachment to beta cells, by analogy to the viral attachment sites of Mengovirus (Luo et al., 1987) and rhinovirus 14 (Rossmann et al., 1985). The EMC VP1s have over 95% sequence homology with that of Mengovirus (Fig. 2), and the 152nd amino acid on VP1 happens to be among the residues in the 'pit', the proposed attachment site for Mengovirus (Luo et al., 1987). Furthermore, the 152nd amino acid in EMC viruses (Ala) lies in the highly conserved, strongly hydrophilic patch of VP1 (Fig. 2) containing three proximal prolines (Pro-Thr-Gly-Thr-Gly-Thr), suggesting that Phe-16 may not play a significant role in the diabetogenicity of EMC-D virus.

Nevertheless, our present data do not completely rule out other possibilities such as certain combinations of mutations that may affect the diabetogenicity of EMC virus in a concerted way. Confirmation for the present molecular assessment of the biological activities is now being pursued by further molecular genetic studies involving production of mutant viruses from the full-length cDNA constructs and site-specific mutagenesis on those potential sites.

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