Gain or Loss of Diabetogenicity Resulting from a Single Point Mutation in Recombinant Encephalomyocarditis Virus

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Molecular pathogenetic mechanisms for virus-induced disease have received considerable attention. Encephalomyocarditis (EMC) virus-induced diabetes in mice has been extensively studied to elucidate the cellular and molecular mechanisms involved in the development of this disease. In this study, we report for the first time that a single point mutation at nucleotide position 3155 or 3156 of the recombinant EMC viral genome, located on the major capsid protein VP1, which causes an amino acid change, results in the gain or loss of viral diabetogenicity. A G base at nucleotide position 3155 (alanine at amino acid position 776 of the EMC virus polyprotein [Ala776]; GCC) results in viral diabetogenicity, whereas the substitution of other bases at the same or next position results in a loss of viral diabetogenicity. This finding provides clear evidence that a point mutation at a critical site in a viral genome affects the ability of the virus to cause a cell-specific disease.

One of the best examples indicating that viruses play an etiological role in the pathogenesis of insulin-dependent diabetes mellitus (IDDM) comes from mice infected with encephalomyocarditis (EMC) virus (8, 13, 21, 22). The M variant of EMC (EMC-M) virus induces diabetes by infecting and destroying pancreatic beta cells in susceptible mice. However, this effect was found to be inconsistent (15, 24). Through plaque purification of EMC-M virus, we isolated two stable variants, the highly diabetogenic EMC-D virus and the nondiabetogenic EMC-B virus (22). We used these two variants to identify the viral gene or genes responsible for the induction of diabetes in mice and to study the long-term complications of virus-induced diabetes (25). Our initial examination of the genomes of these two variants revealed a total of 14 nucleotide differences between them (2). Further studies of 21 different nondiabetogenic and 15 different diabetogenic viruses derived from stocks of the EMC-B and EMC-D variants revealed that only the 776th amino acid, alanine (Ala776), of the EMC virus polyprotein, located on the major capsid protein VP1, is common to all diabetogenic variants. In contrast, Thr at this position (Thr776) is common to all nondiabetogenic variants (1). Identification of this difference between the diabetogenic and nondiabetogenic variants of EMC virus was not sufficient to determine whether Ala776 plays a critical role in the development of IDDM in EMC virus-infected mice.

We next needed to perform functional tests to determine whether the exchange of Ala776 for Thr776, and vice versa, affects viral diabetogenicity. Previously, it was not possible to perform such experiments because of the characteristic long poly(C) tract, which makes it difficult to amplify viral cDNA in bacterial systems (1). We developed a new recombinant RNA technique involving the creation of a chimeric RNA by attaching the poly(C) region of the viral RNA to the 5′-truncated RNA transcript of the EMC viral cDNA (3). We used this technique and site-specific mutagenesis at nucleotide position 3155 of the EMC viral genome to investigate the role of Ala776 in the diabetogenicity of EMC virus in susceptible SJL/J mice.

We first changed the amino acid at position 776 (Ala; GCC) of the polyprotein of diabetogenic EMC virus to Thr (ACC) by site-specific mutagenesis with a Muta-Gen kit (Bio-Rad, Richmond, Calif.). In order to obtain a uracil-containing single-stranded template, an EMC-D viral cDNA fragment covering bases 1870 to 3190 from pd104 (1, 3) was subcloned to the SphI and SstI sites of a phagemid, p7Z19R, and then transfected into Escherichia coli CJ236 (Dut- Ung+). The uracil-containing single-stranded recombinant phagemid was isolated by superinfection of the M13K07 helper phage. After annealing the mutagenic oligonucleotide containing the desired base (Thr776-GCAACTTGGAGGCCACCAAGCACCAGGCAACACCTG to the single-stranded template, a mutant strand was synthesized by T4 DNA polymerase and joined to T4 DNA ligase (3). This heteroduplex recombinant phagemid was introduced into E. coli MV1190 (Ung+). Mutants were screened by the double-stranded deoxy DNA sequencing method using the M13 universal primer (U.S. Biochemicals, Cleveland, Ohio). To obtain mutated transcript RNA, SphI and SstI fragments (nucleotides 1870 to 3190), mutated at nucleotide position 3155, were subcloned to the corresponding site of pDEPHI (1, 3). In vitro transcription was carried out with T7 RNA polymerase after linearization of the plasmid with SfiI. The 5′ viral RNA (nucleotides 1 to 476) was isolated by gel elution after cutting of EMC-D viral RNA between nucleotide positions 476 and 477 with RNase H (17). This 5′ viral RNA (nucleotides 1 to 476) was joined to a mutated transcribed RNA [nucleotide 477 to the 3′ poly(A) tail] by using RNA ligase and a brace primer to construct chimeric RNA (3). The chimeric RNA was transfected into an L929 cell monolayer (6), and progeny viruses were isolated from the L929 cells. The sequence of the mutated region (i.e., GCC [Ala776]→ACC [Thr776]) of the progeny viruses was confirmed by direct RNA sequencing. Similarly, we changed the amino acid at position 776 (Thr; ACC) of the polyprotein of nondia-
TABLE 1. Effects of alanine and threonine at amino acid position 776 of the EMC virus polyprotein on induction of diabetes in EMC virus-infected SJL/J mice

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of mice</th>
<th>Glucose index(^{a}) (mg/dl; mean ± SD)</th>
<th>Pancreatic insulin level(^{b}) (μg/g of pancreas; mean ± SD)</th>
<th>% with diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>32</td>
<td>129 ± 23</td>
<td>383 ± 47</td>
<td>0</td>
</tr>
<tr>
<td>EMC-D</td>
<td>21</td>
<td>436 ± 87</td>
<td>23 ± 8</td>
<td>100</td>
</tr>
<tr>
<td>Ala(^{776}) chimeric</td>
<td>17</td>
<td>406 ± 97</td>
<td>17 ± 9</td>
<td>100</td>
</tr>
<tr>
<td>Thr(^{776}) chimeric</td>
<td>20</td>
<td>153 ± 21</td>
<td>337 ± 36</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{a}\) Blood glucose levels were measured in virus-infected (10\(^5\) PFU/mouse, intraperitoneally) and PBS-injected mice by glucose oxidase assay with o-dianisidine dihydrochloride as the indicator dye and the glucose index was calculated (22, 24). Briefly, for each mouse, nonfasting (NF) glucose levels were measured on days 7 and 14, and glucose tolerance tests (GTT) were performed on days 10 and 17. These four values were then combined to give the glucose index for each mouse in order to eliminate some of the variability associated with individual values which may occur when single glucose determinations are used: \([4 \times \text{NF day 14}] + (3 \times \text{NF day } 7) + (2 \times \text{GTT day } 17) + (1 \times \text{GTT day } 10)]/10. Any mouse with a glucose index greater than 244 mg/dl (5 standard deviations above the mean for nondiabetic control PBS-injected mice) was classified as diabetic (22).

\(^{b}\) Insulin was extracted from the pancreas and measured by radioimmunoassay at 17 days after infection (22).

We also measured pancreatic insulin content by radioimmunoassay (22). Pancreatic insulin concentrations were low for both EMC-D virus-infected and Ala\(^{776}\) chimeric virus-infected mice, with levels of 23 ± 8 and 31 ± 9 μg/g of pancreas, respectively. In contrast, pancreatic insulin concentrations were significantly higher in Thr\(^{776}\) chimeric virus-infected and PBS-injected control animals, with levels of 337 ± 36 and 383 ± 47 μg/g of pancreas, respectively (Table 1).

To determine whether the differences in blood glucose levels and pancreatic insulin content were reflected in the histopathology of the pancreas, groups of experimental and control mice, treated as described above, were sacrificed at 17 days after infection. Microscopic examination of pancreatic sections revealed extensive beta-cell destruction in Ala\(^{776}\) chimeric virus-infected mice (Fig. 1A; Table 2) or PBS-injected control mice. On the basis of blood glucose levels, pancreatic insulin content, and islet histopathology, we conclude that the chimeric virus containing Ala\(^{776}\) induces IDDM as effectively as does the wild-type EMC-D virus. In contrast, the chimeric virus containing Thr\(^{776}\) in place of Ala\(^{776}\) shows no diabetogenicity.

To determine whether the substitution of other amino acids for Ala\(^{776}\) has any effect on the diabetogenicity of EMC virus, we constructed all possible base permutations of either the first or second base position of codon 776, using site-specific mutation with appropriate oligonucleotides (Ala\(^{776}\) CCAACTGG

FIG. 1. (A) A pancreatic section from mice infected with Ala\(^{776}\) chimeric virus shows extensive inflammatory infiltration of the islets and beta-cell necrosis. (B) A pancreatic section from mice infected with Thr\(^{776}\) chimeric virus shows no inflammation. Original magnification, X 400.
colocalized fluorescence in over 50% of the examined islets. Furthermore, this residue is in a highly conserved hydrophilic pit area, the proposed attachment site for mengovirus. Finally, similar mengovirus (12), the 776th amino acid lies in the beta cells (Table 3).

Ala<sup>776</sup> chimeric virus showed EMC virus-specific antigens in their beta cells (Table 3).

The significance of the 776th amino acid on the EMC virus polyprotein in the early stages of EMC virus infection and the pathogenesis of IDDM is not clear. By analogy to the structurally similar mengovirus (12), the 776th amino acid lies in the pit area, the proposed attachment site for mengovirus. Furthermore, this residue is in a highly conserved hydrophilic patch of the EMC virus major capsid protein, VP1, containing three proximal prolines (Pro-Thr-Gly-Thr-Pro-Ala<sup>776</sup>-Lys-Pro). Such sites are proposed as viral attachment sites in picornaviruses (16). Our earlier study showed that an amino acid change from Thr<sup>776</sup> (nondiabetogenic EMC-B virus) to Ala<sup>776</sup> (diabetogenic EMC-D virus) reduced the hydrophilicity of this region by 37% (9). We speculate that a shift away from optimal hydrophilicity may affect the initial interactions between the virus and the beta cells. Thus, more hydrophobic (valine) or more hydrophilic (threonine, serine, proline, and aspartic acid) amino acid substitutions may adversely affect the attachment of the virus to beta cells. In addition, alanine may play an important role in viral penetration, uncoating of hydrophobic patches buried inside the capsid, or release of viral RNA from the capsid. These possibilities remain to be investigated.

Finally, genetic factors may exert a major influence on the development of IDDM (4). However, much evidence suggests that environmental factors are also important, influencing the penetrance of diabetes susceptibility genes (19). For example, the concordance rate for IDDM between monozygotic, genetically identical twins is 40% or less (5). As an environmental factor affecting the induction of IDDM, the virus may be considered a primary agent injurious to pancreatic beta cells and/or a triggering agent of beta-cell-specific autoimmunity, resulting in the onset of IDDM (7, 10, 18). The development of diabetes in mice infected with the EMC virus provides the best evidence that viruses are primary injurious agents inducing diabetes (8, 13, 22). For the past 30 years, EMC virus-induced diabetes has been extensively studied (18). However, the molecular pathogenic mechanisms involved in the development of this disease have not been elucidated. In this study, we have demonstrated for the first time that a single amino acid at

### TABLE 2. Histological changes in the islets of Langerhans from SJL/J mice infected with recombinant chimeric viruses<sup>a</sup>

<table>
<thead>
<tr>
<th>Recombinant chimeric virus</th>
<th>Development of diabetes</th>
<th>Total no. of islets examined</th>
<th>No. of islets (%) with the indicated histological changes&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>M</td>
<td>S</td>
</tr>
<tr>
<td>Ala&lt;sup&gt;776&lt;/sup&gt;</td>
<td>Yes</td>
<td>88</td>
<td>5 (5.7)</td>
</tr>
<tr>
<td>Thr&lt;sup&gt;776&lt;/sup&gt;</td>
<td>No</td>
<td>95</td>
<td>95 (100)</td>
</tr>
<tr>
<td>Ser&lt;sup&gt;776&lt;/sup&gt;</td>
<td>No</td>
<td>87</td>
<td>87 (100)</td>
</tr>
<tr>
<td>Pro&lt;sup&gt;776&lt;/sup&gt;</td>
<td>No</td>
<td>103</td>
<td>103 (100)</td>
</tr>
<tr>
<td>Asp&lt;sup&gt;776&lt;/sup&gt;</td>
<td>No</td>
<td>92</td>
<td>92 (100)</td>
</tr>
<tr>
<td>Val&lt;sup&gt;776&lt;/sup&gt;</td>
<td>No</td>
<td>112</td>
<td>112 (100)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Five- to six-week-old SJL/J mice were infected with recombinant chimeric viruses (10<sup>5</sup> PFU/mouse, intraperitoneally).

<sup>b</sup> N, normal islets with normal morphology; M, mild to moderate degree of insulitis with lymphocytic infiltration of 1 to 49% of islets and well-preserved islet architecture; S, severe insulitis with lymphocytic infiltration of 50 to 100% of islets; A, atrophied, small, retracted islets showing severe beta-cell necrosis, with or without residual lymphocytic infiltrate (11). Results are based on 15 to 20 islets per mouse and five to seven mice per group.

### TABLE 3. Effects of amino acid substitutions at position 776 of the EMC virus polyprotein on the infection of beta cells and the induction of diabetes in SJL/J mice

<table>
<thead>
<tr>
<th>Amino acid at position 776</th>
<th>Nucleotides&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Antiviral antibody titer&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Presence of viral antigen&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Glucose index&lt;sup&gt;d&lt;/sup&gt; (mg/dl, mean ± SD)</th>
<th>Pancreatic insulin level (μg/g of pancreas, mean ± SD)</th>
<th>% with diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>GCC</td>
<td>+ +</td>
<td>407 ± 98</td>
<td>30 ± 91</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>ACG</td>
<td>−</td>
<td>149 ± 19</td>
<td>391 ± 36</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>GCC</td>
<td>−</td>
<td>159 ± 21</td>
<td>387 ± 41</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>GGC</td>
<td>−</td>
<td>142 ± 19</td>
<td>396 ± 43</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>GAC</td>
<td>−</td>
<td>169 ± 21</td>
<td>379 ± 37</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>GTC</td>
<td>−</td>
<td>172 ± 18</td>
<td>398 ± 45</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Point mutations at nucleotide position 3155 or 3156 were made with appropriate synthetic oligonucleotides.

<sup>b</sup> Sera were obtained from mice 17 days after infection, and the titer of neutralizing anti-EMC antibodies was determined. Values are expressed as the mean reciprocal of the highest dilution of serum that inhibited plaque formation by 50%.

<sup>c</sup> At 3 days after infection, sections of pancreas from three randomly selected mice were stained with fluorescein-labelled anti-EMC virus antibody and rhodamine-labelled anti-insulin antibody to determine the presence of viral antigens in the beta cells (14, 20, 23). −, no colocalized fluorescence in the examined islets; +++, colocalized fluorescence in over 50% of the examined islets.

<sup>d</sup> Ten mice per group; 10<sup>5</sup> PFU of recombinant virus/mouse.
position 776 of the EMC virus polyprotein determines viral diabetogenicity. The change of a single amino acid (Ala→Thr) at amino acid position 776 of the polyprotein in diabetogenic EMC virus results in a loss of viral diabetogenicity. Similarly, the change of a single amino acid (Thr→Ala) at the same position in nondiabetogenic EMC virus results in a gain of viral diabetogenicity.

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REFERENCES


