Encephalomyocarditis Virus and Diabetes Mellitus: Studies on Virus Mutants in Susceptible and Non-susceptible Mice

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SUMMARY

The so-called M-variant (especially subtype D) of encephalomyocarditis virus (EMCV) induces a diabetes-like syndrome in certain mouse strains which may serve as a model of insulin-dependent diabetes mellitus (IDDM) in man. The development and course of diabetes was influenced by a number of virus and host factors, among these being virus strain, virus dose, mouse strain, age, sex, and the host's immunological status. In a D-variant stock of EMCV, we found a virus plaque variant (PV 2) diabetogenic for DBA/2 mice, and at least one variant (PV 7) that did not affect carbohydrate metabolism. Although the diabetogenicity of PV 2 proved to be a genetically stable characteristic after further passages in vivo and in vitro, the incidence of diabetes varied somewhat (mean value 65% in 10-week-old DBA/2 mice infected with 10^5 p.f.u.). Both lower (10^1 or 10^3 p.f.u.) and higher (10^7 or 10^8 p.f.u.) virus doses led to a diminished incidence and severity of diabetes. In younger animals (5 weeks) transient hyperglycaemia often appeared, whereas in older animals (20 weeks) there was a higher rate of mortality. Histological examination of the islets of Langerhans in diabetes-susceptible (DBA/2) and resistant (C57BL/6) mice revealed that EMCV-induced hyperglycaemia appeared to develop in parallel to islet cell damage. Even in diabetic animals, some unaffected islets were regularly found. This study demonstrates that EMCV mutants may have completely different biological effects and produce diabetes only in special circumstances. Host factors play a significant role in the development of diabetes.

INTRODUCTION

Encephalomyocarditis virus (EMCV) induces encephalitis and myocarditis in rodents (Warren, 1965). Craighead (1966a) isolated two variants from the EMC strain described by Murnane et al. (1960) which differed in myocardiotropism (M-variant) and neurotropism (E-variant). He found that the M-variant exhibited a particular tropism for the insulin-producing B-cells of the pancreas (Craighead, 1966b). A diabetes-like syndrome (Craighead & McLane, 1968), characterized by hyperglycaemia, can be induced in certain mouse strains (Craighead & Higgins, 1974), and may serve as a model of insulin-dependent diabetes mellitus (IDDM) (Müntefering et al., 1971).

Yoon et al. (1980) isolated two further variants from the EMCV M-pool: a diabetogenic (D) and a non-diabetogenic (B) variant; these were reported to interfere with each other and, therefore, to influence the incidence of diabetes. Although the D-variant has been purified by those authors through several selection steps, its diabetogenicity, in pilot experiments, has not been consistent in our laboratory. The aim of the present study was to isolate and characterize various EMCV variants and to determine factors influencing the incidence and severity of diabetes in mice (Kruppenbacher et al., 1982).
METHODS

Animals. DBA/2 and C57BL/6 mice were obtained from the Zentralinstitut für Versuchstiere, Hannover, F.R.G., and from Bomholtgard, Ry, Denmark, respectively. Unless stated otherwise, 10-week-old male mice were used in all experiments. Normally, five animals were held in a single cage. Mice were fed ‘Haltungsdiet 1324’ purchased from Altromin Spezialfutterwerke, Lage, F.R.G., and allowed free access to food and water.

Virus. The D-variant of EMCV was kindly provided by Dr J. W. Yoon, NIH, Bethesda, Md., U.S.A. Virus stocks were prepared, and titrations were performed in L-cell monolayers. Cell cultures were maintained on Eagle’s MEM with 5% foetal calf serum (FBS). Mice were infected intraperitoneally (i.p.) with 0·5 ml inoculum containing 10^5 p.f.u., unless otherwise stated. Plaque assays were done as described previously (Schürmann & Eggers, 1983) on L-cell monolayers with agarose overlay containing 1% newborn calf serum.

Isolation of the virus variants. Cell monolayers in Petri dishes (6 cm diam.) were inoculated with 40 p.f.u. After 2 days, monolayers were stained with 0·01% neutral red solution. Four h later, eight isolated plaques were picked with Pasteur pipettes according to arbitrary morphological aspects. Subsequently, a second plaque purification was performed with each of the eight plaque variants (PV); virus stocks were then prepared from each PV, and stored in aliquots at -20 °C.

Blood glucose assay. Blood was taken from the retroorbital sinus of each non-fasting animal. The blood glucose values were assayed by the hexokinase method (test combination Gluco-quant®, Boehringer Mannheim) and expressed in mg/dl. Most of the values were determined twice. The blood glucose values were established before infection, on the 3rd, 5th, 7th, 14th and 28th, and finally, in most cases, on the 60th day post-infection. Blood glucose values exceeding the average of uninfected mice by fivefold standard deviation were defined as hyperglycaemic. If hyperglycaemia existed for a period of at least 28 days after infection, mice were defined as diabetic. In some experiments urine was also examined semi-quantitatively for glucosuria (Glukotest®, Boehringer Mannheim).

Histological preparations. Pancreatic tissue was fixed in Bouin’s solution and embedded in Histosec® (E. Merck, Darmstadt, F.R.G.). From the tissue blocks, sections 6 to 10 μm thick, cut longitudinally, were taken. Since it was possible to differentiate the islets of Langerhans from the exocrine pancreas tissue clearly with haematoxylin and eosin staining, special staining methods (van Gieson, Goldner staining) were used only occasionally.

RESULTS

Selection of the virus variants

Initial screening of eight plaque-purified viruses (PVs) showed five to be diabetogenic. For further experiments, four PVs were chosen (PV 2, 3, 5, 7). In summary (Table 1), PV 2 produced diabetes in the majority of DBA/2 mice (65%), whereas PV 7 was consistently non-diabetogenic. It should be mentioned that in addition to the animals classified as diabetic (see Methods), we also found a number of animals with transient hyperglycaemia (e.g. 24% for PV 2). In our experiments, several passages of different virus stocks in vivo (mouse heart) and in cell culture caused no differences in diabetogenicity.

Mortality and diabetogenicity in DBA/2 mice of different ages

DBA/2 mice at the ages of 10 weeks, 20 weeks or 10 months were infected with 10^5 p.f.u. of diabetogenic variant PV 2. In older animals (20 weeks or older) the mortality increased substantially (Table 2a). In 10- and 20-week-old animals diabetes occurred in about 60% of surviving animals (Table 2b).

Dose dependence of diabetes incidence

The effect of virus dose (PV 2) on the development of diabetes and transient hyperglycaemia in DBA/2 mice is shown in Table 3. With a dosage of 10 p.f.u./animal only five of 20 animals developed transient hyperglycaemia. The blood glucose values of the other animals remained normal during the experiment. With 10^3 p.f.u., 16 of 19 animals became hyperglycaemic, but only eight became permanently diabetic. The highest incidence of diabetes was found with 10^5 p.f.u./animal. With doses higher than this, the incidence of diabetes decreased again. Mice infected with 10^8 p.f.u./animal did not become hyperglycaemic at all (Table 3).

Diabetogenic and non-diabetogenic PVs in susceptible and non-susceptible mice

Twenty DBA/2 and twenty C57BL/6 mice were infected with PV 2 or PV 7, respectively, and blood glucose levels were determined (Table 4). Five animals from each group were killed on
EMC virus-induced diabetes in mice

Table 1. Diabetogenicity of isolated EMCV mutants in DBA/2 mice

<table>
<thead>
<tr>
<th>Expt.</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>Total diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9/10*</td>
<td>3/10</td>
<td>7/10</td>
<td>0/10</td>
<td>36/55 (65%)</td>
</tr>
<tr>
<td>2</td>
<td>11/25</td>
<td>11/25</td>
<td>10/25</td>
<td>0/25</td>
<td>14/35 (40%)</td>
</tr>
<tr>
<td>3</td>
<td>16/20</td>
<td>ND†</td>
<td>ND†</td>
<td>0/20</td>
<td>0/55 (0%)</td>
</tr>
</tbody>
</table>

* Number of diabetic animals/number of animals infected.
† ND, Not done.

Table 2. (a) Mortality of EMCV PV 2 infection in DBA/2 mice of different ages

<table>
<thead>
<tr>
<th>Age of mice</th>
<th>Days after virus inoculation</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 weeks</td>
<td>1/197* (0.5%)</td>
<td>19/196 (10%)</td>
<td>38/196 (19%)</td>
<td>44/186 (24%)</td>
<td>51/127 (40%)</td>
<td></td>
</tr>
<tr>
<td>20 weeks</td>
<td>2/51 (4%)</td>
<td>9/51 (18%)</td>
<td>12/31 (39%)</td>
<td>18/31 (58%)</td>
<td>22/31 (71%)</td>
<td></td>
</tr>
<tr>
<td>10 months</td>
<td>0/14 (0%)</td>
<td>10/14 (71%)</td>
<td>13/14 (93%)</td>
<td>13/14 (93%)</td>
<td>13/14 (93%)</td>
<td></td>
</tr>
</tbody>
</table>

* Number of dead/infected animals.

(b) Diabetogenicity of EMCV PV 2 infection in DBA/2 mice of different ages

<table>
<thead>
<tr>
<th>Age of mice</th>
<th>Days after virus inoculation</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 weeks</td>
<td>0/44† (0%)</td>
<td>40/77 (52%)</td>
<td>79/123 (64%)</td>
<td>31/58 (53%)</td>
<td>40/71 (56%)</td>
<td></td>
</tr>
<tr>
<td>20 weeks</td>
<td>2/49 (4%)</td>
<td>22/42 (52%)</td>
<td>17/19 (89%)</td>
<td>2/3 (66%)</td>
<td>4/6 (66%)</td>
<td></td>
</tr>
<tr>
<td>10 months</td>
<td>0/14 (0%)</td>
<td>1/4 (25%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† Number of hyperglycaemic/surviving animals.

Table 3. Influence of virus dose on the development of diabetes in DBA/2 mice (summary of several experiments)

<table>
<thead>
<tr>
<th>Dose (p.f.u./mouse)</th>
<th>Number of mice</th>
<th>Diabetes</th>
<th>Transient hyperglycaemia</th>
<th>Normal glycaemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁴</td>
<td>0</td>
<td>5</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>10³</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>10²</td>
<td>16</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>10¹</td>
<td>1</td>
<td>6</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>10⁰</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Concentration of blood glucose in DBA/2 and C57BL/6 mice after inoculation with diabetogenic and non-diabetogenic EMCV variants

<table>
<thead>
<tr>
<th>Virus variant</th>
<th>Mouse strain</th>
<th>Uninfected</th>
<th>Days after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>PV 2</td>
<td>DBA/2</td>
<td>170 ± 18*</td>
<td>199 ± 95</td>
</tr>
<tr>
<td></td>
<td>C57BL/6</td>
<td>181 ± 18</td>
<td>126 ± 19</td>
</tr>
<tr>
<td></td>
<td>DBA/2</td>
<td>169 ± 11</td>
<td>148 ± 13</td>
</tr>
<tr>
<td></td>
<td>C57BL/6</td>
<td>176 ± 12</td>
<td>146 ± 21</td>
</tr>
</tbody>
</table>

* Mean blood glucose level (mg/dl) ± S.D.
days 3, 5, 7 and 14 for histological studies. Eighty\% of PV 2-infected DBA/2 mice developed hyperglycaemia. The same virus did not cause significant alteration of carbohydrate metabolism in C57BL/6 mice. PV 7 was non-diabetogenic for both mice strains.

**Histological findings**

The endocrine islet tissue of the pancreas of DBA/2 and C57BL/6 mice was quantitatively examined from several Histosec-embedded sections of each pancreas. Each value given in Table 5 is derived from examination of 100 islets. The extent of pyknotic nuclei, necrotic cells and the number of mononuclear infiltrate cells paralleled the incidence and severity of the diabetes syndrome (see also Table 4). Similar but less pronounced differences were detected in exocrine tissue.

**Other symptoms of EMCV infection in DBA/2 mice**

Checking for glucosuria was useful as a simple and quick screening procedure. Furthermore, in all experiments, it was observed that the mean body weight of infected animals, compared with controls, decreased within the first week; however, the differences were not statistically significant. Neurological symptoms appeared only in animals infected with PV 2. Paresis and paralysis of the hind legs were seen between the 5th and 7th day after infection. Altogether, 48 of 310 PV 2-infected animals showed paralysis. Sixty-seven\% of these 48 animals did not become diabetic.

**DISCUSSION**

Yoon et al. (1977) have suggested that there might be at least two virus variants in the EMCV M-pool which differ in diabetogenicity in DBA/2 mice. By several steps of selection they succeeded in isolating a highly diabetogenic EMCV D-variant and a non-diabetogenic EMCV B-variant (Yoon et al., 1980). However, the present work shows that even highly purified diabetes-inducing EMCV D-stock contains mutants of different pathogenicity. From eight PVs cloned from EMCV D, we found at least one mutant, PV 7, which was totally non-diabetogenic for DBA/2 mice. Two other variants (PV 6 and PV 8) also apparently did not affect carbohydrate metabolism, but they have been tested in only a small number of mice.

We were able to show that the pathogenic properties remained constant after further passages in vitro and in vivo in the course of our experiments. Even if new mutants arose, the pathogenic properties of the virus stocks as a whole did not significantly change throughout our experiments.

We found some variability in the incidence of diabetes, with a mean value of 65\%. The variability was about in the same range as in the pilot experiments with the original D-variant (data not shown). Our most diabetogenic virus, PV 2, did not achieve the 95\% incidence of diabetes reported by Yoon et al. (1980). It is reasonable to assume that divergent definitions of diabetes account for the apparent differences of diabetes incidence. If we add the number of animals with transient hyperglycaemia to the number of diabetic animals, then our total percentage increases to about 90\%. The glucose index (Ross et al., 1976) used by Yoon et al. to define diabetes discerns minor hyperglycaemic alterations and provides a simple evaluation of carbohydrate metabolism. However, animals may be diagnosed as diabetic which are only transiently hyperglycaemic.
Craighead & Steinke (1971) found that the EMCV M-variant was less pathogenic in older animals than in younger ones. Our experiments also demonstrated a clear-cut relationship between age and susceptibility. With mutant PV 2, the mortality of mice increased substantially with increasing age. Almost all 10-month-old mice inoculated with $10^5$ p.f.u. of PV 2 succumbed. Histological examination of these animals showed acute necrosis of the pancreas. Myocarditis in moribund animals was as pronounced as in non-moribund animals. The pancreata of the latter, however, were largely intact. The brains of all animals examined (moribund or not) exhibited only minimal or no histological changes. Therefore, it is assumed that involvement of the exocrine pancreas accounts for the mortality in old animals. Due to great variance in their experiments Ross et al. (1976) did not find age-related differences in the incidence of diabetes. Our results are compatible with these findings.

In order to optimize the model, the administered virus dose was varied. When a dose of $10^5$ p.f.u. was inoculated, the highest incidence of diabetes in DBA/2 mice was observed. The percentage of diabetic animals decreased with lower doses. Surprisingly, it also decreased with higher virus doses. With $10^8$ p.f.u., we did not find any disturbance of blood glucose levels. As far as we know, $10^8$ p.f.u. has not been used as infective dose by other investigators and may explain why this phenomenon was not clearly established before (Yoon et al., 1977). At present, we have insufficient data to offer a convincing hypothesis to explain this interesting phenomenon.

The detailed histological examination of pancreata of DBA/2 and C57BL/6 mice validated the previous finding that there is good correlation between the severity of diabetes and the extent of pathological changes of injured islets (Müntefering, 1972; Hayashi et al., 1974). It is shown that (i) PV 2 induced more islet destruction than PV 7 in both mouse strains and (ii) that the susceptible DBA/2 mice exhibited more islet destruction than non-susceptible C57BL/6. On the other hand, all diabetic animals had some morphologically intact islets, and non-diabetic mice showed moderate islet damage. Interestingly, even the exocrine tissue of pancreas is affected to different degrees. These results strengthen the view that the virus variant as well as the mouse strain (genetic factor) are of great significance in the development of diabetes (see also Yoon et al., 1984).

Another interesting observation was that 15% of the DBA/2 mice developed paralysis of the hind legs. Therefore, it is evident that the virus still has neurotropic properties. A disproportionately high number of the paralysed mice did not have elevated blood glucose levels, even though feeding was facilitated to compensate for difficulties of food intake caused by the paralysis.

It has to be assumed that there exist biochemical differences among the EMCV variants which are not yet defined (Ray et al., 1983). On the other hand, host factors, e.g. age and strain of mice, also play an important role in the development of diabetes. Furthermore, a critical virus dose is necessary to produce a high incidence of this disease. Sex (Boucher et al., 1975), immunomodulation (Jansen et al., 1977; Müntefering et al., 1979) and hormones (Morrow et al., 1980) have also been reported to be significant. Therefore, we conclude that only a set of special circumstances favours the development of diabetes.

Only a fraction of IDDM in man may be caused by coxsackie B viruses (Mertens et al., 1983). Our data from virus-induced diabetes in mice offer an explanation of why this disease rarely occurs, despite the occurrence of widespread virus infections. Although there is no final proof for a virus aetiology of IDDM in man, several suggestive observations exist. Further investigations of the animal model of EMCV-induced diabetes might provide clues to an understanding of virus-induced IDDM in man.

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