A genetically determined host factor controlling susceptibility to encephalomyocarditis virus-induced diabetes in mice

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Levels of insulin mRNA in pancreata from SJL/J male mice susceptible to encephalomyocarditis (EMC-D) virus-induced diabetes started to decrease rapidly 24 h after injection with EMC-D virus and only a trace remained 72 h after injection. In contrast, insulin mRNA in pancreata from C57BL/6J male mice resistant to EMC-D virus-induced diabetes did not show any significant changes 0 to 96 h after injection. EMC-D viral RNA in pancreata from SJL/J mice started to increase rapidly 24 h after injection, reached its peak at 48 h and then decreased gradually. In contrast, EMC-D viral RNA in pancreata from C57BL/6J mice was undetectable except for the 24 and 48 h points after injection. EMC-D virus could bind readily to freshly isolated beta cells from SJL/J mice but scarcely bound to beta cells from C57BL/6J mice. In contrast, there was no significant difference between SJL/J and C57BL/6J mice in binding of EMC-D virus to their cultured beta cells. The rate of EMC-D viral attachment to beta cells from C57BL/6J mice increased significantly during the first 24 h culture period and reached the same rate of attachment as that seen for beta cells from SJL/J mice. This suggests that viral receptors on the beta cells derived from strains of mice resistant to EMC virus-induced diabetes are not expressed in vivo, but are expressed during cell culture, rendering the beta cells susceptible to EMC viral infection. On the basis of our previous and present observations, we conclude that a genetic factor controlling susceptibility to EMC-D virus-induced diabetes may operate by modulating the expression of viral receptors on the beta cells.

Insulin-dependent diabetes mellitus (IDDM) results from the destruction of insulin-producing pancreatic beta cells. Genetic factors, autoimmunity and viral infections have been extensively studied as possible causes of IDDM. It is thought that viruses act as primary injurious agents for beta cells or as triggering agents for beta cell-specific autoimmunity (Yoon, 1990). Infection of mice with the D variant of encephalomyocarditis (EMC-D) virus results in the destruction of beta cells, and the clinical picture is characterized by hyperglycaemia and hypoinsulinaemia (Yoon et al., 1980). Several experiments with inbred strains of mice revealed that the development of EMC virus-induced diabetes is genetically determined. Only certain strains of mice such as SJL/J, SWR/J, DBA/2J and NIH/Swiss develop diabetes. In contrast, other strains such as C57BL/6J, AKR/J, CBA/J, LP/J and CE/J do not develop diabetes (Boucher et al., 1975; Craighead, 1975; Notkins et al., 1981; Onodera et al., 1978; Ross et al., 1976; Yoon et al., 1976, 1980; Yoon & Notkins, 1976).

Earlier studies with the M variant of encephalomyocarditis (EMC-M) virus, which contains a mixture of diabetogenic EMC-D and non-diabetogenic EMC-B, suggested that the genetic factors controlling susceptibility to EMC virus-induced diabetes may operate at the level of insulin-producing beta cells (Chairez et al., 1978; Onodera et al., 1978; Yoon et al., 1976; Yoon & Notkins, 1976). After infection in vivo with EMC-M virus, beta cells from susceptible mice showed significantly higher viral titres than beta cells from resistant mice (Yoon et al., 1976; Yoon & Notkins, 1976); however, there was no significant difference in EMC-M viral susceptibility in 9 day cultured beta cells between the susceptible strain (CD-1) and the resistant strain (C57BL/6J) (Wilson et al., 1980). The reason for the difference in susceptibility to EMC virus infection in vivo and in vitro has not been explained. We speculated that the difference in susceptibility to the virus in vivo and in vitro may be due to the expression of viral receptors on the beta cells that are not expressed in animals but are expressed in cell cultures. Thus, the cultured beta cells derived from resistant strains of mice may change and become susceptible to the EMC virus. It is well known that some viruses can grow in monolayer cultures derived from organs from hosts that are ordinarily resistant to these viruses. It has been speculated that the development
of susceptibility in culture is presumably due to induction of viral receptors on the cells (Casto & Hammon, 1969; Crowell et al., 1987; Holland, 1961; Holland & McLaren, 1959; Kunin, 1964). The present investigation was initiated to see whether EMC-D viral receptors would be generated during the cultivation of beta cells freshly isolated from mice resistant to EMC-D virus-induced diabetes and whether the susceptibility of the beta cells to the viral infection would convert from the resistant state to the susceptible state.

Viruses were purified by sucrose and CsCl gradient centrifugation from the supernatant of L929 cell cultures infected with plaque-purified EMC-D virus (Bae et al., 1989; Yoon et al., 1988). The diabetogenic activities of purified EMC-D virus were checked by injecting 30 SJL/J and 30 C57BL/6J male mice (The Jackson Laboratory) with EMC-D (3 × 10^6 pfu/mouse) (Yoon et al., 1980). All SJL/J male mice developed diabetes (mean glucose index 436 ± 37 mg/dl), whereas none of the C57BL/6J male mice became diabetic (mean glucose index 157 ± 19 mg/dl).

To investigate whether there is any correlation between viral infection of beta cells and their destruction, we measured viral RNA and insulin mRNA in pancreata from SJL/J and C57BL/6J mice at different times after viral infection. Briefly, a portion of EMC-D viral cDNA (VPI-cDNA) (Bae et al., 1989; Eun et al., 1988) and rat pre-proinsulin cDNA (Cordell et al., 1979) without the poly(A) tail were radiolabelled with [32P]dCTP by the oligonucleotide-primed synthesis method (Ausubel et al., 1987) and used for probing EMC-D viral RNA and insulin mRNA, respectively. Tail parts of pancreata were removed from SJL/J and C57BL/6J male mice (five mice per group) at 0, 12, 24, 48, 72 and 96 h after injection with EMC-D virus (3 × 10^6 pfu/mouse). Total RNA was extracted from the pooled pancreata by the guanidine HCl method (Chirgwin et al., 1979) and precipitated by adding a half volume of 95% ethanol. The RNA was quantified by reading A_{260}. The RNA was further clarified by phenol–chloroform extraction if the ratio of A_{260}/A_{280} was below 1.8. The extracted RNA from the pooled pancreata was blotted on nylon membranes (100 and 20 μg for insulin mRNA and 30 and 6 μg for EMC-D viral RNA) and hybridized with either the 32P-labelled EMC-D viral cDNA probe or insulin cDNA probe as described previously (Pak et al., 1988). After sequential washing with 2 × SSC and then with 0.2 × SSC containing 0.1% SDS at 55 °C, autoradiography was performed. Each dot was cut, the hybridized RNA was removed by boiling the cut membrane, and the radioactivity was counted.

Insulin mRNA levels in pancreata from SJL/J male mice started to decrease rapidly 24 h after injection with EMC-D virus, and only a trace of insulin mRNA remained 72 and 96 h after injection (Fig. 1a, b). In contrast, insulin mRNA in pancreata from C57BL/6J male mice did not show any significant changes up to 96 h after EMC-D viral infection (Fig. 1c, d). These results suggest that pancreatic beta cells from C57BL/6J mice were saved, but beta cells from SJL/J mice were destroyed when they were infected with EMC-D virus. However, we cannot exclude the possibility that there may be a difference in the rate of cellular RNA degradation in dead beta cells between SJL/J and C57BL/6J mice after infection with EMC-D virus.

EMC-D viral RNA in pancreatic tissue from SJL/J mice started to increase 24 h after injection, reached its peak at 48 h, and decreased gradually thereafter (Fig. 1a, b). In contrast, EMC-D viral RNA in pancreatic tissue from C57BL/6J mice was undetectable at most times tested except at 24 and 48 h (Fig. 1c, d), possibly as a result of the infection of non-beta cells or viraemia. It was previously shown that EMC virus selectively infects and destroys beta cells but not other endocrine cells in the pancreatic islets (Stefan et al., 1978). However, from this study we could not exclude the possibility that EMC virus may infect non-endocrine cells. Even if this...
possibility occurred, the infection was minor as the viral RNA level in C57BL/6J mice was significantly less than that detected in SJL/J mice. Furthermore, in SJL/J mice, there was an inverse correlation between viral RNA and insulin mRNA in the pancreas, suggesting that an increase in EMC viral RNA resulted in a decrease in insulin mRNA synthesis and/or the progressive destruction of insulin-producing beta cells. In contrast, there was no such correlation between viral RNA and insulin mRNA in C57BL/6J mice infected with EMC-D virus, suggesting that EMC-D virus does not affect insulin mRNA levels in C57BL/6J mice.

To see whether the difference in insulin mRNA levels was related to the degree of virus-induced histopathology as well as to the insulin content of beta cells, various measurements were made 4 days after injection with EMC-D virus. Sections of pancreata were prepared and examined microscopically (Yoon et al., 1980). In general, islets from EMC-D-injected C57BL/6J mice showed little pathological change (Fig. 2a); in contrast, islets from EMC-D-injected SJL/J mice showed extensive destruction of beta cells (Fig. 2b). When pancreatic sections were stained with anti-insulin antibody 4 days after EMC-D virus injection as described elsewhere (Yoon et al., 1984; Yoon & Notkins, 1976; Baek & Yoon, 1991), most of the beta cells from C57BL/6J mice stained positively but most of those from SJL/J mice stained negatively. In addition, the concentration of immunoreactive insulin (IRI) in the pancreas was measured as previously described (Yoon et al., 1980; Baek & Yoon, 1990). The concentration of IRI in the pancreata of EMC-D-injected C57BL/6J mice (370 ± 51 µg/g pancreas; n = 10) was not different from that in uninjected C57BL/6J mice (381 ± 47 µg/g pancreas; n = 10). In contrast, the concentration of IRI in EMC-D-injected SJL/J mice (31 ± 7 µg/g pancreas; n = 10) was significantly decreased compared with that of uninjected SJL/J mice (379 ± 49 µg/g pancreas; n = 10).

The data from these three experiments (histology, immunofluorescence and IRI concentration) are consistent with the results for insulin mRNA in pancreata from C57BL/6J and SJL/J mice. It is generally accepted that synthesis of macromolecules, such as RNA and protein, begins to decline within several hours after picornavirus replication in infected cells (Lucas-Lenard, 1979). However, neither the mRNA levels nor the concentration of IRI declined in C57BL/6J pancreata up to 4 days after injection with EMC-D virus. Taken together, these results suggest that EMC-D virus does not destroy pancreatic beta cells in C57BL/6J mice.

It is not known why beta cells from C57BL/6J mice are not infected by EMC-D virus. As the first step in virus infection is binding of virus to cells, we attempted to determine the attachment rate of virus to freshly isolated beta cells, which are similar to beta cells in vivo.

We previously established a method for isolating beta cells from mice by collagenase treatment (Yoon et al., 1984; Yoon & Notkins, 1976) and for labelling and purifying EMC virus (Bae et al., 1989; Eun et al., 1988; Yoon et al., 1988). Briefly, monolayers of L929 cells were adsorbed with EMC-D virus at an m.o.i. of 10. Two hours after adsorption, the medium was removed and replaced with MEM without methionine but containing 5 % dialysed fetal bovine serum (FBS) and 20 µCi per ml of [35S]methionine. Sixteen hours later, the labelled virus was harvested and purified as described previously (Bae et al., 1989; Eun et al., 1988; Yoon et al., 1988; Bae et al., 1990). Before applying the collagenase digestion method for the preparation of pancreatic beta cells, we tested to see whether the collagenase treatment itself would result in a decrease in viral attachment to cells by the loss or modification of cell receptors (McClintock et al., 1983). We treated BMP cells (simian virus 40-transformed baby CD-1 mouse pancreatic cell line) with collagenase (3 mg/ml), and compared the rate of EMC-D viral
attachment to the collagenase-treated and untreated cells. There was no significant difference in the rate of viral attachment.

We performed three additional preliminary experiments for studies on the attachment of EMC-D virus to pancreatic beta cells prior to the main experiments. Firstly, we determined the saturation level for $^{35}$S-labelled viral attachment to cells ($10^6$ in 500 µl) by gradually increasing the virus-to-cell ratio. Attachment was saturable at a ratio of 1000 or more (Fig. 3a). Secondly, we determined the specificity for the EMC-D viral receptor on the beta cells by adding unlabelled EMC-D virus at different virus-to-cell ratios to pancreatic beta cells ($10^6$ in 500 µl) prior to the addition of $^{35}$S-labelled EMC-D virus ($10^8$ p.f.u./10$^6$ cells). Significant inhibition of labelled viral attachment found at an unlabelled virus-to-cell ratio of 1000 (Fig. 3b), and we used this ratio in subsequent experiments. Thirdly, we determined the optimal temperature for binding of EMC-D virus to cells by performing binding experiments ($10^9$ p.f.u. labelled virus; $10^6$ cells; 1 h) at different temperatures. We found that the highest binding occurred at 4 °C (Fig. 3c) and we used this temperature in our subsequent experiments.

To compare the rate of viral attachment to freshly isolated beta cells from SJL/J mice with that to freshly isolated beta cells from C57BL/6J mice, pancreatic beta cells were prepared from 5- to 6-week-old SJL/J or C57BL/6J male mice (30 mice per group) as described previously (Yoon et al., 1984; Yoon & Notkins, 1976). The isolated beta cells were suspended in MEM with 5% FBS and kept for 20 min at 4 °C. The number of pancreatic beta cells was adjusted to 2 x 10$^6$ cells per ml of MEM with 2% FBS. One-thousand p.f.u. of $^{35}$S-labelled purified EMC-D virus per cell was added to 500 µl of beta cell suspension. The mixed suspension was incubated at 4 °C for 1 h with rotation at 100 r.p.m. The cells were washed three times with PBS. Cell pellets were suspended in 500 µl of PBS and mixed with the same volume of 10% TCA. Most of the macromolecules were precipitated by centrifugation at 12,000 r.p.m. after being maintained at 4 °C for 4 to 5 h. The attachment of EMC-D virus to beta cells was determined by counting the radioactivity of the precipitate after dissolving it in 0.15 M-NaOH and mixing it with aqueous cocktail solution. There was a significant difference ($P < 0.01$) in the attachment of the EMC-D virus to beta cells between SJL/J and C57BL/6J mice. Attachment of the total input virus to the beta cells was approximately 39% in SJL/J mice but only 9% in C57BL/6J mice, suggesting that there is a distinct difference in the EMC-D viral receptor on freshly isolated beta cells between strains of mice susceptible (SJL/J) and resistant (C57BL/6J) to the development of EMC-D virus-induced diabetes. How-

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Fig. 3. Attachment of EMC-D virus to isolated pancreatic beta cells prepared from SJL/J mice. (a) Saturation curve of EMC-D viral attachment to pancreatic beta cells. The rate of attachment was calculated as a percentage of total input labelled EMC-D virus. Virus-to-cell ratio is shown on a log scale. (b) Specificity of EMC-D viral attachment to beta cells. The rate of attachment was calculated as for (a). Virus-to-cell ratio is shown on a log scale. (c) Determination of optimal temperature for the attachment of EMC-D virus to beta cells. The rate of attachment was calculated as for (a). Each point shown in (a), (b) and (c) represents the arithmetic mean of duplicate determinations.
ever, an earlier study showed that there is no significant difference in susceptibility to EMC-M virus infection in 9 day cultured beta cells between strains of mice susceptible (CD-1) and resistant (C57BL/6). We speculated that conversion of the resistant state in vivo to the susceptible state in vitro may be attributable to the expression and/or modulation of viral receptor(s) on the beta cells during cell culture. Thus, beta cells derived from resistant strains of mice might change and become susceptible to EMC viral infection.

To investigate whether there is any difference in the attachment of virus to beta cells between freshly isolated cells and cultured cells derived from C57BL/6 mice as well as SJL/J mice, pancreatic beta cells were prepared from 70 (5- to 6-week-old) mice of each strain as described above. The isolated beta cells were divided into six samples. The number of beta cells per sample was adjusted to $2 \times 10^6$ cells per ml of MEM with 5% FBS. Each sample was placed on a plastic dish (35 mm) and incubated at 37 °C in a humidified atmosphere containing 5% CO$_2$. Fourteen hours later, the non-adherent beta cells were collected, thereby eliminating the contaminating fibroblastoid cells that had adhered to the dish (Yoon et al., 1980). EMC-D virus replicated in the cells was determined at different times thereafter. The 0 h culture represents freshly isolated beta cells without cultivation. The attachment of $^{35}$S-labelled purified EMC-D virus to beta cells was carried out as described above.

The data in Fig. 4 represent an average of three separate experiments employing duplicate measurements at each point. In C57BL/6J beta cells, binding of EMC-D virus to freshly isolated cells (0 h) was only about 9% of the total input virus, whereas binding of the virus to 24 h cultured cells increased significantly to 40% ($P < 0.005$). In contrast, there was no significant difference in the binding of EMC-D virus to SJL/J beta cells between freshly isolated cells and 24 h cultured cells. Furthermore, there was no significant difference in the attachment of EMC-D virus to beta cells from C57BL/6J mice between the 24 h and 48 h culture points, although a gradual increase in viral attachment to beta cells was seen in both strains of mice. These results suggest that viral receptors on the beta cells from C57BL/6J mice are not expressed in vivo, but are expressed during the first 24 h of culture and gradually increase thereafter.

To investigate whether there is a difference in EMC-D viral replication when freshly isolated beta cells from susceptible (SJL/J) or resistant (C57BL/6J) mice are infected, a suspension of freshly isolated pancreatic islet cells from each strain was challenged with EMC-D virus from an m.o.i. of 100 (Yoon & Notkins, 1976). After a 1 h adsorption period at 37 °C, the islet cells were washed three times and resuspended in maintenance medium (Eagle's MEM containing 3% heat-inactivated FBS and 70 mg/ml d-glucose). Approximately $1 \times 10^6$ cells were plated onto 35 mm Petri dishes, and the amount of virus in the cells was determined at different times thereafter (Yoon & Notkins, 1976). EMC-D virus replicated in pancreatic islet cells from SJL/J mice; titres reached a peak at 24 h after adsorption and declined thereafter (8 h, $4.2 \times 10^4$; 24 h, $7.3 \times 10^5$; 48 h, $5.8 \times 10^4$; 72 h, $4.3 \times 10^5$). Viral replication also occurred in the C57BL/6J islet cells, although with delayed kinetics of replication (8 h, $3.4 \times 10^4$; 24 h, $4.9 \times 10^4$; 48 h, $5.9 \times 10^4$; 72 h, $4.6 \times 10^5$). The peak titre from C57BL/6J islet cells was much lower than that from the SJL/J mice. It is not known, however, whether replication of the virus occurred in non-beta cells within the suspension of islet cells from each strain or in some EMC-D-susceptible subpopulation of beta cells in the C57BL/6J mouse strain. If we assume that the EMC-D virus attached primarily to non-beta cells from the C57BL/6J mice, then it is clear that once viral attachment occurs, EMC-D replication takes place in these cells. We are currently...
identifying the type of pancreatic islet cell (from C57BL/6J mice) in which the EMC-D virus replicates.

To determine whether there is any difference in EMC-D viral attachment to kidney cells between freshly isolated and cultured cells, kidney cells were prepared from 75 C57BL/6J and 75 SJL/J male mice as described elsewhere (Yoon & Notkins, 1976). Viral attachment was measured as described above. Table 1 shows that in C57BL/6J mice, there was a significant difference (P < 0.005) in the attachment of virus to kidney cells between freshly isolated cells and the 24 h cultured cells; binding of EMC-D virus to freshly isolated kidney cells (0 h) was 11.2% of total input virus, whereas binding of the virus to 24 h cultured kidney cells increased to 37.6%. Similarly, in SJL/J mice binding of EMC-D virus to freshly isolated kidney cells (0 h) was approximately 10.7% of the total input virus, whereas binding of the virus to 24 h cultured kidney cells increased to 37.6%. However, there was no difference in viral attachment to kidney cells between C57BL/6J and SJL/J mice. When kidney sections from SJL/J and C57BL/6J mice infected with EMC-D virus (3 × 10⁵ p.f.u./mouse) for 2 to 5 days were stained with anti-EMC-D viral antibody (Yoon et al., 1980), no viral antigens were found in the kidney sections from either strain, indicating that kidney cells were not susceptible to EMC-D virus infection in vivo, but were susceptible to the virus in vitro after culture (Yoon & Notkins, 1976). On the basis of these observations, we suggest that EMC-D viral receptor(s) in kidney cells are similar to those in beta cells of C57BL/6J mice in that the receptors are not expressed in vivo, but are expressed during the first 24 h of culture.

In our present study, we compared the rate of viral attachment to freshly isolated beta cells from C57BL/6J mice with that to cultured beta cells, and found there was a significant difference between them. Binding of EMC-D virus to freshly isolated C57BL/6J beta cells was low, whereas binding of the virus to cultured beta cells was significantly high, suggesting that EMC-D viral receptors on the C57BL/6J beta cells were expressed. The expression of viral receptors on beta cells which are ordinarily resistant to viral infection could convert the resistant state into the susceptible state. Mendelsohn et al. (1986) showed that it is possible to transfer susceptibility to poliovirus infection from HeLa cells (susceptible to poliovirus infection) to mouse L cells (resistant to poliovirus infection) by DNA (encoding the HeLa cell poliovirus receptor) transformation. More recently, Kaplan & Racaniello (1991) reported that down-regulation of poliovirus receptor RNA in HeLa cells results in resistance to poliovirus infection. Furthermore, our finding was supported by a recent study on the correlation between functional receptors and infectivity of poliovirus (Couderc et al., 1990). In this study, it was shown that 6% of freshly isolated endothelial cells (EC) from human umbilical veins were susceptible to poliovirus infection, whereas 80% of EC were susceptible to poliovirus after 4 days of in vitro primary culture. On the basis of our own information and that of others, we suggest that the difference in susceptibility to EMC virus infection in vivo and in vitro could be due to viral receptor(s) on the beta cells and that the determination of susceptibility to EMC-D virus-induced diabetes depends on the expression of the viral receptor on the pancreatic beta cells.

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### References


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