Factors affecting the infection of the D variant of encephalomyocarditis virus in the B cells of C57BL/6J mice

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Summary. The D variant of encephalomyocarditis virus is capable of infecting most inbred strains of mice. However, only certain strains are susceptible to the diabetogenic effect of this virus. In order to understand why some inbred strains do not become diabetic, the pathogenesis of infection was studied in diabetes-resistant C57BL/6J mice. It was the purpose of the investigation to ascertain whether specific host defense factors might play a crucial role in the mechanism of resistance. To determine whether perturbations of the immune response would alter the resistance of these animals, mice were treated with a high dose (1.15 mmol/kg body weight) of the T- and B-cell toxin cyclophosphamide prior to infection with the D variant. This treatment did not induce overt diabetes or glucose intolerance in the mice tested 7 days after infection. Based on this finding, it appeared likely that resistance to the D variant is conveyed by some factor other than cell-mediated immunity. A likely candidate to control this viral infection is the interferon system. To investigate this possibility,

C57BL/6J mice were infected with the D variant and the concentrations of serum interferon titred at various intervals thereafter. In contrast to previous reports with diabetes susceptible mice, C57BL/6J mice were found to generate a substantial interferon response against this variant, with peak levels found in the serum at 24 h following infection. Additional studies were performed in which mice were treated with antibody to mouse interferon alpha/beta at the time of infection and again 3 days after infection with the D variant. Sixty percent of the animals treated in this manner became glucose intolerant. The results of these studies indicate that the interferon system is a critical determinant of resistance to the diabetogenic effect of the D variant in C57BL/6J mice.

Key words: D variant of encephalomyocarditis virus, C57BL/6J mice, interferon response, B cell destruction, cyclophosphamide treatment.

A viral etiology for Type 1 (insulin-dependent) diabetes mellitus has been suggested by the abrupt onset of clinical symptoms and seasonal variations sometimes seen in this disease [1]. The description of a murine model for Type 1 diabetes induced by encephalomyocarditis virus (EMCV) provided a means for examining experimentally the possible role of viruses in this disease [2–5]. Two strains of EMCV were initially isolated – an E strain that is highly neurotropic, and a M strain that produces myocarditis and little central nervous system involvement [6]. This M strain is capable of inducing a diabetes mellitus-like disease by the selective destruction of pancreatic B cells in certain inbred strains of mice [4]. Other inbred strains of mice are resistant to this diabetogenic effect, even though these animals sustain a viral infection. Further investigations into the M strain resulted in the isolation of two variants – a B variant which is nondiabetogenic, and a D variant which causes this disease [7]. Although

strains of mice have been identified that are resistant to this highly diabetogenic virus, the mechanisms controlling this resistance have yet to be fully identified.

Investigations into the mechanisms of resistance to EMC virus-induced diabetes, as well as the mechanisms of susceptibility, may provide insight into the disease processes involved. Different resistant strains may possess different mechanisms for coping with the infection. It is clear from research with the M strain in resistant C57BL/6J mice that cultured B cells from these mice are susceptible to infection [8], and that in vivo the islets sustain a subtle lesion that is associated with insulinitis [9]. Why these animals are capable of sustaining an islet insult, and yet remain euglycaemic, is unknown. The purpose of these experiments was to determine the mechanism of resistance to the D variant of EMCV in C57BL/6J mice. Specifically, the roles of the immune and interferon systems were explored.

Materials and methods

Animals

Male C57BL/6J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine, USA, and were between 6 to 12 weeks of age at the time of infection. The animals were provided with water and Purina Laboratory Rodent Chow ad libitum, and were housed 6 mice per cage and maintained on a 12-h dark/light cycle.

Virus

Plaque isolated D variant of encephalomyocarditis virus was kindly supplied by Dr. Ji-Won Yoon (Laboratory of Oral Medicine, National Institute of Dental Research, National Institutes of Health (NIH), Bethesda, Md, USA). The virus was titred on secondary mouse embryo cells, then stored at -70°C until use. Dilutions of the virus were prepared in Hank's Balanced Salt Solution (HBSS, Flow Laboratories, McLean, Va, USA) at 1×10^6 plaque-forming units (PFU)/ml. Each animal received 0.1 ml of the virus or diluent intraperitoneally. The virus used for this study was from the original stock obtained from Dr. Yoon and was not passaged. This virus was found to cause overt hyperglycaemia in 100% of diabetes-susceptible SJL/J mice.

Glucose tolerance testing

Glucose tolerance testing (GTT) was performed on non-fasted mice prior to infection and 7 days after infection. Each mouse received an injection of glucose intraperitoneally at a concentration of 11 mmol/kg body weight. Blood samples were obtained prior to the glucose administration and 60 min post-injection. Blood glucose concentrations greater than five standard deviations above mean control values 1 h after the glucose challenge were considered arbitrarily to be diabetic.

Pancreatic insulin determination

Frozen pancreases were weighed and extracted with cold, acidified ethanol, and the concentration of immunoreactive insulin was determined by radioimmunoassay utilizing a back titration method [10]. Results were expressed as the total number of units of insulin per gram wet weight of pancreas.

Histology

At killing, pancreases were removed and bisected along a longitudinal plane. Half of each pancreas was frozen at -70°C for pancreatic insulin determination, while the other half was fixed in Bouin's solution, embedded in paraffin, then sectioned at 6 μm for histological evaluation. These sections were stained by haematoxylin and eosin or immunoperoxidase techniques [11] to demonstrate pancreatic B cells. Hearts also were removed, fixed in Bouin's solution, embedded in paraffin, sectioned at 6 μm and stained by haematoxylin and eosin for histologic evaluation.

Measurement of infectious virus in pancreases

Four days after infection, pancreases were removed from infected mice, weighed, frozen and stored at -70°C . The instruments were rinsed with alcohol and flamed between removal of organs. At the time of titration, the pancreases were thawed and a 10% suspension (wt/vol) in HBSS was prepared by grinding the tissue. The virus was titred on secondary mouse embryo cells in 96 well culture plates and expressed as the tissue culture infectious dose, infecting 50% of the inoculated cultures (TCID₅₀) per gram wet weight of pancreas, using the methods described by Hsiung [12].

Interferon titres

Interferon was titred based on its ability to inhibit the cytopathic effect of 2×10^4 plaque-forming U/ml of Vesicular Stomatitis Virus (VSV) on L-929 cells in 96 well microtitre plates [13]. The quantity of interferon was expressed as the inverse of the dilution that inhibited 50% of the cytopathic effect (CPE) of the challenge virus, and was converted into International Units of interferon according to the titre of the NIH standard (Cat No. G-002-904-511; experimental units = 1.2 IU).

Lymphocyte blastogenic assay

Spleens were aseptically removed and reduced to single cell suspensions in cold RPMI 1640 (Flow Laboratories, McLean, Va, USA). Transformation of isolated lymphocytes by Concanavalin A (Con A, Miles Laboratories, Inc., Elkhart, Ind, USA) or Salmonella typhosa 0901 lipopolysaccharide (LPS, Difco Laboratories, Detroit, Mich, USA) was performed as described previously [14].

Experimental design

The basic design of the experiments in this communication is stated below. C57BL/6J mice were infected with 10^5 plaque-forming units of EMCV-D. Seven days after infection the mice were given a glucose tolerance test and then killed. The pancreas from each animal was removed and saved for determination of insulin content and histological evaluation. Mice receiving treatment with cyclophosphamide received this chemical 24 h before infection. Animals treated with antibody to interferon received this antibody at the time of infection and were retreated again 48 h later. Control mice received only the virus or were treated only with cyclophosphamide or the anti-interferon antibody.

High dose cyclophosphamide treatment of C57BL/6J mice

Each mouse was weighed and then injected intraperitoneally 1.15 mmol/kg body weight with cyclophosphamide (CY) obtained from Mead Johnson Co., Evansville, Ind, USA. Control mice received diluent only (phosphate buffered saline, PBS). The mice were infected with EMCV-D 2 days after CY treatment. Blood glucose levels were measured 2 days prior to CY administration and before virus inoculation on day 0, as well as 7 days after D variant infection. At killing, 7 days after virus treatment, the pancreases were removed for histological evaluation and pancreatic insulin determination.

Treatment with antibody to mouse interferon (α/β)

Rabbit anti-mouse interferon (IFN) (α/β) was obtained from Lee Biomolecular Research, Inc. (San Diego, Calif, USA, Lot 85038). This antibody was reconstituted in sterile water, then diluted with sterile PBS to 20000 units/ml. C57BL/6J male mice were injected intraperitoneally with 2000 units of anti-interferon (anti-IFN) concomitant with 1×10^5 plaque-forming units of EMCV-D. These mice received a further bolus of anti-IFN on day 3 of the infection. Another group of these mice were given anti-IFN intraperitoneally on the same schedule as the first group; however, they did not receive virus. Virus-only infected controls and uninfected, untreated controls were also established. Glucose tolerance testing was performed prior to antibody or virus treatment, and 7 days after infection. The animals were killed at 7 days and their pancreases removed for insulin determinations and histological evaluation.

Statistical analysis

Results were expressed as mean $\pm$ standard error of the mean (SEM). For two group comparisons, significance of differences was assessed using an unpaired Student's *t*-test. For three group comparisons, a single factor analysis of variance [15] was used. In both tests values with a $p < 0.05$ were regarded as significant.

Results

Infection of C57BL/6J mice with EMCV-D

Effect of EMCV-D on blood glucose levels of C57BL/6J mice. Glucose tolerance testing was performed on C57BL/6J mice prior to, and 7 days after, infection with EMCV-D. Determinations of blood glucose concentrations before a glucose challenge were not elevated at 7 days.

Slight increases in blood glucose levels were seen 60 min following a glucose challenge before infection and 7 days after infection. The mean blood glucose levels of these animals, however, did not meet the criteria established at the beginning of the experiments for diabetic GTT (five standard deviations above control means; Fig. 1). Although the mean of all GTT was not in the diabetic range 7 days after infection, 12% of C57BL/6J mice infected with the D variant exhibited diabetic GTT (Table 1).

Histopathological evaluation of EMCV-D infected C57BL/6J pancreas and heart. After infection of C57BL/6J mice with EMCV-D, a diffuse infiltration of leukocytes in the stromal areas of the pancreas was evident. This infiltration became pronounced around the blood vessels, ducts and islets. However, islets appeared to be structurally intact and contained many positive staining B cells (Fig. 2). Following infection, some focal areas of necrosis were seen in the heart (Fig. 3).

Cyclophosphamide treatment of EMCV-D infected C57BL/6J mice

C57BL/6J mice were treated with a single high dose of cyclophosphamide (1.15 mmol/kg body weight) 48 h prior to infection with EMCV-D in order to cause immunosuppression in these mice. The effectiveness of the cyclophosphamide treatment was evaluated by the use of a lymphocyte transformation assay for T- and B-cell responses to mitogens. The immune systems of the animals were found to be significantly impaired ($p < 0.05$) as compared to uninfected controls (Table 2). Histological evaluation showed the leukocyte infiltration to be markedly reduced. Blood glucose concentrations following GTT in cyclophosphamide-treated, infected animals were found not to be significantly ($p < 0.05$) altered from D variant or cyclophosphamide-only treated animals (Fig. 4). Thus, pharmacological immunosuppression of C57BL/6J mice did not

Table 1. Percent of glucose intolerant animals after infection and treatment with cyclophosphamide or anti-interferon antibody

Treatment	% Glucose intolerant	<i>n</i>
EMCV-D	12%	17
CY + EMCV-D	25%	8
aIFN + EMCV-D	60%	15

The effect of treatment with cyclophosphamide (CY) and antibody (aIFN) to mouse interferon alpha/beta in combination with the D variant of encephalomyocarditis virus (EMCV-D) on the occurrence of diabetes in C57BL/6J mice

Fig. 1. Response of C57BL/6J male mice to a glucose challenge prior to infection and 7 days post-infection with the D variant of encephalomyocarditis virus (EMCV-D) [(solid boxes are blood glucose concentrations before the challenge and diagonal boxes are concentrations 60 min after a glucose challenge of 11 mmol/kg body weight, pre-infection 0 min ($n=30$), pre-infection 60 min ($n=15$), 7 days ($n=20$), 7 days 60 min ($n=17$)]. Each point represents the mean $\pm$ SEM

produce alterations in the blood glucose profile of these animals.

Treatment of EMCV-D infected C57BL/6J mice with antibody to interferon

The ability of C57BL/6J mice to produce an interferon response to the D variant of EMCV was determined by assaying blood sera for interferon at various times after infection. This mouse strain was found to generate a substantial interferon response against this variant, with peak levels found in the serum at 24 h following infection (Fig. 5). Control mice did not demonstrate interferon production.

To determine the efficacy of anti-interferon treatment on viral titre, 5 C57BL/6J mice were treated with 2000 units of anti-interferon antibody on the day of infection with EMCV-D and retreated with the same dosage of antibody 48 h later. After 24 h the mice were

Fig.2. C57BL/6J mouse pancreas showing prominent infiltration of leukocytes around blood vessels, ducts and islets 7 days after infection with EMCV-D. Islets are intact, and many insulin-containing B cells are demonstrated by immunoperoxidase staining

Fig.3. C57BL/6J mouse heart showing focal areas of necrosis 7 days after infection with D variant of encephalomyocarditis virus (stained with haematoxylin and eosin)

Fig.4. Blood glucose concentrations in D variant of encephalomyocarditis virus (EMCV-D) infected male mice treated with cyclophosphamide (Cy). Cyclophosphamide and EMCV-D (solid boxes, $n=8$) does not significantly alter the response to a glucose challenge (11 mmol/g body weight) compared to D-variant only (diagonal boxes, $n=17$) and Cy-only (open boxes, $n=5$), treated mice, 7 days after infection (mean $\pm$ SEM)

Fig.5. The effect of infection with the D variant of encephalomyocarditis virus on serum interferon concentrations in C57BL/6J mice. Each point represents the mean $\pm$ SEM of the IU/ml from 3 mice killed at that time interval

Table 2. Effect of cyclophosphamide on the mitogenic response of spleen cells

Mitogen	Cy+EMCV-D	Cy only
Con A	12%	4%
LPS	22%	20%

Lymphocyte transformation assay of spleen cells from cyclophosphamide (Cy)-treated C57BL/6J mice showing reduced mitogenic responses in these mice following exposure to Concanavalin A (ConA) and lipopolysaccharide (LPS). Results are expressed as percent of control values. Both B and T cells are affected by treatment with high doses of cyclophosphamide. There were no differences in mitogenic response between animals treated with Cy only and those given Cy and infected with the D variant of encephalomyocarditis virus (EMCV-D)

killed, and the viral titres in the pancreases of these animals were compared to those of 5 mice receiving EMCV-D only. The results of these studies showed that the 5 anti-interferon treated mice had titres of 1.83×10^7 , 3.98×10^7 , 8.5×10^8 , 1.83×10^7 and 1.00×10^8 TCID₅₀/gm of pancreas compared to titres of 1.26×10^6 , 1.82×10^6 , 1.26×10^6 , 1.26×10^6 and 1.82×10^6 TCID₅₀/gm of pancreas of virus only treated mice.

When interferon activity was ablated by the administration of specific anti-IFN antibody, blood glucose concentrations following GTT in infected C57BL/6J mice were found 7 days later to be significantly greater

(<0.05) than those of D variant-only, or antibody-only treated mice (Fig. 6). The GTT level for these antibody-treated mice was in the diabetic range based upon criteria established at the beginning of the experiments. Neither control group was found to be diabetic. The percentage of animals meeting the criteria for diabetes also increased from 12% in EMCV-D-only treated mice to 60% in anti-IFN treated mice, clearly showing

Fig. 6. Blood glucose concentrations in D variant of encephalomyocarditis virus (EMCV-D) infected male mice treated with antibody to mouse interferon alpha/beta. Treatment with anti-interferon antibody (aIFN) significantly ($p < 0.05$) elevates the response to a glucose challenge (11 mmol/kg body weight) in EMCV-D infected mice (solid boxes, $n = 15$) compared to EMCV-D only (diagonal boxes, $n = 17$) or anti-IFN only (open boxes, $n = 10$) treated mice, 7 days after infection (mean $\pm$ SEM)

that anti-IFN treatment alters the diabetogenic capacity of the virus in resistant mice (Table 1).

Microscopic examination of the heart and pancreas revealed gross pathological changes. Extensive B-cell necrosis accompanied by insulitis was seen throughout the islets (Fig. 7). Severe damage was observed in the myocardium that was characterized by necrosis of myocardial fibres and extensive infiltration of leukocytes (Fig. 8).

The levels of pancreatic insulin present in anti-IFN treated EMCV-D infected animals were found to be reduced as compared to the levels found in nondiabetic, infected controls (Table 3). This supports the presence of a greater B-cell insult in these anti-IFN treated animals as compared to animals receiving EMCV-D alone.

Table 3. Effect of treatment with anti-interferon antibody on the pancreatic insulin concentrations of infected mice

Treatment	Pancreatic insulin		<i>n</i>
	(Mean $\pm$ SEM)	U/g	
Controls	1.66 $\pm$ 0.15		25
EMCV-D	0.96 $\pm$ 0.05		49
aIFN + EMCV-D	0.75 $\pm$ 0.11		7
aIFN Only	1.29 $\pm$ 0.12		10

The effect of treatment with antibody to mouse interferon alpha/beta on the levels of pancreatic insulin in combination with the D variant of encephalomyocarditis virus (EMCV-D) on the levels of pancreatic insulin in C57BL/6J mice. Antibody to mouse interferon reduced the amount of insulin present in the pancreases of the infected mice

Fig. 7. C57BL/6J mouse pancreas showing extensive islet damage 7 days after infection with the D variant of encephalomyocarditis virus and treatment with anti-IFN serum. A marked infiltration of leukocytes is present throughout the islet. The number of insulin containing B cells, as demonstrated by immunoperoxidase staining, is visibly reduced

Fig. 8. C57BL/6J mouse heart showing massive myocardial necrosis 7 days after infection with the D variant of encephalomyocarditis virus and treatment with anti-IFN serum (stained with haematoxylin and eosin)

Discussion

C57BL/6J male mice are resistant to the diabetogenic effect of the M strain of EMCV [16]. The present data show that these mice are also resistant to the D variant of EMCV-D at 7 days post-infection. This time frame was shown to correspond to the peak period of pancreopathy in SWR/J mice infected with EMCV-D [17]. Histologically, the islets of C57BL/6J mice infected with the D variant exhibit insulinitis with relatively few alterations in endocrine cells. The presence of an insulinitis in the absence of any serious islet lesion suggested that this immune response may have a protective effect against the development of diabetes in this strain of mice. Additionally, the recent report of Yoon et al. [18], demonstrating that immune factors play no apparent role in enhancing B-cell destruction, would suggest that these factors may be important for the protection of B cells from the virus. However, the studies with cyclophosphamide indicate that cell-mediated immunity is not the critical factor protecting against virus-induced B-cell injury.

Cyclophosphamide is a powerful immunosuppressive agent with dosage-specific effects. Although early reports suggested that high doses (1.5 mmol/kg body weight) of cyclophosphamide exert a preferential effect on the B containing areas of lymphoid tissue [19, 20], more recent reports have demonstrated convincingly that T cells, as well as B cells, are destroyed by administration of a single, large dose of the compound [21, 22]. The results of the present studies support these latter reports. Both T- and B-cell mitogenic responses were severely attenuated by cyclophosphamide. High dose cyclophosphamide treatment of male C57BL/6J mice did not alter the course of infection in these animals. GTT values were not significantly different from untreated, infected mice. Since this high dose of cyclophosphamide was not effective, lower doses of this compound were not investigated. Therefore, the immune response does not appear to be responsible for resistance in this strain. The presence of the mononuclear cell infiltrate may represent only a reactive insulinitis responding to lymphokines or other signals released by the virus-involved cells.

Since the immune system did not appear to be responsible for the resistance in C57BL/6J mice, the critical factor was sought elsewhere in these animals. Interferon is one of the earliest defenses of cells against viral infection [23, 24]. Generally, alpha and beta interferons are produced by all cells in response to viral stimuli and act within the cell to block further viral replication. The ability to generate an interferon response, and the magnitude of this response to a given stimulus, is a genetically-determined trait. Diabetes-susceptible mice may be unable to produce or react with an interferon response that is capable of inhibiting viral replication, whereas the interferon generated by resistant mice may be sufficient to block further

spread of the virus. This notion is supported by studies which demonstrated a deficient interferon response in diabetes-susceptible SJL/J mice to the D variant [7, 25]. In contrast to the findings utilizing SJL/J mice, the results of the present investigation using diabetes-resistant C57BL/6J mice show that a rapid, substantial interferon response was elicited. This strain of mouse previously has been reported to produce a significant interferon response to other viral infections [26] and, as a result of the present investigation, it can be concluded that this genetically-determined trait also is operable during infections with the D variant of EMC virus.

The studies employing antiserum to interferon underscore the prime importance of the interferon system for the control of infection by the D variant in C57BL/6J mice. Sixty percent of C57BL/6J mice treated with rabbit-anti-mouse alpha/beta interferon serum, administered concomitantly with EMCV-D to suppress the initial interferon response, and again at 3 days post-infection to further reduce interferon levels, were found to be diabetic. The elevated blood glucose levels correlated well with histological findings of severely damaged islets and hearts. Earlier administration of the second dose of antibody (24 h post-infection) resulted in 100% mortality in infected mice, suggesting that this treatment did enhance the virulence of the virus. The mortality was most likely due to overwhelming damage to the myocardium. Early reduction of interferon levels, therefore, was responsible for altering the course of EMCV-D infection in C57BL/6J mice, rendering them less resistant to the diabetogenic effects of the virus.

Gould and coworkers [27] recently published apparently conflicting results. They showed that the addition of alpha/beta or gamma interferon at 4 days post-infection with EMCV-D exacerbated the development of diabetes in C57BL/6J and ICR Swiss mice. However, addition of poly I:C, an inducer of interferon, was shown to decrease the severity of diabetes in ICR Swiss mice; this suggests that endogenous interferon protected, whereas exogenous interferon accelerated, the disease. The time of administration of interferon may be critical in determining the ultimate effect that this compound might have on the host. Recent work with Cocksackie B3-induced myocarditis has shown that administration of interferon or poly I:C could protect against these virus-induced lesions only if they were given within 24 h of virus infection or concomitantly. Administration of interferon at 72 h, rather than protecting as it did earlier, accelerated the development of myocardial lesions [28]. These workers suggest that interferon may play different roles in the viral infection, depending upon the time of administration. Early in the infection, interferon acts classically to reduce the severity of the lesions. Later, interferon exerts a deleterious role in the infection. This could explain the difference between the development of diabetes in these experiments and those of Gould et al. [26]. Early

suppression of the interferon response could allow an increase in B-cell exposure to EMCV-D, thus increasing B-cell damage. An increase in the amount of interferon later in the course of the virus infection could result in B-cell damage via a different mechanism. Late interferon production could facilitate cell-mediated immune responses against the islets, since interferon itself has many influences on the state of the immune system [23].

Previous studies from this laboratory have demonstrated in another strain of mouse (C3H/HeJ), resistant to the diabetogenic effects of the D variant, that the tropism of the virus has changed from B cells to acinar cells [29]. The results of this study suggest that the B cells have lost receptors for the virus and the acinar cells now possess them. In the present study, there was no evidence of exocrine lesions following infection with the D variant, and it is readily apparent that the B cells possess receptors for the virus. Therefore, it can be concluded that there is not one mechanism of resistance to the diabetogenic effects of the D variant, but rather a variety of mechanisms which are genetically determined. Similar heterogeneity in the pathogenic response to the same virus by genetically different individuals could explain why only some individuals become diabetic after infection.

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