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Modification of encephalomyocarditis virus-induced diabetes in mice by antiviral agents

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The current study used a murine model of diabetes induced by the D variant of encephalomyocarditis virus (DEMC) to determine the protective effect of exogenous antiviral agents. Antivirals, which were found to inhibit the development of DEMC virus cytopathic effect in L-929 cell monolayers, were administered intraperitoneally beginning 12 h prior to DEMC virus challenge. Arildone (500 mg/kg per day) or murine interferon (3.2×10^6 IU/kg per day) significantly reduced the incidence of hyperglycemia at 4 days after virus challenge. The incidences of hyperglycemia were 96% in untreated, 62% in arildone, and 0% in interferon treated mice. In other experiments we found that interferon (1.6×10^6 IU/kg per 12 h $\times$ 3) significantly protected mice against diabetes when administered at the time of virus infection or beginning 12 h afterwards. This effect was associated with reductions in average viral titers in the heart and pancreas of infected animals relative to untreated, infected mice. The results of these studies suggest that picornavirus induced diabetes may be prevented or ameliorated by the use of antiviral agents.

diabetes; encephalomyocarditis virus; interferon; antiviral agents

Introduction

Several epidemiologic [5,16] and clinical [18,19] studies implicate picornavirus infection as one cause of diabetes mellitus in humans. Isolates of coxsackievirus B4 [21] and coxsackievirus B5 [2] recovered from patients with recent onset of insulin-dependent diabetes have been shown to induce β cell damage and hyperglycemia in selected strains of mice. Similarly, strains of encephalomyocarditis virus (EMC) [1,3,22] and mouse-adapted coxsackievirus group B [17,23,12] cause experimental infections in certain mouse strains that produce a disease characterized by pancreatic islet cell destruction, insulin deficiency, and hyperglycemia. A plaque-purified, diabe-

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togenic variant of EMC virus termed the D variant (DEMC), has been recently shown to cause prolonged hyperglycemia and long term ocular and renal complications of diabetes in mice [24]. Although the relative importance of picornavirus infections as a cause of human diabetes is still unknown [7,4,8], modalites that prevent or ameliorate picornavirus infections might provide a clinically useful means of reducing the risk of diabetes in certain target populations. In the present study, several antiviral agents were screened for in vitro activity against DEMC virus, and then used in a murine model of DEMC virus infection to determine whether administration of these agents could limit virus replication in the pancreas and alter the development of hyperglycemia.

Methods and materials

Animals Six-week-old male SJL/J mice (Jackson Laboratories, Bar Harbor, Maine), a strain previously shown to be susceptible to EMC virus-induced diabetes [22], were housed in groups of four and allowed unlimited access to standard dry food (Purina Rodent Chow®) and drinking water.

Virus The D variant of EMC virus [22,24] was kindly provided by Dr. Ji-Won Yoon, National Institutes of Health, Bethesda, Maryland. Stock pools of virus were produced by intraperitoneal inoculation of mice with $10^{4.0}$ tissue culture infectious doses 50% ($TCID_{50}$) of virus. Four days after infection, the mice were killed by cervical dislocation, and the pooled hearts were homogenized (10% w/v) in Hank's balanced salt solution, pH 7.2 (HBSS). Following clarification, aliquots were stored at -70°C.

Cell culture L-929 mouse fibroblast cells (American Type Culture Collection, Rockville, Maryland) were grown at 37°C in Eagle's minimal essential medium (EMEM) containing 2 mM glutamine and 10% heat-activated horse serum (Gibco, Grand Island, New York) and maintained in EMEM containing 2% fetal bovine serum (Sterile Systems, Inc., Logan, Utah), 2 mM glutamine, 100 U/ml penicillin G, 50 μg/ml gentamicin, and 1 μg/ml amphotericin B. Viral plaque titrations were performed by inoculation of serial 10-fold dilutions of clarified organ homogenates or virus suspensions onto quadruplicate L-929 cell monolayers grown in 16-mm diameter wells (Costar, Cambridge, Massachusetts). Monolayers were incubated for one hour at 37°C, washed with HBSS, and then overlaid with EMEM containing 1% agarose and antibiotics. After 48 h incubation, the monolayers were fixed with formalin and stained with methylene blue, and the number of plaques counted.

Antiviral agents The antiviral agents tested in the current study were selected because of previously documented activity against picornaviruses in vitro or in experimental animal infections or in both [10,9,6,11,14,13]. Lyophilized murine interferon, a mixture of α and β interferons with specific activity 1.3×10^7 international units (IU)/mg protein, was purchased from Lee Biomolecular, San Diego, California. Arildone (4-[6-(2-chloro-4-methoxy)phenoxyl]hexyl-3,5 heptanedione) was kindly provided by the Sterling-Winthrop Research Institute, Rensselaer, New York; MDL-860 (2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile) by Merrell-Dow Pharmaceuticals, Indianapolis, Indiana; and 2-(α-hydroxy-benzyl) benzimidazole (HBB) by Merck,

Sharp and Dohme Research Laboratories, Rahway, New Jersey. Guanidine was obtained from Eastman Organic, Rochester, New York. For in vitro studies arildone and MDL-860 were initially dissolved in dimethyl sulfoxide (10 mg/ml) and then further diluted in maintenance medium. Interferon was reconstituted with sterile saline prior to use in animal experiments. Arildone was solubilized in warm (37°C), sterile corn oil (40 mg/ml) for animal experiments.

In vitro screening of antiviral compounds Quadruplicate sets of L-929 cell monolayers in 16-mm diameter wells were infected with approximately $10^{2.0}$ $TCID_{50}$ of DEMC. Following adsorption for 1 h, infected and control monolayers were washed with HBSS and then overlaid with maintenance medium containing antiviral compounds at concentrations of 1, 5, or 10 μg/ml or interferon at concentrations of 10^2, 10^3, or 10^4 IU/ml. Monolayers were then incubated at 36°C and observed daily for cytopathic effect (CPE). The inhibition of CPE by test compounds was rated relative to infected, untreated monolayers at 48 h after infection, when these control monolayers typically showed 75–100% cytopathology.

Infection of mice Groups of mice were infected by intraperitoneal injection (0.2 ml) of DEMC virus suspension. On the fourth and/or fourteenth day after infection blood samples were collected from the retroorbital venous plexus into heparinized 250-μl Natelson capillary tubes (Scientific Products, McGaw Park, Illinois). The plasma was separated by centrifugation, frozen at -20°C and later assayed for glucose concentration by an automated glucose oxidase method (Beckman Glucose Analyzer, Beckman Instruments, Inc., Berkeley, California).

Mice were defined to suffer from diabetes if their non-fasting plasma glucose was > 5 SD above the mean of uninfected control animals [22]. The mean (± SD) for uninfected control mice in these experiments was 167 (± 20) mg/dl. Hence, diabetes was considered to be present in mice if the plasma glucose level was ≥ 268 mg/dl.

Antiviral agents which inhibited DEMC virus-induced CPE in vitro were tested for protective activity in the murine model. Beginning 12 h prior to intraperitoneal infection with $10^{2.0}$ $TCID_{50}$ DEMC virus, intraperitoneal injections (0.1 ml/dose) of arildone (250 mg/kg per dose) or interferon (1.6×10^6 IU/kg per dose) were begun and continued every 12 h for a total of 9 doses. Other experiments determined the effect of varying the time of initiating interferon administration relative to the virus challenge.

In certain experiments animals were killed at 4 days after infection to collect hearts and pancreases for titrations of infectious virus content. The organs were harvested, washed with HBSS, and then homogenized (10% w/v) in HBSS containing the protease inhibitor Aprotinin® (Sigma Chemical Co., St. Louis, Missouri) at concentrations of 200 kallikrein inhibitor U/ml. Organ homogenates were frozen at -70°C prior to titrations in L-929 cell monolayers.

Results

The results of in vitro screening of antivirals for CPE inhibitory activity against DEMC virus are summarized in Table 1. Guanidine, HBB, and MDL-860 did not inhibit the development of CPE. Interferon, and to a lesser extent arildone, were

inhibitory in these experiments and were utilized for subsequent studies in mice.

The effect of administering interferon or arildone to mice beginning 12 h prior to DEMC infection is shown in Table 2. Treatment with either agent significantly diminished the frequency of hyperglycemia at 4 days after virus challenge, relative to untreated, infected mice or to infected mice given the corn oil vehicle alone. The protective activity of interferon was significantly greater than that of arildone. Intraperitoneal administration of corn oil in uninfected mice was associated with plasma glucose concentrations (160 ± 27 mg/dl) similar to those of untreated, uninfected mice (167 ± 20 mg/dl).

Table 3 lists the results obtained, when the initiation of interferon treatment was delayed and the duration of treatment shortened. Initiating administration at the time of infection or 12 h afterwards was associated with a significant decrease in the frequency of diabetes at 14 days, compared to infected, untreated mice. Interferon treatment begun 24 h after infection significantly reduced the frequency of hyperglycemia after 4 days, but the majority of these animals went on to develop diabetes by 14 days after infection. Treatment begun at 48 h had no effect.

The titers of DEMC virus in the heart and pancreas of infected animals are listed in Table 4. Untreated animals had a mean titer of approximately 10^4 PFU/0.2 ml homogenate in either organ at 4 days after infection. Compared to these animals, interferon-treated mice had lower viral titers in both heart and pancreas, when treatments were initiated simultaneously with DEMC infection or at 12 h afterwards. Initiation of interferon administration at 24 h was associated with reduced titers in some animals, but no substantial overall decrease, compared to untreated, infected mice.

TABLE 1

Inhibition of encephalomyocarditis (D variant) induced cytopathic effect in L-929 cell monolayers by antiviral agents

Antiviral	Concentration (μg or IU/ml)	Inhibition of CPE[a] (%)
Interferon	10^2	100 (0)
	10^3	100 (0)
	10^4	100 (0)
Arildone	1	3 (9)
	5	69 (35)
	10	75 (27)
MDL-860	1,5,10	0 (0)
Guanidine	1,5,10	0 (0)
2-(α-Hydroxybenzyl) benzimidazole	1,5,10	0 (0)

[a] Values listed are the percent inhibition of cytopathic effect relative to infected, untreated monolayers and represent mean (SD) of observations from 4–8 monolayers at each concentration.

TABLE 2

Effect of prophylactic administration of murine interferon or arildone on the occurrence of hyperglycemia in DEMC virus challenged mice

Treatment	Dose/treatment	Duration of administration (h postinfection)[a]	No. mice hyperglycemic at 4 days/ total no. infected (%)	Mean ± SD plasma glucose (mg/dl)
None	-	-	22/23 (95.7)	498 ± 90
Corn oil	-	-12 to +72	5/5 (100)	488 ± 22
Arildone	250 mg/kg	-12 to +72	13/21 (61.9)[b]	367 ± 190[d]
Interferon	1.6×10^6 U/kg	-12 to +72	0/19 (0)[c]	121 ± 34[e]

[a] Treatments were administered intraperitoneally every 12 h. Male SJL/J mice were inoculated intraperitoneally with $10^{2.0}$ $TCIC_{50}$ D variant of encephalomyocarditis virus at time 0 h.

[b] $P = 0.014$ vs no treatment, two-tailed Fisher exact test.

[c] $P < 10^{-6}$ vs no treatment, $P < 10^{-4}$ vs arildone.

[d] $P < 0.01$ vs no treatment, two-tailed Student's t test.

[e] $P < 0.001$ vs no treatment.

TABLE 3

Effect of varying the time of initiating interferon administration on the development of hyperglycemia in DEMC virus infected mice

Treatment	Duration of administration (h postinfection)[a]	No. mice hyperglycemic/total no. infected (%) on postinfection		Mean ± SD plasma glucose (mg/dl) on postinfection	
		Day 4	Day 14	Day 4	Day 14
None	-	14/29 (58.3)	10/16 (62.5)	327 ± 187	444 ± 212
IFN	0–24	0/24 (0)[b]	4/18 (22.2)[d]	123 ± 43[f]	217 ± 649
IFN	12–36	1/24 (4.2)[c]	3/17 (17.6)[e]	126 ± 63[f]	203 ± 129[f]
IFN	24–48	3/23 (13.0)[d]	11/17 (64.7)	129 ± 108[f]	410 ± 171
IFN	48–72	12/24 (50.0)	11/18 (61.1)	305 ± 108	399 ± 196

[a] Interferon 1.6×10^6 IU/kg was administered intraperitoneally every 12 h for 3 doses. Male SJL/J mice were inoculated intraperitoneally with $10^{1.5}$ $TCID_{50}$ D variant of encephalomyocarditis virus at time 0 h.

[b] $P = 0.0001$ vs no treatment, two-tailed Fisher exact test.

[c] $P < 0.011$ vs no treatment.

[d] $P = 0.014$ vs no treatment.

[e] $P = 0.021$ vs no treatment.

[f] $P < 0.001$ vs no treatment, two-tailed Student's t test.

TABLE 4

Effect of varying the time of initiating interferon administration on viral titers in DEMC virus infected mice

Treatment	Duration of administration (h post infection)	Virus titer (log_{10} PFU/0.2 ml homogenate) at 4 days after infection in		
		n	Heart	Pancreas
None	-	6	4.0 (3.5–4.6)[a]	3.9 (2.9–4.6)
IFN	0–24	6	<1.0 (<1.0)	<1.0 (<1.0)
IFN	12–36	6	2.2 (<1.0–4.0)	1.7 (<1.0–3.9)
IFN	24–48	6	3.5 (2.0–4.0)	3.5 (2.3–4.2)
IFN	48–72	2	4.5 (4.5)	4.5 (4.5)

[a] Values listed as mean (range).

Discussion

The results demonstrate that administration of antiviral agents can prevent picornavirus-induced diabetes in a murine model characterized by β cell destruction and insulin deficiency [22,24]. Specifically, treatment with arildone initiated 12 h prior to virus infection had a modest but significant effect on the development of hyperglycemia. Administration of murine interferon prior to virus challenge prevented the occurrence of hyperglycemia; initiation of interferon administration concurrent with or 12 h after virus challenge was associated with significant reductions in frequency of hyperglycemia. The effect of interferon in preventing hyperglycemia was reflected in reduced viral titers in the heart and pancreas of infected animals.

The antiviral agents which were active in the current studies have previously been demonstrated to inhibit picornaviruses in other types of experimental infections. McKinlay and coworkers demonstrated that arildone, an aryl-diketone compound that selectively inhibits picornavirus uncoating [1], was effective in preventing poliovirus-induced paralysis and death in mice, when administered intraperitoneally or orally in doses similar to those used in the current study [9]. In other studies it was found that interferon affords partial protection against death due to a lethal EMC virus infection [6] and that administration of an interferon inducer is effective in reducing cardiac lesions following coxsackie B3 virus-induced myocarditis in mice [11]. Wilson and coworkers demonstrated that murine interferon reduced viral replication and prevented decreases in insulin release in mouse β cells infected in vitro with EMC virus [20]. Our results also extend the recently reported observations of Yoon and coworkers who found that multiple daily treatments with murine interferon (1 h prechallenge to 72 h postchallenge) reduced the frequency of diabetes in DEMC virus inoculated mice, as compared to no treatment or to single treatments given before virus challenge [25].

In the present study, the results of in vitro screening of antivirals predicted to a limited extent the outcome in DEMC virus-infected mice. Arildone concentrations of

5–10 μg/ml partially inhibited the development of DEMC virus CPE in cell culture. Adequate arildone absorption was confirmed by finding plasma arildone concentrations of 4–6 μg/ml at 4 h after single 170 mg/kg intraperitoneal dosages (assays kindly performed by Dr. Mark McKinlay, Sterling-Winthrop Research Institute, Rensselaer, New York), but arildone had only limited protective effect in the mouse model. Alternative frequencies or methods of administration may have demonstrated greater activity. After intraperitoneal administration of murine interferon at the dosages used in this study, peak serum concentrations range from approximately 400–1000 IU/ml (L. Kronenburg, pers. commun.). These concentrations readily inhibited DEMC virus-induced CPE in vitro, and correspondingly, murine interferon was associated with dramatic protective activity against diabetes in the mouse model. In human studies, peak serum levels of approximately 90–220 U/ml were present in two patients with newly diagnosed, insulin dependent diabetes, who were treated with subcutaneous human leukocyte interferon (3×10^5 U/kg per day)[15]. However, interferon was not associated with therapeutic benefit in these patients, although neither had documented active viral infection [15].

The results of these studies suggest that picornavirus-induced diabetes may be prevented or ameliorated by use of exogenous antiviral agents. These findings provide further impetus to determine the importance of picornavirus infections in human diabetes.

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