Originals

Coxsackie B4 viruses with the potential to damage Beta cells of the islets are present in clinical isolates

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Summary. Infections with Coxsackie viruses (especially Coxsackie B4) are thought to be involved in the pathogenesis of diabetes. Many interdependent variables determine the outcome of an infection with a Coxsackie virus, one of them being the tropism of the virus for a specific tissue. The extent to which Beta cell tropic variants of Coxsackie B4 virus occur naturally was assessed. Human isolates of this virus were tested in an in vitro system in which elevated insulin release from infected islets incubated at a non-stimulatory (2 mmol/l) glucose concentration appears to be related to viral attack. Using this technique, 8/24 isolates tested, im-

paired secretory function in mouse islets. Some strains of Coxsackie B4 virus, therefore, will directly infect mouse islets in vitro leading to changes in islet cell function. In conclusion, these findings confirm that variants of Coxsackie B4 virus with the potential to damage Beta cells occur quite frequently in the natural population. In certain circumstances the damage they inflict on Beta cells may cause destruction of these cells, or precipitate overt diabetes.

Key words: Beta cells, Coxsackie B4 virus, insulin release, islets.

Environmental agents such as viruses are sometimes important in the pathogenesis of insulin-dependent diabetes. Epidemiological [1] and serological [2] data suggest that Coxsackie virus infections, especially with Coxsackie B4 virus, are involved in the disease process and there have been a number of case reports of Coxsackie virus infections which appear to be directly linked to the onset of diabetes [3–6]. In addition, the metabolic functions of islets are impaired following infection with certain non-lytic strains of Coxsackie B4 virus both in vivo [7–9] and in vitro [10]. Many factors, both host and viral, influence the outcome of an infection. Importantly, the virus exists as a heterogeneous mixture of virions of different tropisms and pathogenicities [11, 12]. It is also subject to a high rate of mutation [13] which may alter tropism and lead to a particularly severe attack on a specific organ.

Coxsackie B4 viruses are pathogenic in mice as well as humans and thus the murine model has proved to be of great value in studies of virus-induced diabetes. Earlier studies from other groups have shown that there are strains of Coxsackie B4 virus in the natural population which can induce glucose abnormalities in mice [14, 15]. Our observation that in vitro some strains of virus induce a 'leakage' of insulin from mouse islets incubated at a non-stimulatory glucose concentration [10] prompted us to examine the possibility of using this relatively quick

method to identify isolates which contain virions that might directly affect Beta cells. These strains do not necessarily cause Beta cell lysis [8, 9] but nevertheless cause Beta cell dysfunction.

Materials and methods

All chemicals, including bovine serum albumin, (Fraction V) and collagenase (Type 1 A and Type V) were of the highest grade, obtained from Sigma Chemical Co. (Poole, U.K.). Insulin antiserum and human insulin standards were supplied by Wellcome Diagnostics Ltd., (Beckenham, Kent, U.K.).

Random samples of human Coxsackie B4 isolates were obtained from Public Health Service Laboratories in England. The viruses had been isolated from patients of varying ages, ranging from 1 week to 21 years, presenting with various symptoms such as headaches, febrile fits, sore throats and meningitis. None of the samples came from a patient with diabetes. The origin of the isolates was the throat, faeces or cerebrospinal fluid. The viruses had been passaged a maximum of three times in Vero cell culture and upon receipt received one further passage through Vero cells before use in the in vitro assay. The resulting virus stocks (and virus preparations) were titred by plaque assay on Vero cell monolayers and stored in aliquots at -20°C .

Prototype Coxsackie B4 virus (JVB strain) was passaged through tissue culture eight times, and the stocks were prepared and titred as above to produce CB4-1-CB4-3. Plaque purification of CB4-1 and CB4-3 was carried out on Vero cell monolayers and virus

Table 1. The effect of laboratory preparations of Coxsackie B4 virus and encephalomyocarditis virus on the basal rate of insulin release from mouse islets in vitro 48 h post-inoculation

Virus	% change ^a	<i>p</i> value
Encephalomyocarditis virus, myocardial (EMC1) ^b	+ 688	< 0.001
Encephalomyocarditis virus, myocardial (EMC2) ^b	+ 50	< 0.05
Encephalomyocarditis virus, neurotropic (EMC3)	+ 2	NS
Cloned prototype Coxsackie B4 virus	– 19	NS
Coxsackie B4, tissue culture-adapted (CB4-1)	+ 47	NS
Coxsackie B4, mouse pancreas-adapted (CB4-2)	+ 290	< 0.01
Coxsackie B4, mouse pancreas + B-cell-adapted (CB4-3)	+ 80	< 0.001

^a % change calculated as % increase or decrease in rate of insulin release from infected islets compared to equivalent control islets (5–10 replicates per observation); ^b diabetogenic in mice

yields from islets were assessed by plaque assay. Encephalomyocarditis virus (M and E strains) was prepared and titred using a modification of previously described methods [16].

Isolation of islets

Islets were isolated from the pancreases of male DBA/2 mice, aged 10–12 weeks, by collagenase digestion [17] and maintained in tissue culture in RPMI 1640 medium (supplemented with 5 mmol/l glucose, 20 mmol/l Hepes, 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin sulphate) for 18–24 h at 37°C in a 5% CO₂ in air atmosphere.

Infection of islets

Islets were washed with fresh RPMI 1640 (as above but without serum) and then inoculated with virus suspension at a multiplicity of infection of 1:10.

Dilutions of the viral suspension when required were made in serum-free RPMI 1640. Control islets were treated identically throughout except that cell or tissue extracts without virus were used for sham-inoculations. The virus was allowed to adsorb for 1 h at 37°C, the islets were then washed twice with RPMI 1640 medium with 10% fetal calf serum and cultured in fresh medium for 24 or 48 h at 37°C in a 5% CO₂:95% air incubator.

Incubation of islets

After the culture period, the islets were washed in a bicarbonate buffer (as above) [18]. Groups of 5–10 islets per tube were preincubated in fresh buffer for 30 min at 37°C. The islets were centrifuged at 500 rev/min for 5 s then incubated for 1 h at 37°C in 150 µl buffer containing 2 mmol/l glucose in the case of infected islets, and 2 or 20 mmol/l glucose in the case of controls (to test islet function). The incubation buffer was collected to assay insulin release using a standard radioimmunoassay [19]. The mean rate of insulin release from control islets was 2.8 ± 0.5 pg islet⁻¹ · min⁻¹ (*n* = 9) at 2 mmol/l glucose concentration rising to 15.4 ± 3.9 pg islet⁻¹ · min⁻¹ at 20 mmol/l glucose concentration (*n* = 9) in the series of incubations carried out after 48 h in culture. Samples were heat-treated for 5 min at 65°C to

inactivate the virus. This treatment was shown to have no effect on subsequent radioimmunoassay.

Statistical analysis

Release of insulin from islets infected with virus, was compared to equivalent control islets and statistical significance of differences assessed using Student's *t*-test.

Results

Table 1 shows the effects of infection of mouse islets with three strains of encephalomyocarditis virus (a murine pathogen) and different laboratory preparations of Coxsackie B4 virus in the test system used in the present study. Two myocardial (diabetogenic) preparations [20] of encephalomyocarditis virus (EMC1 and EMC2) caused significantly higher amounts of insulin to be released from islets following infection compared to control islets. The neurotropic (non-diabetogenic) strain (EMC3) had no effect. These results validate this test system.

A cloned prototype Coxsackie B4 virus, derived from the prototype known to be non-diabetogenic in mice [21] also had no significant effect on insulin release. The prototype Coxsackie B4 virus which had been serially passaged through Vero cells (CB4-1) produced no effect on mouse islets. The same isolate, after serial passage through mouse pancreas (CB4-2), or through mouse pancreas and then mouse Beta cells (CB4-3), induced varying degrees of insulin 'leakage' at basal glucose concentration.

Table 2 shows the effects of 24 clinical isolates on insulin release from infected islets. Eight of the isolates produced a significant increase in the rate of insulin release compared to equivalent control islets, ranging from 42–316%. The remainder of the isolates produced both increases and decreases in insulin release but the values were not significantly different from controls. Four of the 24 viruses were isolated from the faeces, three were isolated from throat swabs and one was isolated from the cerebrospinal fluid. One of the isolates (Isolate 12, Table 2) induced a marked threefold increase in insulin release.

Beta cell tropic variants of CB4 virus infect and replicate in islets. Plaque purified preparations derived from CB4-1 and CB4-3 (Table 1) were used to infect islets at a high multiplicity of infection (1 virion:1 Beta cell). The amount of virus released into the culture medium was assessed by plaque assay (see Methods) at various time points. At 24-h post-infection islets infected with CB4-1 derived virus yielded < 0.1% of the initial viral inoculum dose and islets infected with CB4-3 derived virus yielded < 0.3% of the initial viral inoculum dose. By 4.5 days post-infection there was a 9-fold and a 20-fold increase in the amount of virus (compared to the inoculum dose) measured in the incubation medium in the two preparations respectively. Virus has also been found (by plaque assay) in both groups of islets from 24-h post-infection onwards following pretreatment with anti-CB4 antibody to neutralise any extracellular virus and after lysis of the islet cells.

Table 2. The effect of clinical isolates of Cocksackie B4 virus on basal insulin release from mouse islets in vitro 48 h post-inoculation

	Source of isolate	% change ^a	p value
Isolate CB4	1 Faeces	+ 30	NS
	2 Throat swab	- 1	NS
	3 Cerebrospinal fluid	- 12	NS
	4 N/G	+ 92	< 0.02
	5 N/G	+ 33	NS
	6 N/G	+ 12	NS
	7 N/G	- 30	NS
	8 N/G	- 2	NS
	9 Faeces	+ 169	< 0.01
	10 Faeces	+ 42	< 0.05
	11 Throat swab	+ 33	NS
	12 N/G	+ 316	< 0.001
	13 N/G	- 37	NS
	14 N/G	+ 39	NS
	15 N/G	0	NS
	16 Throat swab	+ 43	< 0.01
	17 Throat swab	- 23	NS
	18 Throat swab	- 29	NS
	19 Throat swab	- 23	NS
	20 Throat swab	- 1	NS
	21 Faeces	+ 51	< 0.05
	22 Faeces	+ 40	NS
	23 Faeces	+ 43	< 0.05
	24 Throat swab	+ 89	< 0.01

^a % change as % increase or decrease in rate of insulin release from infected mouse islets compared to equivalent control islets (5–10 replicates per observation). N/G not given

Discussion

In earlier work we have shown that some strains of virus damage the Beta cells of islets inducing excessive insulin release, even in the absence of cytolytic change [10]. In the present study it has been assumed that the increased release of insulin as an intracellular protein is an index of the capacity of the virus to attack Beta cells.

Two earlier studies investigating the diabetogenic potential of clinical isolates in vivo in mice have been recorded. Kuno et al. [14] examined the effects of 12 Cocksackie B4 clinical isolates on the blood glucose of mice and found that 7 of 12 produced hypoglycaemia within 2–4 days of inoculation, suggestive of Beta-cell damage and insulin 'leakage'. This has been observed with some of the preparations (CB4-2) used in this study [9]. In the second study Jordan et al. [15] estimated that 8 of 20 clinical isolates of Cocksackie B4 virus produced abnormalities in glucose metabolism in mice examined over a 17-day period. The study carried out demonstrates the existence of viral variants with the potential to impair islet cell function in the Cocksackie B4 virus pool, and suggests that they are quite common. These variants have direct effects on islets

and could act as initiators of autoimmunity directed against the Beta cells or perhaps inflict damage which though not leading to overt diabetes could be important. Beta-cell dysfunction in which there is only limited damage to cells is known to occur quite frequently in man [22, 23]. In some individuals with a depleted Beta-cell reserve this type of attack could precipitate insulin insufficiency.

The cloned prototype Cocksackie B4 virus did not induce insulin 'leakage' from islets. Also, a preparation of virus which had been extensively passaged through tissue culture (CB4-1) did not have any significant effect on insulin release from mouse islets. Adaptation of the virus to mouse pancreas (CB4-2) or to mouse pancreas and Beta cells (CB4-3) increased tropism for the Beta cells and resulted in elevated insulin release in this system. These preparations (CB4-2 and -3) have been shown to impair insulin synthesis and release from mouse islets in vivo [9] and in vitro [10]. Previous workers have also shown that after passage of prototype Cocksackie viruses through Beta cells, transient hyperglycaemia can be induced in animals [24, 25].

The methods by which the virus causes 'leakage' without cytolysis is as yet undetermined, but virus-induced changes in permeability could be involved [26], or alternatively normal secretory mechanisms may be disrupted such that precursors of insulin (measured as 'insulin' in the RIA) are released.

In conclusion, these observations show that Beta-cell tropic variants are present in the general population of Cocksackie B4 viruses and suggest that in specific circumstances they could play some role in the pathogenesis of diabetes. The genome of the prototype Cocksackie B4 virus has been recently sequenced [27] and studies are now under way to compare the structure of Beta-cell tropic variants of the virus to the non-diabetogenic prototype, in order to identify molecular determinants of diabetogenicity.

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