Cloning and expression of the VP1 major capsid protein of diabetogenic encephalomyocarditis (EMC) virus and prevention of EMC virus-induced diabetes by immunization with the recombinant VP1 protein

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The development of diabetes in mice induced by encephalomyocarditis (EMC) virus provides the best experimental evidence that viruses have an aetiological role in the pathogenesis of this disease. The major capsid protein (VP1) of EMC virus is important for both the attachment of the virus to pancreatic beta cells and for the determination of antigenicity. This experiment was initiated to clone the gene for the major capsid protein, VP1, of the diabetogenic EMC (EMC-D) virus, express the VP1 protein, and test whether the recombinant VP1 protein can prevent development of EMC-D virus-induced diabetes in mice. We successfully cloned the VP1 gene of the EMC-D virus in the expression vector pRSET and subsequently expressed the protein in Escherichia coli. The recombinant VP1 protein was then purified by affinity chromatography. Five- to six-week-old male SJL/J mice were immunized intraperitoneally with purified VP1 protein and then challenged after various intervals with highly diabetogenic EMC-D virus. None of the VP1-immunized mice developed diabetes, irrespective of the interval between immunization and virus challenge, whereas 80 to 95% of the EMC-D virus-infected control mice did develop diabetes. All of the VP1-immunized mice showed intact pancreatic islet architecture, whereas most of the infected control mice showed severe beta cell necrosis and lymphocytic infiltration of their pancreatic islets. On the basis of these observations, we conclude that the recombinant VP1 protein of EMC-D virus can completely prevent the development of EMC-D virus-induced diabetes in mice.

Introduction

Insulin-dependent diabetes mellitus (IDDM) results from the almost complete destruction of insulin-producing pancreatic beta cells. Genetic factors, various immune system alterations, viral infections, and diet have all been extensively studied as possible causes of IDDM (Rossini et al., 1993; Scott & Marliss, 1991; Todd & Bain, 1992; Yoon & Park, 1993). While genetic susceptibility is thought to be a prerequisite for the disease, the 64% discordance rate for IDDM between monozygotic (genetically identical) twins suggests that environmental factors may influence the clinical expression of genetic susceptibility (Todd & Bain, 1992; Yoon, 1990). Viruses, as one environmental factor affecting the induction of diabetes, may act either as primary injurious agents of beta cells or as triggering agents for beta-cell-specific autoimmunity in man and animals (Yoon & Park, 1993).

Evidence for virus-induced diabetes comes largely from experiments with animals, such as the induction of diabetes in susceptible mice by encephalomyocarditis (EMC) virus (Craighead & McLane, 1968; Yoon et al., 1976). The D variant of EMC virus (EMC-D) can induce diabetes in over 90% of genetically susceptible mice by infecting and destroying pancreatic beta cells (Yoon et al., 1980). One of the major capsid proteins of the EMC virus, VP1, appears to be involved in the attachment of the virus to the cell and contains the main neutralizing immunogenic site for the virus (Eun et al., 1988; Kang & Yoon, 1993).

This investigation was initiated to clone and express the VP1 gene of the diabetogenic EMC-D virus and to test whether immunization with the recombinant VP1 protein could prevent the development of EMC-D virus-induced diabetes in mice. We now report that immunization with recombinant VP1 protein of EMC-D virus can completely prevent the development of EMC-D virus-induced diabetes by the induction of neutralizing antibodies.

Methods

Mice. Five-week-old SJL/J male mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and housed under specific-pathogen-free conditions at the animal facility at The University of Calgary. All...
animals were maintained on Purina-NIH mouse ration, containing 5% fat and 23% protein. Only male SJL/J mice were used, as sex differences in susceptibility to EMC virus-induced diabetes exist in this strain. Ninety percent of male SJL/J mice consistently develop diabetes when infected with EMC-D virus (Yoon et al., 1980). All animals were handled in accordance with the guidelines established by the Medical Research Council of Canada.

**Virus.** EMC-D viruses were grown in L929 cells as described previously (Yoon et al., 1988) and the virus was assayed on monolayers of L929 cells and the titre expressed in p.f.u. when infected with EMC-D virus (Yoon 1980, 2% fat and 2-3% protein. Only male SJL/J mice were used, as sex differences in susceptibility to EMC virus-induced diabetes exist in this strain. Ninety percent of male SJL/J mice consistently develop diabetes when infected with EMC-D virus (Yoon et al., 1980). All animals were handled in accordance with the guidelines established by the Medical Research Council of Canada.

**Preparation of viral proteins VP1, VP2 and VP3, and measurement of neutralizing antibodies.** The EMC-D virus was purified as described elsewhere (Yoon et al., 1980, 1988). The virus was concentrated by ultracentrifugation at 35,000 r.p.m. (Beckman 60Ti rotor) for 2 h. The virus pellet was resuspended in PBS containing 1% Nonidet P-40 (Sigma) and was sonicated. The suspension was layered onto a 15-50% sucrose gradient and centrifuged at 250,000 r.p.m. for 6 h in a Beckman LS ultracentrifuge with a SW28 rotor. Ten fractions were harvested, precipitated with 10% trichloroacetic acid, and subjected to SDS-PAGE to identify those fractions containing high concentrations of viral protein. Fractions containing viral capsid protein were pooled and subjected to SDS-PAGE. The gel was soaked in cold 0.1 M-KCl solution to visualize the protein bands. Each VP1, VP2 or VP3 band, as identified by molecular mass, was cut out of the gel and homogenized in a minimum volume of PBS. Mice were injected intraperitoneally with 200 µl of gel suspension containing 50 µg of one viral capsid protein (4 mice/group). Three weeks later, the mice were boosted, and 2 days later the mice were bled. Control groups included mice that were injected with polyacrylamide gel suspension alone (control) for the effects of the gel, 4 mice) and untreated mice (control, 4 mice).

Western blots were performed as previously described (Ko et al., 1991). Protein samples from EMC-D virus were separated by SDS-PAGE and transferred to nitrocellulose paper (BA83; Schleicher & Schuell). After blocking with 5% skim milk for 1 h, the membrane was incubated in mouse serum (1:200) in PBS for 5 h. Serum was obtained from mice injected with one of the three viral capsid proteins and from the control groups described above. Following washing with 0.05% Tween-20/PBS, the membrane was incubated with alkaline phosphatase-conjugated rabbit anti-mouse IgG (1:1000). After washing in 0.05% Tween 20/PBS, the reaction product was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad) in carbonate buffer.

For the in vitro neutralization assay, serum from each group of mice was serially diluted in 5-fold steps with MEM supplemented by 2% fetal calf serum (FCS) and 20 µl of each dilution was placed in a 96-well microtiter plate. The VP1 band was visualized by soaking in cold 0.1 M-KCl and was cut out of the gel and suspended in a minimal volume of PBS by sonication. Fractions containing the recombinant VP1 protein were pooled and dialysed in 50 mM-Tris-HCl, 1 mM-CaCl$_2$ pH 8.0. Enterokinase was added at a ratio of 1:100 (enzyme:substrate). The mixture was incubated overnight at 37°C. The identity of the VP1 protein was confirmed by SDS-PAGE and Western blotting as described above, except that the membrane was incubated with the VP1-specific monoclonal antibody (MAb) ED-HJ 23 (Yoon et al., 1988).

**Cloning of the VP1 gene in expression vector pRSET.** The VP1 gene, which consists of 831 nucleotides encoding a 277 amino acid protein (Eun et al., 1988), was used for subcloning into the expression vector pRSET (Fig. 1). The pRSET vector containing the T7 promoter was purchased from Invitrogen. The VP1 gene was inserted just after the enterokinase recognition sequence Asp-Asp-Asp-Asp-Lys-Asp. For the correct open reading frame (ORF) of the VP1 gene, pRSET-B was chosen and digested with BamiHI. Following treatment with Klenow in the presence of dA, dG and dT, the remaining flanking nucleotide C was removed by mung bean nuclease and the vector was then digested with EcoRI. The VP1 gene was amplified by PCR with pED$_{100}$ (cDNA clone containing the full-length EMC-D gene) (Bae & Yoon, 1993) using the upstream primer VP1F (5'GGAGTGGAGAATGGCTGAGAAGAGGCGGTT 3') and downstream primer VP1L (5'GGAAATTCAACGCGCTTTGGC 3'). The downstream primer was designed to contain a stop codon for translation termination (bold) and an EcoRI site (underlined) for the cloning. PCR was cycled 30 times at 93°C for 1 min, 54°C for 1 min, and 72°C for 1 min. The amplified VP1 gene was treated with Klenow and completely digested with EcoRI. The vector and the insert were ligated with T4 DNA ligase and the ligation mixture was transformed in JM109 cells. Recombinant clones that had the insert were analysed by automated sequencing.

**Expression, purification and identification of the recombinant VP1 protein.** The recombinant VP1 protein was expressed and purified as previously described (Kroll et al., 1993). BL21 (DE3) cells (Studier et al., 1990) were transformed with the plasmid containing the VP1 gene. In BL21 (DE3) cells, the T7 polymerase expresses under the control of the lac UV5 promoter, therefore, the polymerase can be induced by IPTG. To produce the recombinant proteins, the expression was induced with 0.5 mM-IPTG when the cell culture (50 ml) reached an OD$_{600}$ of 0.6-0.8; then the culture was incubated for an additional 5 h at 37°C. The cells were suspended in 10 ml of 20 mM-sodium phosphate, pH 7.4, and lysed by sonication with three 30 s pulses of about 100 W at 0°C. The soluble and insoluble cell fractions were separated by centrifugation of the cell homogenate at 8000 g at 4°C (Sorvall RC 5B, SS-34 rotor) for 10 min. The pellet containing the insoluble VP1 protein was dissolved in 10 ml of 6 M-guanidinium hydrochloride, 500 mM-NaCl, 20 mM-sodium phosphate pH 7.8, and directly applied to ProBond resin (Invitrogen), which was charged with nickel ions. The column was washed with buffer A (8 M-urea, 20 mM-sodium phosphate, 500 mM-sodium chloride) in a pH-step gradient of pH 7.8, pH 6.0, and pH 5.3, then eluted with buffer A of pH 4.0. The fractions were collected and a small aliquot of each fraction was analysed by SDS-PAGE. Proteins were visualized by Coomassie blue staining. Fractions containing the recombinant VP1 protein were pooled and dialysed in 50 mM-Tris-HCl, 1 mM-CaCl$_2$ pH 8.0. Enterokinase was added at a ratio of 1:100 (enzyme:substrate). The mixture was incubated overnight at 37°C. The identity of the VP1 protein was confirmed by SDS-PAGE and Western blotting as described above, except that the membrane was incubated with the VP1-specific monoclonal antibody (MAb) ED-HJ 23 (Yoon et al., 1988)

**Test of the recombinant subunit vaccine for the induction of neutralizing antibodies.** To determine whether the purified VP1 protein induces anti-EMC-D virus antibodies that are able to neutralize diabetogenic EMC-D virus, 10 SJL/J male mice (5 weeks old) were immunized intraperitoneally with 50 µg of purified VP1 protein in 200 µl of gel suspension in PBS. Purification of the recombinant VP1 protein was accomplished by SDS-PAGE. Following electrophoresis, the VP1 band was visualized by soaking in cold 0.1 M-KCl and was cut out of the gel and suspended in a minimal volume of PBS by homogenizing the gel slice in a tissue grinder. Sera from these immunized animals were collected at 14 days after injection. An in vitro neutralization test was performed as described above, except that sera were diluted in 2-fold steps.

**Vaccination of SJL/J mice with recombinant VP1 protein and measurement of blood glucose.** To see whether the recombinant VP1 protein could prevent EMC-D virus-induced diabetes, 70 male SJL/J mice (5 weeks old) were intraperitoneally immunized with 50 µg of recombinant VP1 protein in 200 µl of gel suspension. We chose to use this amount of recombinant VP1 protein for immunization on the basis of our kinetic studies on the induction of neutralizing antibodies (unpublished data). Fifty µg of recombinant VP1 protein was the
minimum amount able to induce a sufficient titre of neutralizing antibodies. As a control, age-matched male SJL/J mice were injected with an equal amount of PBS (control, 10 mice) or polyacrylamide gel suspension (carrier control, 70 mice). Two weeks later, the mice were boosted with another 50 μg of recombinant VP1 protein in 200 μl of gel suspension. Control animals were again injected with an equal amount of PBS or polyacrylamide gel suspension. The VP1-immunized mice were intraperitoneally challenged with $5 \times 10^5$ p.f.u. of EMC-D virus at 10, 30, 60 (20 mice/group), or 90 (10 mice/group) days after boosting. Gel-injected mice were infected at the same times, whereas PBS-injected
mice were not infected. Glucose indices were measured as previously
described (Yoon et al., 1977, 1980). Briefly, glucose levels were
measured in blood by use of a glucose oxidase assay with O-dianisidine
dihydrochloride as the indicator dye. Blood for glucose tolerance tests
(GTT) was obtained 60 min after intraperitoneal injection of 2 mg of
glucose/g body weight. Non-fasting (NF) glucose levels were measured
7 and 14 days after infection, and GTT were performed 10 and 17 days
after infection. GTT and NF glucose measurements were performed at
the same ages in PBS-injected non-infected mice. The four values
obtained were then combined as follows to give the glucose index
for each mouse. Glucose index = (4 × NF day 14) + (3 × NF day 7) + (2 × GTT day 17) + (1 × GTT day 10)/10. This formula for the
index was used to eliminate some of the variability associated with
individual glucose determinations and weighted to emphasize non-
fasting hyperglycaemia and persistence of abnormal glucose levels. The
justification for the index is found elsewhere (Ross et al., 1976).
Individual mice were classified as diabetic when their blood glucose
index was 235 mg/dl (5 SD above the mean blood glucose indices of
PBS-treated, non-infected control mice). (Yoon et al., 1980).

Histological examination. Pancreata were examined from VP1-
immunized mice challenged 10 days after the last immunization and
from the two control groups. Five to seven mice from each group were
killed 10 days after virus infection and 50% of each pancreas was fixed
in 10% buffered formalin phosphate. Paraffin-embedded sections were
stained with haematoxylin and eosin (HE) and 15-20 pancreatic
islets/mouse were examined under a light microscope (Buek & Yoon,
1990). Histological changes in the pancreatic islets were subjectively
classified as either normal, as having a mild to moderate degree of
insulitis, as having severe insulitis, or as atrophied. Normal islets were
defined as islets with normal morphology. A mild to moderate degree
of insulitis was defined as lymphocytic infiltration of between 1-49 %
of the islets, with the islet architecture remaining well preserved. Severe
insulitis was defined as lymphocytic infiltration of 50-100 % of the
islets. At this stage, the islets were small and retracted, showing severe
beta cell necrosis, with or without residual lymphocytic infiltrate.

Extraction of insulin from pancreas and measurement of insulin levels.
Insulin was extracted from the pancreata of VP1-immunized/EMC-D
virus-infected mice, PBS-injected/uninfected mice, and gel-injected/
EMC-D virus-infected mice (10 mice/group) as previously described
(Davoren, 1962; Yoon & Notkins, 1976). Briefly, frozen pancreatic
tissue (50% of each pancreas) was placed into PBS and then 1-5 ml
extraction solution A (380 ml absolute alcohol, 20 ml H2O, 8 ml
concentrated HCI and some drops of alcoholic phenol red) was added.
This mixture was homogenized with polytron type PT 10-20-350D in
glass tubes for 30 s at position 5. The homogenized material in the
tubes was incubated at 4 °C for 10 h. At the end of the incubation
period, the homogenized material was microcentrifuged at 800 g for
5 min for clarification. The supernatant was saved. The pellet was then
incubated for 4 h at 4 °C in 1 ml of extraction solution B (356 ml
absolute alcohol, 124 ml water and 7-5 ml HCl) and homogenized as
described above. The homogenized material was microcentrifuged at
4 °C for 5 min at 800 g. The supernatant was pooled with the
supernatant from the first extraction. The pooled supernatant was
neutralized with concentrated NH4OH (about 10 μl) until the phenol
red turned purple. The neutralized supernatant was precipitated at 4 °C
and then microcentrifuged at 4 °C for 5 min at 800 g. The supernatant
was used for the measurement of insulin by radioimmunosassay (Hales
& Randle, 1963; Yoon et al., 1980; 1984). The concentration of
immunoreactive insulin was measured using mouse insulin as a
standard. Briefly, the extracted samples were mixed with 125I-labelled
insulin and anti-insulin antibody raised in guinea-pig. The mixture was
incubated for 2 h at room temperature, then anti-guinea-pig IgG raised
in sheep was added and this mixture incubated for 30 min at room
temperature. At the end of the incubation period, the samples were
microcentrifuged at 4 °C for 10 min at 1500 g. The supernatant was
removed and the radioactivity of the pellets measured. The activity (%) was
calculated as follows: percentage activity = (counts of standard or
sample/count of blank) × 100. On the basis of the activity in standards,
a curve was constructed and the concentration from each sample read
against the curve.

Results

Determination of the candidate capsid protein for the
construction of the expression vector

To see which capsid protein, from among the three
exposed EMC-D viral capsid proteins, had the strongest
antigenicity and could induce the highest titre of
neutralizing antibodies against the EMC-D virus, we
isolated the VP1, VP2 and VP3 proteins by SDS-PAGE
and then immunized groups of SJL/J mice with one of the
three proteins. Specific antibodies against VP1, VP2,
and VP3 proteins were produced and reacted with their
respective viral capsid protein (Fig. 2). An in vitro
neutralization assay was performed using diluted sera
from each group of mice. We found that neutralizing
activity appeared in the sera from the VP1- (1:625) and
VP2- (1:5) immunized mice, with the highest titre
appearing in the VP1-immunized group. Sera from VP3-
immunized mice had no neutralizing effect. We therefore
selected the VP1 protein for the construction of an
expression vector.
Prevention of diabetes by subunit vaccine

Fig. 3. Nucleotide sequence of the fused EMC-D VP1 gene and its predicted amino acid sequence. The N-terminal fused leader peptide contains a hexahistidyl sequence for purification on Ni\(^{2+}\)-affinity resins and an enterokinase (EK) proteolytic cleavage site for leader peptide removal. The intact VP1 gene is composed of 831 nucleotides, which encode 277 amino acids.

Construction of the expression vector for the VP1 gene, expression of the VP1 gene, and identification of the expressed VP1 protein

The VP1 gene of the EMC-D virus was cloned into a prokaryotic expression vector, pRSET. This vector has a strong inducible T7 promoter and encodes recombinant proteins as fusions with a multifunctional leader peptide containing a hexahistidyl sequence for purification using Ni\(^{2+}\)-affinity chromatography and an enterokinase proteolytic cleavage site for leader peptide removal (Kroll et al., 1993). The cloned gene was checked to see whether the correct ORF of the fusion protein was present in the vector by DNA sequencing using T7 primer. We found that the cloned gene contained the correct ORF of the fusion protein (Fig. 3). The constructed vector (Fig. 1) that contained the VP1 gene, named pRSET-VP1, was transformed into BL21 (DE3) cells (Studier et al., 1990) and the recombinant VP1 protein was overexpressed, mainly in the insoluble fraction. Using this system, we obtained an expression yield of 54 mg of protein/100 ml of culture. The overexpressed VP1 protein was purified...
Fig. 4. Expression and purification of recombinant VP1 protein. Proteins were visualized by Coomassie brilliant blue staining (a) and analysed by Western blotting using anti-VP1 antibodies (ED-HJ-23) (b). Lane M, molecular size markers; lane 1, insoluble fractions of cell extract; lane 2, affinity-purified fusion protein; lane 3, recombinant VP1 protein after enterokinase cleavage; lane 4, purified EMC-D viral protein. Arrow indicates the intact VP1 protein of EMC-D virus.

Table 1. Prevention of virus-induced diabetes by immunization of SJL/J male mice with a recombinant subunit vaccine (VP1 protein of EMC-D virus)

<table>
<thead>
<tr>
<th>Time after immunization (days)</th>
<th>Challenged gel-injected mice*</th>
<th>Challenged VP1-immunized mice†</th>
<th>Non-infected PBS-injected control mice‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose index (mg/dl)†</td>
<td>Glucose index (mg/dl)†</td>
<td>Glucose index (mg/dl)†</td>
</tr>
<tr>
<td></td>
<td>Diabetes (%)</td>
<td>Diabetes (%)</td>
<td>Diabetes (%)</td>
</tr>
<tr>
<td>10</td>
<td>417±63</td>
<td>163±15</td>
<td>158±15</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>384±71</td>
<td>154±14</td>
<td>161±16</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>378±82</td>
<td>162±12</td>
<td>159±15</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>90</td>
<td>341±83</td>
<td>164±15</td>
<td>162±14</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Mice received 200 µl of gel suspension twice at 5 and 7 weeks of age; n = 70.
† Mice were immunized twice with 50 µg of a recombinant subunit vaccine (VP1 protein of EMC-D virus) in 200 µl of gel suspension at 5 and 7 weeks of age; n = 70.
‡ Mice received 200 µl of PBS twice at 5 and 7 weeks of age and were not challenged with EMC-D virus; n = 10.
§ Number of days after last immunization with VP1 protein or after last injection of gel suspension that mice were challenged with EMC-D virus. Non-infected PBS-injected control mice were tested at the same ages.
¶ Any mouse with a glucose index above 235 mg/dl, which is 5 sd above the mean glucose index of uninfected control mice (160 ± 15 mg/dl), was considered to have diabetes. Each group contained 10 to 20 mice.
¶¶ Glucose indices were calculated as described in Methods.

and the fused leader peptide was removed by enterokinase cleavage. The purified recombinant VP1 protein was identified by both Coomassie blue staining and by immunoblotting using antibodies against the VP1 protein (Fig. 4). The molecular mass of the purified recombinant VP1 protein after enterokinase cleavage was 33 kDa, identical to the molecular weight of EMC-D viral VP1 protein.

Prevention of EMC-D virus-induced diabetes by immunization with recombinant VP1 protein

To see whether the recombinant VP1 protein could induce neutralizing antibodies against EMC-D virus, we immunized 10 male SJL/J mice once with 50 µg of recombinant VP1 protein, and then sera were harvested 14 days after immunization. We measured neutralizing
Prevention of diabetes by subunit vaccine

Fig. 5. Histological examination of the islets of Langerhans. (a) Sections of pancreas from VP1-immunized mice after EMC-D virus infection showing intact islet architecture. (b) Sections of pancreas from PBS-injected, uninfected mice showing intact islet architecture. (c) Sections of pancreas from gel-injected EMC-D virus-infected mice showing extensive inflammatory infiltrate with mononuclear cells in the islets and some beta cell necrosis. (d) Sections of pancreas from gel-injected EMC-D virus-infected mice showing insulitis with severe beta cell necrosis. Bar marker in (d) represents 50 μm.

antibody titres in the sera and found the average titre to be 1:665.6 (range 1:256 to 1:1024).

To see whether the recombinant VP1 protein could prevent the development of EMC-D virus-induced diabetes, we immunized SJL/J male mice with recombinant VP1 protein at five and seven weeks of age, then challenged groups of mice with EMC-D virus at various times after the last immunization. We found that none of the VP1-immunized animals developed diabetes, irrespective of their age at challenge. In contrast, 80–95% of the gel-injected EMC-D virus-infected mice became diabetic (Table 1). The incidence of diabetes in the gel-injected, EMC-D virus-infected mice was slightly lower when the mice were challenged with the virus when they were older.

To see whether the VP1-immunized mice had any pathological changes in their pancreatic islets, we examined HE-stained sections of pancreata obtained at 10 days after virus challenge from VP1-immunized mice challenged with EMC-D virus at 10 days after immunization. The pancreatic islets from VP1-immunized EMC-D virus-infected mice showed intact islet architecture without any lymphocytic infiltration, similar to the condition of islets from PBS-injected, non-infected control mice (Fig. 5a and b; Table 2). In contrast, pancreatic sections from the gel-injected EMC-D virus-infected mice showed extensive inflammatory infiltrate with mononuclear cells in the islets and beta cell necrosis (Fig. 5c and d; Table 2).

To see whether there were any differences in pancreatic immunoreactive insulin content between VP1-immunized, EMC-D virus-infected mice and PBS-injected,
Table 2. Histological changes in the islets of Langerhans from VP1-immunized and gel-injected SJL/J male mice infected with EMC-D virus*

<table>
<thead>
<tr>
<th>Group</th>
<th>Development of diabetes</th>
<th>Total islets examined</th>
<th>Histological changes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N (x)</td>
<td>M (x%)</td>
</tr>
<tr>
<td>Challenged gel-injected mice†</td>
<td>yes</td>
<td>85</td>
<td>5 (5.9)</td>
</tr>
<tr>
<td>Challenged VP1-immunized mice§</td>
<td>no</td>
<td>125</td>
<td>125 (100.0)</td>
</tr>
<tr>
<td>Non-infected PBS-injected control mice¶</td>
<td>no</td>
<td>73</td>
<td>73 (100.0)</td>
</tr>
</tbody>
</table>

* Mice were infected with EMC-D virus 10 days after last immunization or injection of gel.
† Values are the number of islets with percentage in parentheses. N, normal islets with normal morphology; M, mild to moderate degree of insulitis with lymphocytic infiltration of between 1-49% of the islets and well-preserved islet architecture; S, severe insulitis with lymphocytic infiltration of 50-100% of the islets; A, atrophied, small retracted islets showing severe beta cell necrosis, with or without residual lymphocytic infiltrate. 15-20 islets/mouse and 5-7 mice/group were examined.
§ Mice received 200 μl of gel suspension twice at 5 and 7 weeks of age.
¶ Mice were immunized twice with 50 μg of a recombinant subunit vaccine (VP1 protein of EMC-D virus) in 200 μl of gel suspension at 5 and 7 weeks of age.

uninfected control mice, we extracted insulin from pancreata and measured insulin content by radioimmunoassay. We found that there was no difference in pancreatic insulin content between the two groups of mice. The mean insulin content in pancreata of VP1-immunized, EMC-D virus-infected mice was 158 ± 27 μg/g pancreas, whereas the mean insulin content in pancreata of PBS-injected, uninfected control mice was 161 ± 28 μg/g pancreas. In contrast, gel-injected EMC-D virus-infected mice had a mean insulin content of 19 ± 7 μg/g pancreas.

Discussion

The best experimental evidence indicating that viruses have an aetiological role in the pathogenesis of IDDM comes from studies on mice infected with EMC virus (Craighead, 1975; Notkins et al., 1981; Yoon & Park, 1993). As with other picornaviruses, the EMC-D virus has four capsid proteins, three of which (VP1, VP2, and VP3) are exposed on the surface of the particle. We determined that the VP1 protein, of the three exposed capsid proteins, induced the highest titre of neutralizing antibodies against the EMC-D virus. Therefore, we selected the VP1 protein for the construction of an expression vector for the VP1 gene and expression of this protein for use as a recombinant subunit vaccine in an animal model.

We successfully cloned the VP1 gene of the EMC-D virus in the expression vector pRSET, expressed the protein in Escherichia coli, then purified it by affinity chromatography. When SJL/J mice were immunized intraperitoneally with the purified VP1 protein and then challenged after various intervals with highly diabetogenic EMC-D virus, none of the VP1-immunized mice developed diabetes, irrespective of the interval between immunization and virus challenge. In addition, all of the VP1-immunized mice showed intact pancreatic islet architecture. In contrast, most of the gel-injected, EMC-D virus-infected control mice developed diabetes and showed lymphocytic infiltration of their pancreatic islets and severe beta cell necrosis. These results are consistent with other studies showing successful immunization with synthetic vaccines using linear antigenic sites on the VP1 protein of other viruses (Arnon, 1991; Homa et al., 1993).

Subunit vaccines have several advantages over live vaccines. They provide increased safety and lower toxicity, as the potential hazards of live virus vaccines are eliminated. Immunization with an attenuated live vaccine may result in progeny virus that have acquired more virulent properties, rendering them capable of causing disease. Subunit vaccines are also easier to produce in abundant quantities by recombinant techniques. There are, however, some disadvantages to subunit vaccines. These vaccines are much less immunogenic than live vaccines, and usually produce a much lower antibody titre as compared to live vaccines. In this study, however, inoculation of mice with VP1 protein resulted in the production of relatively high antibody titres against the EMC-D virus and protection from EMC-D virus-induced diabetes. Therefore, this recombinant protein can be used for protection against virus-induced diabetes.

In humans, there is a great deal of circumstantial
evidence associating coxsackie B viruses with IDDM. Much of the evidence has come from epidemiological studies linking recent-onset IDDM with coxsackie B virus infections (reviewed in Yoon & Kominek, 1995). In addition to epidemiological studies, there have been several case reports in which coxsackie B viruses have been isolated from patients with newly diagnosed IDDM (Champsaur et al., 1982; Gladisch et al., 1976; Yoon et al., 1979), as well as many anecdotal reports describing the development of IDDM in patients with a recent or concurrent coxsackie B virus infection (Ahmad & Abraham, 1982; Asplin et al., 1982; Jenson et al., 1980; Negro et al., 1986; Niklasson et al., 1985; Orchard et al., 1982; Palmer et al., 1981; Wilson et al., 1977). The situation is more definitive in animals, where coxsackie B viruses induce a diabetes-like syndrome in genetically susceptible animals, including several strains of mice (Coleman et al., 1973; 1974; Toniolo et al., 1982; Yoon et al., 1978) and Patas monkeys (Yoon et al., 1986). From results of studies on humans and animals, it is speculated that coxsackie B viruses, especially the B4 serotype, may play a role in some cases of IDDM, either by directly initiating the development of the disease, or by operating as the final insult to beta cells in individuals in whom ongoing autoimmune beta cell destruction has already been taking place (Yoon & Kominek, 1995). Our current development of a safe recombinant subunit vaccine to prevent EMC-D virus-induced diabetes in an animal model may have potential implications for the prevention of coxsackie B virus-associated diabetes in humans, as we have clearly demonstrated that EMC-D virus-induced diabetes in mice can be completely prevented by recombinant VP1 protein as a safe subunit vaccine.

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References


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