Originals

Expression of a viral gene in insulin-producing cell lines renders them susceptible to immunological destruction

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Summary. The gene coding for the glycoprotein D of herpes simplex virus type 1 was cloned into plasmids under the transcriptional control of the SV40 promoter-enhancer or the rat insulin 1 promoter-enhancer sequences. These plasmids were transfected into rat insulinoma cells (RINm5F) and mouse NIH/3T3 cells and the expression of glycoprotein D was examined using cell surface immunofluorescence. The rat insulin 1 promoter-enhancer sequences directed efficient expression in RINm5F cells, but not in NIH/3T3 cells. In contrast, the SV40 promoter-enhancer sequences worked well in NIH/3T3 cells, but not in RINm5F cells. Expression of glycoprotein D did not interfere with insulin production by

RINm5F cells. When stable cell lines expressing glycoprotein D were exposed to anti-herpes simplex virus type 1 antibodies and complement, they were destroyed. These studies provide additional evidence that specific promoter-enhancer elements are required for efficient gene expression in certain cell types and demonstrate that the expression of foreign antigens on the surface of insulin-producing cells can lead to their immunological destruction.

Key words: RINm5F, insulin promoter-enhancer, herpes simplex virus type 1 glycoprotein D, antiviral antibody, immunological destruction.

Type 1 (insulin-dependent) diabetes mellitus is due to destruction of B cells in the pancreatic islets of Langerhans. In the majority of the cases of Type 1 diabetes, immunological factors are thought to be responsible for B-cell destruction [1–3]. Precisely what triggers the immune response to B cells is not known. A variety of factors have been proposed, including viral infections [4, 5]. One of the several mechanisms by which viruses might trigger an immune response to B cells is by inserting viral antigens into the cell membrane. This could occur during the course of acute or persistent viral infections.

The present experiments were initiated to develop, by molecular cloning techniques, insulin-producing B cell lines expressing a viral antigen. We prepared plasmids containing the gene coding for the glycoprotein D (gD) of herpes simplex virus type 1 (HSV-1). gD is found in the viral envelope as well as on the surface of HSV-1 infected cells and it stimulates the production of high-titered virus-neutralizing antibody [6]. The gD gene was placed under the control of the rat insulin 1 promoter-enhancer or the SV40 promoter-enhancer sequences. By transfecting rat insulinoma cells (RINm5F) with these plasmids, we succeeded in developing stable

cell lines that expressed HSV-1 gD on their surface. The efficacy of different promoter-enhancer sequences, the capacity of transfected cells to produce insulin and the susceptibility of transfected cells to immunological destruction were evaluated.

Materials and methods

Cells

NIH/3T3 cells were grown in Dulbecco's modified Eagle medium with 4.5 g/l glucose (DMEM) (Whittaker, MA Bioproducts, Walkersville, Md, USA) containing 10% heat-inactivated calf serum (Colorado Serum Co, Denver, Colo, USA). The rat insulinoma cell line (RINm5F) was established by Gazdar et al. [7]. RINm5F cells were grown in RPMI 1640 medium (Quality Biological, Inc, Gaithersburg, Md, USA) with 10% heat-inactivated fetal bovine serum (GIBCO, Grand Island, NY, USA).

Construction of recombinant plasmids

A 2.2-kilobase (kb) BglII-BamHI fragment containing the bacterial aminoglycosyl-3'-phosphotransferase gene (neo gene) was excised from pSV2neo [8] and inserted into the BamHI site of pGEM1 (Pro-

Fig. 1a-c. Structure of recombinant plasmids. **a** Plasmid pIPEno (5.47 kilobases (kb)) is a derivative of pGEM1. It contains the neo gene, as a 2.9-kb HindIII-BamHI fragment from pSV2neo [8], cloned at the BamHI site and the rat insulin 1 promoter-enhancer sequences (PvuII site at -410 to RsaI site at +51) cloned at the SacI site using SacI linkers. **b** Plasmid pSVgD (7.79 kb) was constructed from pSV2neo by replacing the neo gene with glycoprotein D (gD) gene. pSV2neo was cut with HindIII and BamHI and the 3.4-kb fragment containing the SV40 promoter-enhancer was ligated to a 4.4-kb HindIII-BamHI fragment containing the gD gene excised from a plasmid containing the HSV-1 EcoRI H fragment [10]. **c** Plasmid pIPEgD (7.77 kb) is a derivative of pGEM1. It contains the rat insulin 1 gene regulatory sequences (-410 to +51) cloned at the SacI site using SacI linkers and the gD gene, as a 4.4-kb HindIII-BamHI fragment from pSVgD, cloned at the EcoRI site using EcoRI linkers

mega Biotech, Madison, Wisc, USA) to generate plasmid pOneo. The PvuII-RsaI fragment from the rat insulin 1 gene containing the rat insulin 1 gene promoter-enhancer sequences (-410 to +51) [9] was inserted into the SacI site of pOneo located 16 base pairs upstream of the neo gene by using SacI linkers to generate plasmid pIPEno (Fig. 1a).

A plasmid containing the HSV-1 EcoRI H fragment cloned in pBR325 [10] was cut with HindIII and BamHI. The 4.4-kb fragment containing the gD gene without the viral regulatory sequences [11] was ligated to a 3.4-kb HindIII-BamHI fragment derived from pSV2neo. The resultant plasmid, pSVgD, encodes the gD gene under the control of the SV40 early promoter (Fig. 1b).

A plasmid containing the rat insulin 1 gene regulatory sequences (PvuII site at -410 to RsaI site at +51) cloned with SacI linkers at the SacI site of pGEM1 was cut with EcoRI and ligated to the 4.4-kb fragment containing the gD gene which was excised from pSVgD with HindIII and BamHI, flush-ended, and ligated to EcoRI linkers. The resulting plasmid, pIPEgD, encodes the gD gene in an appropriate form for expression under the control of the rat insulin 1 promoter-enhancer sequences (Fig. 1c).

DNA transfections and isolation of cells expressing glycoprotein D

The DNA transfections were performed by the calcium phosphate precipitation technique [12] as modified by Wigler et al. [13] and Barbacid [14]. Briefly, NIH/3T3 cells were plated in 10 cm dishes at a density of $1-2 \times 10^5$ cells/dish the day before transfection. RINm5F cells were plated in 10 cm dishes at a density of $7-8 \times 10^5$ cells/dish at two days before transfection and the medium was replaced with DMEM containing 10% fetal bovine serum during transfection. A calcium phosphate precipitate of DNA was prepared by adding dropwise a 0.5 ml aliquot containing 20 μ g of DNA in

250 mmol/l CaCl₂ to 0.5 ml of twofold concentrated HNP buffer (50 mmol/l Hepes, pH 7.1, 280 mmol/l NaCl, 1.4 mmol/l phosphate) with constant mixing. The amount of plasmid DNA per dish was 1 μ g for pSV2neo and pIPEno, and 5 μ g for pSVgD and pIPEgD. The total amount of DNA per dish was adjusted to 20 μ g using carrier DNA from host cells. The DNA-calcium-phosphate mixtures were incubated at room temperature for 40 to 50 min, and then added to the cells. After the cells were incubated at 37°C for 16 to 18 h, DNA precipitates were removed and the cells were refed with fresh medium. RINm5F cells were glycerol-shocked at room temperature for 2 min using 2 ml of 15% glycerol in HNP buffer. The pre-selection expression time was 24 h for NIH/3T3 and 48 h for RINm5F. Cells were then subcultured into selective media, which was DMEM containing 10% calf serum and 300 μ g/ml of G418 (GIBCO) for NIH/3T3 cells and RPMI 1640 containing 15% fetal bovine serum and 300 μ g/ml of G418 for RINm5F cells. Selective media were changed twice a week. After 10-11 days for NIH/3T3 cells or 20-25 days for RINm5F cells, colonies were trypsinized with cloning cylinders, expanded in selective media, and screened by cell surface immunofluorescence using rabbit anti-HSV-1 antibodies and fluorescein-conjugated swine anti-rabbit immunoglobulins (Dako Co, Santa Barbara, Calif, USA). In some experiments colonies were stained with 1% methylene blue in 70% ethanol and counted.

Immunofluorescence

Subconfluent cell monolayers grown in tissue culture chamber/slides were washed with Dulbecco's phosphate buffered saline without calcium and magnesium (PBS) and fixed with 100% ethanol for 3 min at room temperature. The fixed cells were incubated for 30 min at 37°C with a 1:200 dilution in PBS of monoclonal antibody 4S specific for HSV-1 gD, kindly provided by Dr. M. Zweig [15]. The cells were washed with PBS and then incubated for 30 min at 37°C with fluorescein-conjugated goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Inc, Gaithersburg, Md, USA) diluted 1:20 in PBS. After washing with PBS, the slides were mounted in 50% (volume/volume) glycerol in PBS and viewed by fluorescence with a Zeiss microscope. Cell surface immunofluorescence was carried out as described above except that viable suspended cells, without fixation, were incubated with the antibodies at 4°C for 1 h.

Fluorescence-activated cell sorter (FACS)

Cells were treated with 10 mmol/l EDTA in PBS to make single cell suspensions and stained with rabbit anti-HSV-1 antibodies (1:200) followed by fluorescein-conjugated swine anti-rabbit immunoglobulins (1:200) at 4°C for 15 min and analysed by cell sorting on a

Fig. 2 A-D. Immunofluorescence of transfected RINm5F cells (**A, C**) and NIH/3T3 cells (**B, D**). Viable cell suspensions (**A, B**) or ethanol-fixed cells (**C, D**) were incubated with HSV-1 glycoprotein D specific monoclonal antibody and then with fluorescein-conjugated goat anti-mouse IgG antibody ($\times 168$)

440 FACS cell sorter (Becton Dickinson, Mountain View, Calif, USA).

³⁵S-methionine labelling of cells, immunoprecipitations, and gel electrophoresis

Immunoprecipitations were performed as previously described [16] with slight modifications. Cells were labelled with 200 μ Ci/ml of ³⁵S-methionine (New England Nuclear, Boston, Mass, USA) in methionine-free medium for 15 min or were labelled with ³⁵S-methionine for 15 min and then washed twice with medium and incubated with medium containing 1.0 mmol/l methionine for 60 min. The cells were washed twice with cold 50 mmol/l Tris-HCl, pH 7.5, containing 100 mmol/l NaCl (Buffer A) and were extracted at 4°C with 0.5 ml of Buffer A containing 1% Nonidet P-40 (Sigma, St. Louis, Mo, USA), 0.5% sodium deoxycholate (DOC) (Sigma), 1.0 mmol/l phenylmethylsulfonylfluoride (Sigma), 1 mg of aprotinin (Sigma) per ml, and 0.1% bovine serum albumin (Sigma). The lysates were frozen and thawed three times and were centrifuged for 30 min in a Beckman microfuge. For immunoprecipitation, 0.2 to 0.5 ml of the lysates were mixed with 5 μ l of rabbit anti-HSV-1 antibody or 5 μ l of normal rabbit immunoglobulin (Dako), and subsequently with 5 to 10 mg of protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ, USA) for 1 h at 4°C. The protein A-Sepharose beads were washed twice in Buffer A containing 1% Nonidet P-40 and 0.5% DOC, once in Buffer A containing 0.1% sodium dodecyl sulfate (SDS), 1% DOC, and 1% Triton X-100, and once in 50 mmol/l Tris-HCl, pH 6.8, containing 0.1% SDS. The precipitated proteins were eluted from the beads by adding 50 μ l of twofold concentrated sample buffer (100 mmol/l Tris-HCl, pH 6.8, 4% SDS, 4% 2-mercaptoethanol, 20% glycerol) and heating at 100°C for 5 min. Samples were analysed by electrophoresis in 8% SDS-polyacrylamide gels [17]. Gels were fixed in 50% methanol - 7% acetic acid, treated with Enlightening (New England Nuclear), and then exposed to Kodak XAR film.

Measurement of insulin

Insulin concentrations were measured quantitatively with a ¹²⁵I radioimmunoassay kit (Amersham Co, Arlington Heights, Ill, USA) according to the instructions provided by the manufacturer.

Cytotoxicity assay

Complement mediated cytotoxicity assay was performed as described previously [18]. Briefly, cells in 96-well tissue culture plates were labelled with 1 μ Ci of ⁵¹Cr-sodium chromate (New England Nuclear) per well for 5 h. Cells were then incubated with rabbit anti-HSV-1 antibodies at 37°C for 1 hr, followed by incubation at 37°C for 40 min with a 1:5 dilution of rabbit complement (Low-Tox-H) (Accurate Chemical and Scientific Co, Westbury, NY, USA). The supernatants were removed and counted in a Beckman model 9000 gamma counter. The percent ⁵¹Cr release was determined by the following equation: % ⁵¹Cr release = $100 \times [(^{51}\text{Cr release in the presence of antibody and complement}) - (^{51}\text{Cr release with complement alone})] / [(^{51}\text{Cr release by Triton X-100}) - (^{51}\text{Cr release with complement alone})]$. Release in the absence of antibody was less than 10% of total ⁵¹Cr release.

Results

Transfection efficiency of plasmid constructs

The cell-specific expression of insulin results from the interaction of pancreatic B-cell-specific transcriptional factors with cis-acting regulatory sequences in the insulin gene [9, 19]. One might expect that optimal ex-

Table 1. Transfection efficiency of pIPEno and pSV2neo in different cells

Plasmids	G418 resistant colonies ^a	
	RINm5F	NIH/3T3
pIPEno	10	10
pSV2neo	1	450

^a RINm5F and NIH/3T3 cells were transfected with 1 μ g of pSV2neo or pIPEno and selected by G418. Colonies were stained with methylene blue and the number of G418-resistant colonies is shown per 1×10^5 cells transfected

Fig. 3a-d. FACS analysis of transfected and untransfected NIH/3T3 cells and RINm5F cells. Cells were incubated with rabbit anti-HSV-1 antibody (solid line) or normal rabbit immunoglobulin (dashed line) followed by fluorescein-conjugated anti-rabbit immunoglobulins. The dotted line represents background fluorescence of cells incubated with the fluorescein-conjugated antirabbit immunoglobulins alone. **(a)** Untransfected NIH/3T3 cells, **(b)** transfected NIH/3T3 cells, **(c)** untransfected RINm5F cells, **(d)** transfected RINm5F cells

pression of a foreign gene in cultures of B-cell origin would occur when that gene is placed under the regulation of the insulin gene promoter-enhancer sequences. To test this hypothesis we constructed four plasmids representing all combinations of two marker genes (neo gene and HSV-1 gD gene) and two promoter-enhancer sequences. Plasmids pIPEneo and pIPEgD have the rat insulin 1 promoter-enhancer as their transcriptional control element (Figs. 1a and 1c). Plasmids pSV2neo and pSVgD have the marker genes under the control of the SV40 early promoter-enhancer sequences. The transfection efficiency of the two plasmids carrying the

neo gene, which confers resistance to G418 toxicity, were determined in NIH/3T3 cells and in RINm5F cells. In agreement with our hypothesis, pIPEneo was ten times more efficient than pSV2neo in establishing G418 resistant colonies in RINm5F cells, whereas the converse was true with NIH/3T3 cells, in which pSV2neo was 45 times more efficient than pIPEneo (Table 1).

Construction of cell lines expressing HSV-1 gD

To construct cell lines stably expressing gD, RINm5F cells and NIH/3T3 cells were co-transfected with two plasmids, one conferring resistance to G418 and the other containing the HSV-1 gD gene. pIPEgD contains the HSV-1 gD gene under the control of the rat insulin 1 promoter-enhancer and pSVgD contains the HSV-1 gD gene under the control of the SV40 early promoter-enhancer (Figs. 1c and 1b). G418-resistant colonies were tested for expression of gD on the cell surface by immunofluorescence using rabbit anti-HSV-1 antibodies. In agreement with our previous findings, pIPEgD was efficiently expressed in RINm5F cells, but not in NIH/3T3 cells. In contrast, pSVgD was expressed in NIH/3T3 cells, but only minimally in RINm5F cells (Table 2). However, the highest expression ratio was obtained when two plasmids were controlled by different transcriptional control elements.

Cell lines expressing gD were established by two additional cloning cycles and used in subsequent experiments. The expression of gD was confirmed by immunofluorescence using HSV-1 gD specific monoclonal antibody. Expression of gD is evident both on the surface and in the cytoplasm of the transfected RINm5F

Fig. 4. Immunoprecipitation of glycoprotein D from transfected NIH/3T3 and RINm5F cells. Monolayers of cells in 3.5 cm tissue culture dishes were labelled with ^{35}S -methionine for 15 min (lanes 1 and 2) and chased for 60 min (lane 3). The cell extracts were immunoprecipitated by normal rabbit immunoglobulin (lane 1) or rabbit anti-HSV-1 antibody (lanes 2 and 3) with protein A-Sepharose beads. The immunoprecipitates were analysed by 8.0% polyacrylamide gel electrophoresis and fluorography

Fig. 5a and b. Transfected NIH/3T3 (**a**) and RINm5F (**b**) cells destroyed by antiviral antibody and complement. Transfected cells were labelled with ^{51}Cr and incubated with rabbit anti-HSV-1 antibody plus complement (open circle) or with rabbit anti-HSV-1 antibody only (solid circle). % specific cytolysis was determined as described in Materials and Methods. The spontaneous release of ^{51}Cr in the presence of complement was less than 10% of the total release

and NIH/3T3 cells, and is accentuated at the Golgi apparatus and nuclear membrane (Fig. 2). Quantitative analysis by FACS revealed that all the transfected NIH/3T3 cells (B) and most of the transfected RINm5F cells (D), but not the untransfected cells (A and C), had appreciable amounts of gD on their surface (Fig. 3).

To analyse the gD protein produced by the RINm5F and NIH/3T3 lines, cells were labelled with ^{35}S -methionine, followed by radioimmunoprecipitation with rabbit antibodies to HSV-1 and Protein A-Sepha-

rose beads (Fig. 4). When cells were pulsed for 15 min, a 53 kilodalton (kDa) band which corresponds to the precursor form of gD [20, 21] was obtained from transfected NIH/3T3 and RINm5F cells. Immunoprecipitations of untransfected cell extracts with anti-HSV-1 antibody (not shown) and transfected cell extracts with normal rabbit immunoglobulin did not show a 53 kDa band. When pulse-labelled transfected cells were chased for 60 min, the precursor form was converted into mature gD of approximately 58 kDa. The band from the RINm5F cells, however, was considerably more diffuse than the band from the NIH/3T3 cells.

To determine whether the RINm5F lines expressing gD continued to produce insulin, the insulin concentration in the culture media was measured by radioimmunoassay. Five out of six RINm5F clones continued to produce insulin at a level equal to, or greater than, that of untransfected RINm5F cells (Table 3).

Table 2. Expression of glycoprotein D (gD) in cells co-transfected with different plasmids

Plasmids ^a	Colonies ^b	
	RINm5F	NIH/3T3
pIPEgD + pIPEneo	10/39 (26%)	0/34 (0%)
pIPEgD + pSV2neo	6/12 (50%)	0/48 (0%)
pSVgD + pIPEneo	3/68 (4%)	15/24 (63%)
pSVgD + pSV2neo	0/6 (0%)	5/18 (28%)

^a Cells were co-transfected with two plasmids and selected by resistance to G418 toxicity. Resultant colonies were tested for expression of gD by cell surface immunofluorescence using rabbit antibodies to herpes simplex virus type I. ^b Number colonies expressing gD/total colonies tested

Table 3. Insulin production by RINm5F cells expressing glycoprotein D

Cells	Transfection	Clone ^a	Insulin ($\mu\text{U}/\text{ml}$) ^b
RINm5F	pIPEgD + pIPEneo	1	ND
		2	73
RINm5F	pIPEgD + pSV2neo	1	83
		2	> 160
RINm5F	pSVgD + pIPEneo	1	> 160
		2	> 160
RINm5F	none		50
Medium only			ND

^a Colonies derived from experiment described in Table 2 and cloned a total of 3 times. ^b Culture media from semiconfluent cells (3 days after plating cells) were tested for insulin by radioimmunoassay. ND: not detected

Destruction of RINm5F cells expressing gD by antiviral antibody and complement

Cells expressing gD were labelled with ^{51}Cr and incubated with anti-HSV-1 antibody and complement. As seen in Figure 5, both NIH/3T3 and RINm5F cells were readily destroyed although the NIH/3T3 cells were somewhat more susceptible to destruction than the RINm5F cells. Neither of the cells were lysed by normal rabbit immunoglobulin plus complement. Untransfected cells were not susceptible to lysis by anti-HSV-1 antibody plus complement (not shown).

Discussion

We have established RINm5F cell lines constitutively expressing a viral surface antigen as an in vitro model system of antibody mediated destruction of B cells which express a foreign protein. The gD antigen of

HSV-1 was previously expressed in mouse L cell and Chinese hamster ovary cell lines using the SV40 promoter-enhancer as a transcriptional control element [22, 23]. However, when insulin-producing B cells are targeted to express a gene, it is important to select an appropriate transcriptional control element because of cell specific regulation of gene expression [9, 19]. Cell specific expression of the chloramphenicol acetyl transferase gene has been demonstrated in HIT cells (an insulin producing hamster cell line) using the insulin promoter-enhancer in a transient expression system. In the present experiments we compared the efficacy of the SV40 and insulin promoter-enhancers as transcriptional control elements in stable transfection of B cells and fibroblasts.

The rat insulin 1 promoter-enhancer worked efficiently in RINm5F cells, as demonstrated by expression of the gD gene in RINm5F cells transfected with pIPEgD, and by the greater efficiency of pIPEneo over pSV2neo to confer resistance to G418 in RINm5F cells. Conversely, the SV40 promoter-enhancer worked efficiently in NIH/3T3 cells and poorly in RINm5F cells. These findings provide additional evidence that specific transcriptional control elements are required for efficient stable expression of a foreign gene in these cell types. It is of interest that the expression of gD is decreased when co-transfected plasmids have the same transcriptional regulatory elements. Most likely this is the result of promoter competition, a phenomenon well-documented in transient expression systems [24] but not fully described in stably transfected cells.

Several studies have suggested that viral infections, particularly persistent viral infections, might alter the function of cells without producing morphological changes [25]. Cells engineered to express a single viral protein would be useful in dissecting the effect of viral infections on cell function. In the present study, five of the six RINm5F cell lines expressing gD produced as much or more insulin than untransfected RINm5F cells. Thus, the expression of gD in RINm5F cells did not negatively affect the amount of insulin released into the cell-free media. Further studies, however, are required to measure more subtle changes such as the actual rate of insulin synthesis and secretion. In addition to being a model for studying the effect of foreign proteins on insulin synthesis and secretion, RINm5F cells expressing gD might be useful in studying how foreign proteins are processed.

HSV gD has a hydrophobic region that is inserted into the cell membrane [11]. It is produced as a precursor form and then converted into a mature form by the addition of N-linked and O-linked oligosaccharides [26–28]. In our immunoprecipitation experiments, precursor gD showed a band at 53 kDa in both NIH/3T3 and RINm5F cells. The mature form of gD was about 58 kDa in both NIH/3T3 and RINm5F cells, but the band was far more diffuse in RINm5F cells. This dif-

ference is most likely due to cell specific glycosylation [29]. RINm5F cells expressing gD, thus, may provide a useful model for studying cell specific glycosylation in insulin-producing cells.

Apart from the effect that expression of a viral antigen might have on the functional capacity of insulin-producing cells, our studies show that expression of a viral antigen on the surface of insulin-producing B cells makes them susceptible to complement-mediated destruction by antiviral antibody. These *in vitro* findings point to the possibility that the expression of viral antigens may be one of the factors that triggers an immune response to B-cells in Type 1 diabetes.

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