Infection of Cultured Human Pancreatic B Cells with Reovirus Type 3

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Summary. The capacity of reovirus type 3 to infect insulin-producing B cells was studied in human pancreatic cell cultures. Antibody to reovirus was labelled with fluorescein isothiocyanate and antibody to insulin was labelled with tetramethyl rhodamine isothiocyanate. By using a double-labelled immunofluorescent antibody technique, it was shown that only about 6% of the insulin-containing human B cells in culture became infected when inoculated with unpassaged reovirus type 3. However, by repeated passage of the virus in human pancreatic B cell cultures, the percentage of infected B cells increased to 27%, and the virus titre in cultures rose from 8.0×10^4 pfu/ml in the first passage, to 4.9×10^6 pfu/ml in the 5th passage. As measured by radioimmunoassay, the intracellular immunoreactive insulin began to decrease at 24 h after infection. This decrease roughly paralleled the increase in virus titre. In contrast, there was relatively little change in immunoreactive insulin in cultures inoculated with unpassaged reovirus type 3. These studies show that the ability of reovirus type 3 to infect human B cells is enhanced by serial passage in human pancreatic cell cultures and that the infection resulted in the destruction of B cells and release of insulin.

Key words: Virus-induced diabetes mellitus, human pancreatic B cell cultures, double-labelled immunofluorescent antibody staining, reovirus type 3, virus passage.

The possibility that viruses might cause some cases of insulin-dependent diabetes (IDD) by infecting and destroying pancreatic B cells has received considerable attention. Of the numerous viruses implicated, mumps and members of the Coxsackie virus B group (particularly B4) have been most often suggested as possible causal agents of IDD [1–6]. In a recent

study, it was shown that a Coxsackie virus B4 variant, isolated from the pancreas of a 10-year-old child who died of diabetic ketoacidosis, produced diabetes in mice by infecting and destroying B cells [7].

It is difficult, however, to demonstrate *in vivo* that viruses replicate in human B cells and produce diabetes in man. As a practical model, an *in vitro* system has been developed to determine if viruses are capable of infecting human B cells. By use of a double-labelled immunofluorescent antibody technique, it has been shown that human B cells grown in culture were susceptible to infection by mumps [8], Coxsackie virus B3 [9], and Coxsackie virus B4 [7].

We have previously shown that reovirus type 3, passaged in murine pancreatic B cell cultures, produced an insulinitis when inoculated into 1 to 2 week old mice [10]. In the present investigation, the double-labelled immunofluorescent antibody technique has been used to determine whether or not human B cells grown in culture were permissive to reovirus type 3, and whether the capacity of the virus to infect human B cells was enhanced by repeated passage in human B cell cultures. Radioimmunoassay for immunoreactive insulin was used to evaluate the effect of the infection on intracellular and extracellular insulin.

Materials and Methods

Pancreatic B Cell Cultures

Four aseptically removed human pancreases from normal subjects 17, 18, 24, and 40 years old (Table 1) were obtained from the Division of Transplantation Surgery, Naval Research Medical Institute, Bethesda, Md. Monolayer cultures enriched for pancreatic B cells were prepared by a technique described previously [11]. Briefly, 5 g of the tail of the pancreas were washed 5 to 7 times in phosphate-buffered saline without Ca^{++} and Mg^{++} (PBS; 0.1 mol/l; pH 7.4) at 4 °C, and containing penicillin (400 IU/ml) and streptomycin (100 mg/l). The pancreas was minced into small pieces and 70 ml of cold PBS was added. The tissue was

Fig. 1. **A** Human pancreatic cells grown on coverslips and stained with TRITC-labelled anti-insulin antibody ($\times 480$). Specific immunofluorescence can be seen in the cytoplasm of B cells. **B** Human fibroblasts (WI-38) grown on coverslips and stained with TRITC-labelled anti-insulin antibody ($\times 480$). None of the cells stained positively

allowed to settle for 1 min and the supernatant fluid containing floating debris was discarded. This procedure was repeated 15 to 20 times until pieces of pancreas less than 1.0 mm in diameter were obtained. The pancreatic tissue was then transferred to a flask containing 50 ml of prewarmed collagenase solution (12 mg/ml in PBS; 190 U/mg). Enzymatic digestion was carried out for 20 to 25 min at 37 °C with vigorous shaking or magnetic stirring. The collagenase-treated material (8 ml) was then transferred to a conical tube containing 35 ml of PBS and centrifuged for 1 to 2 min at speeds just sufficient to allow the cells to settle. The supernatant fluid was carefully removed so as not to disturb the pellet. Each pellet was resuspended in fresh PBS. The cells were centrifuged and washed three more times and filtered through sterile gauze to remove large aggregates. The filtered cells then were centrifuged at 1200 rpm for 7 min and the pellet was suspended in prewarmed chemically defined growth medium [MPNL 65/C [12] plus 7% heat-inactivated fetal calf serum] [11] and adjusted to 1.0×10^5 viable cells/ml. Plastic tissue culture dishes (35 mm) then were seeded with 2.5 ml of cell suspension and incubated at 37 °C in a humidified atmosphere of 5% CO₂. Approximately 12–15 h later, the non-adherent cells were decanted, thereby eliminating most of the fibroblastoid cells which had adhered to the dish. The decanted cells were seeded in 35 mm plastic tissue culture dishes and at the end of 48 h, the medium was replaced with fresh growth medium plus thiomersal (0.7 mg/litre) [13] (Aldrich Chemical, Milwaukee, Wisc). The cells were refed at 2 day intervals with the thiomersal

containing growth media. The monolayers were then used for experiments at 5 to 12 days after culture.

For immunohistochemical staining, each 35 mm plastic tissue culture dish had a sterile coverslip (22 mm square Corning Glass No. 1 coverslip) placed in it. Human pancreatic cells were added to the dishes to overlay the coverslip (2.5 ml containing 5×10^2 cells). The procedures for decantation and change of media were the same as described above. Cells grown on the coverslips for 5 days were then stained (see section on double-labelled immunofluorescent antibody technique). About 15 to 20% of the cells grown on coverslips without using the decantation step proved to be B cells as evaluated by staining with tetramethyl rhodamine isothiocyanate (TRITC)- or fluorescein isothiocyanate (FITC)-labelled anti-insulin antibody. In contrast, over 30% of the cells in cultures prepared with the decantation step proved to be B cells (Fig. 1).

Virus

Reovirus type 3 (Abney strain) previously passed 12 times in cultures of Rhesus monkey kidney cells was originally obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, USA. This prototype virus was serially passaged in human pancreatic B cell cultures using a virus to cell ratio of 10:1. Each passage of the virus was frozen and used for experiments as described in Results. In addition, reovirus type 3 previously passaged 20 times in cultures of murine pancreatic B cells in our laboratory [10] was also used for comparison against the human B cell passaged virus.

Plaque Assay

The infectious titre of reovirus type 3 was determined by inoculating confluent L-929 cell monolayers (60 mm dishes) with 0.2 ml of appropriate dilutions of the virus sample. After adsorption for 1 h at 37 °C, the monolayers were overlaid with agarose medium [1% (w/v) agarose with Eagle's MEM and 5% (v/v) fetal bovine serum]. Monolayers were stained 6 days later with 1:40,000 dilution of neutral red. Plaques were counted within 24 h, and the virus titre expressed as plaque-forming units (pfu/ml).

Double-labelled Immunofluorescent Antibody Technique

Antibody to reovirus type 3 was prepared by repeated IP inoculation of 6 week old guinea pigs with 10^6 pfu of reovirus type 3 at weekly intervals for two weeks. Serum was collected seven days after the final immunization. The 50% neutralization titre of the antiserum to reovirus type 3 was 1:1000. The γ -globulin fraction of the serum, purified by ammonium sulphate precipitation and DEAE cellulose column chromatography, was conjugated with FITC [14]. Unconjugated FITC was removed by gel filtration through Sephadex G-25. The labelled γ -globulin was then adsorbed with acetone-treated mouse liver powder to eliminate non-specific fluorescence. The specificity of the labelled antiserum was established by inhibition tests using unlabelled antisera to reovirus type 3.

The γ -globulin fraction of antibody to porcine insulin, obtained from Miles Laboratories, Elkhart, Ind., was labelled with TRITC [14]. The specificity of this labelled antibody was demonstrated by staining frozen acetone-fixed sections of human pancreas as previously described [8, 9]. Only insulin-containing B cells in the islets stained bright orange, while the surrounding acinar cells did not stain. Absorption of the labelled antibody with purified porcine insulin completely blocked staining. Furthermore, as a control for specificity, it was found that diploid human fibroblast cells (WI-38) were unstained (Fig. 1B) when tested with TRITC-labelled anti-insulin antibody. In addition, none of the

Table 1. Host specificity in susceptibility to reovirus^a

Donor ^b	Years	Virus	No. of rhodamine positive cells ^c	No. of fluorescein positive cells ^d	No. of both positive cells ^e	% of virus-infected B cells ^f
1	17	NP ^g	61	179	3	5
		MBP ^h	79	185	4	5
2	40	NP	65	192	4	6
		MBP	81	241	6	7
3	24	NP	69	149	4	6
		MBP	65	152	4	6
4	18	NP	83	158	6	7
		MBP	103	186	8	8

^a The data represent observations accumulated from 3 or 4 coverslips in each of the groups

^b All donors were male caucasian

^c Insulin-containing B cells stained with rhodamine-labelled anti-insulin antibody

^d Reovirus-infected cells stained with fluorescein-labelled anti-reovirus type 3 antibody

^e Reovirus-infected insulin-containing B cells stained with both antibodies

^f Percent of both positive cells ($f = e/c$)

^g Unpassaged reovirus type 3 (stock virus from ATCC)

^h Murine pancreatic B cell passaged reovirus type 3

cells stained positive when uninfected human pancreatic cells were stained with FITC-labelled anti-reovirus antibody.

To identify reovirus type 3 infected B cells, cells grown on coverslips were infected for two days and then fixed in acetone and stained with both TRITC-labelled anti-insulin antibody and FITC-labelled anti-reovirus antibody overnight at 4 °C. Coverslips then were washed three times with PBS, mounted with Elvanol [11] and examined for immunofluorescence with a Zeiss microscope illuminated by an HBO 200 mercury lamp. Double-stained cells were identified by examining the coverslips first with rhodamine filters (BP 546/9 excitation filter and an LP 590 barrier filter), and then with fluorescein filters (KP 490 and BG 12 excitation filters and an LP 528 barrier filter). About 150–300 insulin-containing B cells were examined.

Measurement of Immunoreactive Insulin

Monolayers of human pancreatic B cells were maintained in Eagle's medium containing glucose, 100 mg/100 ml for three days before infection with virus [9, 15]. Insulin was extracted from the monolayers at different times after infection as well as from uninfected controls by methods described elsewhere [16]. Extracellular insulin was measured from supernatant samples from the media of the monolayer cultures (triplicate). The concentration of immunoreactive insulin was measured by radioimmunoassay with porcine insulin used as a standard [17].

Statistical Analysis

The statistical significance of the differences between the means of the sample groups was tested by Student's t-test with the Welch adjustment for unequal variances where necessary [18]. The significance level of $p < 0.001$ was used as a criterion of differences.

Results

Susceptibility of Human B Cells to Unpassaged Reovirus Type 3

Cultures enriched in human B cells were grown on coverslips and inoculated with reovirus type 3

(Abney strain) at a virus-to-cell ratio of 1:1. Their susceptibility to infection was determined by the double-labelled immunofluorescent antibody technique. Human B cells were found to be relatively resistant to reovirus type 3 (Table 1). At 2 days after infection, approximately 6% of the insulin-containing B cells contained viral antigens. There was no significant difference in the susceptibility of B cells obtained from different human pancreases to both the unpassaged and the murine B cell passaged reovirus type 3 (Table 1).

Passage of Reovirus Type 3 in Human B Cell Cultures

Reovirus type 3 was serially passaged in human B cell cultures. Virus was harvested 72 h after infection, and the titre of infectious virus was determined. Virus titres in cultures showed that the peak titre rose from 8.0×10^4 pfu/ml (1st passage) to 4.9×10^6 pfu/ml (5th passage). Since these cultures were not pure B cells, an increase in viral titre cannot be only attributed to replication of the virus in B cells. By use of the double-labelled immunofluorescent antibody technique, the percent of infected B cells was determined. Figures 2A, B and C are photomicrographs of human pancreatic cell cultures infected with the fifth passage of reovirus type 3 and stained 30 h later with TRITC-labelled anti-insulin antibody and FITC-labelled anti-reovirus type 3 antibody.

When rhodamine filters were used to examine the cells (Fig. 2A), a diffuse orange colour was found in the cytoplasm but not in the nucleus. When the same three cells were examined for viral antigens with fluorescein filters (Fig. 2B), a green colour was seen in the cytoplasm. Viral antigens were not found in the nucleus, but they did appear to be relatively evenly distributed in the cytoplasm. Fig. 2B also shows

Fig. 2A–C. Human pancreatic cell cultures infected with reovirus type 3 at a virus-to-cell ratio of 1:1. At the end of 30 h, the cultures were stained with TRITC-labelled anti-insulin and FITC-labelled anti-reovirus antibody. **A** Photograph taken with rhodamine filters. **B** Photograph of same area, but taken with fluorescein filters. Arrows point to same cells as seen in Figure 2A. **C** Photograph of same area, but taken first with rhodamine filters and then with fluorescein filters. Arrows point to same cells as seen in Figure 2A ($\times 720$)

reovirus-antigens in two adjacent cells. The lack of fluorescence in the corresponding positions in Fig. 2A when the rhodamine filters were used indicates that these cells did not contain insulin, and probably represent one of the other cell types (e. g., A, D, pancreatic polypeptide-producing, acinar, ductal or fibroblasts) present in the culture. Fig. 2C shows three insulin-containing B cells infected with reovirus type 3 (5th passage) identified by the presence of orange and green fluorescence in the cytoplasm.

Table 2 shows the effect of repeated passage of reovirus type 3 on the infectivity to insulin-contain-

Table 2. The effect of passages of reovirus on infectivity to human B cells^a

Number of passages	Total no. of cells examined	No. of rhodamine positive cells	No. of fluorescein positive cells	Number of both positive cells	Percent of virus-infected B cells
0 ^b	1390	278	678	17	6
1	884	172	389	12	7
3	790	157	327	19	12
5	960	193	405	52	27

^a The different passages of reovirus type 3 were inoculated into the monolayers of pancreatic B cell cultures and stained with rhodamine-labelled anti-insulin antibody and fluorescein-labelled anti-reovirus antibody

^b Unpassaged reovirus type 3 (stock virus obtained from ATCC)

Table 3. The effect of virus passages on the intra- and extracellular insulin levels in human pancreatic B cell cultures three days after infection^a

Passage number	Intracellular IRI (ng/ml)	Extracellular IRI (ng/ml)
Uninfected control	911 $\pm$ 11 ^b	415 $\pm$ 9
0 ^c	855 $\pm$ 14	374 $\pm$ 3
1	788 $\pm$ 42	375 $\pm$ 3
3	576 $\pm$ 4	296 $\pm$ 13
5	358 $\pm$ 41	220 $\pm$ 8

^a The concentration of immunoreactive insulin (IRI) in human pancreatic B cell cultures infected with different passages of reovirus type 3 for three days. Approximately 8×10^5 cells were inoculated with the virus at a virus-to-cell ratio of 10:1 after each passage of virus was concentrated. Statistically significant ($p < 0.001$) differences in both intracellular and extracellular IRI were obtained between (1) passages 1 and 5; (2) uninfected controls and passage 3; and (3) uninfected controls and passage 5

^b Mean $\pm$ standard deviation (SD)

^c Unpassaged reovirus type 3 (stock virus from ATCC)

ing B cells. The percentage of infected B cells increased from 6 to 27% by the 5th passage. The effect of different passages of reovirus type 3 on insulin content is shown in Table 3. Intracellular insulin in cultures (triplicate) containing approximately 8×10^5 cells was 911 ± 11 ng/ml in the uninfected cultures. At three days after infection, there was relatively little change in immunoreactive insulin in cultures inoculated with unpassaged reovirus type 3. However, the intracellular immunoreactive insulin significantly decreased ($p < 0.001$) in cultures inoculated with fifth passaged reovirus type 3. The concentration of extracellular insulin roughly paralleled the decrease in the intracellular insulin content.

Table 4. The concentration of intra- and extracellular immunoreactive insulin (IRI) in human B cell cultures infected with the fifth passaged reovirus type 3

Days after infection	Intracellular IRI (ng/ml)		Extracellular IRI (ng/ml)	
	Infected cultures	Uninfected cultures	Infected cultures	Uninfected cultures
0 ^a	1154 ± 85 ^b		453 ± 11	
1	804 ± 38	1054 ± 51	473 ± 4	445 ± 6
2	ND ^c	ND	493 ± 11	452 ± 5
3	443 ± 38	943 ± 13	284 ± 10	427 ± 8
5	353 ± 29	800 ± 12	231 ± 11	403 ± 6
7	287 ± 13	760 ± 29	195 ± 7	387 ± 2

^a Before infection^b Mean ± SD^c Not done

Measurements of Immunoreactive Insulin in Pancreatic B Cell Cultures Infected with Reovirus Type 3 (5th Passage)

The effect of reovirus type 3 on intracellular and extracellular insulin is shown in Table 4. Insulin in cultures (triplicate) containing about 10^6 cells was 1154 ± 86 ng/ml at the time of viral infection. Within 3 days after inoculation, immunoreactive insulin declined to 443 ± 38 ng/ml, which was less than 50% of the uninfected controls. The concentration of insulin in the extracellular fluid increased slightly during the first 2 days of the infection, probably reflecting release from damaged B cells. The concentration of extracellular insulin then declined, roughly paralleling the decrease in the intracellular insulin content. Analysis of the data from these experiments revealed statistically significant differences ($p < 0.001$) between the infected and uninfected groups on days 3, 5 and 7 in intracellular insulin and on days 5 and 7 in extracellular insulin.

Light Microscopy

By light microscopy, approximately 30–40% of the reovirus (5th passage) inoculated monolayers were destroyed within 72 h after infection, while the uninfected monolayers showed no evidence of cytopathology. Infectivity assays revealed a steady increase in viral titre, peaking at about 5×10^6 pfu/ml 72 h after infection.

Discussion

We have shown for the first time that the capacity of a virus to infect human pancreatic B cells can be enhanced by repeated passage in cultures. In the present study, only 6% of cultured human pancreatic B cells were susceptible to infection by unpassaged and murine B cell-passaged reovirus type 3 (when inocu-

lated at a virus-to-cell ratio of 1:1), as compared to 60% for unpassaged mumps [8], 30% for unpassaged Coxsackie virus B3 [9], and 37% for a diabetogenic variant of Coxsackie virus B4 isolated from the pancreas of a child with diabetic ketoacidosis [7]. However, serial passage of reovirus type 3 in human B cell cultures increased the percentage of infected B cells to 27%. Adaptation of reovirus to infect human B cells was also reflected by immunochemical tests. Immunoreactive insulin levels in the human B cell cultures significantly decreased when infected with passaged virus as compared to unpassaged reovirus or murine B cell-passaged virus.

The precise mechanism by which serial passage of reovirus type 3 in cultures enriched for human B cells enhances its infectivity is not known. The two most likely possibilities are selection of variants of human B-cell tropic virus and non-genetic adaptation of reovirus type 3 to human B cells. Selection of variants could have occurred in two ways. First, the original stock virus pool may have contained three populations of virus: one tropic for human B cells; one tropic for mouse B cells [10]; and the other not tropic for either. Growing the stock virus in cultures enriched for human B cells may have favoured the replication of the human B-tropic virus. Similarly, passaging the stock virus in murine B cell cultures may have enhanced the multiplication of mouse B-cell tropic virus [10]. Second, mutation or recombination may have taken place during serial passage of the virus in cultures, and the presence of human B cells may have favoured the selection of mutated or recombinated B-cell tropic virus. Alternatively, non-genetic adaptation may have occurred because of host-controlled alterations in viral antigen or coat. Thus, the capacity of the virus to bind to human B cells would be increased. In any event, it may be possible to distinguish between a stable mutant and non-genetic adaptation by plaque-purifying the virus [19] and by testing the virus in B cell cultures derived from humans and mice.

Neither the present study with human B cell-passaged reovirus type 3 nor earlier studies with mumps [8] and Coxsackie virus B3 [9] proves that any of the viruses can actually infect and destroy B cells *in vivo*. It is known that some viruses will grow in cultured cells derived from animals that are resistant to infection [20–24]. Differences in susceptibility to *in vitro* and *in vivo* infection may be related to the host defence mechanisms, induction of viral receptors, and/or the requirement for cell division. Thus, the *in vitro* susceptibility of B cells may not be a true reflection of their *in vivo* susceptibility. Nonetheless, when Coxsackie viruses B3 and B4 and reovirus type 3 were passaged multiple times in murine pancreatic B cell cultures, these viruses infected and destroyed B cells to produce a diabetes-like syndrome in susceptible mice [10, 25, 26]. These studies suggest that under appropriate circumstances, a number of viruses can infect and destroy insulin-producing B cells. The circumstances which might lead to the generation of a large number of B-cell tropic viruses in a susceptible host are not known. Recently, a variant of Coxsackie virus B4 isolated from the pancreas of a child with diabetic ketoacidosis was shown to infect cultured human B cells prepared from the pancreas of a non-diabetic [7]. These studies showed that, at least in animal models, the *in vitro* susceptibility of B cells to infection by virus reflected the diabetogenic capability of the virus *in vivo*. However, it is not known if B-cell tropic variants of reovirus type 3 can infect human B cells *in vivo* and cause abnormal glucose metabolism in man.

These data suggest that B-cell tropic variants of reovirus type 3 can be selected by repeated passaging in human B cell cultures. It lends further support to the idea that if some cases of human IDD are caused by viruses, the passage history of the virus may play an important role.

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