Brief Notes and Comments

Virus-induced Diabetes Mellitus

XI. Replication of Coxsackie B3 Virus in Human Pancreatic Beta Cell Cultures

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SUMMARY

The capacity of Coxsackie B3 virus to infect insulin-containing beta cells was studied in human pancreatic cell cultures. Antibody to Coxsackie B3 virus was labeled with fluorescein isothiocyanate, and antibody to insulin was labeled with rhodamine. By use of a double-label antibody technique, three populations of cells were identified: uninfected insulin-containing beta cells, which stained only with rhodamine-labeled anti-insulin antibody; Coxsackie-infected (noninsulin-containing) cells, which stained only with fluorescein-labeled anti-Coxsackie antibody; and Coxsackie-infected insulin-containing beta cells, which stained with both antibodies. Radioimmunoassay showed that intracellular immunoreactive insulin decreased rapidly beginning at 24 hours after infection, and the decrease in insulin roughly paralleled the increase in viral titer. It is concluded that, under in vitro conditions, human beta cells are susceptible to Coxsackie B3 virus. DIABETES 27:778-81, July, 1978.

Epidemiologic studies and case reports have suggested that a temporal relationship may exist between the onset of certain viral infections, particularly mumps and Coxsackie, and the subsequent development of juvenile-onset-type diabetes mellitus.[1-5] Proof that these viruses actually replicate in beta cells and produce diabetes, however, was not established. Recently, by use of a double-label antibody technique it was shown that human beta cells grown in culture were susceptible to infection by mumps.[6] This technique employed rhodamine-labeled antibody to insulin, which stained insulin-containing beta cells orange, and fluorescein-labeled antibody to mumps, which stained mumps-infected cells green. Insulin-containing beta cells infected with mumps were identified by the presence of both orange and green fluorescence in the cytoplasm. In the present investigation the double-label antibody technique was used to determine whether human beta cells grown in culture were permissive to Coxsackie B3 virus, and radioimmunoassay for immunoreactive insulin was used to evaluate the effect of the infection on intracellular and extracellular insulin.

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MATERIALS AND METHODS

Three human pancreases from subjects 15, 18, and 34 years old were obtained within six hours after death from the Division of Transplantation Surgery, Naval Research Medical Institute, Bethesda. Monolayer cultures enriched for pancreatic beta cells (about 15 to 20 per cent) were prepared by slight modification (manuscript in preparation) of a method described earlier.[7] Cells that had been in culture for four days were infected with Coxsackie B3 virus (Nancy strain), obtained from the American Type Culture Collection, Rockville, Md. The virus was passaged 10 times in secondary mouse embryo cells and the titer determined in plaque-forming units (PFU).

Antibody to insulin was made in guinea pigs and the γ-globulin fraction was labeled with tetramethyl rhodamine isothiocyanate (TRITC).[8] Antibody to Coxsackie B3 virus was made in rabbits and the γ-globulin fraction was labeled with fluorescein isothiocyanate (FITC). The specificity of these antibodies was demonstrated by methods described previously.[6] To identify Coxsackie B3 virus-infected beta cells, coverslips containing infected cells were fixed in cold acetone and stained with both TRITC-labeled

anti-insulin antibody and FITC-labeled anti-Coxsackie B3 virus antibody overnight at 4° C. Coverslips then were washed, mounted, and observed for immunofluorescence. Double-stained cells were identified by examining the coverslips first with rhodamine filters and then with fluorescein filters.[6] About 100 insulin-containing beta cells were examined.

Monolayers of pancreatic beta cells were maintained in Eagle's medium containing glucose at a concentration of 100 mg. per deciliter.[7] Insulin was extracted from the monolayers at different times after infection as well as from uninfected controls by methods described elsewhere.[9] The concentration of immunoreactive insulin was measured by radioimmunoassay with porcine insulin used as a standard.[10]

The data in this report comes from studies on the pancreas of a 15 year old white male whose HLA type was A2, A3, B7, B15.

RESULTS

Figures 1A and 1B are photomicrographs of human pancreatic cultures infected with Coxsackie B3 virus that were stained 24 hours later with TRITC-labeled anti-insulin antibody and FITC-labeled anti-Coxsackie B3 virus antibody. When rhodamine filters were used to examine the cells (figure 1A), a diffuse orange color was found in the cytoplasm but not in the nucleus. When the same two cells were examined for viral antigens with fluorescein filters (figure 1B), a green color was seen in the cytoplasm. Viral antigens were not found in the nucleus nor did they appear to be as evenly distributed in the cytoplasm as insulin. Figure 1B also shows Coxsackie viral antigens in several adjacent cells. The lack of fluorescence in the corresponding positions in figure 1A when the rhodamine filters were used indicates that these cells do not contain insulin, and probably they represent one of the other cell types (e.g., alpha cells, acinar

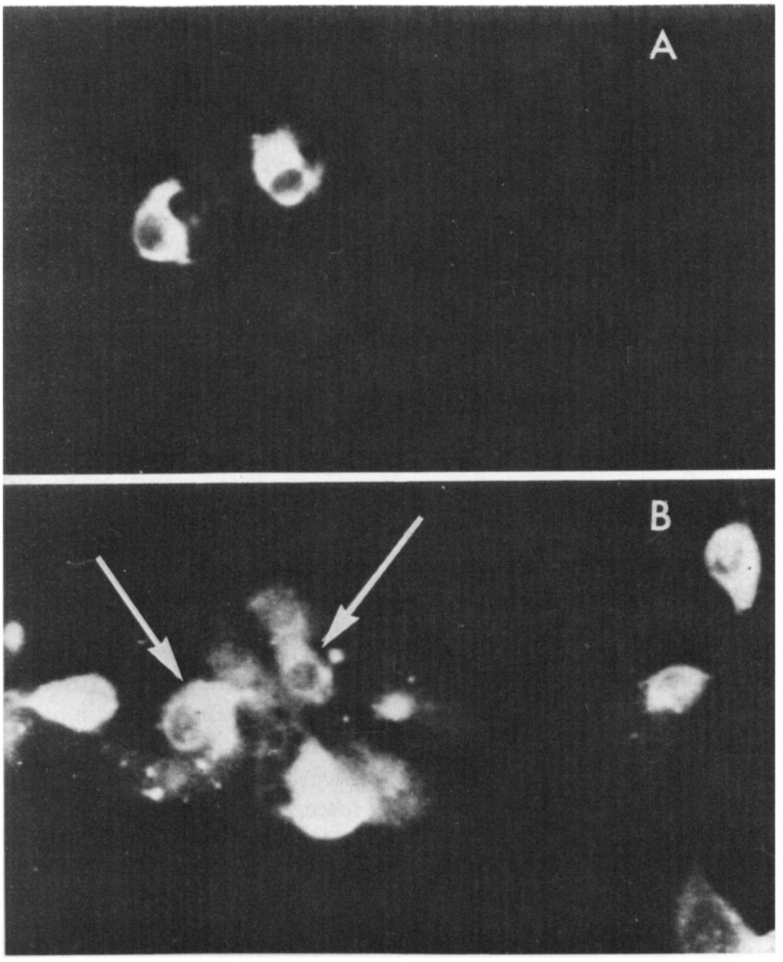

FIGURE 1

Human pancreatic cultures infected with Coxsackie B3 virus at a virus-to-cell ratio of 1.0. At the end of 24 hours, the cultures were stained with TRITC-labeled anti-insulin and FITC-labeled anti-Coxsackie B3 virus antibodies. (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 1A. Magnification (× 480).

cells, or fibroblasts) present in the culture. At 24 hours after infection, viral antigens were found in about 30 per cent of the insulin-containing cells.

The effect of Coxsackie virus on insulin is shown in figure 2. Intracellular insulin in cultures containing about 3×10^5 cells was close to 1,600 ng. (figure 2A) at the time of viral inoculation. Within 72 hours after inoculation, intracellular insulin declined to less than 500 ng., while insulin in the uninfected cultures remained at close to 1,400 ng. The concentration of insulin in the extracellular fluid (figure 2B) increased slightly during the first 48 hours of the infection, probably reflecting release from damaged beta cells. The concentration of extracellular insulin then sharply declined, roughly paralleling the decrease in the intracellular insulin content. Similar data were obtained from monolayer cultures prepared from the two other human pancreases studied (data not shown). Analysis of the data from all three experiments revealed statistically significant differences ($p < 0.001$) between the infected and uninfected groups on days 3 and 5 in the case of intracellular insulin (2A) and on days 4 and 5 in the case of extracellular insulin (2B).

By light microscopy, almost 50 per cent of the Coxsackie virus-inoculated monolayers were destroyed within 72 hours after infection, while the uninfected monolayers showed no evidence of cytopathology. Infectivity assays revealed a steady increase in viral titer, peaking at 1.7×10^6 PFU per milliliter at 72 hours after infection (data not shown).

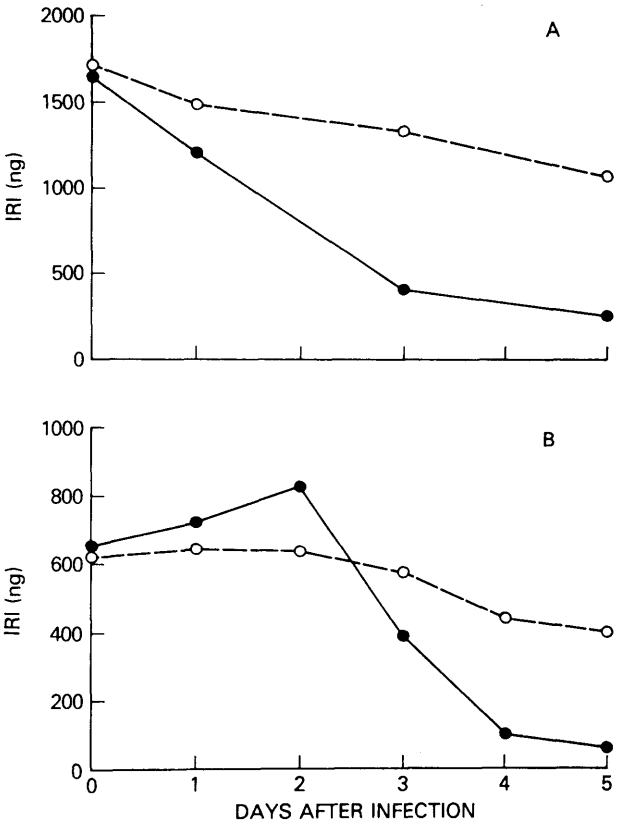

FIG. 2. The mean concentration of immunoreactive insulin (IRI) in human pancreatic cultures infected with Coxsackie B3 virus. Approximately 3×10^5 cells were inoculated with Coxsackie B3 at a virus-to-cell ratio of 10. Media were changed 24 hours before cells and supernatant fluids were harvested for measurement of insulin. Data are expressed as nanograms per culture. (A) Intracellular insulin. (B) Extracellular insulin. (—●—) infected with Coxsackie B3 virus; (--o--) uninfected.

DISCUSSION

Studies on the interaction of virus with insulin-containing cells have been restricted by the difficulty in preparing cultures enriched in beta cells. Less than 20 per cent of the cells in our cultures proved to be beta cells, as evaluated by staining with TRITC-labeled anti-insulin antibody. Since these cultures are predominantly nonbeta cells, an increase in viral titer cannot be attributed to replication of the virus in beta cells. By use of the double-label antibody technique, however, it was possible to show that the beta cells in these cultures were susceptible to infection by Coxsackie B3 virus. In addition, by radioimmunoassay it was shown that the infection markedly altered the insulin content of the beta cells. The combination of these techniques may prove useful in studying other viruses in cultures containing low numbers of beta cells.

Neither the present study with Coxsackie B3 nor the earlier study with mumps[6] proves that either of these viruses actually infect and destroy beta cells in vivo. It is known that some viruses will grow in cultured cells derived from organs that, when in the host, are resistant to the virus.[11-15] The development of susceptibility in culture has been attributed to a variety of factors, including the induction of viral receptors, the requirement for cell division, and the lack of host defense mechanisms. Thus, the in vitro susceptibility of beta cells may not be a true reflection of the in vivo status of these cells. Nonetheless, at least in the case of inbred mice, quantitative differences among strains in the susceptibility of their beta cells to infection with the M-variant of encephalomyocarditis virus can be demonstrated under in vitro conditions and these differences parallel in vivo susceptibility.[7,16] Whether beta cells from different individuals will prove to be equally susceptible to Coxsackie B3 or

whether the degree of permissiveness will vary depending on the genetic background of the individual (e.g., HLA type) remain to be determined.

In addition to the susceptibility of cultured human beta cells to Coxasackie B3 virus and mumps, mouse beta cells grown in culture are susceptible to the M-variant of encephalomyocarditis virus,[7] Coxsackie B3 virus,[17] Coxsackie B4 virus, and reovirus type 3 (unpublished data). Moreover, by passage of reovirus type 3 and Coxsackie B4 virus through murine beta cell cultures, it has been possible to obtain variants that, when inoculated into mice, infect beta cells and produce hyperglycemia and/or abnormal glucose tolerance tests (unpublished data).[19] Thus, whether a virus will infect beta cells depends on the strain of the virus, the passage history of the virus, and the genetic background of the host.[5,18] Taken together, these studies in animals and in cell cultures, suggest that, under appropriate conditions, a number of viruses might be capable of infecting insulin-containing beta cells.

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