Human cytomegalovirus in the pancreas of patients with type 2 diabetes: is there a relation to clinical features, mRNA and protein expression of insulin, somatostatin, and MHC class II?*

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Summary. Human cytomegalovirus (HCMV) was recently demonstrated in the pancreas of about half the patients with type 2 diabetes mellitus in the absence of mumps, rubella or Coxsackie B virus. The present study addresses the question as to whether type 2 diabetes with an HCMV-positive pancreas differs from those with HCMV-negative pancreases with respect to age, sex, treatment, duration of disease, volume densities of B-cells and D-cells, mRNA levels of insulin and somatostatin, islet amyloid peptide deposits and major histocompatibility complex (MHC) class I and class II gene transcription, and protein expression. HCMV-positive type 2 diabetic patients showed a tendency towards a shorter duration of disease and significantly increased levels of MHC class II on RNA. In addition, expression of MHC class II product (HLA-DR) was identified in duct epithelial cells and/or islet cells in 9 diabetic pancreases and in 2 non-diabetic glands. No MHC class I expression could be detected. No other clinical differences between HCMV-positive and HCMV-negative glands were found. All 10 HCMV-positive diabetics showed a strong expression of MHC class II mRNA in the pancreas. By immunocytochemistry, 4 of 10 demonstrated expression on the islets; three of ten also expressed MHC DRβ on ductal cells. This finding might be related to the viral infection, as only 2 of the 9 HCMV-negative patients were HLA-DRβ positive and none of the non-diabetic controls showed increased levels of MHC class II mRNA. These data suggest that HCMV infection in the pancreas is associated with type 2 diabetes. However, no conclusions as to a role of this virus in the aetiology of type 2 diabetes can be drawn at present.

Key words: Type 2 (non-insulin-dependent) diabetes mellitus – Human cytomegalovirus – Major histocompatibility complex II – Islet amyloid polypeptide

Introduction

Diabetes mellitus is a syndrome with heterogeneous pathogenesis (Klöppel 1984; Lefèbvre 1988) and amongst the various aetiological factors that may be involved in the development of diabetes are viruses (Rayfield et al. 1976; Notkins et al. 1984; Leiter et al. 1989). From experimental work and studies in children with fatal virus infections, it is known that the encephalomyocarditis virus, and reovirus or Coxsackie B4 viruses can cause necrosis of the endocrine cells of the pancreas (Craighead and Steinke 1971; Jenson et al. 1980; Ujevich and Jaffe 1980; Ahmad and Abraham 1982; Onodera et al. 1983; Yoon et al. 1986; Szopa et al. 1990). Most pancreases from patients with diabetes, however, lack signs of lytic islet cell necrosis, but display changes that are either compatible with autoimmune destruction of the β-cells, as in type 1 diabetes (Klöppel et al. 1991), or B-cell dysfunction, as in type 2 diabetes (Klöppel et al. 1991). Yet viruses might still play a role in the pathogenesis of diabetes by either triggering autoimmune B-cell destruction via expression of major histocompatibility complex (MHC) proteins (Botazzo et al. 1985; Jennings et al. 1985; Fouliès and Farquharson 1986; Suzumura et al. 1986; Campbell et al. 1988) or, alternatively, by persisting in B-cells and impairing their specific function (Oldstone et al. 1984; Oldstone 1989). The latter hypothesis has been investigated in depth in animal studies using lymphocytic choriomeningitis virus (LCMV) and Venezuelan equine encephalitis (VE) virus as models. LCMV infection has been shown to decrease insulin production (Oldstone et al. 1984; Rodriguez et al. 1985) without any evidence of cell injury or inflammation. This was also observed with VE virus (Rayfield et al. 1976) and Coxsackie B4 virus (Yoon...
et al. 1986). Finally, patients with the congenital rubella syndrome have been shown to develop growth retardation (Preece et al. 1977) and diabetes (Ginsberg-Fellner et al. 1985) in the absence of any inflammatory or necrotic islet changes.

Human cytomegalovirus (HCMV) has also been implicated in the aetiology and pathogenesis of diabetes. Diabetes developed in a child with congenital HCMV infection at the age of 13 months (Ward et al. 1979). Nuclear inclusions in islet cells and insulitis were found in infants who died of disseminated HCMV infection (Hultquist et al. 1988). In a recent study we found HCMV genome was demonstrated in 15% of newly diagnosed type 1 diabetes who had islet cell antibodies as well (Pak et al. 1988). In a recent study we found HCMV nucleic acids in the islets of Langerhans in 44% of patients with type 2 diabetes mellitus, but not the nucleic acids of mumps, rubella or Coxsackie B (Löhr and Oldstone 1990). The nucleic acid sequences were detected in fixed, paraffin-embedded pancreatic tissue using slot blot and in situ hybridization as well as polymerase chain reaction derived DNA sequencing. To analyse the significance of this finding for the development of type 2 diabetes further, two subgroups of diabetics were formed from the original study group in which detailed clinical data were available, one HCMV positive and one HCMV negative. These were compared with one another and the healthy controls with regard to several variables such as treatment and duration of diabetes, mRNA levels of insulin and somatostatin, volume density of B-cells and D-cells, transcription of class I and II MHC genes, protein expression of HLA-DR, and islet amyloidosis.

### Materials and methods

Well-preserved tissue obtained at autopsy within 24 h of death from the pancreas of 19 patients with type 2 (non-insulin-dependent) diabetes mellitus and 18 normoglycaemic patients without evidence of pancreatic disease, was retrieved as blocks of formalin-fixed or Bouin-fixed, paraffin-embedded material from the files of the Departments of Pathology at the University of Hamburg, FRG; the University of Essen, FRG; the University of Brussels, Belgium; and the University of California at San Diego. The blocks of all 18 controls and the diabetics were subjected to RNA analysis (see below); in 15 of the 18 normoglycaemic patients detailed clinical data were available and blocks of those patients were therefore used for further studies. The clinical criteria for type 2 diabetes mellitus were abnormal glucose tolerance and elevated fasting blood glucose levels. No HLA typing was performed in these patients. Information on clinical data (age, sex, treatment with diet and/or oral hypoglycaemics or with insulin, and duration of diabetes) was extracted from the patients’ records and summarized in Table 1. Ten patients with a duration of the disease between 1 and 24 years received insulin (mean, 5 years). All other patients were treated with diet and/or oral hypoglycaemics.

Preparation of RNA from paraffin-embedded tissue was carried out as described in previous studies (Löhr and Nerenberg 1990; Löhr and Oldstone 1990). In brief, tissue was cut, digested with proteinase K in the presence of a chaotropic agent, sodium dodecyl sulphate (SDS), mixed with GTC, subjected to ultracentrifugation with caesium chloride, extracted with phenol/chloroform, precipitated and finally assessed for quality and quantity by mini-gel electrophoresis and spectrophotometrical readings.

For slot-blot analysis, 25 μg RNA aliquots were denatured at 65°C for 15 min in 6 x SSC (1 x SSC=0.15 M sodium chloride 0.015 M trisodium citrate), 7.4% formaldehyde, then serially diluted in 15 x SSC. Samples were applied to nitrocellulose membranes using a 72-slot minifold blot apparatus. Nitrocellulose membranes were baked 2 h at 80°C, prehybridized 4 h at 37°C in 50% deionized formamide, 5 x SSC, 2.5 x Denhardt's solution, 100 μg/ml boiled, sonicated salmon sperm DNA and then hybridized for 24 h at 37°C with the respective labelled probes (see below). After hybridization, membranes were washed in 2 x SSC, 0.1% SDS at 37°C for 30 min, in 0.1 x SSC, 0.1% SDS at 55°C or 65°C for
After several washes and a 30 min incubation with the avidin-bio-
0.1% bovine serum albumin. Incubation took place at room tem-
perature for 30 min. After shaking off the blocking serum, the
slides were incubated in phosphate-buffered saline (PBS). For
rewashing, reacted with 2,2 diaminobenzidine, rewashed, and
tinylated antibody (rat anti-mouse IgG, Boehringer Mannheim)
was applied at a 1:50 dilution for 30 min at room temperature.
Class II MHC expression was identified by a monoclonal
antibody A II (gift from Dr. A. Foulis, Glasgow, UK). This anti-
body was known to react with paraffin-embedded tissue (Epenetos
et al. 1985; Löh and Klöppel 1987) and raised against the purified
human class II antigens, DR and DQ (Neefjes et al. 1986). After
deparaffinizing, the endogenous peroxidase was blocked with 3%
hydrogen peroxide in methanol. After further dehydration and
washes in phosphate-buffered saline (PBS), the slides were incubat-
ed with 10% normal porcine serum in a humid chamber at room
were used to compare the different diabetic groups among each
other and with the controls. Data were entered in an Apple Macin-
tosh computer and analyzed with StatView (Abacus Concepts, Ber-
keley, Calif., USA).

All probes were obtained as plasmids and transformed in com-
petent bacteria (E. coli, DH5α) (Maniatis et al. 1982). The HCMV
DNA probes (pCM3, pJN201) were a gift from Dr. J. Nelson
(Schrier et al. 1985). cDNA probes for insulin (gift from Dr. P.
Southern, Minneapolis, Minn., USA; Cordell et al. 1979), somatos-
tatin (gift from Dr. W. Rutter, San Francisco, Calif., USA; Lipkin
et al. 1988), non-polymorphic region of human class I and class
II MHC (pdp1 and pDRβ, gift from Dr. S. Lawrence, La Jolla,
Calif., USA (Curtsinger et al. 1987)) were used as well as a probe to
the 28S ribosomal RNA (S138, gift from Dr. P. Southern;
Lipkin et al. 1988). Probes were prepared by the random hexo-
primer method with 32P (Feinberg and Vogelstein 1983) and specif-
ic activity of greater than 5 × 108 cpm/ml. For each hybridization,
1–2 × 106 cpm/ml hybridization mix were used.

Immunocytochemistry was performed (Löh and Klöppel 1987)
on 3 μm serial sections using a monoclonal antibody against insulin
(1:5000; Biogenex via Camon, Wiesbaden, FRG) and polyclonal
antibodies against glucagon, somatostatin (1:2000 and 1:3000;
both INC, Stillwater, Minn., USA), pancreatic polypeptide
(1:1000; gift from Dr. R.E. Chance, Indianapolis, Ind., USA) and
islet amyloid polypeptide (1:2000; rat IAPP12–37, kindly donated
by Dr. O. Madsen, Hagedorn Res. Laboratories, Gentofte, Den-
mark). Class II MHC expression was identified by a monoclonal
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human class II antigens, DR and DQ (Neefjes et al. 1986). After
deparaffinizing, the endogenous peroxidase was blocked with 3%
hydrogen peroxide in methanol. After further hydration and
washes in phosphate-buffered saline (PBS), the slides were incubat-
ed with 10% normal porcine serum in a humid chamber at room
temperature for 30 min. After shaking off the blocking serum, the
monoclonal antibodies were applied diluted in PBS containing
0.1% bovine serum albumin. Incubation took place at room tem-
perature for 18 h. After several washes in PBS, the secondary bio-
tinylated antibody (rat anti-mouse IgG, Boehringer Mannheim)
was applied at a 1:50 dilution for 30 min at room temperature.
After several washes and a 30 min incubation with the avidin-bio-
tin-complex (ABC, Vectastain) at room temperature, the slides were
rewashed in PBS, reacted with 2,2 diaminobenzidine, rewarmed,
dehydrated and mounted for microscopic evaluation. Specificity
of the antibodies was demonstrated by systematically including
positive and negative controls. Human tonsillar tissue was used as
a positive control for the HLA-DR antibody. For identification
of islet amyloid, sections adjacent to those immunostained for
IAPP were stained with Congo red. The percentage of islets con-
aining extracellular IAPP deposits was estimated and graded ac-
cording to a semi-quantitative score ranging from grade 0 (no ex-
tracellular IAPP present in the islets), grade 2 (10%–50% of all islets
positive for extracellular IAPP) to grade 3 (more than 50% of the
islets contain extracellular IAPP).

Determination of the volume density of the immunoreactive
area of the insulin-containing B-cells and the somatostatin contain-
ing D-cells was performed as described (Kloppel et al. 1985; Löh
et al. 1989). Briefly, H & E-stained sections were evaluated at a
final magnification of × 22 for the volume densities (volume frac-
tions) of the mesenchymal tissue and parenchyma expressed rela-
tive to the whole pancreas according to established principles
(Oberholzer 1983). Volumes of mesenchymal tissue and parenchym-
a of the pancreas, including the endocrine tissue, were calculated
as described (Kloppel et al. 1985; Löh et al. 1989). In a second
step, volume densities of the immunochemically stained endocrine
cells were measured at a total magnification of ×400 by a semi-
automatic image analyser (Löh et al. 1989). In this analysis we
report only data on B-cells and D-cells, because we could relate
these data to the mRNA values of insulin and somatostatin.

Statistical evaluation was performed with the Mann-Whitney
test. Two-sided p-values ≤ 0.05 were considered statistically signifi-
cant.

Results

As part of the former study group of 32 pancreases from patients
with type 2 diabetes, 44% of which we reported to contain HCMV sequences (Löh and Oldstone 1990), we
selected the samples from 19 patients from whom we had further information on duration of disease and
treatment. Of these, 10 were positive for HCMV by slot-
blot filter hybridization to pancreatic RNA, whereas
none of 18 age- and sex-matched controls yielded a posi-
tive signal (Fig. 1). There was no difference in age, sex,
or treatment of diabetes (diet and/or oral hypoglycaemic
versus insulin) between the patients containing HCMV
nucleic acids in the pancreas and those who did not
(Table 1). However, patients expressing HCMV tended
to have a shorter duration of diabetes when compared
to the HCMV-negative type 2 diabetes (two-sided P <
0.06). The volume densities of B-cells and D-cells in
HCMV-positive diabetic patients did not differ signifi-
cantly from those of the HCMV-negative diabetics or
from the non-diabetic patients (Table 1). In diabetics
the mean volume density of B-cells tended to be lower
than in non-diabetics; no such difference was noted bet-
ween the mean volume densities of D-cells in diabetics
and non-diabetics (Table 1).

Insulin and somatostatin mRNA was measured by
quantitation of autoradiographs. Although the densito-
metrically read value of the hybridization signal for insu-
lin was slightly lower in the HCMV-positive type 2 dia-
etics (Table 1: insulin mRNA/28S RNA), a statistically
significant level was not reached, due to interindividual
variation. Similar results were obtained on the protein
level (Table 1). No changes could be observed for soma-
tostatin, on either the RNA or protein level.

Extracellular IAPP was found in the islets of 15 of
19 (79%) type 2 diabetics and 0 of 15 non-diabetic con-
trols (Fig. 2). The number of HCMV-positive diabetics
and HCMV-negative diabetics with extracellular deposi-
tion of IAPP in the islets did not differ significantly
(Table 2). Likewise, there was no obvious difference
within the IAPP-negative group. The amount of extra-
cellular islet deposits of IAPP varied from islet to islet
and from case to case. There was no difference in the
grades of IAPP positivity between the HCMV-positive
Table 2. Immunoreactivity for extracellular IAPP deposits in HCMV-positive or HCMV-negative type 2 diabetics, and in non-diabetics

|                  | IAPP+ cases | IAPP score |
|------------------|-------------|------------|
|                  | 1           | 2          | 3          |
| Non-diabetics    | 0/15        | 1          | 2          | 3          |
| Diabetics HCMV+  | 8/10        | 4          | 4          | 0          |
| Diabetics HCMV-  | 7/9         | 4          | 2          | 1          |

Grading of percentage of islets containing extracellular IAPP: 1 < 10%; 2 10–50%; 3 > 50% of islets

and HCMV-negative diabetics (Table 2). In islets without extracellular IAPP all or some of the B-cells also stained for IAPP. This intracellular IAPP immunoreactivity disappeared from the B-cells with the occurrence of significant extracellular IAPP deposits.

None of the diabetic nor any of the non-diabetic control pancreases showed a RNA signal for hybridization with the MHC class I probe (data not shown). Twelve of 19 diabetic patients expressed various levels of class II MHC RNA in the pancreas, and 10 of these were HCMV positive (Fig. 1, Table 3). None of the 15 age- and sex-matched control patients was positive for class II MHC RNA. The DRβ probe did not cross-hybridize with HCMV (Fig. 1).

Expression of class II MHC antigen (HLA-DR) was identified on endothelial cells as well as scattered mononuclear cells in all specimens from the diabetic patients as well as the non-diabetic control patients (Fig. 3a). In 7 of the diabetic and in 2 of the non-diabetic patients there was positive staining on duct cells lining small ducts, and/or islet cells (Table 3). When adjacent serial sections were stained with insulin antiserum, the results suggested that the endocrine cells expressing class II MHC belonged to the group of insulin-producing cells (Fig. 3b, c). There was no clear relationship between
the class II MHC RNA expression level and the degree and distribution pattern of class II MHC antigens in the diabetic and non-diabetic pancreases (Table 3). Expression of class II MHC antigens on ducts and/or islets was found in 5 of 10 HCMV-positive diabetic pancreases and 2 of 9 HCMV-negative diabetic pancreases. It is noteworthy that one of the pancreases with the highest expression level of class II MHC RNA, which exhibited no class II MHC positivity on ducts or islets but was HCMV positive, revealed a discrete lymphocytic infiltration throughout the pancreatic tissue.

Discussion

Recently, we reported that HCMV nucleic acids are present in the endocrine pancreas in 44% of patients with type 2 diabetes mellitus (Löhr and Oldstone 1990). In the present study we examined whether any of seven variables, age, sex, treatment of diabetes, duration of disease, volume density of B-cells and D-cells, expression of insulin and somatostatin RNA levels, HLA class II (DRβ) mRNA or islet amyloid, correlated with the HCMV positivity of the diabetic pancreases. The only findings which showed a relation with the HCMV status of the patients were a tendency towards shorter duration of diabetes and a more frequent expression of HLA-DR mRNA in the HCMV-positive patients.

From experimental studies in mice and hamsters infected with the LCM or VE virus it is known that these animals may develop a type 2-like diabetes without any obvious morphological damage to the islets. In these animals' diabetes, the possibility has been considered that the virus selectively suppresses the specific function of the pancreatic B-cells but leaves their cellular structure unaffected (Oldstone et al. 1984; Oldstone 1989). The only islet change associated with LCM infection was islet hypertrophy in animals with diabetes of short duration. This was interpreted as an attempt of the endocrine pancreas to increase insulin production during the early stage of disease. In our series of HCMV-positive type 2 diabetics with a mean duration of 11 years, we found neither B-cell hypertrophy nor any increase in volume density of B-cells. Instead, the B-cells showed a tendency towards a decrease in volume density as well as insulin RNA/ribosomal RNA ratio, which rather suggests a reduction in their number. However, even these B-cell changes appear to be unrelated to any specific damage caused or mediated by HCMV, since they were also found in HCMV-negative patients.

The tendency of the HCMV-positive patients to have a shorter mean duration of diabetes could imply that HCMV infection aggravates diabetes by further impairing B-cell function. However, as the length of the disease varied considerably from patient to patient, this finding lacked statistical significance and did not allow us to draw any firm conclusions with respect to an additive effect of HCMV in the development of type 2 diabetes.

In view of the results obtained with transgenic mice expressing a class II MHC molecule in the pancreas and developing diabetes mellitus in the absence of cellular injury or infiltration of the islets (Lo et al. 1988), we tested our samples with a probe to the non-polymorphic region of the human class II MHC DRβ gene. Nine of 10 type 2 pancreases positive for HCMV expressed various levels of MHC class II RNA, but none of 18 controls and only 2 of 9 pancreases of HCMV-negative diabetics. At the protein level we found an expression of class II MHC (HLA-DRβ) on endothelial cells, some mononuclear cells and duct cells in the non-diabetic as well as in the diabetic pancreases. These findings confirm the results from from other studies (Daar
Fig. 3a–c. Class II MHC antigen (HLA-DR) expression in the pancreas from type 2 diabetics. 

a Positive staining on endothelial cells surrounding an islet and small ducts (D) (case no. 83/83). 

b Positive staining on endocrine cells of an islet (case no. 92/87). 

Arrows indicate HLA-DR-positive cells which also appear to contain insulin (INS) on a consecutive section stained for insulin (c). Immunostaining for HLA-DR (a, b) and insulin (c). ×250
I and class II expression (Jennings et al. 1985; Rosenthal et al. 1985; Suzumura et al. 1986; Gaulton et al. 1989).

Islet amyloidosis, the deposition of extracellular IAPP as amyloid within the islets is a morphological indicator lesion for type 2 diabetes in the pancreas (Westmark et al. 1987; Clark et al. 1990; Klöppel et al. 1991). Yet its significance for the pathogenesis of the disease is unknown. We found insular IAPP deposits in about 80% of the diabetic pancreases confirming the data of other studies (Westmark et al. 1987; Clark et al. 1990), but failed to demonstrate any relationship with the HCMV status in the patients.

In summary, this study revealed no association between HCMV infection and clinical or morphological variables characterizing patients with type-2 diabetes. The only finding that might be related to the presence of HCMV is positivity for MHC class II RNA. The possible significance of the HCMV infection for the function of the endocrine pancreas in man remains unclear. Preliminary results in transgenic mice using the major promoter of the HCMV immediate early gene hooked to a β-galactosidase reporter gene demonstrated targeting of this gene to the islets of Langerhans (Jay A. Nelson, personal communication). Using this model, it should be possible to study the biology of HCMV infection in mammalian islets in more detail.

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| Case no. | HLA-DR-positivea | DR β-positiveb | HCMV-positive |
|---------|------------------|----------------|--------------|
|         | E    | D     | I    |                       |
| Type 2 diabetics |
| 52/80   | 2    | 1     | 3    | pos.                   |
| 27/81   | 4    | 1     | 1    | pos.                   |
| 95/82   | 2    | 2     | 3    | pos.                   |
| 39/83   | 2    | 1     | 1    | pos.                   |
| 82/84   | 4    | 2     | 2    | pos.                   |
| 18/83   | 2    | 1     | 1    | pos.                   |
| 83/83   | 4    | 1     | 1    | pos.                   |
| 35/81   | 3    | 2     | 1    | pos.                   |
| 74/85   | 3    | 1     | 2    | pos.                   |
| 106/84  | 2    | 4     | 1    | pos.                   |
| 90/85   | 4    | 1     | 3    | pos.                   |
| 101/86  | 4    | 2     | 2    | pos.                   |
| 114/86  | 2    | 2     | 1    | pos.                   |
| 115/86  | 2    | 2     | 1    | pos.                   |
| 109/87  | 4    | 1     | 2    | pos.                   |
| 92/87   | 4    | 3     | 2    | pos.                   |
| 96/87   | 4    | 2     | 1    | pos.                   |
| 97/87   | 4    | 3     | 3    | pos.                   |

Nondiabetic patients

| Case no. | HLA-DR-positivea | DR β-positiveb | HCMV-positive |
|---------|------------------|----------------|--------------|
|         | E    | D     | I    |                       |
| 26/83   | 4    | 1     | 2    |                       |
| 29/83   | 3    | 1     | 1    |                       |
| 43/87   | 4    | 2     | 3    |                       |
| 65/87   | 3    | 2     | 1    |                       |
| 67/87   | 2    | 2     | 2    |                       |
| 71/87   | 4    | 2     | 3    |                       |
| 72/87   | 3    | 1     | 1    |                       |
| 91/87   | 4    | 1     | 1    |                       |
| 93/87   | 2    | 1     | 1    |                       |
| 94/87   | 2    | 1     | 1    |                       |
| 95/87   | 4    | 1     | 1    |                       |
| 98/87   | 2    | 1     | 1    |                       |
| 99/87   | 4    | 1     | 1    |                       |
| 100/87  | 2    | 1     | 1    |                       |
| 102/87  | 2    | 1     | 1    |                       |
| 103/87  | 4    | 2     | 1    |                       |
| 33/88   | 2    | 1     | 1    |                       |

a Grading of percentage of endothelial cells/macrophages (E), ducts (D) and islets (I) expressing HLA-DR: 1 < 10%; 2 10–30%; 3 30–50%; 4 > 50%
b RNA-DR/β expression score: 1, weak; 2, moderate; 3, strong

et al. 1984a, b; Fouilis and Farquharson 1986; Löh and Klöppel 1987; Dib et al. 1988). However, we also observed expression on islet cells and in particular B-cells in 1 control and 7 diabetic pancreases, findings which have so far been considered to be specific for type 1 diabetes of recent onset (Fouilis and Farquharson 1986). Why there was no clear relationship between class II MHC mRNA and its product is poorly understood. Technical problems or yet unknown variations in the interplay between transcription and translation may be important. However, the fact that diabetic HCMV-positive pancreases expressed class II MHC mRNA more frequently than HCMV-negative diabetic glands may be related to the fact that viruses are known to induce class
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