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Viral infection induces cytokine release by beta islet cells

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SUMMARY

Viral infection has been suggested to play a triggering role in the pancreatic beta cell destruction which occurs in insulin-dependent diabetes (IDDM). However, the underlying mechanism of this phenomenon is unknown. In this study a human insulinoma cell line has been infected with measles, mumps and rubella viruses since a temporal association is reported between the clinical onset of IDDM and diseases caused by these viruses. The infection with measles and mumps viruses induced the release of interleukin-1 (IL-1) and interleukin-6 (IL-6) by the cell line as assessed by a bioassay and up-regulated the expression of human leucocyte antigen (HLA) class I and class II antigens as evaluated by cytofluorimetric analysis. Stimulation with rubella virus induced the release of IL-6 only and had no effect on HLA antigen expression. These data show for the first time that IL-1 and IL-6 secretion by an insulinoma cell line may occur after viral infection and suggest that cytokine release and increased expression of HLA molecules by beta cells may act to induce the immune response towards beta cells in IDDM.

INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) develops as the result of an autoimmune process that selectively destroys pancreatic beta islet cells.[1] In individuals genetically susceptible to IDDM a triggering event often takes place before the clinical onset of the disease and evidence is accumulating that a viral infection of beta cells may trigger the disease process;[2,3] however the pathway of disease induction by the viral agents is unknown.

The hypothesis that viruses may be implicated in the aetiopathogenesis of IDDM derives from both clinical and experimental observations; thus, a temporal relationship exists between the clinical onset of IDDM and the occurrence of some viral diseases;[4] furthermore, in few cases viruses have been isolated from the pancreas of children with diabetic ketoacidosis[5] and data from experimental studies show that IDDM can be induced in animals by infecting beta cells with some viruses.[6,7] However, at least in humans, non-specific viral infection seems to be a precipitating factor of an already on-going autoimmune process and not a causative agent.[3]

Hyperexpression of class I major histocompatibility complex (MHC) molecules alone or together with aberrant expression of class II MHC molecules by beta cells are also pathological features in the early stages of the disease both in rats and humans[8,9] and it has been speculated that beta cells can acquire the function of antigen-presenting cells through the abnormal expression of such proteins.[10]

Recently, it has been demonstrated that beta cells can be induced to produce interleukin-6 (IL-6),[11] which is known to play a key role in immune activation, suggesting that following a triggering event the beta cell itself may take an active part in the autoimmune process occurring in IDDM. Furthermore the expression of beta cell antigens has been recently shown to be regulated by other cytokines such as IL-1, tumour necrosis factor (TNF) and interferon-gamma (IFN-γ).[12]

In the present study we have investigated the effect of an *in vitro* infection with viruses on an established human insulinoma cell line.[13] The viruses used for these experiments were mumps, measles and rubella, since a close temporal association has been described between the clinical onset of IDDM and the corresponding virally induced diseases. Particular attention has been paid to cytokine release and MHC protein expression by the cell line.

MATERIALS AND METHODS

Cell line

The human insulinoma cell line CM[13] used in this study originated from a patient undergoing surgery for severe hypoglycaemia in 1987; this cell line grows as a monolayer culture in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS). The cells used for this study were from an early passage.

Insulin and C-peptide production by the cell line was determined by radioimmunoassay using commercially available

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kits (Biodata, Milan, Italy) under basal culture conditions (11 mM glucose) and after 30 min incubation with somatostatin (20 and 100 μg/ml).

Before inoculation with viruses, cells were grown to confluence in culture flasks.

Viruses

CM cell cultures were inoculated with measles virus [Schwarz strain $\log_{10}$ 5·90 plaque-forming units (PFU)/ml], mumps virus (Urabe strain $\log_{10}$ 5·09 PFU/ml) and rubella virus [RA27/3 strain $\log_{10}$ 4·05 tissue culture infective dose $(TCID)_{50}$/ml]. The multiplicity of infection was 0·05 with measles virus, 0·02 with mumps virus and 0·015 with rubella virus. After 2 hr incubation, cell monolayers were washed and the virus suspension replaced with fresh medium containing 2% FCS. Cells were then incubated for 3 days at 37° in a 5% CO_2 atmosphere. Aliquots of supernatant and cells were collected 24, 48 and 72 hr after inoculation and stored at −70° until required. Viral infection was assessed by standard plaque assay. Measles and mumps titres were determined as plaque-forming units in green monkey kidney (VERO) cells. Rubella virus was assayed in rabbit kidney (RK13) cells.

CM cells were also stained with two polyclonal sheep antisera against measles and mumps viruses using indirect immunofluorescence in order to determine the percentage of cells infected.

Evaluation of MHC protein expression

After 72 hr culture, cell monolayers were trypsinized, washed twice, resuspended in fresh medium and incubated for 1 hr at 37° to allow recovery.

Staining of cells for MHC proteins was performed using the indirect immunofluorescence technique using non-polymorphic monoclonal antibodies to HLA class I (W6/32) and class II (L203) antigens. Briefly, 5×10^5 cells were centrifuged in 5 ml plastic tubes; 50 μl of a 1/50 dilution of the antibodies were added to the resuspended pellets and then incubated at 4° for 60 min. Cells were washed twice and incubated with 50 μl of a 1/50 dilution of fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG for 30 min; cells were finally washed three times, fixed with formalin and analysed using a flow cytometer.

Cytokine assays

IL-1 was measured by a two-stage bioassay using the EL-4 NOB-1 mouse thymoma cell line which produces IL-2 in response to IL-1 and the CTLL mouse lymphocyte cell line which proliferates in response to IL-2.[14] Briefly, duplicate sample aliquots and the WHO 1st International Standard for IL-1β were diluted in 96-well plates to a final volume of 100 μl/well; 100 μl containing 10^5 NOB-1 cells were then added to each well and incubated for 24 hr. Fifty microlitres of supernatant from each well were transferred to another 96-well plate and 50 μl of 10^5/ml CTLL cells were added to each well and incubated at 37° for 24 hr. Tritiated thymidine (0·5 μCi) was then added to each well and after 4 hr the cells were harvested onto filter mats. Cell proliferation was evaluated by measuring the radioactivity incorporated into DNA by scintillation counting. The sensitivity of this assay is approximately 0·2 pg/ml.

IL-6 was measured by a proliferation assay using the murine hybridoma cell line B9 which proliferates specifically in response to IL-6.[15] Briefly, duplicate sample aliquots and the standard were diluted in a 96-well plate; 100 μl containing 5×10^4 cells were added to each well and incubated at 37° for 72 hr; 20 μl of tetrazolium salt (MTT) solution [5 mg/ml in phosphate-buffered saline (PBS)] were added to each well and the cells cultured for another 5 hr. Ten microlitres of 10% SDS/0·02 N HCl (SDS solution) were then added to each well and plates left in darkness. After 2 hr the absorbance at 620 nm was measured using an ELISA reader. The sensitivity of this assay is approximately 0·2 pg/ml.

For neutralization experiments, appropriate dilutions of specific polyclonal antisera raised against recombinant human IL-1 and IL-6 were preincubated with samples for at least 1 hr before the addition of cells.

IL-2 was measured using the CTLL proliferation assay as described above.[16] The sensitivity of this assay is approximately 0·2 IU/ml. TNF-γ and -β and IFN-γ were measured using two-site ELISA with a sensitivity of approximately 1 U/ml as previously described.[17,18] Biological activity of IFN was determined by bioassay using the Hep2/C cell line as described.[19] This assay has a sensitivity of approximately 100 pg/ml.

Granulocyte–macrophage colony-stimulating factor (GM-CSF) was assayed by ELISA using a kit (Insight™ GM)[20] distributed by 'Medical Resources Limited'; the sensitivity was found to be approximately 20 pg/ml.

RESULTS

C-peptide and insulin were detectable in the CM cells cultured in 11 mM glucose medium (C-peptide = 40 ± 1 ng/ml; insulin = 536 ± 1 μIU/ml). Somatostatin at concentrations of 20 and 100 μg/ml was able to reduce C-peptide after 30 min (17 ± 1 ng/ml and 15 ± 1 ng/ml, respectively) and insulin (336 ± 30 μIU/ml and 190 ± 5 μIU/ml, respectively).

Stimulation with mumps, measles and rubella viruses for up to 72 hr had no cytopathic effects on the cell line as assessed by light microscopy. Measurement of the virus titre in the supernatants and in the cell extracts showed that growth of both measles and mumps viruses occurred in the CM cells whilst growth of rubella virus was not detectable. An eclypse phase was not detected for either virus since both were present in CM cells at Day 1 post-infection. However a latent phase was evident with measles virus since released virus was not present in the supernatant until Day 2 following infection. This was not the case with mumps, where virus was detected in the supernatant from Day 1.

The percentage of infected cells detected by immunofluorescence ranged between 50 and 75% with measles virus and between 75 and 90% with mumps virus.

Both measles and mumps viruses were inoculated at a similar multiplicity of infection, yet mumps appeared to produce a more vigorous infection of CM cells. No growth of rubella virus was detected in this study. This virus generally grows less well *in vitro* then measles and mumps and hence a lower titred inoculum was available and a lower multiplicity of infection used. It is possible that CM cells might be susceptible to infection with this virus if a higher infecting dose is used.

Secretion of cytokines

IL-1, IL-2, IL-6, TNF, IFN-γ and GM-CSF were all undetectable in the supernatants of non-infected cells. IL-1 production

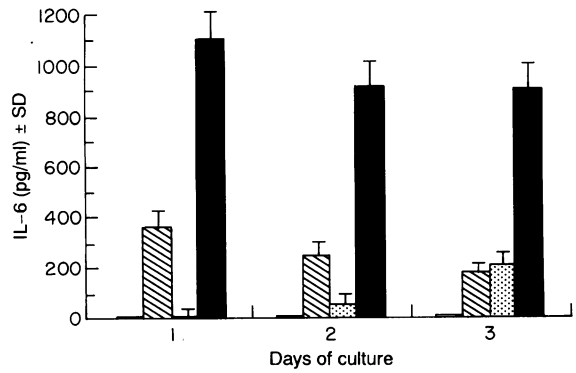

Figure 1. Secretion of IL-6 by the CM cell line following the viral infection with: measles (■); mumps (▧); rubella (▨). Control (non-infected cells) (□).

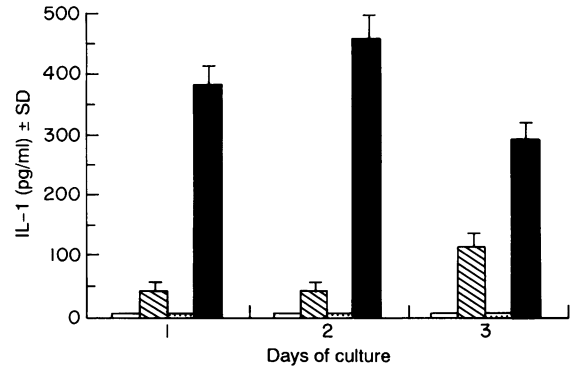

Figure 2. Secretion of IL-1 by the CM cell line following the viral infection with: measles (■); mumps (▧); rubella (▨). Control (non-infected cells) (□).

was stimulated by infection of CM line with mumps and measles viruses, whilst IL-6 production was stimulated by all three viruses (Figs 1 and 2).

Inclusion of potent specific neutralizing antibodies against IL-6 and IL-1β resulted in almost complete reduction of biological activity of IL-6 and IL-1β respectively (data not shown), confirming that the bioactivity present in the supernatants were indeed due to IL-1 and IL-6. IL-2, TNF, IFN and GM-CSF were still undetectable in the CM cell line supernatants 24, 48 and 72 hr after the infection with the different viruses.

HLA antigen expression

Class I and class II HLA antigens were constitutively expressed by the CM cell line. Following infection with mumps and measles viruses, staining for HLA class I and class II antigens increased compared to the non-infected cells. No change in fluorescence intensity was observed before or after stimulation with rubella virus (Figs 3 and 4).

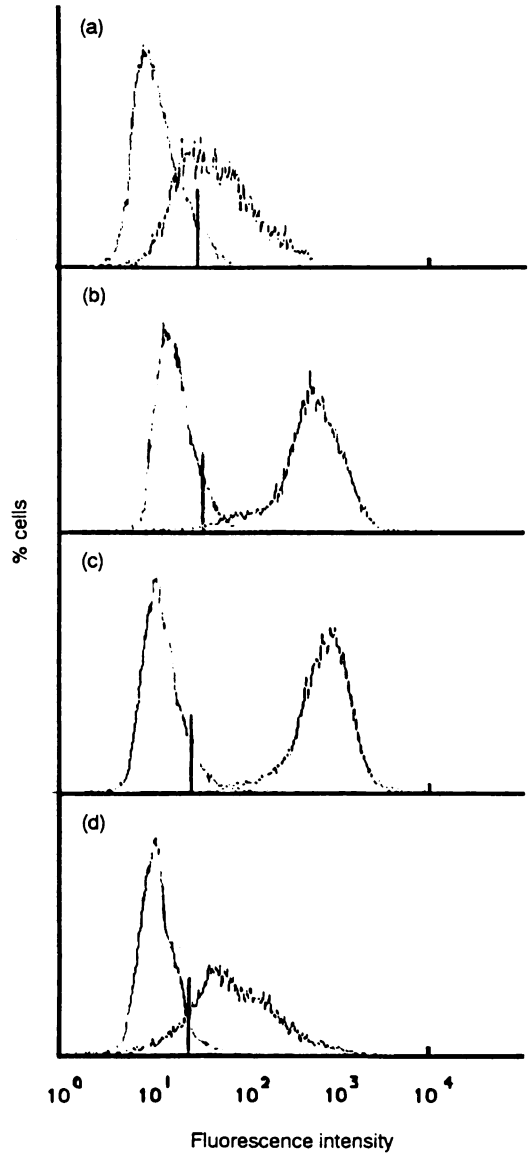

Figure 3. Expression of HLA class I proteins by the CM cell line following stimulation with different viruses as evaluated by cytofluorimetric analysis. Whilst infection with measles and mumps viruses increased fluorescence intensity indicating hyperexpression of HLA class I antigens, stimulation with rubella virus did not modify this pattern. The fluorescence intensity is compared with that of an irrelevant antibody. Uninfected (a); measles (b); mumps (c); rubella (d).

DISCUSSION

Our data demonstrate that infection with mumps and measles viruses induced production of IL-1 and IL-6 and up-regulated the expression of HLA class I and class II antigens by a human insulinoma cell line. Rubella virus did not infect the insulinoma cells and had no effect on HLA antigen expression and IL-1 secretion. However incubation of the cell line with rubella virus induced secretion of IL-6, which may be due to passive (i.e. non-infective) stimulation.

Several studies have shown that pancreatic beta cells of patients with IDDM at diagnosis hyperexpress class I antigens and aberrantly express class II antigens,[9,21] and that inappropriate expression of such proteins may account for the enhanced killing potential of autoreactive T cells.

Unlike normal beta cells, the cell line used for our experiments constitutively expresses HLA class II antigens and

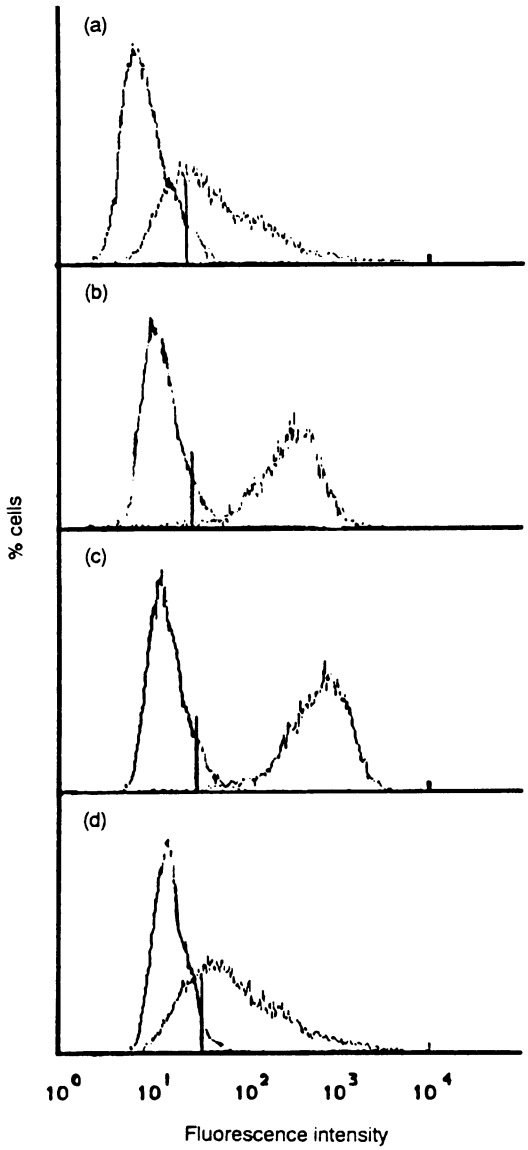

Figure 4. Expression of HLA class II proteins by the CM cell line following stimulation with different viruses as evaluated by cytofluorimetric analysis. Whilst infection with measles and mumps viruses increased the fluorescence intensity, indicating hyperexpression of HLA class II antigens, stimulation with rubella virus did not modify this pattern. The fluorescence intensity is compared with that of an irrelevant antibody. Uninfected (a); measles (b); mumps (c); rubella (d).

therefore the *in vitro* system we established may not exactly reflect the *in vivo* situation. However, this study demonstrates that viral infection can up-regulate HLA class II expression on beta cells in addition to that of class I proteins, which has been previously reported with other viruses.[22]

The mechanism by which the viral infection induces enhanced expression of HLA proteins is unknown. To determine whether cytokines could be responsible for this effect, we investigated the *in vitro* ability of different cytokines to up-regulate HLA protein expression on CM cells. Among the cytokines tested (IL-1, IL-2, IL-6, IL-4, TNF, GM-CSF and IFN-γ), only TNF, IFN-γ and GM-CSF were capable of enhancing the expression of class I and class II HLA proteins by the cell line (data not shown). However, of all the cytokines assayed only IL-1 and IL-6 were detected in the cell supernatants following viral infection, suggesting that cytokines are probably not involved in the process leading to the increased expression of MHC proteins induced by the viral infection. A direct up-regulation of MHC class I genes transcription by reovirus in cultured islet cells has been reported.[22] A role for cytokines in the pathogenesis of IDDM has been postulated.[23] In particular IL-1, TNF and IFN-γ are cytotoxic for beta cells *in vitro* and have recently been suggested as possible inducer factors of beta cell antigens[12] in addition to effector molecules in the process of beta cell killing.[24,25]

The observation that insulinoma cells can be induced by viral infection to produce cytokines such as IL-1 and IL-6 may be relevant for the pathogenesis of IDDM. Thus, IL-1 and IL-6 play a central role in the immune network by promoting both cell growth and activation and by inducing the secretion of several other cytokines by cells of the immune system.[26,27] If such cytokines are produced by beta cells following viral infection, they may act as inducers of an immune response in the pancreatic islets, therefore facilitating the local expansion of autoreactive T-cell clones.

It is possible that in subjects susceptible to IDDM the long latency phase preceding the clinical onset of the disease[28] may be shortened by a viral infection of the beta cells. Thus, if periinsulitis is present in human prediabetes, as has been shown in animal models,[29] the secretion of IL-1 and IL-6 by virus-infected beta cells may act as an extra source of cytokines which potentially can promote the growth of clones of autoreactive lymphocytes already present in the pancreatic infiltrate. In the case of such infection in non-susceptible individuals, the release of cytokines by virally infected beta cells should not trigger the autoimmune response as lymphocytes are not detectable in the islet area.

In conclusion, elucidation of the mechanisms which are responsible for beta-cell destruction can lead to attempts aimed to prevent the disease. In this direction the observation that viral infection may trigger cytokine release by beta cells points out to the importance of early vaccination to protect against measles and mumps in individuals susceptible to IDDM.

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