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Cytokines in sera from insulin-dependent diabetic patients at diagnosis

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SUMMARY

Cytokines are known to play an important role in autoimmunity and have been suggested to be involved in the pathogenesis of insulin-dependent diabetes (IDDM). In the present study we have measured IL-1, IL-2, IL-4, IL-6, interferon-gamma (IFN-γ) and tumour necrosis factor (TNF) (using both immunoassays and bioassays) in sera from 50 patients affected by IDDM at the time of clinical diagnosis and 51 age and sex matched controls. Detectable levels of IL-1, IL-2, IL-6 and IFN-γ were found in the serum of a small percentage of subjects and were not significantly different between patients and controls. IL-4 was detectable in a higher number of both patients and controls and circulating TNF-α (>1 U/ml) was found in a percentage of patients (24%) significantly higher than controls ($P<0{\cdot}01$). Raised levels of TNF-α were detectable using an immunoenzymatic assay whereas TNF bioactivity in these samples was negligible. We conclude that the presence of immunoreactive TNF-α in the patient's sera may reflect an increased localized production of this cytokine at pancreatic level. However, the measurement in serum of other cytokines does not add information on the role that they may play in the pathogenesis of IDDM.

Keywords IL-1 IL-2 IL-4 IL-6 interferon-gamma tumour necrosis factor insulin-dependent diabetes

INTRODUCTION

Insulin dependent diabetes (IDDM) is an autoimmune disease resulting from a cell-mediated immune destruction of pancreatic islet beta cells. Several immunological abnormalities have been described in patients with IDDM at the time of diagnosis including autoantibodies against islet-cell components and presence of activated lymphocytes in peripheral blood (Pozzilli *et al.*, 1983; Herold *et al.*, 1984; Hitchcock *et al.*, 1986; DeBernardinis *et al.*, 1988). These alterations are the expression in the circulation of the pathological hallmark of the disease which is the infiltration of the pancreatic islets by mononuclear cells (insulitis) (Bottazzo *et al.*, 1985). Thus, lymphocytes and monocytes are likely to play a key role in the pathogenesis of this disease, although the precise events leading to the cytotoxic damage of beta cells are still unclear.

Cytokines include regulatory proteins of the immune system and are produced by different cell types including lymphocytes and monocytes. Their pleiotropic activities critically affect the level of the immune response and they have therefore been implicated in the pathogenesis of several autoimmune diseases including IDDM (Mandrup-Poulsen *et al.*, 1986).

Recent studies have shown that cytokines such as IL-1, tumour necrosis factor alpha (TNF-α) and interferon-gamma (IFN-γ) are directly cytotoxic to beta cells *in vitro* (Mandrup-Poulsen *et al.*, 1986; Campbell, Iscaro & Harrison, 1988; Mandrup-Poulsen *et al.*, 1989); in addition TNF and IFN-γ stimulate HLA antigen expression and may therefore facilitate the recognition of beta cell antigens by cells of the immune system; TNF and IFN-γ also induce the synthesis of intracellular adhesion molecules by beta cells (Campbell *et al.*, 1989a), whereas IL-1 and IL-6 can regulate insulin secretion and thus modulate antigen expression associated with the secretory capacity of beta cells (Campbell *et al.*, 1989b).

Conversely, administration of large quantities of IL-1 to animals susceptible to type 1 diabetes prevents the development of hyperglycaemia (Peterson *et al.*, 1990).

As only limited data are available on the circulating levels of cytokines in diabetes, in the present study we have measured serum levels of IL-1, IL-2, IL-4, IL-6, TNF and IFN-γ in patients with IDDM at diagnosis when it is expected that raised values might be found as a consequence of the on-going autoimmune process involving beta cells.

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PATIENTS AND METHODS

Subjects

Patients were randomly selected amongst those attending the out-patient Diabetic Clinic of the II Clinica Medica, University of Rome 'La Sapienza' where they were first diagnosed during 1989.

Venous blood was collected in the morning at 9 a.m. from 50 patients affected by IDDM before their first insulin injection (30 females and 20 males, mean age 15 ± 7 s.d. years) and from 51 normal subjects matched for age and sex (26 females and 25 males, mean age 20 ± 9 s.d. years). IDDM was diagnosed according to the Standards of National Diabetes Data Group. Mean blood glucose levels were 17·5 mM/$l \pm 3{\cdot}9$ s.d.; 35% of patients also showed ketonuria with mean blood glucose levels of 20·1 mM/$l \pm 4{\cdot}1$ s.d. No apparent infections were reported at the time of blood sampling. A group of 15 long-standing diabetic patients was also included in the study (9 females and 6 males; mean age 59 ± 11 s.d. years; mean blood glucose levels 16·2 mM/$l \pm 4{\cdot}2$ s.d.) as control patients with hyperglycaemia, but long after diagnosis.

Serum was separated within 1 h from sampling and stored at -20°C until required (for different manipulation of sera, see section on cytokine bioassays). Due to the large number of assays to be performed only IL-2, IFN-γ and TNF were measured in all patients' sera, whereas the other cytokines were assayed according to the amount of serum available.

Cytokine bioassays

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-2 mouse lymphocyte cell line which proliferates in response to IL-2 (Gearing *et al.*, 1987). Briefly, duplicate sample aliquots and the WHO 1st International Standard were diluted in 96-well plates to a final volume of 100 μl per well; 100 μl containing 10^5 NOB-1 cells were then added to each well and incubated for 24 h. Supernatant (50 μl) from each well were transferred to another 96-well plate and the IL-2 released was then measured using the CTLL-2 cell line proliferation assay (Gillis *et al.*, 1978). The sensitivity of the IL-1 bioassay is approximately 20 mU/ml; 1 U of IL-1 is equivalent to 10 pg.

The bioactivity of IL-2 was measured using the bioassay described by Gillis *et al.* (1978), based on the ability of IL-2 to induce the proliferation of the CTLL-2 cells. Briefly, duplicate sample aliquots and the IL-2 International Standard were diluted in 96-well plates to a final volume of 50 μl per well; 50 μl of 10^5/ml CTLL were added to each well and incubated at 37°C for 24 h. Tritiated thymidine was then added to each well and after 4 h the cells were harvested onto filter mats. Cell proliferation was evaluated by measuring the radioactivity incorporated into DNA by scintillation counting. (Assay sensitivity: approximately 0·1 U/ml; 1 U of IL-2 is equivalent to approximately 7·6 ng.)

IL-4 bioactivity was measured using the ability of the cytokine to cause proliferation of the TF1 erythroleukaemia cell line (Kitamura *et al.*, 1989). Briefly, duplicate sample aliquots and the standard were diluted in a 96-well plate; 100 μl of RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) containing 1×10^5 cells were added to each well and incubated at 37°C for 48 h; 0·5 μCi of tritiated thymidine was then added to each well and after 4 h the cells were harvested onto filter mats and the cell proliferation was evaluated by measuring the radioactivity incorporated into DNA by liquid scintillation counting. (Assay sensitivity: approximately 0·2 U/ml; 1 U of IL-4 is equivalent to approximately 100 pg.)

For IL-6 bioactivity, sera were first heat inactivated at 56°C for 30 min and IL-6 content was determined by a proliferation assay using the murine hybridoma cell line B9 which proliferates specifically in response to IL-6 (Helle, Boeije & Aarden, 1988). 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°C for 72 h; 20 μl of a tetrazolium salt (MTT) solution (5 mg/ml in phosphate-buffered saline (PBS)) were added to each well and the cells cultured for another 5 h. Ten microlitres of 10% SDS/0·02 M HCl (SDS solution) were then added to each well and plates left in the darkness. After 2 h the absorbance at 620 nm was measured using an ELISA reader. (Assay sensitivity: approximately 1 mU/ml; 1 U of IL-6 is equivalent to 200 pg.)

TNF bioactivity was measured using the L929 cell line cytotoxicity assay (Meager *et al.*, 1987). Briefly, 100 μl containing 2×10^5 trypsinized L929 cells were aliquoted in each well of a 96-well plate and incubated at 37°C for 24 h; titrations of the TNF standard and dilutions of the samples in duplicate in medium containing 2 μg/ml actinomycin D were added to the cells and incubated 24 h at 37°C; the cells were then washed, stained with Naphthol Blue Black for 30 min and fixed with formalin. The absorbance at 620 nm was determined using an ELISA reader. (Assay sensitivity: approximately 0·1 U/ml; 1 U of TNF is equivalent to 25 pg.)

Cytokine immunoassays

Immunoreactive levels of IL-1α were assayed using a two-site immunoradiometric assay (IRMA) as previously described (Thorpe *et al.*, 1988) (assay sensitivity: approximately 4 U/ml).

IL-4 levels were measured by a two-site IRMA (Bird, Madhwa & Thorpe, 1991) (assay sensitivity: approximately 0·2 U/ml).

TNF and IFN-γ levels were measured using two-site ELISA as previously described (Meager *et al.*, 1987; Meager, 1987) (assay sensitivity: approximately 1 U/ml).

IL-6 was measured by a two-site IRMA (assay sensitivity: approximately 20 mU/ml). All assays were calibrated using WHO International Standards or reference reagents distributed by the National Institute for Biological Standards and Control. Assays were validated to establish whether serum contained interfering substances, by enclosing a titration of the reference reagents diluted in human serum in each assay (Thorpe *et al.*, 1988).

All the cytokine assays used in this study were found to be reproducible. The interassay variability was $< 10\%$ for all the methods.

Statistical analysis

Statistical analysis includes the Mann–Whitney U Test and the χ^2 test with Yates' correction, where appropriate. The correlation coefficient was calculated for analysis of blood glucose levels in relation to different cytokine values.

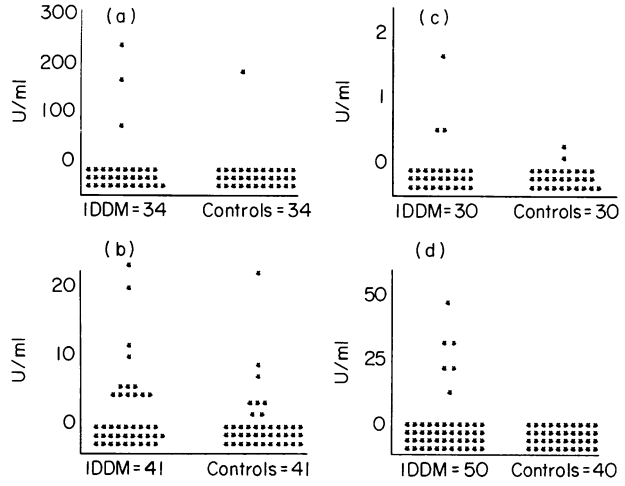

Fig. 1. (a) IL-1α values in the serum of 34 nd IDDM patients and 34 control subjects, as detected by immunoradiometric assay. (b) IL-4 values in the serum of 41 nd IDDM patients and 41 control subjects as detected by immunoradiometric assay. (c) IL-6 values in the serum of 30 nd IDDM patients and 30 control subjects as detected by immunoradiometric assay. (d) IFN-γ values in the serum of 50 nd IDDM patients and 40 control subjects as detected by an ELISA method. Difference between groups not significant.

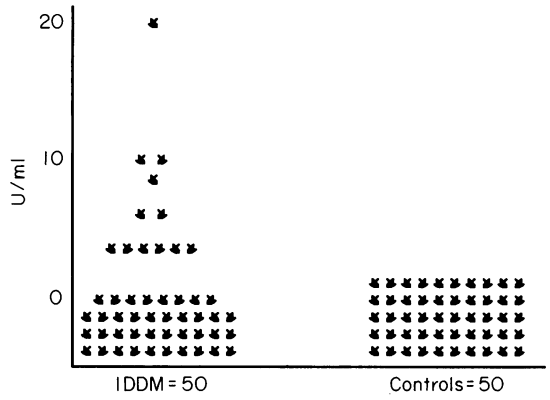

Fig. 2. TNF-α values in the serum of 50 nd IDDM patients and 50 control subjects as detected by an ELISA method. Difference between groups is statistically significant ($P<0.01$, χ^2 with Yates' correction).

RESULTS

Sera from newly diagnosed IDDM patients (nd IDDM), long-standing diabetics and control subjects were compared for levels of tested cytokines. Detectable levels of IL-1α were found in a small percentage of subjects (8·8% nd IDDM, 6·6% long-standing diabetics and 2·9% control subjects) using the immunoradiometric assay for IL-1α (Fig. 1); however, only one patient and one control subject showed a corresponding bioactivity for IL-1 as assessed by EL4-NOB1 cell line-based bioassay.

IL-2, as evaluated by a bioassay, was not detectable in any of the sera. Measurable levels of IL-4 (>0·2 U/ml) were found in 31·7% of nd IDDM patients, 6·6% long-standing patients and in 19·5% of control sera using the IRMA (Fig. 1), but no corresponding bioactivity was observed using the bioassay. Detectable levels of IL-6 (>20 mU/ml) were observed in 10% of nd IDDM, 6·6% long-standing patients and 6·6% of control sera using IRMA (Fig. 1), but only one patient's serum showed a corresponding biological activity.

TNF-α levels (>1 U/ml) were found in 24% of nd IDDM, and in none of long-standing diabetic patients and controls using the ELISA and this difference was statistically significant (χ^2 test ($P<0.01$)) (Fig. 2). However, no TNF biological activity was detected using the L929 bioassay.

No correlation was found between TNF-α levels and blood glucose values (correlation coefficient = 0·089). A correlation with blood glucose and other cytokine levels was not observed.

Detectable levels of IFN-γ (>1 U/ml) were found in 12·5% of nd IDDM sera but in none of the long-standing patients and the controls using the ELISA (Fig. 1).

DISCUSSION

Over the past few years several reports have suggested a possible role for cytokines in the pathogenesis of Type 1 diabetes (Mandrup-Poulsen *et al.*, 1986; Campbell *et al.*, 1988; Mandrup-Poulsen *et al.*, 1989); however, evidence for cytokine involvement in the disease process is mainly based on *in vitro* studies, whilst evidence for their *in vivo* detection is at present lacking.

This study reports apparently for the first time an investigation to establish if abnormal levels of relevant different cytokines are present in sera from patients with IDDM at the time of diagnosis, when it is known that the beta cell cytotoxic process is operative.

Our results show that no significant differences exist between the percentage of newly diagnosed IDDM patients and control subjects or long-standing diabetic patients with detectable serum levels of IL-1, IL-2, IL-4, IL-6 and IFN-γ, indicating that the presence of these cytokines in peripheral blood is not a distinctive feature of IDDM at diagnosis. In other autoimmune diseases, raised levels of IL-1, IL-2 and IFN-γ have been reported (Eastgate *et al.*, 1988; Kramer & Wick, 1989), suggesting that production of these cytokines at the site of target organ may be reflected by measurable levels in circulation.

However, the percentage of newly diagnosed diabetic patients with detectable serum TNF-α was significantly higher than both the normal and the long standing diabetic controls. TNF is a cytokine with a wide range of biological activities, including modulation of the immune response and cytotoxicity as well as metabolic effects. The levels of TNF-α detected in the patients' sera did not correlate with their blood glucose levels, suggesting that hyperglycaemia was probably not the cause of raised serum TNF. However, it may be possible that other factors (e.g. increased hormone secretion associated with stress of IDDM onset) may be important in increasing TNF levels. Although TNF-α was found in 24% of the diabetic sera using the immunoenzymatic assay, no corresponding biological activity was found using the L929 bioassay. We cannot exclude that this discordance may be due to the effect of metabolites present in the sera of patients with poor metabolic control which may inhibit the bioassay. However, a similar discrepancy in bioassay and immunoassay results was observed in most cases with the other cytokines. The lack of correlation between the results

obtained with the two methods could be due to the fact that the immunoassays may detect biologically inactive material organizing from degradation processes such as denatured proteins, aggregates of the molecules or cytokines bound to carriers or soluble receptors, which specifically react with the antibody but do not have biological activity.

Alternatively, the presence of cytokine inhibitors in serum may be postulated to explain the lack of bioactivity in the samples.

Antibodies to cytokines have been reported in the serum of patients with autoimmune diseases, and this also may account for the loss of cytokine activity (Bendtzen *et al.*, 1990).

Finally, the recent demonstration of soluble TNF receptors which can compete with the membrane receptor for the binding of TNF strongly suggests that they may function as *in vivo* inhibitors for TNF bioactivity (Engelmann, Novick & Wallack, 1990).

In conclusion, the finding that immunoreactive TNF-α can be detected in serum from patients with Type 1 diabetes at diagnosis is suggestive of an increased production rate which may take place at pancreatic level and be reflected by measurable levels in the circulation. If this observation is confirmed in a larger number of cases, TNF levels could become a very useful marker to monitor the disease process.

However, the measurement of the other cytokines in peripheral blood does not give a useful indication of their possible role in the process of beta cell destruction and cannot be used as a marker for the disease.

Unfortunately, evaluation of cytokines is not possible in human pancreas where their measurement may be useful for understanding their involvement in the disease process. Thus, information on the type and amount of cytokines released at the site of beta cell lymphocytic infiltration can be used to exploit ways of controlling the cytotoxic process.

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