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High serum levels of soluble CD8 in insulin-dependent diabetes

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SUMMARY

In type 1 (insulin-dependent) diabetes mellitus (IDDM) $CD8^+$ T cells represent the majority of lymphocytes which infiltrate the pancreatic islets during β cell destruction. Soluble CD8 antigen (sCD8) has been shown to correlate with CD8 cell subset activation. In this study we measured by ELISA sCD8 levels in sera from: 33 newly diagnosed IDDM patients; 29 type 1 diabetics with duration of disease more than 1 year; 37 healthy siblings of IDDM patients; 19 healthy controls. Sera from both groups of IDDM patients and from healthy siblings exhibited soluble CD8 mean levels significantly higher than controls ($P=0{\cdot}0001$, $P<0{\cdot}003$, $P<0{\cdot}03$ respectively). Soluble CD8 levels above the normal range (mean ± 2 s.d. of controls) were found in a percentage of newly diagnosed subjects (54·5%) significantly higher than in subjects with a long-standing duration of disease (6·9%, $P<0{\cdot}0005$) and healthy siblings (16·2%, $P<0{\cdot}002$). Our results suggest that the raised levels of soluble CD8 near to diabetes onset may indicate the activation of $CD8^+$ T cells probably responsible for the autoimmune β cell destruction.

Keywords soluble CD8 type 1 (insulin-dependent) diabetes lymphocyte activation

INTRODUCTION

Type 1 (insulin dependent) diabetes mellitus (IDDM) is the result of an autoimmune destruction of pancreatic β cells. Antibodies against β cell antigens (i.e. islet cell antibodies, insulin autoantibodies, glutamic acid decarboxilase antibodies, etc.) and peripheral activated lymphocytes, such as $HLA\text{-}DR^+$ and $IL\text{-}2R^+$ cells, in both $CD4^+$ and $CD8^+$ T subsets, precede the onset of type 1 diabetes and decline rapidly after diagnosis [1–7]. These peripheral immunological abnormalities may reflect the ongoing infiltration of pancreatic islets by mononuclear cells (insulitis) probably responsible for the β cell destruction. In an animal model of type 1 diabetes (NOD mouse), insulitis is a progressive process which requires the sequential interaction between $CD4^+$ activated lymphocytes, prominent in the early stages of islet infiltration, and $CD8^+$ lymphocytes, predominant after the onset of β cell destruction [8,9]. Interestingly, these findings are consistent with the observations that human diabetic pancreas, obtained from rare autopsies of IDDM patients who died at time of diagnosis, and biopsies of isografted pancreas have shown a predominance of activated $CD8^+$ T lymphocytes in the 'end-stage' islet infiltration [10–12].

It has been reported that $CD8^+$ lymphocytes spontaneously release a soluble form of their membrane CD8 antigen (sCD8) and that its quantity is related to the activation of this T cell subset both *in vitro* and *in vivo* [13,14]. Since the serum levels of sCD8 probably reflect the activity of $CD8^+$ cell subset involved in β cell destruction, we measured sCD8 serum levels in newly diagnosed type 1 diabetics and in patients with IDDM of more than 1 year duration. In addition, we examined the same parameter in a group of siblings of IDDM patients thought to be at increased risk of developing the disease [15,16].

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PATIENTS AND METHODS

Subjects

The subjects were selected among type 1 diabetics and their first-degree relatives attending the Endocrinology Unit of the Department, taking into account good metabolic control, duration of disease, age and sex. They were distributed in three groups as follows: 33 newly diagnosed (<3 months) type 1 diabetics (15 males and 18 females, mean age $16{\cdot}2 \pm 7{\cdot}1$ years) (Group A); 29 type 1 diabetic patients of more than 1 year duration (range 1–12 years) (11 males and 18 females, mean age $18{\cdot}5 \pm 5$ years) (Group B); 37 healthy siblings of type 1 diabetics (13 males and 24 females, mean age $15{\cdot}2 \pm 7{\cdot}8$ years) (Group C). Blood samples were collected and sera were separated and stored at $-30°C$ until use. A subsequent blood sample was collected in eight newly diagnosed diabetics during a follow-up period ranging from 4 to 18 months. In addition, peripheral $CD8^+HLA\text{-}DR^+$ lymphocyte subset was analysed in the same blood sample of 12 Group A patients. Blood samples from 19 healthy subjects (seven males and 12 females, mean age 25 ± 8 years) served as controls. No apparent infectious diseases were reported at the time of blood sampling.

Islet cell antibodies

Islet cell antibodies (ICA) were detected by an indirect immunofluorescence assay using frozen sections of unfixed human pancreas. Quantification of ICA was performed by dilution of sera until ICA could not be detected and results were converted to Juvenile Diabetes Foundation (JDF) units, by a standard curve based on the international JDF reference serum sample [17].

Measurement of soluble CD8 levels in sera

The CD8 levels in the sera were measured by an enzyme immunoassay test kit (Cell Free T8 Kit; T Cell Sciences, Cambridge, MA). Briefly, the 96-well microplates were coated with murine MoAb to CD8 antigen for 20 h at 4°C. After coating the plate was washed three times with washing buffer. The plate was then incubated for 2 h at 37°C with 300 μl of blocking solution per well, followed by three washings. Sera diluted 1:10 were applied to the plate and the plate was incubated for 90 min at 37°C. After three washings the plate was incubated with horseradish peroxidase (HRP)-conjugated murine MoAb directed against a different epitope of human CD8. After the removal of unbound HRP conjugate by washings, *o*-phenylediamine was added to each well and incubated for 30 min. The reaction was stopped by addition of 2 N H_2SO_4 and the absorbance measured at 490 nm. The results, expressed as U/ml, were determined from the standard curve obtained with five CD8 standard samples ranging from 0 to 2000 U/ml.

Measurement of the peripheral blood CD8$^+$HLA-DR$^+$ cells

The number of CD8$^+$HLA-DR$^+$ lymphocytes was determined by two-colour flow cytometric analysis on peripheral blood samples as previously described [18]. Briefly, whole blood samples were stained with both isothiocyanate-conjugated CD8 (Leu-2) and PE-conjugated HLA-DR murine MoAbs (Becton Dickinson Monoclonal Centre, San Jose, CA). After lysis of erythrocytes, analysis of prepared samples was performed using a FACScan flow cytometer (Becton Dickinson Immunocytochemistry System, San Jose, CA). Quality control criteria were applied to ensure the inclusion of at least 95% of the lymphocytes and no greater than 10% non-lymphocyte events in the analysis gate. Results were expressed as absolute counts/mm^3 of blood.

Statistical analysis

Data were expressed as mean $\pm$ s.d. Study groups were compared using the Mann–Whitney *U*-test and χ^2 test with Fisher's exact method. The correlation coefficient was calculated for analysis of sCD8 levels in relation to the absolute number of CD8$^+$HLA-DR$^+$ lymphocytes.

RESULTS

The mean levels of the soluble CD8 were significantly higher in newly diagnosed diabetics (535 $\pm$ 154 U/ml; $P=0{\cdot}0001$) compared with healthy subjects (339 $\pm$ 108 U/ml), diabetics with at least 1 year of duration of the disease (450 $\pm$ 153 U/ml; $P<0{\cdot}02$) and healthy siblings (441 $\pm$ 179 U/ml; $P<0{\cdot}02$). Interestingly, both Groups B and C showed sCD8 serum levels significantly higher than controls ($P<0{\cdot}003$ and $P<0{\cdot}03$ respectively). The mean $\pm$ 2 s.d. of sCD8 levels observed in the healthy controls was considered within normal range. Soluble CD8 levels higher than 555 U/ml (upper limit of the normal range) were found in 54·5% (18/33) of Group A, in 6·9% (2/29) of Group B and 16·2% (6/37) in Group C (Fig. 1). Differences were statistically significant when Group A was matched with Groups B and C (χ^2 test respectively $P<0{\cdot}0005$ and $P<0{\cdot}002$); no significant difference was observed between Groups B and C.

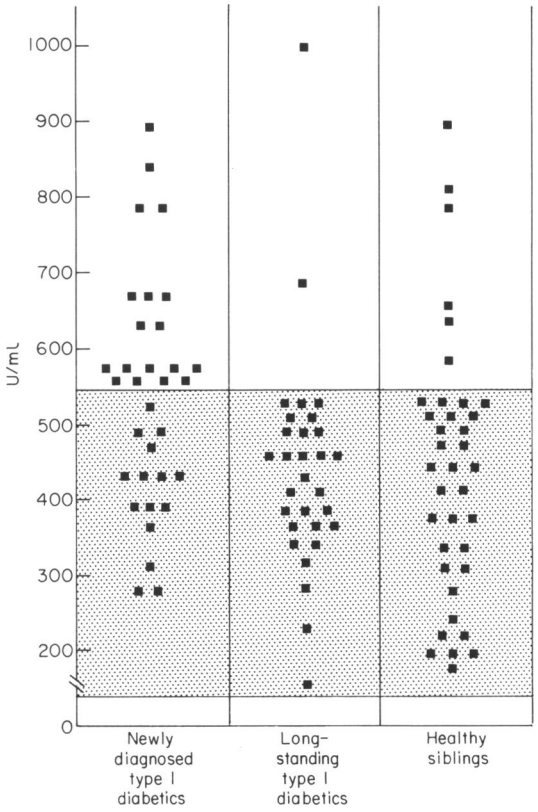

Fig. 1. Soluble serum CD8 levels in patients with varying disease duration and in healthy siblings of type 1 diabetics. Shaded area indicates the mean $\pm$ 2 s.d. of soluble CD8 levels in 19 healthy controls. χ^2 test showed significant differences in the prevalence of CD8 levels above the normal range within the three groups studied (newly diagnosed *versus* long-standing and healthy siblings respectively $P<0{\cdot}0005$ and $P<0{\cdot}002$).

The ICA positivity of the three groups studied is shown in Table 1. No correlation was observed between sCD8 values and ICA positivity when results were considered either '*in toto*' or 'per group'.

The follow up included eight newly diagnosed diabetics with sCD8 starting values either above ($n=4$) or within ($n=4$) the normal range. All these patients were comparable with regard to the insulin requirement that did not vary during the follow up. Three of four patients with high sCD8 values showed a decrease throughout the disease, while the other remained persistently

Table 1. Islet cell antibody (ICA) positivity

Groups	ICA$^+$	ICA$^-$
Newly diagnosed type 1 diabetics ($n=33$)	22	11
Long standing type 1 diabetics ($n=29$)	10	19
Healthy siblings ($n=37$)	0	37

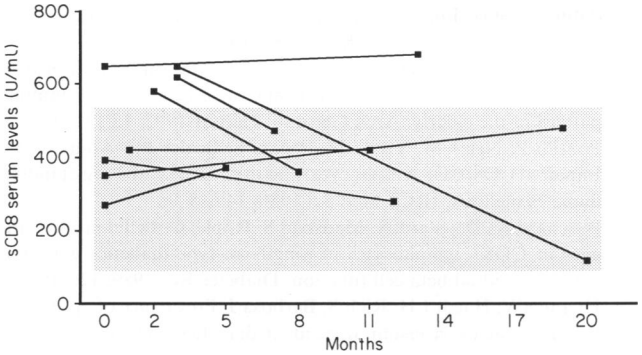

Fig. 2. Follow up of serum sCD8 values in eight newly diagnosed type 1 diabetics. Shaded area indicates the mean ± 2 s.d. of soluble CD8 levels in 19 healthy controls.

elevated. The remaining four maintained their sCD8 levels within the normal range throughout the follow up (Fig. 2).

The $CD8^+HLA\text{-}DR^+$ peripheral lymphocytes were measured in 12 newly diagnosed diabetics. Collected data showed an increased absolute number of double positive peripheral lymphocytes ($136{\cdot}9 \pm 34$) in comparison with controls ($17{\cdot}3 \pm 9{\cdot}8$; $P = 0{\cdot}0001$). No correlation was found between sCD8 serum levels and number of $CD8^+HLA\text{-}DR^+$ peripheral lymphocytes.

DISCUSSION

Soluble CD8 serum levels were higher in newly diagnosed IDDM patients in comparison with diabetics of more than 1 year duration, showing a trend similar to the other immunological abnormalities widely occurring at type 1 diabetes onset and gradually disappearing after diagnosis [1]. In fact, the prevalence of sCD8 serum levels above the upper limit of normal range dramatically declines from 54·5% observed in newly diagnosed patients to 6·9% found in long-standing diabetics. This result prompted us to verify with a follow up of eight newly diagnosed diabetics the observed drop of sCD8 values throughout the disease (Fig. 2). Interestingly, three out of four patients with sCD8 starting levels above the normal range showed a decrease of their values, while the other one remained persistently elevated. The sCD8 levels of the other four remained within the normal range throughout the follow up. These observed variations occurred in well controlled diabetics whose insulin requirements were comparable at recruitment and persisted unmodified during the follow up.

The maintenance of elevated sCD8 values in a low percentage of long-standing diabetics is in line with the reported persistence of active humoral and cellular immune phenomena [1,19]. In particular, with respect to the cellular abnormalities, the presence of a higher percentage of $CD8^+$ (suppressor/cytotoxic) lymphocytes in diabetics with preserved β cell function has been described [20], while in terms of humoral phenomena, the persistence of ICA in a relatively low number of long-standing type 1 diabetics has been widely reported [1]. The determination of ICA in the sera of our subjects provided us with the most reliable tool for the study of IDDM-related humoral abnormalities, allowing us to study a possible correlation between ICA and sCD8. The absence of correlation between these two parameters in all the groups studied supports the concept that ICA are merely immunological markers of β cell destruction, mediated by effector cells [10–12]. The presence of activated T cell subsets in the peripheral blood of newly diagnosed IDDM patients [6,7] led to speculation that they might be considered as circulating markers of the activated lymphocytes which infiltrate the pancreatic islets, destroying the β cells. It is of note that, in our study, we observed an increased number of $CD8^+HLA\text{-}DR^+$ peripheral lymphocytes in 12 newly diagnosed diabetics studied by flow cytometry, but, when we matched these results with the sCD8 serum levels, which are considered to fit well the activation of $CD8^+$ T cells, no correlation was found. Therefore, the lack of correlation probably reflects the segregation of insulitis at the pancreatic level or differential shedding of sCD8 by individual activated $CD8^+$ cells.

Soluble CD8 levels above the upper limit of the normal range were found in six out of 37 ICA^- healthy siblings. First degree relatives of IDDM patients, who share many genetic and environmental factors with diabetics, present an increased incidence of immune abnormalities [15,16,21,22]. Significant changes of peripheral T lymphocyte subsets were also observed in ICA^- subjects of this healthy population compared with a group of normal subjects without a family history of IDDM [23]. In addition, in high risk subjects, such as ICA^+ siblings, increased circulating $CD8^+HLA\text{-}DR^+$ T cells have been described [24,25]. Interestingly, when we quantified sCD8 levels in five siblings with circulating ICA, two out of five showed high values (data not shown). During a 1 year period of observation, neither ICA^+ nor ICA^- siblings, irrespective of low or high levels of sCD8, progressed to IDDM. Only sCD8 follow up in these subjects and in prediabetics could validate the effectiveness of sCD8 levels in monitoring ongoing islet infiltration, and may be of great interest in getting inside the 'end stage' of β cell destruction.

The membrane-associated CD8 antigen is formed by two subunits (α and β) [26], and its soluble form is recognized as the α chain subunit [27]. Despite the large bulk of evidence attesting to raised sCD8 serum levels in autoimmune diseases [28–30], malignancies [31] and viral infections [32,33] such as infectious mononucleosis [14], the role of this soluble molecule has not been completely defined. In contrast, it is well defined that the CD8 antigen expressed on T lymphocyte membranes is involved in the recognition of the HLA class I molecule–antigen complex on the cell surfaces [34]. Soluble CD8 is largely reputed to be an indicator of the $CD8^+$ T subset activity, although an immunoregulatory function cannot be completely excluded. It is worth noting that sCD8 could interfere in the bridging between $CD8^+$ T lymphocytes and the cells expressing the HLA class I molecule–antigen complex, thus affecting cellular immune cooperation.

At this point we can speculate on the possible mechanisms determining the increased sCD8 levels in IDDM. It is noteworthy that β cell destruction is a cell-mediated phenomenon where cytokines probably play a key role, being directly cytotoxic to β cells *in vitro* [35,36]. In addition, the observation of high tumour necrosis factor (TNF) levels in the sera of IDDM patients at diagnosis suggests the use of soluble molecules in monitoring the lymphocytic infiltration of pancreatic islets [37]. Moreover, TNF-α potentiates the $CD8^+$ T cell function and increases the release of sCD8 from synovial fluid mononuclear cells of patients with rheumatoid arthritis [28]. Consequently, increased production of TNF-α at the pancreatic level, during islet cell infiltration, could represent a stimulus for increased

sCD8 release from CD8+ T cells during insulitis. The hyperexpression of HLA class I molecules in islet cells during lymphocytic infiltration [38] has also been described, thus implying that an immunoregulatory function of the sCD8 in the interaction between effector cells (CD8+) and targets (β cells hyperexpressing HLA class I molecules) cannot be ruled out.

In conclusion, this study provides the first evidence for the quantification of sCD8 in type 1 diabetes. Such a parameter may provide a non-invasive method for *in vivo* monitoring of activated CD8+ T cells responsible for the 'end stage' of insulitis, and may represent an important tool in the comprehension of the immunopathology of type 1 diabetes.

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