Int J Clin Lab Res (1996) 26: 55–59

ORIGINAL

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Antibody response to islet antigens in anti-CD4/prednisolone immune intervention of type 1 diabetes

Received: 25 September 1995 / Accepted: 12 January 1996

Abstract Cytoplasmic islet cell antibodies, glutamic acid decarboxylase autoantibodies, spontaneous insulin autoantibodies, and insulin-induced antibodies were analyzed in a 1-year follow-up study of 12 newly diagnosed patients with insulin-dependent diabetes mellitus aged 14 ± 2 years (range 7–20 years) who had been initially treated with either multiple injections of insulin alone (control group) or, in addition, anti-CD4 monoclonal antibody/prednisolone (treatment group). Despite individual variations in islet cell antibody titers, there were no significant differences in the prevalence or changes in the mean titers between the two groups. Glutamic acid decarboxylase autoantibodies remained almost unchanged, but correlated with levels of islet cell antibodies. While at initiation of treatment only 50% of the patients from both groups had spontaneous insulin autoantibodies, all patients developed insulin-induced antibodies upon conventional insulin therapy during the course of follow-up. This was not related to islet cell antibody or glutamic acid decarboxylase antibody levels. The insulin requirement was markedly reduced through the period of follow-up, but did not significantly differ between the two groups. A correlation between islet cell antibody levels and insulin requirement was observed in the control group but not in the treatment group. Plasma levels of the antibodies were not associated with changes in stimulated C-peptide or hemoglobin A_1 concentrations. Activated T-lymphocytes persisted in both groups of patients, but their mean levels were not significantly different. The reason for the absence of statistically significant differences between treatment and control groups could be due to the small number of patients in the study. In conclusion, short-term immune intervention with anti-CD4 monoclonal antibody in addition to insulin therapy did not suppress autoimmune reactions towards the beta cells.

Key words Islet cell antibodies · Glutamic acid decarboxylase autoantibodies · Insulin (auto) antibodies · Anti-CD4 therapy · Type I diabetes

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Introduction

Insulin-dependent diabetes mellitus (IDDM) is induced by a T-cell-mediated destruction of pancreatic beta cells. At onset of the disease, the mononuclear cell infiltrate seen in the islets consists of $CD4^+$ helper/inducer and $CD8^+$ cytotoxic/suppressor T-lymphocytes, in addition to macrophages. While earlier studies reported a predominance of $CD8^+$ cells among the islet-infiltrating lymphocytes [1, 2], a recent investigation has demonstrated abundant $CD4^+$ lymphocyte infiltrates, with relatively small numbers of $CD8^+$ cells present [3]. Pilot clinical trials have shown anti-CD4 monoclonal antibody (mAb) to have beneficial effects in several autoimmune diseases [4–7], but such trials have not yet been performed in IDDM patients.

Consistent with the suggestion that autoreactive $CD4^+$ helper/inducer T-lymphocytes may play a role in the induction of an immune response towards the pancreatic beta cells is the outcome of immune intervention trials in new-onset IDDM, using the T-lymphocyte blocking agent cyclosporin A, where remission rates of 30%–50% were achieved [8]. Immunosuppression with cyclosporin A caused the disappearance of islet cell antibodies (ICA) or a reduction in the prevalence or mean titer, but reappearance of ICA was also seen under therapy [9]. However, neither ICA nor glutamic acid decarboxylase autoantibodies (GAD_{65}-AAb) proved useful for monitoring the effectiveness of treatment or predicting disease remission [10].

We have recently carried out the first randomized controlled trial of the murine anti-CD4 mAb MAX.16H5 in combination with prednisolone in children with newly diagnosed IDDM [11]; this antibody has been effective in the treatment of inflammatory and autoimmune diseases [4–6]. With the exception of two patients, the treatment failed to induce significant clinical improvement or remission of IDDM through the 1-year follow-up, although all patients benefitted from the treatment [11]. We have extended our previous study and report here the effects of the treatment on autoantibodies to islet antigens and T-lymphocyte activation.

Patients and methods

Patients

Twelve patients with newly diagnosed IDDM aged 14±2 years (range 7–20 years) entered the trial. The treatment regimen and the clinical characteristics have already been described in detail elsewhere [11]. In brief, on admission, all patients received intensive insulin therapy, with initial doses of 0.71±0.15 U/kg per day for 72 h to attain euglycemia. Patients were randomly assigned to a treatment or control group. The treatment group ($n=6$) received, in addition to insulin therapy, 0.5 mg/kg per day i.v. infusions of anti-CD4 mAb in combination with 1.0 mg/kg per day prednisolone over 5 consecutive days, and thereafter prednisolone orally for another 5 days. The control group was treated with multiple injections of regular insulin alone. Two weeks after commencing the therapy, all patients were put on a conventional therapy, consisting of two daily insulin injections, and were followed for 1 year. The mean age of the treatment group was 12±2 years (range 7–17 years) versus 15±1 years (range 12–20 years) for the control group. The age difference was not statistically significant.

Islet cell cytoplasmic antibodies

ICA were determined by immunocytochemistry on frozen sections of blood group 0 human pancreas using horseradish peroxidase-conjugated secondary antibody, as described previously [12, 13]. Conversion of the end-point titers was done by double dilutions of the 80 U JDF standard, as recommended by the International ICA Workshop. The detection limit of our assays was <5 Juvenile Diabetes Foundation (JDF) units, and our laboratory participates regularly in the ICA Workshops. In the 10th IDW ICA Proficiency Program, validity was 89%, sensitivity 90%, and specificity 88%.

Glutamic acid decarboxylase autoantibodies

GAD_{65}-AAb were determined by radioimmunoassay [13] employing ^{125}iodine (I)-labelled recombinant GAD_{65}. The test results achieved at the recent second International GAD Antibody Workshop were: 87% sensitivity and 84% specificity. The relative content of GAD-AAb in the sera is expressed in GAD-AAb units, calculated from the binding of negative control and positive GAD-AAb serum to the iodinated GAD_{65} tracer [13].

Insulin autoantibodies and Insulin antibodies

Spontaneous insulin autoantibodies (IAA) were determined by a radiobinding displacement assay [14]. The assay used monoiodinated human insulin and a charcoal precipitation technique to separate antibody-bound from free insulin. Our laboratory also participates in the IDW proficiency program for IAA, and according to the results of this program, our assay has a 100% specificity and 80% sensitivity. Those insulin antibodies induced by insulin treatment (IAb) were measured by a similar method [14], but this latter assay used iodinated porcine insulin in place of human insulin. In brief, 50-µl serum samples were thoroughly mixed with 50 µl A14-mono-^{125}I-porcine insulin (0.1 nmol/l in 0.04 mol/l phosphate buffer, pH 7.4, containing 0.9% sodium chloride, 0.3% albumin, and 0.03% sodium azide). Samples run in parallel contained, in addition to the radioactive tracer, 333 nmol/l unlabelled porcine insulin (Sigma, Germany). Following a 3-day incubation at 4 °C, 1 ml of 1.25% charcoal and 1% dextran were added and the samples centrifuged at 1,500×g for 10 min to separate bound from free ^{125}I-insulin. All samples were run in duplicate and sera were considered antibody positive if the difference in the percentage of tracer bound in the absence and presence of unlabelled insulin exceeded the mean+3 SD value of age-matched healthy control subjects.

T-cell analysis

Peripheral blood mononuclear cells were isolated by Ficoll/Visotrast density gradient centrifugation. Staining of lymphocyte surface antigens was performed with fluorescein isothiocyanate (FITC)-conjugated anti-HLA-DR mAb and phycoerythrin (PE)-conjugated anti-CD3 mAb from Dianova Immunotech (Hamburg, Germany). Analysis on an EPICS Profile II Flow Cytometer (Coulter Electronics, Hialeah, Fla., USA) was performed as previously described [11].

Plasma C-peptide and hemoglobin A_1

Plasma C-peptide levels were measured by radioimmunoassay [15] and glycated hemoglobin (HbA_1) by high-pressure liquid chromatography (Diamat, Bio-Rad, Munich, Germany).

Statistics

Results are given as the mean±SD. Linear regression analysis, Mann-Whitney rank-sum test and Student's t-test were used for evaluation of data. $P<0.05$ was chosen for the level of significance.

Results

Islet cell antibodies

Prior to therapy, 83% (5/6) of the treatment group and 67% (4/6) of the control group had ICA. One patient in the treatment group became ICA positive shortly after initiation of anti-CD4/prednisolone therapy, while 1 patient in the control group became positive and another remained negative (Fig. 1). The mean levels of ICA were 102 JDF units in the treatment group and 62 JDF units in the control group, but the decreases in the mean titers (12 and 24 JDF units, respectively) were not significantly different between the two groups. ICA levels were not related to basal or stimulated C-peptide values, either at the time of commencing therapy or after 1 year of follow-up. An association between ICA levels and the decrease observed in HbA_1 was also lacking, whereas a positive correlation ($r=0.92$, $P<0.01$) between ICA levels and insulin requirement was found at the end of the follow-up in the control group only.

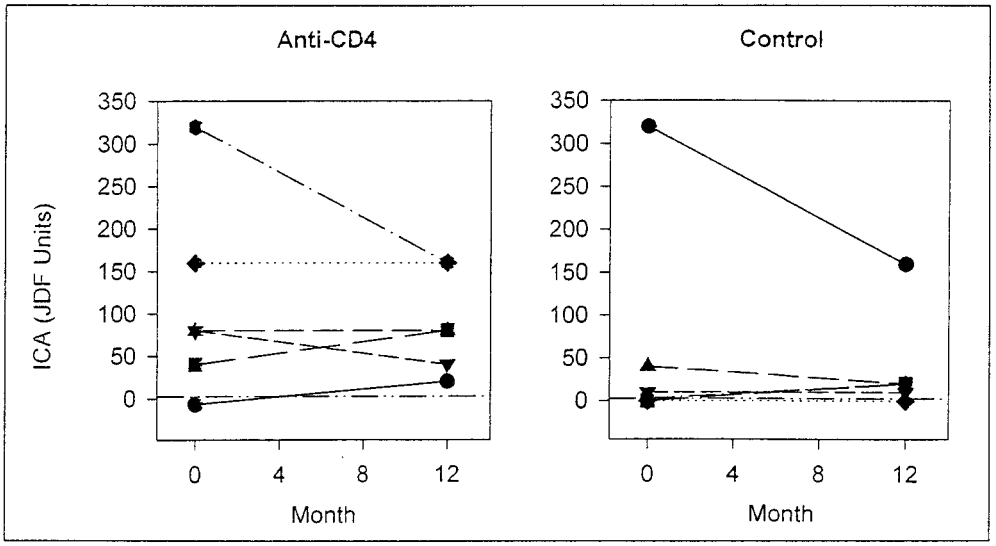

Fig. 1 Individual levels of cytoplasmic islet cell antibodies (*ICA*) in two groups of insulin-dependent diabetes mellitus (IDDM) patients prior to initial therapy with anti-CD4/prednisolone (anti-CD4) or with multiple insulin injections alone (control) and after 12 months of conventional insulin therapy. Levels are expressed in Juvenile Diabetes Foundation (*JDF*) units, as recommended by the IDW ICA Proficiency Program. Values below the *dashed horizontal line* indicate ICA negativity. The corresponding symbols indicate individual patients

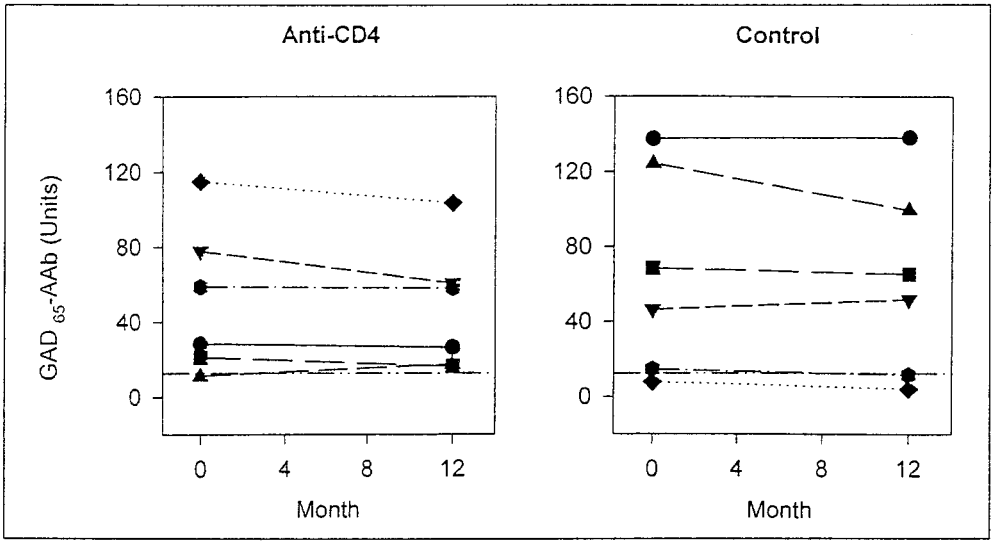

Fig. 2 Individual levels of glutamic acid decarboxylase autoantibodies (GAD_{65}-*AAb*) in newly diagnosed IDDM patients before initial therapy with anti-CD4/prednisolone (anti-CD4) or with insulin alone (control) and after 12 months of conventional insulin treatment. Levels are expressed in units according to the second International GAD Antibody Workshop. Values below the *dashed horizontal line* indicate GAD-AAb negativity

Glutamic acid decarboxylase autoantibodies

At the time of diagnosis, 83% (5/6) were positive for GAD_{65}-AAb in the treatment as well as the control group. One patient in the control group who had a low GAD_{65}-AAb level converted from positive to negative during the follow-up (Fig. 2). In contrast to ICA, individual GAD_{65}-AAb levels did not markedly change, but the levels prior to therapy and after the follow-up period correlated well with the ICA levels ($r=0.50$, $P<0.02$ and $r=0.54$, $P<0.002$, respectively). However, GAD_{65}-AAb levels did not correlate with insulin requirement, nor were they related to C-peptide or to HbA_1 values.

IAA and IAb

Low-level IAA were present in 50% (3/6) of treatment and control group patients at the time of diagnosis. The mean levels (expressed as percentage binding) were not significantly different between the groups. Since all patients, regardless of the treatment regimen, received insulin, IAb were induced, and their binding values increased through the follow-up period up to 28.1% ± 12.3% in the treatment and 38.2% ± 8.5% in the control group (Fig. 3).

T-lymphocyte activation

T-cell activation was increased during follow-up in the treatment and control groups. The percentage of activated T-cells ($HLA\text{-}DR^+CD3^+$) varied greatly among individual patients, but the mean percentage of the control and treatment groups was not significantly different by the end of the study (Table 1). The increase in T-cell activation was not related to the level of autoantibodies or to the insulin-induced IAb.

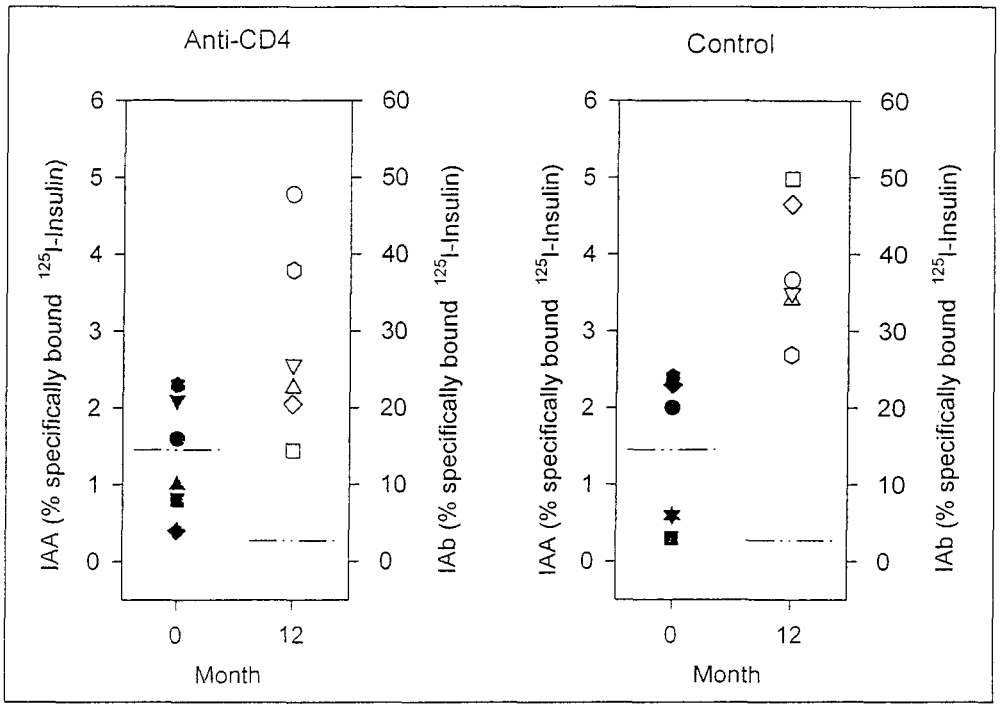

Fig. 3 Insulin autoantibodies (*IAA*) in newly diagnosed IDDM patients before anti-CD4/prednisolone (anti-CD4) or intensive insulin treatment alone (control) and insulin-induced antibody (*IAb*) levels after 12 months of conventional insulin therapy. Antibody levels are expressed as percentage radioiodinated insulin specifically bound to the patients serum. Values below the *dashed horizontal line* indicate antibody negativity

Effects of anti-CD4/prednisolone treatment on metabolic parameters

Compared with the control group, anti-CD4/prednisolone treatment had no significant effects on the above immune parameters or on metabolic parameters, such as stimulated C-peptide secretion, HbA_1, and insulin requirement (Table 1). The small number of patients studied could account for the absence of any difference between the two groups.

Discussion

Our results suggest that in newly diagnosed IDDM patients initial treatment with anti-CD4 mAb/prednisolone together with intensive insulin therapy, followed by a conventional treatment regimen, does not result in a significant reduction in mean titers or prevalence of ICA or GAD_{65}-AAb. Although stimulated C-peptide levels did not increase in the treatment group, improvement of metabolic control was achieved in both groups of patients, as indicated by the decrease in HbA_1 and insulin requirement, but these changes are obviously not associated with the levels of ICA.

These results are in agreement with immune intervention trials with cyclosporin A [10]. While cyclosporin A reduced the prevalence and mean titers of ICA in individual patients, anti-CD4/prednisolone did not have such an effect. Furthermore, the continued presence of autoantibodies together with the formation of IAb in both groups indicates that anti-CD4/prednisolone does not suppress the humoral response either to self islet antigens or to exogenous insulin. Using azathioprine and prednisolone for 1 year, others reported satisfactory metabolic outcomes – 3 of 20 immunosuppressed patients were insulin independent at 1 year [16] – whereas another trial with azathioprine alone achieved good metabolic control but no sustained C-peptide responses [17]. While in the former trial, a decreased titer of ICA was associated with a favorable response to intervention, in the latter, as in our present pilot study, the frequency of ICA and insulin antibodies did not differ between the azathioprine-treated or placebo group. These results suggest that generalized immunosuppression or semi-specific immunotherapy at diabetes onset seem to have only minimal effects, if at all, on humoral anti-islet cell reactions.

As we have previously reported [11], the patients improved their metabolic control regardless of the treatment regimen. This was certainly due to the initial intensive in-

Table 1 Changes in biochemical parameters and T-cell activation in anti-CD4/prednisolone-treated and control new-onset insulin-dependent diabetes mellitus patients at 12 months (+ increase, – decrease)[a]

Biochemical parameters	Control group ($n = 6$)	Treatment group ($n = 6$)
Stimulated C-peptide (nmol/l)	+0.23 ± 0.30	−0.14 ± 0.44
Glycated hemoglobin A_1 (%)	−5.24 ± 3.18	−3.72 ± 3.32
Insulin requirement (U/kg per day)	−0.21 ± 0.12	−0.13 ± 0.41
T-cell activation (% $CD3^+HLA\text{-}DR^+/CD3^+$)	+8.45 ± 7.94	+6.50 ± 10.1

[a] Values represent mean ± SD

sulin therapy administered to both groups. It is interesting, however, that 2 patients from the treatment group have maintained their increased stimulated C-peptide levels beyond the 1-year follow-up period (for 2.5 years so far), despite the persistence of ICA, GAD_{65}-AAb, and activated T-lymphocytes.

The present results do not support previous suggestions that short-term treatment with anti-CD4 T-cell mAbs might be preferable to cyclosporin A in inducing remission of IDDM in man. Although we have recently shown that anti-CD4 treatment caused a transient depletion of $CD4^+$ cells in treated patients and modulated the relative CD4 antigen density on residual cells [11], it was obviously not sufficient to inhibit T-helper cell-dependent activation of the autoantibody-producing B lymphocyte clones for a long time. As in various other autoimmune diseases which have been treated with anti-CD4 mAbs [4–7], we found no long-term benefits after short-term treatment. It should be emphasized that the short period of treatment may account for the lack of an effect, but other predictors of the immune response need to be identified to maximize the risk-to-benefit ratio of long-term anti-CD4 immune therapy. In addition to the duration of treatment, onset of treatment, antibody dose, and epitope specificity of the antibody should be investigated as variables in future animal experiments.

Acknowledgements We thank Edeltraud Schallock and Martina Strebelow for skilled technical assistance. This work was supported by grants from the KAI-WIP program (projects no. 018293/M and 020120/M) of the Federal Government of Germany and by the BMFT (project no. 01ZU8607).

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