Clin Exp Immunol 1997; **107**:341–346

21-hydroxylase autoantibodies in adult patients with endocrine autoimmune diseases are highly specific for Addison's disease

A. FALORNI*†, S. LAURETI*, A. NIKOSHKOV†, M. L. PICCHIO*, B. HALLENGREN‡, C. L. VANDEWALLE§, F. K. GORUS§, BELGIAN DIABETES REGISTRY¶, C. TORTOIOLI*, H. LUTHMAN†, P. BRUNETTI* & F. SANTEUSANIO* *Department of Internal Medicine and Endocrine & Metabolic Sciences, University of Perugia, Perugia, Italy, †Department of Molecular Medicine, Karolinska Institute, Stockholm and ‡Department of Endocrinology, University of Lund, Malmö, Sweden, §Department of Metabolism and Endocrinology, Diabetes Research Centre, Vrije Universiteit Brussel and ¶BDR, Brussels, Belgium

(Accepted for publication 23 September 1996)

SUMMARY

The diagnostic specificity of recombinant 21-hydroxylase autoantibodies (21OH-Ab) for Addison's disease was tested in adult patients with either Graves' disease (GD), insulin-dependent diabetes mellitus (IDDM), or polyendocrinopathy, as well as in healthy controls. Using a radiobinding assay with *in vitro* translated recombinant human 21-hydroxylase, we found 21OH-Ab in 24/28 (86%) idiopathic Addison patients, and using an immunofluorescence assay we found adrenal cortex autoantibodies (ACA) in 12/28 (43%) patients ($P = 0{\cdot}002$). All the 12 ACA-positive sera were also positive for 21OH-Ab and ACA were found in 11/15 (73%) patients with less than 15 years and in 1/13 (8%) patients with 15–38 years of disease duration ($P = 0{\cdot}002$). 21OH-Ab were present in 3/92 (3%) patients with GD, in 1/180 (0·6%) with IDDM and in 0/106 healthy subjects. The 21OH-Ab-positive GD and IDDM patients were also positive for ACA. None of 17 patients with polyendocrinopathy, but without Addison's disease, had 21OH-Ab. None of the 180 Belgian IDDM patients had Addison's disease or developed an adrenal insufficiency at follow up. In two out of three Graves patients, the presence of 21OH-Ab was associated with clinical and biochemical signs of adrenal insufficiency. Of the 89 21OH-Ab-negative patients with GD none had Addison's disease at the time of blood sampling, and 79 were followed up for 5·6–7·5 years and none developed clinical signs of adrenal insufficiency. We conclude that the presence of 21OH-Ab in patients with endocrine autoimmune diseases is highly specific for Addison's disease.

Keywords adrenal autoantibodies Addison's disease polyendocrinopathy radioimmunoassay recombinant autoantigen

INTRODUCTION

A large body of evidence supports the hypothesis that, in Western countries, most cases of idiopathic Addison's disease are caused by autoimmune destruction of the adrenal cortex [1,2]. Although the prevalence of Addison's disease is very low, only 39–60 per million individuals [3,4], the observation that acute adrenal insufficiency associated with mononuclear cell infiltration of the adrenal cortex may be life-threatening [5] strengthens the importance of developing simple and reliable diagnostic assays to identify accurately individuals at high risk for Addison's disease. This is particularly important in the clinical management of adult patients with endocrine autoimmune disorders often associated with Addison's disease, such as Hashimoto's thyroiditis, Graves' disease (GD), insulin-dependent diabetes mellitus (IDDM), or premature menopause [4,6–8]. In this context, it is noteworthy that about 50% of idiopathic Addison patients have a concomitant autoimmune endocrine disease (this combination is referred to as autoimmune polyendocrine syndrome (APS)) [7,9].

The enzyme steroid 21-hydroxylase (21OH) is a major adrenal autoantigen in Addison's disease [10,11], and we [12] and others [10,13,14] have shown that 21OH autoantibodies (21OH-Ab) are present in 70–90% of idiopathic Addison patients.

Because of the high diagnostic sensitivity (percentage of antibody-positive patients) of 21OH-Ab for Addison's disease, this immune marker can be useful for predicting adrenal insufficiency in endocrine autoimmune patients. However, because of the low prevalence of Addison's disease in patients with endocrine autoimmune diseases, the diagnostic specificity (percentage of antibody-negative subjects without the disease) must approach nearly 100% for the antibody assay to be highly predictive. Typically,

Correspondence: Alberto Falorni MD, PhD, Department of Internal Medicine and Endocrine & Metabolic Sciences (DiMISEM), Via E. Dal Pozzo, 06126 Perugia, Italy.

prediction of endocrine autoimmune diseases is limited by low specificity of the autoantibody assay used. For example, IDDM-associated autoantibodies occur frequently in other autoimmune diseases [15,16] and only the combination of both genetic and immune markers can guarantee an adequate specificity for the disease [17].

So far, the diagnostic specificity of 21OH-Ab for Addison's disease has not been elucidated in a large number of patients with endocrine autoimmune diseases. Additionally, it is still unclear whether the 21OH-Ab assay should only be used in combination with—or can be used in substitution of—the classical immunofluorescence assay for adrenal cortex autoantibodies (ACA).

In the present study, we therefore evaluated the prevalence of 21OH-Ab in a large group of adult patients with GD or IDDM in addition to healthy individuals, using our recently developed radiobinding assay with full-length recombinant human 21OH [12]. Additionally, a separate group of polyendocrinopathy or polyautoimmune adult patients was studied for presence of 21OH-Ab. To evaluate the predictive value of our 21OH-Ab assay for Addison's disease, the presence of circulating autoantibodies was correlated with the appearance of clinical signs of adrenal insufficiency at follow up. We also compared the prevalence of 21OH-Ab in our group of idiopathic Addison patients with that of ACA detected in an indirect immunofluorescence assay.

PATIENTS AND METHODS

Serum samples

We analysed serum samples from (i) 28 idiopathic Addison patients (age 26–70 years, F/M ratio 0·9; 19 with 'isolated' Addison's disease and nine with APS II) (group 1) previously used to develop our 21OH-Ab assay [12]; (ii) 92 patients with newly diagnosed GD (age 17–85 years, F/M ratio 3·0) (group 2), recruited between May 1988 and April 1990 at the Department of Endocrinology, Malmö University Hospital, University of Lund, Sweden, and previously studied to evaluate the frequency of GAD65 autoantibodies (GAD65Ab) and islet-cell antibodies (ICA) in patients with untreated hyperthyroidism [15]; (iii) 180 patients with newly diagnosed IDDM (age 19–39 years, F/M ratio 0·5) (group 3), recruited by the Belgian Diabetes Registry and previously characterized for prevalence of GAD65Ab, ICA, and insulin autoantibodies (IAA) [18,19] as well as for HLA and insulin genotype [20]; (iv) 17 patients with polyautoimmune/polyendocrine diseases (age 17–85 years, F/M ratio 4·7) (group 4) diagnosed between 1994 and 1995 at the Department of Internal Medicine and Endocrine & Metabolic Sciences, University of Perugia, Italy; (v) 106 healthy subjects (96 Belgians, previously used [19] as part of a control group of the IDDM patients; and 10 Italians, never tested before for presence of autoantibodies) (age 16–55 years, F/M ratio 1·2) (group 5).

The idiopathic Addison patients were selected from a group of patients with primary adrenal insufficiency after exclusion of patients with adrenocortical tuberculosis, or adrenoleukodystrophy or those bilaterally adrenalectomized by surgery. Autoantibody analyses were carried out after clinical diagnosis was made and were not used for patient recruitment.

The serum samples from the Belgian IDDM patients (group 3) used in this study were selected based on the following criteria: of the 747 IDDM patients consecutively reported to the Belgian Diabetes Registry between May 1989 and April 1993, a serum sample at clinical onset and a standard questionnaire with clinical and demographic data filled in by the treating physician were available for 312 patients. Of these 312 patients, serum samples from every patient with an age >18 years ($n = 180$) were studied. The Italian polyautoimmune/polyendocrine patients (group 4) were recruited on the basis of the concomitant presence of at least two autoimmune diseases (one at least being an endocrine disease, other than Addison's disease), with no other exclusion criteria. Among those patients, 12 had Hashimoto's thyroiditis, three GD, 10 IDDM, one early menopause, four vitiligo, one pernicious anaemia, one systemic lupus erythematosus (SLE), and two had alopecia.

Five of the Swedish GD patients also had or later developed IDDM, one had myasthenia gravis and one pernicious anaemia. Among the Belgian IDDM patients, three had GD or hyperthyroidism, one had Hashimoto's thyroiditis, one anti-thyroglobulin antibodies without thyroid clinical symptoms and one Crohn's disease.

Serum samples were kept frozen at −20°C until 1995, when they were analysed for 21OH-Ab.

To test the predictive value of 21OH-Ab for Addison's disease in adult patients with endocrine autoimmune diseases, samples from groups 2 and 3 were analysed blindly, i.e. without knowing which samples (if any) were from patients with Addison's disease.

21OH-Ab assay

21OH-Ab in human serum were determined using a radiobinding assay with *in vitro* transcribed and translated recombinant ^{35}S-21OH in a multiwell-adapted (Millipore Co, Bedford, MA) procedure previously described [12,21]. The full-length cDNA for human 21OH used in this study was a kind gift of Dr Bon-Chu Chung (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan) and was subcloned into pcDNA II (Invitrogen, San Diego, CA) under the control of the SP6 promoter. Autoantibody titres were expressed as a relative index (21OH index) using a 21OH-Ab-positive Addison serum and two 21OH-Ab-negative healthy sera as described elsewhere [12,21]. In sera with 21OH index >0·8, titration of antibody levels was carried out using scalar dilutions (1:25–1:2500) of the serum and the positive and negative standard sera.

The present study used different standard sera than in our initial report [12], and the upper limit of the reference interval for 21OH-Ab was reassessed by analysing a large number of sera from healthy subjects, as well as the same sera from idiopathic Addison patients previously used to develop our assay [12].

Adrenal cortex autoantibodies

ACA were determined in an indirect immunofluorescence assay using cryostatic sections of bovine adrenal glands and following standard procedures [22].

Statistical analysis

Differences in prevalence of 21OH-Ab between different subject groups as well as differences between prevalence of 21OH-Ab and prevalence of ACA were tested with χ^2 analysis with Yate's correction whenever appropriate. Differences in antibody titre (21OH index) were analysed by the non-parametric Mann–Whitney *U*-test.

The positive predictive value of 21OH-Ab for Addison's disease (PPV) was calculated using the formula: $(P \times sens)/((P \times sens) + ((1 - P) \times (1 - spec)))$, where $P =$ prevalence of

Addison's disease, *sens* = diagnostic sensitivity, and *spec* = diagnostic specificity.

RESULTS

Prevalence and titre of 21OH-Ab and ACA in Addison patients and healthy subjects

The 21OH index in 106 healthy subjects was 0·027 ± 0·013 (range −0·02–0·059). In accordance with previous studies with similar autoantibody assays [12,19,21,23] the upper level of normal was defined as the mean +3 s.d. and was 0·066. None of the 106 healthy sera had a 21OH index above the estimated upper level of normal (Fig. 1).

Using this upper level of normal, 24/28 (86%) idiopathic Addison patients were positive for 21OH-Ab (Fig. 1) (the median of the positive samples was 0·620 and the range 0·112–4·550). This frequency is identical to that observed in our previous study [12] that used the same Addison serum samples but different standard sera.

ACA were found in 12/28 (43%) idiopathic Addison patients. This prevalence was significantly lower ($P = 0·002$) than that of 21OH-Ab in the same population. All ACA-positive sera were also positive for 21OH-Ab (Fig. 2). ACA were found in 11/15 (73%) idiopathic Addison patients with <15 years and in 1/13 (8%) patients with 15–38 years of disease duration ($P = 0·002$) (Fig. 2). Interestingly, ACA were found in all patients with 21OH index >0·6 ($n = 12$) and in no patient with 21OH index <0·6 ($n = 16$) ($P < 0·001$). None of 35 healthy subjects was found positive for ACA.

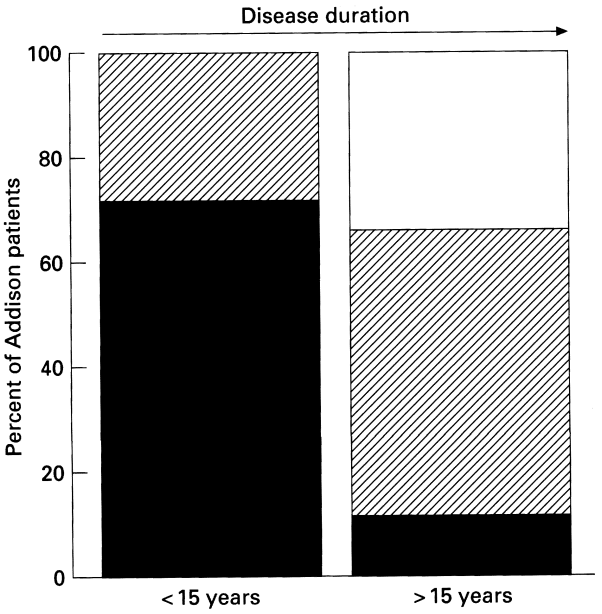

Fig. 2. Prevalence of adrenal cortex autoantibodies (ACA) and/or 21-hydroxylase autoantibodies (21OH-Ab) in idiopathic Addison patients, in relation to disease duration. □, Neither 21OH-Ab nor ACA; ▨, 21OH-Ab only; ■, both 21OH-Ab and ACA.

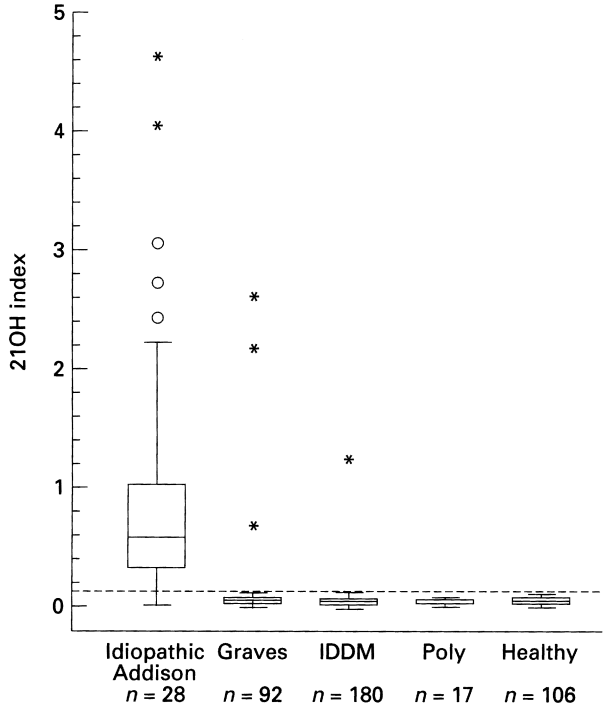

Fig. 1. Box-and-whisker plot of 21-hydroxylase (21OH) index in patients with idiopathic Addison, Graves, insulin-dependent diabetes mellitus (IDDM), or polyautoimmune/polyendocrine diseases (Poly) and in healthy subjects. The upper end of each box is the 75th percentile, and the lower end is the 25th percentile. The horizontal line inside each box is the median. Extreme values (*) are those more than three boxlengths above the 75th percentile or below the 25th percentile, and outliers (○) are those more than 1·5 boxlengths above the 75th percentile or below the 25th percentile. Whiskers are lines from the upper end of the box to the highest observed value or from the lower end of the box to the lowest value that is no outlier. The dotted line shows the upper level of normal in the assay.

Since the diagnostic sensitivity of 21OH-Ab for Addison's disease was higher than that of ACA, serum samples from patients with endocrine (or polyendocrine) autoimmune diseases were first analysed for the presence of 21OH-Ab. Antibody-positive samples were subsequently analysed for the presence of ACA.

Prevalence and titre of 21OH-Ab and ACA in GD, IDDM and polyautoimmune/polyendocrinopathy patients

Among the Swedish GD patients, 3/92 (3%) were positive for 21OH-Ab. The three antibody-positive Graves patients were also positive for ACA and had a 21OH index of 0·698, 2·229 and 2·639, respectively (Fig. 1). Only 1/180 (0·6%) Belgian IDDM patients was positive in our assay, with a 21OH index of 1·211 (Fig. 1). This patient was also found positive for ACA. None of the Italian patients with polyautoimmune/polyendocrine diseases had a 21OH index above the upper level of normal (Fig. 1).

The 21OH index was significantly higher in Addison patients than in GD, IDDM or polyautoimmune/polyendocrinopathy patients ($P < 0·001$ in all comparisons), but not statistically different between healthy controls and either GD or IDDM or polyautoimmune/polyendocrinopathy patients. Similarly, the prevalence of 21OH-Ab was significantly different between GD, IDDM, or polyautoimmune patients and idiopathic Addison patients ($P < 0·001$), but not between GD, IDDM or polyautoimmune/polyendocrinopathy patients and healthy controls. Furthermore, the autoantibody titre in 21OH-Ab-positive patients did not vary significantly among the study groups.

Follow up of GD, IDDM and polyautoimmune/polyendocrinopathy patients

Of the 92 Swedish patients with GD in this study, one 21-year-old male and one 29-year-old female (2%) had Addison's disease at the time of blood sampling, and the male also developed IDDM 2 years later. Both these patients had 21OH-Ab (21OH index 2·229–2·639), with steady titres during a 0·5–1·0 year follow-up period. Further follow-up serum samples were not available. The third GD serum sample positive for 21OH-Ab (21OH index 0·698) was from a 85-year-old woman with pernicious anaemia, but no clinical or laboratory signs of Addison's disease. No follow-up serum samples were available from this patient after her diagnosis with GD. This patient died 4 years later because of cerebrovascular infarction. During the last 4 years of follow up after blood sampling the patient did not show signs of adrenal insufficiency. Of the 89 GD patients without 21OH-Ab, none had Addison's disease at the time of blood sampling and 79 were followed up for 5·6–7·5 years, and none developed adrenal insufficiency during this period.

Assuming that the prevalence of Addison's disease in our population of 92 Swedish GD patients was 2% (2/92), and that the diagnostic sensitivity of 21OH-Ab for Addison's disease would be 100% (2/2) and the diagnostic specificity 98·9% (1/90 21OH-Ab-positive GD patients without Addison's disease), the positive predictive value of our 21OH-Ab assay for Addison's disease in the tested GD population was 66%. When the diagnostic sensitivity observed among the 28 idiopathic Addison patients (86%) was used for the same calculation, the positive predictive value was 63%.

None of the 17 Italian polyautoimmune/polyendocrinopathy patients developed clinical signs of adrenal insufficiency during a 2–12 months follow-up period after the blood sampling for this study. Similarly, none of the 180 adult Belgian IDDM patients either had Addison's disease at the time of blood sampling or developed signs of adrenal insufficiency during a 2·6–6·5 year follow-up period. Accordingly, the diagnostic specificity of our 21OH-Ab assay for Addison's disease in IDDM patients is 99·4% (1/180 antibody-positive patient without Addison's disease). The single 21OH-Ab-positive IDDM patient was a 23-year-old male without other autoimmune or endocrine diseases. The titre of 21OH-Ab remained sustained during the 2 years after onset of IDDM, and the patient did not show clinical signs of adrenal insufficiency in a 5-year follow-up period. Further follow-up serum samples were not available.

In our study, a total of 0/13 patients with Hashimoto's thyroiditis, 1/96 (1%) with GD, 1/194 (0·5%) with IDDM and 1/29 (3%) patients with polyendocrinopathy and/or polyautoimmune diseases was positive for 21OH-Ab in absence of clinical signs of adrenal insufficiency (Table 1).

Table 1. Diagnostic sensitivity and specificity of 21-hydroxylase autoantibodies (21OH-Ab) for Addison's disease in patients with endocrine autoimmune diseases

Diagnosis	Diagnostic sensitivity*	Diagnostic specificity†, %
Addison's disease‡	87% (26/30)	NA§
Graves' disease¶	NA	99 (95/96)
IDDM¶	NA	99·5 (193/194)
Hashimoto's thyroiditis¶	NA	100 (13/13)
Polyautoimmune Polyendocrine¶	NA	97 (28/29)
Healthy	NA	100 (106/106)

*Diagnostic sensitivity of 21OH-Ab is prevalence of Ab-positive patients with Addison's disease.

†Diagnostic specificity of 21OH-Ab is prevalence of Ab-negative subjects without Addison's disease.

‡Total patients with Addison's disease from groups 1, 2 and 3.

§NA, Not applicable.

¶Total patients without Addison's disease from groups 2, 3 and 4.

DISCUSSION

In the present study we show that (i) the presence of 21OH-Ab is a marker at high diagnostic sensitivity for autoimmune Addison's disease, and higher than that of ACA; (ii) ACA and 21OH-Ab have a similarly high diagnostic sensitivity in patients with short–medium disease duration (<15 years), but only 21OH-Ab can be detected in patients with long disease duration (>15 years); and (iii) the presence of 21OH-Ab in patients with GD or IDDM is highly specific for adrenal insufficiency.

In a recent study [12], we demonstrated that 86% of idiopathic Addison sera, and 100% of those from patients with < 20 years of disease duration, were positive for 21OH-Ab. It seemed therefore that the presence of 21OH-Ab is a marker at high diagnostic sensitivity for autoimmune adrenal disease. In the present study we confirmed our previous observation, and we found that ACA could be detected only in patients with a high 21OH index and a short–medium disease duration. Two recent studies [13,14] have tested the frequency of ACA and 21OH-Ab in Addison patients and found a high concordance. However, in those studies no information was given on the correlation between antibody levels and disease duration. Indeed, in our group of patients with < 15 years of disease duration, ACA were present in 73% of cases. This prevalence is similar to the observed prevalence of 21OH-Ab (100%) and to that of ACA in other studies [23]. However, only 21OH-Ab (but not ACA) could be detected at high frequency among Addison patients with long-term disease duration (> 15 years), in our study.

Other adrenal autoantigens have been shown to be the target of autoantibodies in patients with Addison's disease associated with APS I [24,25]. However, 17-hydroxylase-Ab and side chain cleavage enzyme-Ab appear only sporadically in cases of isolated Addison's disease [14,26]. We have found that 21OH-Ab occur as frequently in patients with isolated Addison's disease as in APS II patients [12], and our results can be interpreted to indicate that 21OH is the major target for autoantibodies in Addison's disease, and that the 21OH-Ab assay can be used as an alternative to the classical indirect immunofluorescence technique for ACA.

As a result of the high diagnostic sensitivity and specificity of our radiobinding assay, the predictive value of 21OH-Ab for adrenal insufficiency in GD patients was > 60%. In our study we also determined 21OH-Ab in 180 adult Belgian IDDM patients. As only one subject was found positive, the diagnostic specificity of our assay for Addison's disease was 99·4% in adult IDDM patients. Additionally, in a mixed group of 14 Swedish and Italian polyendocrinopathy patients with IDDM, only the patient with concomitant Addison's disease was positive for 21OH-Ab. The high diagnostic specificity of our autoantibody assay for Addison's disease is also demonstrated by the absence of 21OH-Ab in a group

of 17 Italian polyautoimmune/polyendocrinopathy patients without clinical signs of adrenal insufficiency, as well as in a group of 106 healthy subjects.

The pathogenic role of autoantibodies in Addison's disease is still controversial. 21OH-Ab associated with Addison's disease reduce the enzymatic activity of steroid 21-hydroxylase *in vitro* [27]. However, in a study of subjects with early adrenal dysfunction and adrenal autoantibodies [28], normal levels of 17OH-progesterone were detected, which is inconsistent with a block at the level of 21-hydroxylase. Although the results of our study cannot provide direct information on the pathogenic role of 21OH-Ab, they can be interpreted to demonstrate that the presence of these autoantibodies is strongly associated with autoimmune destruction of the adrenal cortex.

In conclusion, we have demonstrated that the appearance of circulating 21OH-Ab is a highly sensitive and specific marker of adrenal insufficiency. We believe that the use of assays similar to our 21OH-Ab radiobinding assay can be useful for clinical trials for the identification of subjects at high risk for Addison's disease, as well as for improving our understanding of the pathogenic mechanisms of this autoimmune disease.

ACKNOWLEDGMENTS

We wish to thank Dr John Robertson and Professor Åke Lernmark for critical review of this manuscript, Dr Bon-Chu Chung (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan) for the gift of human 21OH cDNA. The following members of the Belgian Diabetes Registry have contributed to the recruitment of patients and control subjects or to the handling of samples for the present study: Edmond Balasse (Brussels), Andre Bodson (Jumet), Jean-Luc Coolens (Hasselt), Willy Coucke (Roeselare), Etienne Couturier (Brussels), Jean-Claude Daubresse (Charleroi), Ivo De Leeuw (Antwerp), Paul Decraene (Bonheiden), Colette Delvigne (Antwerp), Francoise Fery (Brussels), Clementine Gillet (Brussels), Jacques Guiot (Seraing), Bart Keymeulen (Brussels), Georges Krzentowski (Jumet), Gerard Lamberights (Brugge), Michel Letiexhe (Liege), Jan Monballyu (Ekeren), Dany Nicolaij (Kortrijk), Frank Nobels (Aalst), Raoul Rottiers (Gent), Andre Scheen (Liege), Guy Somers (Brussels), Luc Terriere (Hoboken), Kristien Van Acker (Antwerp), Paul Van Crombrugge (Aalst), Luc Van Gaal (Antwerp), Sylva Van Imschoot (Brugge), Patricia Van Rooy (Antwerp), Stefan Vanneste (Zoersel), Jan Vertommen (Antwerp). This study was supported by Swedish Medical Research Council Grants (19X-10408, 19X-09109), Associazione Italiana Lions per il Diabete, the Tore Nilssons Foundation, the Swedish Diabetes Association, the Nordisk Insulin Fund.

REFERENCES

1 Bottazzo GF, Todd I, Mirakian R, Belfiore A, Pujol-Borrell R. Organ-specific autoimmunity: a 1986 overview. Immunol Rev 1996; **94**:137–69.

2 Weetman AP. Autoimmunity to steroid-producing cells and familial polyendocrine autoimmunity. Baillieres' Clinical Endocrinol Metab 1995; **9**:157–74.

3 Stuart Mason A, Meade TW, Lee JAH, Morris JN. Epidemiological and clinical picture of Addison's disease. Lancet 1968; **II**:744–7.

4 Nerup J. Addison's disease—clinical studies. A report of 108 cases. Acta Endocrinol (Copenh) 1974; **76**:127–41.

5 Ringstad J, Rodge S, Loland W, Rode L. Rapidly fatal Addison's disease: three case reports. J Intern Med 1991; **230**:465–7.

6 Irvine WJ, Barnes EW. Addison's disease, ovarian failure, and hypoparathyroidism. Clin Endocrinol Metab 1975; **4**:379–434.

7 Papadopoulos KI, Hallengren B. Polyglandular autoimmune syndrome Type II in patients with idiopathic Addison's disease. Acta Endocrinol (Copenh) 1990; **122**:472–8.

8 Zelissen PMJ, Bast EJEG, Croughs RJM. Associated autoimmunity in Addison's disease. J Autoimmun 1995; **8**:121–30.

9 Bottazzo GF, Doniach D. Polyendocrine autoimmunity: an extended concept. In: Volpe R, ed. Autoimmunity and endocrine disease. New York: Marcel/Dekker, 1985:375–403.

10 Winqvist O, Karlsson FA, Kämpe O. 21-hydroxylase, a major autoantigen in idiopathic Addison's disease. Lancet 1992; **339**:1559–62.

11 Bednarek J, Furmaniak J, Wedlok N *et al.* Steroid 21-hydroxylase is a major autoantigen involved in adult onset autoimmune Addison's disease. FEBS 1992; **309**:51–55.

12 Falorni A, Nikoshkov A, Laureti S *et al.* High diagnostic accuracy for idiopathic Addison's disease with a sensitive radiobinding assay for autoantibodies against recombinant human 21-hydroxylase. J Clin Endocrinol Metab 1995; **80**:2752–5.

13 Colls J, Betterle C, Volpato M, Prentice L, Smith BR, Furmaniak J. Immunoprecipitation assay for autoantibodies to steroid 21-hydroxylase in autoimmune adrenal diseases. Clin Chem 1995; **41**:375–80.

14 Chen S, Sawicka J, Betterle C, Powell M, Prentice L, Volpato M, Rees Smith B, Furmaniak J. Autoantibodies to steroidogenic enzymes in autoimmune polyglandular syndrome, Addison's disease, and premature ovarian failure. J Clin Endocrinol Metab 1996; **81**:1871–6.

15 Hallengren B, Falorni A, Landin-Olsson M, Lernmark Å, Papadopoulos KI, Sundkvist G. Islet cell and glutamic acid decarboxylase antibodies in hyperthyroid patients—at diagnosis and following treatment. J Intern Med 1996; **239**:63–68.

16 Tuomi T, Björses P, Falorni A, Partanen J, Perheentupa J, Lernmark Å, Miettinen A. Antibodies to glutamic acid decarboxylase and insulin-dependent diabetes in patients with APS 1 (autoimmune polyendocrine syndrome type 1). J Clin Endocrinol Metab 1996; **81**: 1488–94.

17 Hagopian WA, Sanjeevi CB, Kockum I *et al.* Glutamate decarboxylase-, insulin- and islet cell-antibodies and HLA typing to detect diabetes in a general population-based study of Swedish children. J Clin Invest 1995; **95**:1505–11.

18 Vandewalle CL, Decraene T, Schuit FC *et al.* Insulin autoantibodies and high titre islet cell antibodies are preferentially associated with the HLA DQA1*0301-DQB1*0302 haplotype at clinical onset of Type 1 (insulin-dependent) diabetes before age 10 but not at onset between age 10 and 40. Diabetologia 1993; **36**:1155–62.

19 Vandewalle CL, Falorni A, Svanholm S, Lernmark Å, Pipeleers DG, Gorus FK, Belgian Diabetes Registry. High diagnostic sensitivity of glutamate decarboxylase autoantibodies in insulin-dependent diabetes mellitus with clinical onset between age 20 and 40 years. J Clin Endocrinol Metab 1995; **80**:846–51.

20 Van Der Auwera B, Schuit F, Lyaruu I, Falorni A, Svanholm S, Vandewalle CL, Gorus FK, Belgian Diabetes Registry. Genetic susceptibility for insulin-dependent diabetes mellitus in Caucasians revisited: the importance of diabetes registries in disclosing interactions between HLA-DQ- and insulin gene-linked risk. J Clin Endocrinol Metab 1995; **80**:2567–73.

21 Falorni A, Örtqvist E, Persson B, Lernmark Å. Radioimmunoassays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies using ^{35}S or ^{3}H recombinant human ligands. J Immunol Methods 1995; **186**:89–99.

22 Betterle C, Scalici C, Presotto F, Pedini B, Moro L, Rigon F, Mantero F. The natural history of adrenal function in autoimmune patients with adrenal autoantibodies. J Endocrinol 1988; **117**:467–75.

23 Falorni A, Grubin CE, Takei I *et al.* Radioimmunoassay detects the frequent occurrence of autoantibodies to the Mr 65,000 isoform of glutamic acid decarboxylase in Japanese insulin-dependent diabetes. Autoimmunity 1994; **19**:113–25.

24 Krohn K, Uibo R, Aavik E, Peterson P, Savilahti K. Identification by molecular cloning of an autoantigen associated with Addison's disease as steroid 17a-hydroxylase. Lancet 1992; **339**:770–3.

25 Winqvist O, Gustaffson J, Rorsman F, Karlsson FA, Kämpe O. Two

different cytochrome P450 enzymes are the adrenal antigens in autoimmune polyglandular syndrome type I and Addison's disease. J Clin Invest 1993; **92**:2377–85.

26 Uibo R, Aavik E, Peterson P, Perheentupa J, Aranko S, Pelkonen A, Krohn KJ. Autoantibodies to cytochrome P450 enzymes P450scc, P450c17, and P450c21 in autoimmune polyglandular diseases types I and II and in Addison's disease. J Clin Endocrinol Metab 1994; **78**:323–8.

27 Furmaniak J, Kominami S, Asawa T, Wedlock N, Colls J, Smith BR. Autoimmune Addison's disease: evidence for a role of steroid 21-hydroxylase autoantibodies in adrenal insufficiency. J Clin Endocrinol Metab 1994; **79**:1517–21.

28 Boscaro M, Betterle C, Sonino N, Volpato M, Paoletta A, Fallo F. Early adrenal hypofunction in patients with organ-specific autoantibodies and no clinical adrenal insufficiency. J Clin Endocrinol Metab 1994; **79**:452–5.