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T and B cell reactivity to adrenal antigens in autoimmune Addison's disease

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SUMMARY

Autoimmune Addison's disease is a rare condition of uncertain pathogenesis. To delineate potential autoantigens, an adrenal homogenate was fractioned by SDS–gel electrophoresis and used in immunoblotting and T cell proliferation assays. Antibodies specific for adrenal proteins with approximate molecular weights of 70, 55 and 45 were found in five out of 20 consecutive Addison's disease patients at routine follow-up. Five sera also reacted with a 52-kD protein shared with liver. T cells from six out of 10 Addison's disease patients proliferated in response to a range of adrenal-specific antigens including one, in particular, with a molecular weight of 18–24 kD. T and B cell reactivity to adrenal antigens did not appear to correlate and there was no relationship with time since diagnosis, associated autoimmunity or HLA-DR type. These results show that the autoimmune response to adrenal antigens in Addison's disease is heterogeneous and that such autoreactivity can only be inconsistently documented using these techniques and circulating antibodies or T cells.

Keywords Addison's disease autoimmunity adrenal gland immunoblotting

INTRODUCTION

Addison's disease is a rare condition (prevalence 30–60 per million in the UK) resulting from adrenal failure. It is caused by autoimmune adrenalitis in about 80% of British patients [1]. In European patients with autoimmune Addison's disease there is a modest association with HLA-DR3 [2,3]. Histology shows adrenal cortical atrophy, with variable degrees of lymphocytic infiltration and fibrosis [4]. However, little is known of the autoimmune mechanisms which produce disease.

It has not been possible to study the infiltrating lymphocytes. A high percentage of circulating activated T cells (as judged by expression of HLA-DR molecules) has been observed in these patients, in common with many other autoimmune disorders [5]. Sensitization of circulating T cells to an adrenal antigen extract has been assessed using two methods: by the migration inhibition assay, reactive T cells were found in 46% of patients [6]; a similar antigen preparation was unable to stimulate T cell proliferation in a blastogenesis assay [7].

Adrenal antibodies have usually been measured by immunofluorescence and have been found in between a half to two-thirds of patients [1,8,9]. Elucidating the nature of the antigen(s) involved has proved difficult, although anatomical localization to the microsomes, plasma membrane or both seems likely [10]. By immunoprecipitation, 57% of patients were found to have antibodies against an adrenal-specific protein of 55 kD, which were also present by immunoblotting [11], although another study using immunoprecipitation reported reactivity against a 38-kD antigen [12].

In view of these conflicting data on T and B cell reactivity in autoimmune Addison's disease, we have performed immunoblotting studies to identify adrenal autoantigens which bind antibodies and stimulate T cell blastogenesis in such patients.

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SUBJECTS AND METHODS

Patients

Sera were collected from 20 consecutive Caucasian patients with Addison's disease (14 women, six men) being followed up in a single endocrine outpatient clinic; peripheral blood mononuclear cells (PBMC) were obtained from the second half of this group ($n=10$). Their mean age was 51 years (range 18–84) and the mean time since diagnosis 13 years (range 1 month–36 years). Four patients had been identified within the preceding year. The diagnosis of autoimmune Addison's disease was based on the simultaneous presence of other autoimmune disorders (15 patients), a strong family history of autoimmunity (one patient), absence of adrenal calcification and exclusion of other potential causes of adrenal failure (three patients), and in the last patient serum had been assayed for adrenal antibodies by immunofluorescence and these were positive. Associated autoimmune disorders included autoimmune hypothyroidism ($n=9$), Graves' disease ($n=4$), type I diabetes mellitus ($n=2$), pernicious anaemia ($n=2$) and premature ovarian failure ($n=1$). Controls were healthy laboratory staff with no personal or family history of autoimmune disorders.

Immunoblotting

An adrenal gland and biopsy from liver were obtained from a kidney donor and immediately snap frozen. The specimens were later thawed and homogenized at 4°C in PBS, pH 7·4, containing protease inhibitors (1 mM TPCK, 1 mM PMSF and 0·5 mM pepstatin A; Sigma, Poole, UK). Debris was removed by centrifugation at 500 *g* for 20 min at 4°C and the supernatants used for electrophoresis after dilution 1 : 1 in sample buffer (0·1 M Tris–HCl, 10% β-mercaptoethanol, 5% SDS; 0·002% bromophenol blue) and boiling for 5 min. SDS–PAGE was carried out as described in detail elsewhere using a 5–15% gradient [13]. The antigen concentration run on gels was determined from preliminary experiments to give optimal loading; by spectrophotometric estimation, using Coomassie blue G250 [14], 8 mg of each antigen preparation were loaded per 30 ml gel (combs 13 cm wide and 1·5 mm thick). Molecular weight standards (Pharmacia, Milton Keynes, UK) in the same sample buffer were run on each gel.

Electrophoretic transfer to nitocellulose paper (NCP) was performed exacly as detailed elsewhere [13], and the transfer was verified by amido black staining of the NCP and Coomassie blue staining of the gel. For immunoblotting with serum, free sites on the NCP were blocked with PBS, 0·05% Tween 20, 3% bovine serum albumin and the paper then cut into strips 0·5 cm wide. Strips were incubated with a 1:20 dilution of serum (in PBS, 0·05% Tween 20) overnight at 4°C, washed and then incubated with anti-human IgG–alkaline phosphatase conjugate (1:500, Sigma) for 2 h at room temperature. After further washing, the strips were incubated with substrate (25 mg *o*-dianoside tetrazotized, 25 mg β-naphthyl acid phosphate, 1·2 mg $MgSO_4$ in 100 ml 0·06 M sodium borate, pH 9·7) according to the method of Dao [15]. Bands were determined by direct inspection.

T cell proliferation assay

PBMC were isolated by Ficoll–Hypaque gradient centrifugation and incubated at 10^5 cells/well (96-well flat-bottomed plates) in RPMI 1640 containing 10% fetal calf serum (GIBCO, Paisley, UK). Fractioned adrenal and liver antigens were prepared using the method of Abou-Zeid *et al.* [16]. Briefly, antigens were electrophoresed and transferred to NCP as described above and then the NCP was divided into 4-mm band fractions by cutting into 14 pieces, each 4 mm deep and 10 mm wide. The NCP pieces were dissolved in dimethyl sulphoxide and resuspended, in particulate form, by the drop-wise addition (with vortexing) of 1 ml carbonate buffer, pH 9·6. The fractioned antigen suspensions were added in triplicate at 20 μl/well to the PBMC. NCP without antigen was used as a control. After 4 days of culture ^{3}H-thymidine was added at 1 μCi/well and the cells harvested onto filters 16 h later. ^{3}H-thymidine incorporation was assessed by liquid scintillation counting and T cell proliferation expressed as a stimulation index (SI) according to the formula

$$SI = \frac{\text{ct/min produced by antigen on NCP}}{\text{ct/min produced by NCP alone}}$$

HLA-DR typing

This was performed by restriction fragment length polymorphism analysis exactly as described elsewhere [3].

Statistical analysis

Differences between groups were analysed using 2×2 contingency tables and the χ^2 test (two-tailed).

RESULTS

Immunoblotting

All 10 control and 20 Addison's disease sera reacted strongly with three distinct adrenal components with approximate molecular weights of 100 kD, 75 kD and 50 kD (Fig. 1). In addition, weaker reactivity was seen with a doublet at 120 kD and another at 30 kD. Several sera from Addison's disease patients (but none from the controls) reacted with additional components: five sera reacted with a protein at 52 kD, three with a protein at 45 kD and single patients each reacted with proteins at 55 or 70 kD. The patient and control sera all reacted with liver components at 120, 100, 75, 50 and 30 kD; the five sera that reacted with a protein at 52 kD in the adrenal preparation also reacted with a similar component in the liver immunoblot; the additional bands seen with the remaining five sera were unique to the adrenal immunoblot. These five sera all came from women whose disease had been diagnosed 6 months, 1 year, 14 years ($n=2$) and 36 years previously; two had autoimmune hypothyroidism (with diabetes in one), one Graves' disease and another pernicious anaemia. Of note, two patients whose disease had been diagnosed in the preceding month did not have antibodies reacting in the immunoblot. Thirteen of the present patients were tested for adrenal antibodies by indirect immunofluorescence at time of diagnosis and seven (54%) were positive. Two of these positive samples reacted in the immunoblotting experiment, with bands at 55 kD and 45 kD. The five patients who did not react by immunoblotting had all been diagnosed 1–6 years previously.

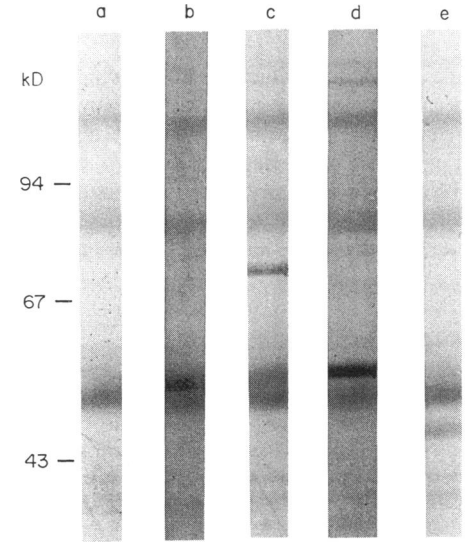

Fig. 1. Representative immunoblots of sera against adrenal antigen Lane a, control serum; lanes b–e, individual Addison's disease ser showing extra bands at 52 kD (lanes b and c), 70 kD (lane c), 55 kD (lan d) and 45 kD (lane e). Molecular weights of standards are shown.

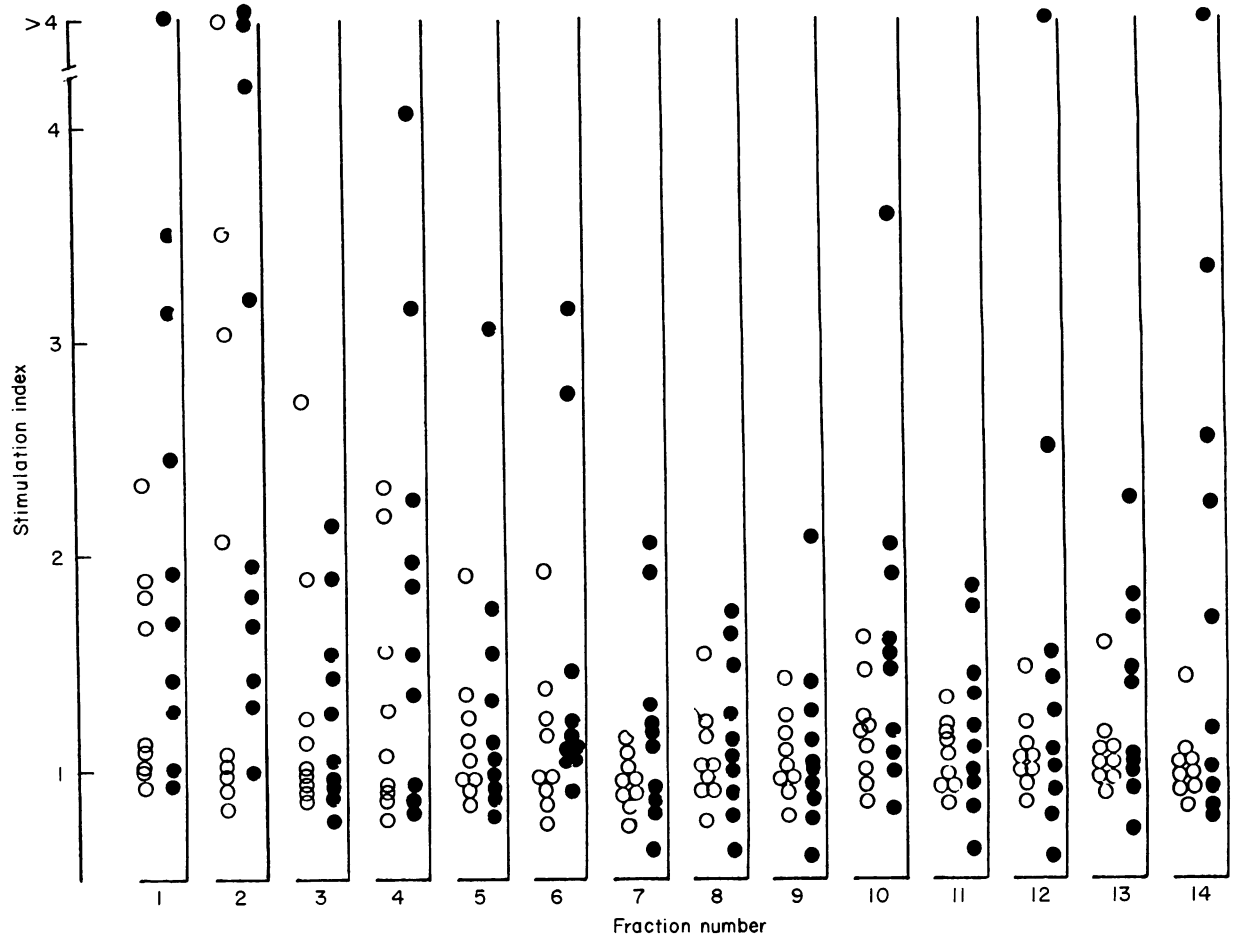

Fig. 2. T cell proliferation in response to 14 fractions of adrenal antigen. Proliferation is shown as a stimulation index; the open circles are individual control PBMC cultures and the closed circles are PBMC cultures from patients with Addison's disease.

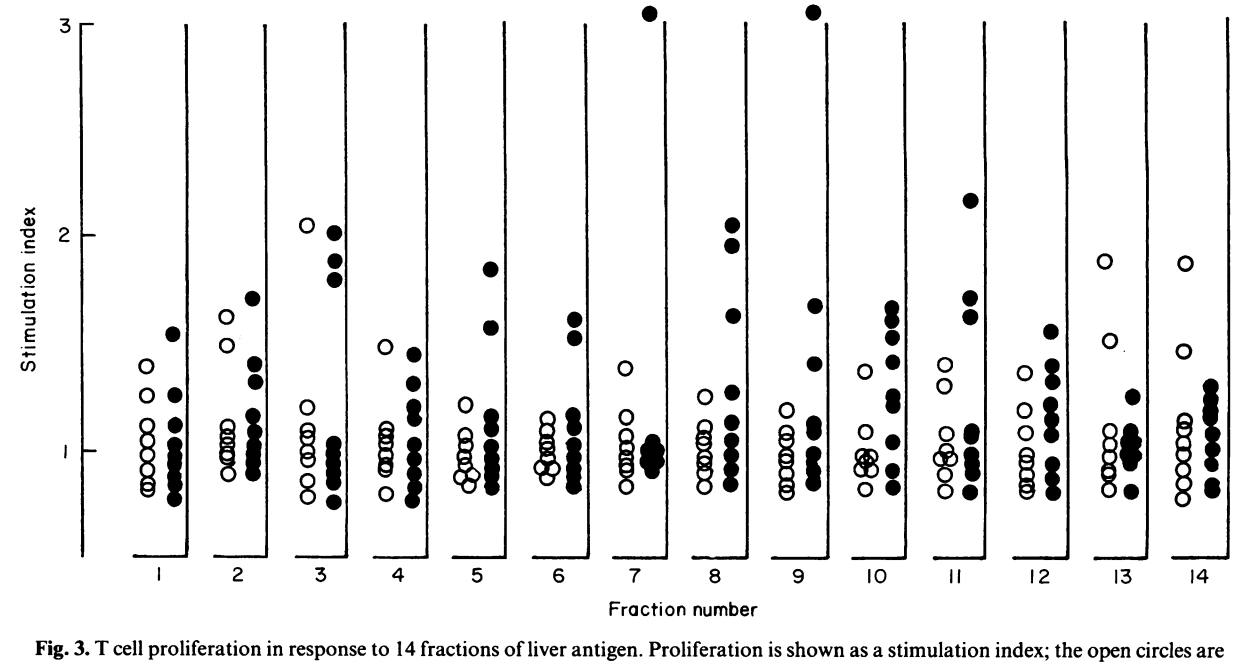

Fig. 3. T cell proliferation in response to 14 fractions of liver antigen. Proliferation is shown as a stimulation index; the open circles are individual control PBMC and the closed circles are PBMC cultures from patients with Addison's disease.

Table 1. Details of patients with Addison's disease and their PBMC proliferative responses to adrenal antigen fractions 5–14

Patient no.	Associated disease	Time since disease (year)	HLA-DR type	Fractions giving SI > 2·0*	Fractions giving SI > 3·0	Immunoblot reactivity (kD)†
1	GD	5	2, 3	10, 12, 14	10, 12, 14	Nil
2	AH, PA	29	2, 3	12, 14	12	Nil
3	POF	0·1	3, 4	14	14	Nil
4	GD	15	3, 4	5, 6	5, 6	70, 52
5	AH	31	3, 3	10, 14	0	45
6	PA	3	2, 3	7	0	52
7	AH, DM	1	3, 4	0	0	45
8	Nil	5	2, 3	0	0	52
9	GD	15	3, 3	0	0	Nil
10	Nil	25	3, 4	0	0	52

GD, Graves' disease; AH, autoimmune hypothyroidism; PA, pernicious anaemia; POF, premature ovarian failure; DM, type I diabetes mellitus; SI, stimulation index.

* Approximate molecular weights of fractions giving a positive response: 5, 65–71 kD; 7, 54–60 kD; 10, 36–42 kD; 12, 30–36 kD; 14, 18–24 kD.

† Molecular weights of bands giving reactivity with serum antibodies by immunoblot: the 52-kD reactivity was shared with liver.

T cell proliferation

The results of T cell proliferation in response to fractionated adrenal and liver antigens are shown in Figs 2 and 3. Because the two gels were run simultaneously, the molecular weights of the proteins in each 4-mm fraction of the NCP were similar; the 14 fractions represent a range of molecular weights from 90 kD, in 6 kD decrements. It can be seen that there was a broad range of reactivity to the adrenal fractions in controls and patients; in particular, fractions 1–4 stimulated PBMC from both groups. However, lower molecular weight fractions (numbers 5, 6, 7, 10, 12 and 14) only stimulated the Addison's disease PBMC.

PBMC from six patients with Addison's disease responded with a SI > 2·0 to one or more of these fractions, a significant difference from the lack of any response > 2·0 in the nine controls ($\chi^2 = 7{\cdot}01$, $P < 0{\cdot}01$). Four of Addison's disease patients had PBMC which gave SI > 3·0 with one or more of the adrenal fractions 5–14 ($\chi^2 = 5{\cdot}46$, $P < 0{\cdot}05$ compared with controls). These results and the patients' characteristics are shown in detail in Table 1. There were no striking differences between responders and non-responders in terms of disease duration, associated autoimmunity or HLA-DR type, although both of the patients tested with no associated autoimmune endocrinopathy failed to respond. There was also no correlation with the presence of antibodies detected by immunoblotting.

By contrast, there was little T cell reactivity against liver antigens (Fig. 3) which was assessed using PBMC from eight of the controls and nine of the patients; there were not sufficient PBMC available from the other two subjects. One each of the controls and patients gave proliferative responses greater than 2·0 to liver fraction 3 (these also gave the highest responses to adrenal fraction 3). Another patient (number 7 in Table 1) showed responsiveness to fractions 7, 8, 9 and 11. There was no T cell response against adrenal antigens in this diabetic patient, who had normal liver function tests.

DISCUSSION

These results demonstrate a heterogeneous T and B cell response to adrenal antigens in autoimmune Addison's disease. Adrenal-specific antibodies were detected by immunoblotting in five (25%) patients, although five others reacted with a 52-kD protein shared with liver; this may represent reactivity against a cytoskeletal component such as desmin [11]. In one previous study, an unstated number of Addison's disease sera were said to precipitate an adrenal-specific antigen of 38 kD [12]; whether this in fact corresponds to the band observed at 45 kD in three of our subjects is not clear. It is possible that immunoblotting may fail to identify antibodies that do not react with denatured antigen, in contrast to immunoprecipitation, but this does not appear to be the case for the 55-kD antigen reported previously as sera recognized this by both techniques [12]. Only one of the 20 present sera reacted with an adrenal component at 55 kD, in contrast with 57% out of 23 sera tested in that study. Immunoblotting was chosen for the present study, as immunoprecipitation also has limitations, and direct comparison between the techniques in analysing antibody responses to infection has shown that immunoblotting reveals a much wider spectrum of antibody response [17].

The reasons for this discrepancy are unclear, but in previous studies the procedure for patient selection is not stated. The differences in this and the time since diagnosis may be critical in determining the frequency of autoantibodies detected. However, the present series of consecutive clinic follow-up patients included four subjects in whom the diagnosis was made within a year of their sera being tested and only two of these had antibodies that reacted with the 45-kD (but not 55-kD) protein. Moreover, adrenal antibodies detected by immunofluorescence disappear slowly, becoming undetectable in only 17% of patients within 5 years, and may persist for at least 20 years [9

There was no obvious correlation between immunofluorescence results and binding by immunoblotting in the experiments we performed. This may have been due to the titre of antibody present. Indirect immunofluorescence assays for adrenal antibodies are often negative in autoimmune Addison's disease (approximately 30–40% of patients) and generally require high concentrations of serum [1,2]. Using higher serum concentrations in the immunoblot experiments created a very high background and was therefore not pursued. It thus seems likely that differences in sensitivity account for the varying frequencies of antibodies reported, although this does not explain the more heterogeneous B cell response observed in the present study in which three specific adrenal proteins (70, 55 and 45 kD) were found to react with Addison's disease sera.

T cell sensitization to adrenal antigens was found more frequently than antibodies (60% of patients with a SI > 2·0; 40% > 3·0) and could be detected 0·1–31 years after diagnosis. Once again there was heterogeneity in the antigens recognized, although fraction 14 (molecular weight 18–24 kD) produced a positive response in four out of the six responders. These proliferative responses appeared to be adrenal antigen-specific as fractions of liver antigen failed to produce blastogenesis in parallel cultures of PBMC from the Addison's disease patients; one unique response against liver antigens was observed in a patient with concurrent autoimmune hypothyroidism and type I diabetes mellitus.

These results confirm and extend previous studies on T cell sensitization in Addison's disease using a migration inhibition assay; 46% of patients gave positive responses and these did not correlate with the presence of adrenal antibodies [6]. They also show, in contrast to a previous investigation [7], that the proliferation assay can be used to detect T cell responses to adrenal antigens. It is possible that the fractionated (and thus semi-purified) antigen preparation used, together with its presentation in particulate form, may have enhanced the sensitivity of this assay in the present study.

We have detected heterogeneous T and B cell responses in autoimmune Addison's disease: several separate adrenal antigens react with autoantibodies and stimulate circulating T cells in these patients. Knowledge of the number and molecular weights of these proteins is an important step is identifying the nature of the key autoantigens in this condition. The lack of T or B cell responses in some patients may be related to localization of the autoreactive lymphocyte population within the target organ; there is a precedent for this in seronegative Hashimoto's thyroiditis [18].

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