Adrenal and ovarian autoimmunity

C Betterle and M Volpato
Institute of Semeiotica Medica, Chair of Clinical Immunology and Allergy, University of Padova, Via Ospedale Civile 105, 35128 Padova, Italy
(Correspondence should be addressed to C Betterle)

Autoimmune adrenal insufficiency

Addison’s disease, described first by Thomas Addison (1), is quite rare and has been described in 40–110 cases per 1 000 000 inhabitants (2–4). Currently, in the developed countries the most common cause of primary adrenal failure is autoimmunity, causing up to 75–80% of the cases of adrenal insufficiency, while tuberculosis is the second most frequent cause, responsible for up to 15–20% of the cases. Many other rare causes are involved in 5–7% of patients (5, 6). Consequently, autoimmune Addison’s disease (AAD) can be considered as a synonym of the idiopathic form of adrenal failure.

About 50–60% of AAD patients have or develop during their life other autoimmune disorders; in the remaining cases the adrenal failure manifests itself as an isolated disease (7).

Four main types of polyglandular autoimmune disease (PGAD) have been classified by Neufeld & Blizzard (8) and AAD is a fundamental disease of PGAD types 1 and 2. AAD can present in three main clinical forms: (i) PGAD type 1, (ii) PGAD type 2, (iii) isolated (9–11).

PGAD type 1

PGAD type 1 consists of chronic hypoparathyroidism, chronic candidiasis and AAD. PGAD type 1 has also been called autoimmune poly-endocrinopathy, candidiasis, ectodermal dystrophy (APECED) (12).

To define this syndrome, at least two of these major components need to be present (8, 13, 14). The three main diseases occur, in general, in a fairly precise chronological order, and they are present all together only in one-third of the observations (12, 14, 15). Candidiasis is usually the first manifestation to appear, mostly before the age of 5, followed by hypoparathyroidism, usually before the age of 10, and later by Addison’s disease, before the age of 15. Thus, PGAD type 1 occurs for the most part in childhood and the complete evolution takes place in the first 20 years of life (8, 12, 14, 15). In addition, other minor components, often appearing later in life, can be present such as: (i) autoimmune endocrinopathies (hypergonadotropic hypogonadism, insulin-dependent diabetes mellitus, autoimmune thyroid diseases); (ii) autoimmune or immuno-mediated gastrointestinal diseases (chronic atrophic gastritis, pernicious anemia, coeliac disease); (iii) chronic active hepatitis; (iv) autoimmune skin diseases (vitiligo, alopecia); (v) ectodermal dystrophy; (vi) keratoconjunctivitis; (vii) immunological defects (T cell defect, IgA deficiency, asplenia) (8, 12–14, 16). PGAD type 1 is a very rare disorder in the world except in Finland where, probably as a result of a founder gene effect, the estimated frequency is about 1/25 000 inhabitants. The ratio of females/males varied from 0.8 to 2.4 in the different studies (8, 12, 14). PGAD type 1 is a condition occurring sporadically or among siblings (15–18). The mode of presentation argues for an autosomal recessive inheritance (17, 19). In the initial studies association with human leukocyte antigen (HLA)-A28 was found to be more frequent in patients with this syndrome than in normal controls, and association with HLA-A3 was more frequent in those with PGAD type 1 and ovarian failure than in those with normal ovarian function (20). A study of 14 Finnish families with PGAD type 1 has shown that the gene responsible for this condition is located on chromosome 21 (21). Recently, we found a high frequency of association with HLA-DR5, with a relative risk of 2.85 (14).

PGAD type 2

PGAD type 2, also known as Schmidt’s syndrome, is characterized by the constant presence of AAD and either thyroid autoimmune disease and/or insulin-dependent diabetes mellitus (IDDM) (7, 8, 13, 22, 23). In addition, other diseases may be present such as hypergonadotropic hypogonadism, vitiligo, alopecia, chronic atrophic gastritis with or without pernicious anemia, and hypophysitis (13).

IDDM, when present, develops before Addison’s disease, whereas thyroid autoimmune disease can develop before, contemporary with or after adrenal insufficiency (7, 23). PGAD type 2 occurs in about 15–45 cases per 1 000 000 inhabitants, at all ages and in both sexes, but it is most common in middle-aged females (7, 11). PGAD type 2 most often occurs in many generations of the same family, and shows a pattern of inheritance consistent with autosomal
Adrenal and ovarian autoimmunity

The natural history of AAD progresses through 4 different stages of adrenal cortex hypofunction; in stage 1, an increase in plasma renin activity is associated with a normal or low serum aldosterone level, which suggests that, initially, the zona glomerulosa is affected. After several months or years, a dysfunction of the zona fasciculata becomes evident which is manifested at first by a decrease of the plasma cortisol response to adrenocorticotropic hormone (ACTH) (stage 2), followed by an increased plasma ACTH level (stage 3) and finally by a decreased plasma cortisol level and overt clinical symptoms (stage 4) (34). Subsequently, it has been reported that ACA in some patients without clinical AAD can disappear spontaneously or after corticosteroid therapy with restoration of the previous impaired adrenal function (35). However, recent follow-up studies of 58 ACA-positive patients (children and adults) indicated that ACA were persistent markers of progression towards overt or subclinical AAD (31, 32). The risk of progression to AAD was high in the group of ACA-positive children (cumulative risk of 100% at 11 years) (32) compared with a lower risk in the group of ACA-positive adults (cumulative risk of 31.6% at 11 years) (31). In the case of ACA-positive adults the presence of a high risk of progression to clinical AAD (33). The natural history of AAD progresses through 4 different stages of adrenal cortex hypofunction; in stage 1, an increase in plasma renin activity is associated with a normal or low serum aldosterone level, which suggests that, initially, the zona glomerulosa is affected. 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titer of antibodies and HLA-DR3 were additional risk factors for clinical AAD (31).

**Steroid-producing cells autoantibodies**

In addition to ACA, some of the patients showed a reaction with cytoplasmic antigens common to other steroid-producing cells such as Leydig cells of the testis, theca cells of the ovary and syncytiotrophoblasts of the placenta. These antibodies are called steroid-producing cells autoantibodies (36). There is a strong association between steroid cell autoantibodies and ACA, and the former can only be detected in the presence of ACA. Steroid cell autoantibodies are polyclonal IgGs that can be distinguished from ACA by preabsorption tests with homogenates of any of the steroid-producing target organs which remove steroid cell autoantibodies reactivity, whereas ACA recognize exclusively the adrenal gland (37). The prevalence of steroid cell autoantibodies varied in different studies and in ACA-positive patients with AAD they were found in 26% of the women and 4% of the men (38), compared with 60–100% of patients with PGAD type 1, 25–40% of patients with PGAD type 2 and 18% of patients with isolated AAD (6, 7, 11, 39). The presence of steroid cell autoantibodies generally correlated with the presence of primary gonadal failure (hypergonadotropic hypogonadism) (7, 39, 40–42). The high prevalence of steroid cell autoantibodies in PGAD type 1 presumably explains why gonadal failure more frequently occurs in patients with this syndrome compared with those with PGAD type 2 (10). In female patients without gonadal failure, the presence of steroid cell autoantibodies confers a high risk of progression towards clinical hypergonadotropic gonadal failure (predictive value 30%) (43, 44).

**ACTH-receptor autoantibodies**

Some autoantibodies have the ability to bind to the cell receptor and affect their function either through mimicking normal ligand action or blocking the ligand binding site on the receptor as reviewed by Wilkin (45). Serum IgG fraction from a woman with AAD was reported to block the ACTH-induced release of cortisol from guinea pig adrenal cells in vitro. This study suggested that adrenal cortex cell function could be diminished as a result of autoantibody binding to the ACTH receptor (46). IgGs capable of inhibiting both ACTH-induced adrenal DNA synthesis and cortisol production by guinea pig adrenal segments were reported in over 90% of the patients with clinical AAD (47). However, a subsequent study performed on guinea pig adrenal cells did not confirm these results, and the inhibitory effects seemed most likely to be attributable to the effects of non-IgG components of the IgG preparations (48). At present, the existence of ACTH receptor-blocking autoantibodies is still under discussion and needs further investigation.

**Adrenal autoantigens**

Investigations on target autoantigens in AAD were initiated by the identification of a microsomal antigen of 55 kDa (49). This was followed, in 1992, by the identification, independently in two laboratories, of steroid 21-hydroxylase (21-OH) as a major adrenal autoantigen (50–52). In the same year screening of a human fetal adrenal cDNA library with the sera from patients with AAD in the context of PGAD type 1 allowed isolation of clones with high homology to steroid 17α-hydroxylase (17α-OH) and this study indicated that 17α-OH was an autoantigen associated with the AAD in the context of PGAD type 1 (53). Reactivity of sera from patients with PGAD type 1 with cytochrome P450 side chain cleavage enzyme (P450scc) was reported soon afterwards (52). Reports on identification of 21-OH as a major adrenal autoantigen were confirmed by studies in several laboratories using different methods, including Western blotting or immunoprecipitation analyses based on native or recombinant 21-OH expressed in bacteria, yeast, mammalian cells or in vitro transcription/translation system (54–57). In addition, recent studies have shown that 21-OH is the major adrenal autoantigen in AAD irrespective of whether the disease presents as isolated AAD or PGAD type 1 or type 2 or in ACA-positive patients without overt AAD (58, 59). Furthermore, a good agreement between the results of ACA (measured by indirect immunofluorescence) and 21-OH autoantibodies (Abs) indicates that 21-OH Abs are the major component of the ACA (56–60). Consequently, 21-OH/ACA have been shown to be markers of progression towards overt AAD in ACA-positive adults and children as described above (31–32).

There were, however, some discrepancies in the different studies regarding the reactivity and the prevalence of autoantibodies to 17α-OH and P450scc in patients with PGAD type 1, type 2 or isolated AAD (52, 53, 55, 57, 58, 61). The reasons for these differences could be related, at least in part, to the sources of autoantigens used to detect autoantibodies (62). A recent study by Chen et al. showed that 17α-OH and P450scc Abs in patients with PGAD type 1, type 2, isolated AAD, and ACA-positive patients without overt AAD were found at prevalences lower than 21-OH Abs (58). Furthermore, 32/33 sera (97%) which were positive for 17α-OH and/or P450 scc Abs were also positive for 21-OH Abs. Following comparison with steroid cell autoantibodies, 17α-OH and P450scc appear to be the major components of steroid cell autoantibodies measured by indirect immunofluorescence (58). Reactivity against other steroidogenic enzymes has been studied but none of the sera from patients with AAD was found to react with 11β-hydroxylase (11β-OH) and 3β-hydroxysteroid dehydrogenase (3β-HSD) (54, 57, 63), aromatase and adrenodoxin (57).
Studies relating to the autoepitopes recognized on 21-OH by sera from patients with AAD were performed by Western blot analysis using 21-OH expressed in an in vitro transcription/translation system, in bacteria or yeast: 90% of the sera reacted with the central portion (residues 165–379) of the protein and the majority of them identified an epitope located between residues 281–379, where the proposed steroid binding site is located (54, 64, 65). Furthermore, half of the sera reacted with the central and C-terminal portion (residues 380–494). Also, 21-OH Abs reacted in a markedly weaker fashion with 21-OH containing amino acid mutations within the central and the C-terminal, regions, which are associated with diminished 21-OH enzyme activity (59, 65). Overall, studies using modified 21-OH proteins containing amino acid deletions or single amino acid mutations indicate that autoantibody epitopes on human 21-OH are conformational and are formed by central and C-terminal parts of the molecule and suggest a close relationship between 21-OH amino acid sequences important for 21-OH enzyme activity and the autoantigen binding site(s). The study of the patients with different forms of AAD, either isolated or in the context of PGAD types 1 and 2 or with subclinical or potential AAD, confirmed these observations and did not demonstrate significant differences between the epitopes recognized by 21-OH Abs in different patient groups (66).

**Enzymes and corresponding autoantibodies in the pathophysiology of AAD and other autoimmune diseases**

The three enzymes recently recognized as target autoantigens in AAD are members of the cytochrome P450 family, which is located in the endoplasmic reticulum; its activity depends on NADPH cytochrome P450 reductase, which is not expressed on the cell surface. Of the three enzymes, only 21-OH is adrenal-specific, 17α-OH being expressed in adrenals and in gonads, and P450scc is present in adrenals, gonads and placenta. These enzymes are involved in the synthetic pathway of glucocorticoids, mineralocorticoids and sex hormones (28, 60, 67). The zona glomerulosa is the major source of mineralcorticoids, the zona fasciculata and the zona reticularis are thought to act as a functional unit in the production of cortisol and androgens (28).

The demonstration that steroidogenic enzymes are target autoantigens in AAD supported the hypothesis of a role of these autoantibodies in the pathogenesis of the disease. An in vitro study demonstrated that IgGs from patients with AAD and antibodies to 21-OH were able to inhibit 21-OH enzyme activity (68). In an attempt to confirm these data in vivo, patients with antibodies to 21-OH and clinical or subclinical AAD were stimulated with ACTH i.v., but no increment of 17-hydroxypregesterone was found, indicating a lack of the inhibiting effect of these autoantibodies in vivo (69). This may be consistent with the report that the passive transfer of IgG from the pregnant mother with AAD to the fetus has never been shown to induce a transient hypoadrenalism.

**New tests for adrenal autoantibodies**

ACA have been detected by indirect immunofluorescence using different animal and human adrenal cortex sections, and by ELISA and RIA methods using adrenal microsomes. However, only the indirect immunofluorescence has been a reliable technique for many years, as reviewed by Betterle et al. (6). Recently, the laboratory diagnosis of AAD has been enriched by use of specific recombinant autoantigens. Two different immunoprecipitation assays for the detection of autoantibodies to 21-OH (21-OH Abs) have been developed, one based on 35S-labeled 21-OH produced in an in vitro transcription-translation system (56, 70), the other based on 125I-labeled recombinant 21-OH produced in yeast (59). Immunoprecipitation assays based on 35S-21-OH are highly specific and sensitive with a good reproducibility but there are limitations associated with the use of DNA and 35S-labeled material. Assays based on 21-OH labeled with 125I, however, in addition to high specificity, sensitivity and a good precision have the added advantage of being convenient and easy to use in routine laboratories. There is a good agreement between results of 21-OH Abs in these assays and ACA by indirect immunofluorescence (56, 58, 59). In contrast, Falorni et al. found some discrepancies between 21-OH Abs and ACA (71). The reasons for these discrepancies are not clear at present but may be related to technical aspects of autoantibodies assays, in particular the indirect immunofluorescence test, which require considerable experience, great care and stringent control procedures to be observed.

To avoid discrepancies in adrenal autoantibodies measurements, an international standardization and proficiency program should be performed in the near future, similar to that successfully performed for islet-cell autoantibodies (72–74).

**Cellular immunity**

In the last few years, very little progress has been made in the understanding of the precise cellular mechanisms leading to AAD. Evidence for an antigen-specific T-lymphocyte response comes from early studies of migration inhibition tests using adrenal cortex antigens (75, 76). Furthermore, a non-specific reduction of the suppressor T-lymphocyte function has been demonstrated (77, 78). The study of the peripheral cells demonstrated an increased percentage of activated T-lymphocytes in patients with recent onset disease.
compared with those with longstanding AAD (79). More recently, a proliferative T-cell response to a fraction with molecular weight of 18–24 kDa has been demonstrated (80).

In an animal model, transfer of spleen cells from mice immunized with adrenal extracts led both to the development of adrenal cortex infiltrates and autoantibodies in healthy recipients but without development of adrenal insufficiency (81). Studies of human adrenocortical tissue from patients with AAD, with characterization of the infiltrating cells, their isolation and cloning, should allow the identification of T-cells autoepitopes and the further progression in our understanding of the cellular mechanisms involved in this disease.

**Gonadal insufficiency**

**Definition**

Premature ovarian failure (POF) is defined as the cessation of ovarian function after puberty and before the age of 40. POF implies that the ovaries have undergone differentiation and have functioned but their function has failed before the expected time of menopause. So, the typical presentation is as secondary amenorrhea but, in rare cases, it can also be as primary amenorrhea (82). The prevalence of POF is found in 0.2% of the female population and an autoimmune origin represents approximately 20% of all cases (11, 83).

**Autoimmune POF**

The diagnosis of autoimmune POF is quite difficult and is based mainly on the exclusion of the other known causes and the demonstration of one or more of the criteria for autoimmune diseases. Two distinct entities are identifiable: (i) autoimmune oophoritis and (ii) resistant ovary syndrome, also called Savage’s syndrome (84).

Genetic assessment has been performed in a group of Caucasian females with isolated secondary amenorrhea without autoantibodies or autoimmune diseases and has allowed the demonstration of HLA-B35 in 37% of the cases with a relative risk of 2.99, and HLA-DR3 in 53% of the cases with a relative risk of 4.3 (85).

Autoimmune POF can present in the following forms: (i) associated with AAD or adrenal cortex autoantibodies and (ii) isolated or associated with autoimmune diseases other than AAD (44, 86).

**POF associated with AAD**

The association between clinical AAD and POF was documented many years ago when POF was reported in 6/77 (8%) patients with AAD; 5 of the 6 (83%) were steroid cell autoantibodies-positive (40). Subsequently, it has been observed that the prevalence of POF ranged from 17% to 50% in patients with PGAD type 1 (8, 12) and from 3.6% to 7% in those with PGAD type 2 (7).

The histological descriptions of the ovaries from these patients are rare, and those reported are based mainly on observations obtained on scarce biopsy material. Oophoritis at biopsy was documented in 18/18 patients with POF and AAD (40, 87–96). Autoimmune oophoritis is characterized by lymphocytic and plasma cell infiltration of the endocrine hilar cells, theca interna of growing follicles and corpora lutea. In the majority of the cases there is a lack of ovarian follicles and sometimes the glands appear fibrotic (streak ovaries) (16, 38, 90). Immunohistochemical analysis of the lymphocytic oophoritis reveals that the inflammatory cells consist mainly of T-lymphocytes (CD4+ and CD8+), a few B cells with a large number of plasma cells, macrophages and NK cells (91). These data underline the close relationship between AAD and autoimmune POF due to oophoritis.

**Autoantibodies and autoantigens**

All the patients with POF associated with AAD are steroid cell autoantibodies-positive (7, 37, 39, 40, 44). In general, POF follows AAD in patients with PGAD type 1 and precedes it in those with PGAD type 2 (44). Steroid cell autoantibodies have also been found in 20–34% of AAD patients in the absence of POF (38, 39, 44). The follow-up of these patients revealed a high risk of gonadal failure in females but not in males (43, 44). Furthermore, steroid cell autoantibodies have also been found in 7.3% of patients with POF without AAD (44) and these patients have a high risk of development of clinical AAD (31, 32).

Sera from fifteen patients with POF in the context of PGAD type 1 were studied by immunoblotting: 60% reacted with P450scc, 40% with 17α-OH, and only 33% with 21-OH (55). These data were subsequently confirmed by immunoprecipitation studies (57). Winqvist et al. (97) using Western blot, studied sera from patients with AAD reacting with Leydig cells and found that the steroid cell antigen consisted mainly of P450scc (in 80% of the sera), and a 51 kDa protein of unknown function (in 60% of the sera); 21-OH was recognised by only 40% of the sera. We studied, by immunoprecipitation assay, 26 steroid cell autoantibodies-positive patients with clinical or subclinical AAD, 11 of whom had POF, and found that 26/26 (100%) of the steroid cell autoantibodies-positive sera reacted with 21-OH, 16/26 (61.5%) with 17α-OH and 17/26 (65%) with P450scc (58). Again, in this group of patients a good correlation was found between ACA and 21-OH and between steroid cell autoantibodies and 17α-OH and/or P450scc. The differences in reactivity to steroidogenic enzymes among POF patients could be due to the different origin of the patients; however they indicate the
necessity of standardization and proficiency programs as suggested in the case of 21-OH Abs.

**POF isolated or associated with autoimmune diseases other than AAD**

Besides AAD, numerous other autoimmune diseases, both endocrine and non-endocrine, frequently occur in association with POF. They include autoimmune thyroid diseases, myasthenia gravis, chronic candidiasis, hypoparathyroidism, vitiligo, alopecia, diabetes mellitus, idiopathic thrombocytopenic purpura, autoimmune hemolytic anemia, pernicious anemia, hypothyroid diseases, myasthenia gravis, chronic candidiasis, primary biliary cirrhosis, chronic active hepatitis, malabsorption, systemic lupus erythematosus, and rheumatoid arthritis (98). A second autoimmune disorder is present in 10–39% of patients with POF, in the majority at a subclinical level (44, 86, 99, 100).

Lymphocytic oophoritis is an exceptional finding in these patients as it has been described in only 6/198 patients with POF not associated with AAD, while in the majority, follicular activity without evidence of lymphocytic infiltration was found (95, 101–116). These data indicate that the absence of lymphocytic oophoritis cannot be regarded as conclusive evidence against an autoimmune aetiology, as this could equally be due to immunological mechanisms, such as blocking antibodies hypothesized for Savage’s syndrome. This syndrome is characterized by amenorrhea in association with hypergonadotropism and apparently normal ovarian follicular apparatus with immature follicles (84).

**Autoantibodies and autoantigens**

Steroid cell autoantibodies are very rare in sera from patients with POF without AAD (39, 44). Recently, many other autoantibodies to gonads have been detected by ELISA in patients with POF isolated or associated with autoimmune diseases excluding AAD. Using corpora lutea extracts, autoantibodies binding protein complexes of 2–36 kDa and 70 kDa have been demonstrated, the latter possibly corresponding to the unoccupied receptor for luteinizing hormone/human chorionic gonadotropin (LH/hCG) (117). These antibodies were present in patients with primary or secondary sterility with or without endometriosis (117). However, the presence of these was not confirmed in various types of POF in another study (118). Autoantibodies to either ovary or oocytes were detected in 66% of patients with POF alone, in 75% of POF associated with other autoimmune diseases, and in 78% of POF with previous pelvic surgery (119). Using two different preparations of human ovarian antigens, reactivity was found in 24% and 60% of the patients with various types of POF (iatrogenic POF, Turner’s syndrome, idiopathic POF and POF associated with AAD) and a frequent cross-reactivity to fallopian tube antigens was also found (118). These results suggest that ovarian antibodies are common in POF, but their specificity and pathogenic role are questionable. Recently, an antibody to 3β-HSD was found in 10/48 (21%) POF patients without other autoimmune diseases, all except one were steroid cell autoantibodies-negative (63).

Follicle-stimulating hormone (FSH) receptor-blocking antibodies were first identified in 3 patients with myasthenia gravis complicated by amenorrhea due to gonadotropin-resistant ovary syndrome as documented by the presence of high levels of gonadotropin and numerous cortical primordial follicles at biopsy (120, 121). A common immunological mechanism mediated by blocking antibodies to acetylcholine receptors and against FSH receptors was hypothesized in these two diseases. Using Feulgen’s cytochemical bioassay, IgGs capable of blocking FSH-induced granulosa cell DNA synthesis were demonstrated in 21/26 (81%) women with POF (14/15 with Savage’s syndrome and 7/11 with isolated POF) (122). Subsequently, a mouse adrenal cell line transfected with human recombinant gonadotropin receptor genes was employed for studying the effects of IgG from patients with POF on progesterone production and cAMP responses. No blocking effects were demonstrated in this experiment (123). Thus, antibodies against LH and FSH receptors may exist but their precise role and prevalence require further studies, including use of cells transfected with recombinant human receptors.

The involvement of T cells in human oophoritis was suggested when migration inhibitory factor production towards ovarian and testicular tissue was found in the serum from a patient with thyroiditis, adenitis and POF (124). CD4/CD8 ratios have been found to be decreased, increased or normal (86). It was demonstrated that circulating activated T-lymphocytes were increased in patients with POF (11, 90, 125, 126), this increment was partially reduced under estrogen substitution indicating that the endocrine defect can contribute to this phenomenon (11). Recently, the number of T-cell subsets have also been found to be in the normal range in POF with elevated FSH levels (127). As yet, no consistent pattern of cell-mediated immunity in POF has emerged from studies in vitro.

**Concluding remarks**

Autoimmune Addison’s disease can present in various forms. Adrenal-cortex autoantibodies are found in the majority of these patients and 21-OH Abs appear to be the major component of these antibodies irrespective of whether the disease presents as isolated AAD or PGAD type 1 or type 2 or potential or subclinical AAD. There is a good agreement between the results of ACA (measured by indirect immunofluorescence) and 21-OH Abs (by immunoprecipitation assays). Furthermore, 17α-OH and P450scC appear to be the major components of steroid cell autoantibodies and are correlated with the presence of POF.
An international standardization and proficiency program should be performed in the near future for adrenal autoantibodies measurements, similar to that successfully performed for other main autoantibodies.

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Adrenal and ovarian autoimmunity


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