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15

Autoimmunity to Steroid-Producing Cells

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1. Introduction

Addison's disease, named after the English physician who provided its full description in 1855, is the result of the destruction or impaired function of adrenocortical cells. Of the 11 cases described by Addison, 10 were likely subsequent to an infiltrative disease (the most common being the secondary localization of *Mycobacterium tuberculosis* to the adrenal gland) and 1 was clinically idiopathic (likely of an autoimmune origin, on the basis of the current knowledge of disease mechanisms). In recent years the ethiologic spectrum of the disease has considerably expanded to include genetic causes not present in the 11 cases described by Thomas Addison. Accordingly, the definition of primary adrenal insufficiency (PAI) appears today more correct. Nevertheless, Autoimmune Addison's Disease (AAD) and post-tuberculosis Addison's disease are still adequate definitions according to the clinical characteristics of the cases described in 1855.

Prevalence of PAI is estimated at 120-160 cases per million in western countries, corresponding to 1 case every 7,000-7,500 individuals (Laureti *et al*, 1999; Løvås *et al*, 2002). The clinical manifestations of the disease result from the glucorticoid, mineralcorticoid and androgen deficiency (Oelkers, 1996). In western countries and Japan, an autoimmune process is responsible for the destruction of the adrenocortical cells and for the clinical manifestations of PAI in around 70-90% of cases (Betterle *et al*, 2002; Nomura *et al*, 1994).

AAD occurs frequently in concomitance with other organ-specific and non-organ-specific autoimmune diseases in the so-called autoimmune polyendocrine syndromes (APS). Since at least two-thirds of patients with AAD have one or more other manifestations of an ongoing autoimmune process against other endocrine glands or different tissues, AAD can be considered a paradigmatic disease for the study of endocrine autoimmunity. On the basis of the type of diseases present in the same patient, different APSs are recognized. APS 1 is caused by mutations of the AIRE (AutoImmune REgulator) gene, which is located on chromosome 21 (The Finnish-German APECED Consortium, 1997). This syndrome is characterized by the concomitant presence of at least two of three diseases: chronic mucocutaneous candidiasis, hypoparathyroidism and AAD. In first-degree relatives of APS1 patients a single disease manifestation is sufficient to formulate the diagnosis. No general agreement exists for the classification of the remaining APSs. Some authors discriminate APS 2, APS 3 and APS 4 according to the different combination of autoimmune diseases present in the same patients. With this classification, APS 2 would identify the association of clinical or pre-clinical AAD with thyroid autoimmune diseases

and/or type 1 diabetes mellitus (T1DM). APS 3 would be the association of thyroid autoimmune diseases with other endocrine and non-endocrine autoimmune manifestations (with the exception of AAD) and APS 4 would include all other possible combinations. No sound pathophysiological and/or genetic background, however, supports this classification, as the different endocrine autoimmune diseases tend to share a common genetic background and the same subject that might be initially be classified as APS 3 or APS 4 would be reclassified as APS2 if clinical or subclinical signs of adrenal or thyroid autoimmunity occur at a later stage. Throughout this chapter we will then refer to APS 2 to include all combinations of AAD with other immuno-mediated diseases, different from hypoparathyroidism or chronic mucocutaneous candidiasis.

Most frequently AAD is associated with Hashimoto's thyroiditis or Graves's disease, one or the other being present in over 50% of patients with autoimmune adrenal insufficiency (Betterle *et al*, 2002; Nerup, 1974; Kong *et al*, 1994; Irvine et al, 1967, 1979; McHardy-Young *et al*, 1974; De Rosa *et al*, 1987; Papadopoulos *et al*, 1990; Kasperlik-Zaluska *et al*, 1991; Zelissen *et al*, 1995; Söderbergh *et al*, 1996; Neufeld *et al*, 1981). Atrophic gastritis is present in 20-25% of AAD patients, while type 1 diabetes mellitus (T1DM) has been reported in 1-20% of addisonian patients (Betterle *et al*, 2002; Nerup, 1974; Kong *et al*, 1994; Irvine et al, 1967, 1979; McHardy-Young *et al*, 1974; De Rosa *et al*, 1987; Papadopoulos *et al*, 1990; Kasperlik-Zaluska *et al*, 1991; Zelissen *et al*, 1995; Söderbergh *et al*, 1996; Neufeld *et al*, 1981). Hypergonadotropic hypogonadism is present in 4.5-19% of AAD women. Other autoimmune diseases found associated with AAD at lower frequency include: vitiligo, alopecia, celiac disease, pernicious anemia, multiple sclerosis, inflammatory bowel disease, Sjögren's syndrome, chronic hepatitis and lymphocytic hypophysitis (Betterle *et al*, 2002; Nerup, 1974; Kong *et al*, 1994; Irvine et al, 1967, 1979; McHardy-Young *et al*, 1974; De Rosa *et al*, 1987; Papadopoulos *et al*, 1990; Kasperlik-Zaluska *et al*, 1991; Zelissen *et al*, 1995; Söderbergh *et al*, 1996; Neufeld *et al*, 1981).

2. Adrenal autoimmunity

The human adrenal autoimmune process is made evident by the apperance of circulating autoantibodies directed against the adrenal cortex or its components, which can be detected in the serum of affected individuals.

The first demonstration of adrenal cortex autoantibodies (ACA), detected by a complement-fixation test in the sera from patients with AAD, was provided in 1957 (Anderson *et al*, 1957). The use of the indirect immunofluorescence (IIF) approach, introduced by Blizzard and Kyle, 1963, has subsequently enabled the development of more sensitive assays for the detection of ACA (Blizzard *et al,* 1963, 1967; Goudie *et al,* 1966; Andrada *et al,* 1968; Wuepper *et al*, 1969; Nerup, 1974; Irvine & Barnes, 1974; Sotsiou *et al*, 1980; Elder *et al*, 1981; Papadopoulos *et al*, 1990). ACA have been detected in 40-75% patients with clinically idiopathic Addison's disease (Blizzard *et al,* 1963, 1967; Goudie *et al,* 1966; Andrada *et al,* 1968; Wuepper *et al*, 1969; Nerup, 1974; Irvine & Barnes, 1974; Sotsiou *et al*, 1980; Elder *et al*, 1981; Stechmesser *et al*, 1985; Kosowicz *et al*, 1986; Papadopoulos *et al*, 1990). Differences in the substrate used for detection of autoantibodies (human vs. animal adrenals), in disease duration and in selection of patients are responsible for the high variability in ACA frequency in patients with PAI.

In spite of the very well known technical limitations, because of the relative simplicity of the assay and the high diagnostic sensitivity, ACA-IIF have represented the gold standard for

adrenal autoantibody determination until the middle of the '90s. ACA react with cytoplasm autoantigen(s) of cells located in all three layers of the adrenal cortex. More recent studies have detected ACA in approximately 80% of patients with AAD, representing over 90% of patients with recent-onset disease and 79% with long-standing disease (>2 years of disease duration) (Betterle *et al*, 1999). ACA are more frequent in patients with APS1 or APS2 than in patients with isolated Addison's disease (Betterle *et al*, 1999).

Attempts to identify the molecular targets of ACA started during the 1980's, which led to the initial identification of a 55-kDa autoantigen present in human adrenal microsomes (Furmaniak *et al*, 1988). Subsequent studies of the beginning of the 1990's have identified steroid-21-hydroxylase (21OH) as the main autoantigen target of ACA (Winqvist *et al*, 1992; Baumann-Antczak *et al*, 1992; Bednarek *et al*, 1992). Around the same time, steroid-17α-hydroxylase (17OH) (Krohn *et al*, 1992) and P450 side chain cleavage (P450scc) enzyme (Winqvist *et al*, 1993) were identified as additional autoantigens.

The discovery of the molecular targets of ACA led to development of radiobinding assays with radiolabelled human autoantigen. The use of either in vitro translated ^{35}S-21OH (Falorni *et al*, 1995, 1997; Colls *et al*, 1995) or 125 I-21OH (Tanaka *et al*, 1997) has enabled the demonstration that 21OH autoantibodies (21OHAb) have a high diagnostic sensitivity and specificity for AAD, being detected in over 95% of cases with clinically idiopathic adrenal insufficiency. The major epitopes recognised by human 21OHAb are located in the central and COOH-terminal domains of the enzyme. In addition, naturally occurring mutations, associated with the development of congenital adrenal hyperplasia, inhibit the binding of human autoantibodies to recombinant 21OH (Chen *et al*, 1998, Nikoshkov *et al*, 1999). *In vitro* studies (Furmaniak *et al*, 1994) have shown that 21OHAb may inhibit the enzymatic activity of the autoantigen, but this finding has not been confirmed *in vivo*, during the natural history of the disease (Boscaro *et al*, 1996; Laureti *et al*, 2002).

Similarly to other organ-specific autoantibodies, 21OHAb have no major pathogenic role in the development of adrenal insufficiency, as they can be detected in approximately 1/200 healthy subjects who do not necessarily progress towards clinical adrenal insufficiency. During pregnancy, 21OHAb cross the placental barrier, but do not determine any sign of clinical or pre-clinical adrenal insufficiency in the newborn (Betterle *et al*, 2004). Hence, 21OHAb are a highly senstive and specific immunological marker of the ongoing adrenal autoimmune process, but do not act as an effector of the destructive autoimmune process.

Our group has recently shown that 21OHAb are predominantly IgG1, with a minor expression of IgG2 and IgG4, which demonstrates a Th1-oriented type of immune response (Brozzetti *et al*, 2010a). However, approximately 10% of AAD patients express IgG4-21OHAb in the absence of IgG1 (Brozzetti *et al*, 2010a). This type of IgG subclass selection reveals a more Th2-oriented type of activation. At present, it is still unclear whether this distinct subgroup of patients with a more Th2-oriented type of immune response represents a different population of AAD subjects or is the expression of a different stage of the natural history of the disease.

Although 17OHAb and P450sccAb can be detected in a small fraction of patients with AAD, these markers are more frequently present in patients with APS 1 or in women with autoimmune oophoritis which causes autoimmune ovarian insufficiency (*see paragraph on Ovarian Autoimmunity*) (Betterle *et al*, 1999; Perniola *et al*, 2000; Falorni *et al*, 2002a).

Diagnosis of PAI is based on clinical ground and hormonal determination, but the ethiologic diagnosis of AAD requires detection of circulating 21OHAb, by using a radiobinding assay.

Autoantibody titer is also important, because the presence of low titre adrenal autoantibodies does not enable the unequivocal diagnosis of AAD in all cases (Falorni *et al*, 2004). ACA and 21OHAb have sporadically been found also in patients with unequivocal post-tuberculosis Addison's disease (Nomura *et al*, 1994; do Carmo Silva *et al*, 2000). A flow-chart for the etiological classification of PAI, that takes into consideration immunological, biochemical and imaging data, has been developed by the Italian Addison Network (Falorni *et al*, 2004). Published data indicate that the diagnostic accuracy of the 21OHAb assay is higher than that of the ACA-IIF (Falorni *et al*, 2004), and 21OHAb is currently the gold standard for detection of adrenal autoantibodies. In the case of a simultaneous presence of both ACA and 21OHAb, the probability of an accurate diagnosis of AAD is higher than 99% (Falorni *et al*, 2004). All patients with AAD should undergo additional autoantibody analyses, such as thyroid autoantibodies, GAD autoantibodies (GADA), steroid-cell autoantibodies (StCA), 17OHAb, P450sccAb or parietal cell autoantibodies, as approximately two thirds of AAD patients show clinical or pre-clinical signs of another autoimmune disease. Autoimmune AAD can be diagnosed also in the presence of medium-high levels of either 21OHAb or ACA (Falorni *et al*, 2004). In the case of patients positive only for 21OHAb or ACA at low levels, as well as in autoantibody-negative subjects, adrenal imaging should be performed to exclude an infiltrative form of adrenal insufficiency (such as post-tuberculosis, sarcoidosis, mycosis or metastatic localization of non-adrenal tumours) (Falorni *et al*, 2004). In male patients negative for both adrenal autoantibody measurement and adrenal imaging analysis, determination of plasmatic very long chain fatty acids (VLCFA) must be performed to exclude X-linked adrenoleukodystrophy (ALD).

At present, no clear and standardised cut-off is available to discriminate between low and medium-high level autoantibody titers and worskhops for the standardization of adrenal autoantibody assays are strongly needed. In a first international serum exchange to compare the results of 21OHAb determination in four independent laboratories, a good positive-negative concordance was observed, but major discrepancies emerged in quantitation of autoantibody titre (Falorni *et al*, 2011). A European program for the standardization of 21OHAb assay is currently being organised, and is expected to be completed within 2012.

Screening of subjects with endocrine autoimmune diseases for the presence of 21OHAb enables the identification of subjects with so-called *preclinical AAD*, which is characterised by the presence of adrenal autoantibodies in the absence of clinical signs of adrenal insufficiency and in the presence of normal basal cortisol concentrations. In patients with autoimmune diseases, 21OHAb can be detected in around 0.5-1.5% T1DM patients, 0.5-1.5% patients with autoimmune thyroid diseases, 0.5-1.0% patients with vitiligo and in 4-8% women with primary ovarian insufficiency (Betterle *et al*, 2002). 21OHAb-positive individuals exhibit a variable degree of adrenal dysfunction that can be classified in four different stages (Betterle *et al*, 1988).

S*tage 0* indicates the apperance of adrenal autoantibodies, as a sign of the ongoing adrenal autoimmune process. 21OHAb appears before ACA, and lasts longer than ACA, during the natural history of the disease.

Often the first dysfunction observed as a consequence of the progression of the autoimmune process is an initial increase of plasmatic renin activity (PRA), at a time when normal ACTH-cortisol axis response is still present (*stage 1*). In *stage 2*, an impaired response to the ACTH stimulation test is documented, while the final *stage 3* represents the pre-clinical stage characterized by increased basal ACTH levels, along with the dysfunctions observed in stage 1 and 2.

No preventative therapy is yet available to delay or block the progressive destruction of adrenocortical cells in pre-clinical AAD. An Italian study has shown that an immunosuppressive therapy with high-dose corticosteroids for the treatment of Graves' ophthalmopathy had been able to revert a stage 2 pre-clinical AAD in a 21OHAb-positive individual (De Bellis *et al*, 1993). As a result of the treatment, disappearance of adrenal autoantibodies and normalization of both ACTH-cortisol and PRA-aldosterone axis function was observed and maintained for over 10 years at follow-up (De Bellis *et al*, 1993). However, the possibility to revert an advanced pre-clinical adrenal insufficiency by high-dose steroid immunosuppression has not yet been confirmed in other independent studies and it is not recommended for the sole purpose of preventing the progression towards clinical AAD.

Among the factors associated with progression towards clinical adrenal insufficiency are adrenal autoantibody levels that tend to increase during the progression of the adrenal dysfunction (Betterle *et al*, 1997).

Approximately 80% of patients with stage 0 and stage 1 do not progress to clinical AAD and may show a spontaneous remission of the pre-clinical dysfunction (Laureti *et al*, 1998). On the other hand, no such spontaneous remission seems to occur in patients with stage 2 and stage 3, in whom progression to clinical AAD is observed in over 95% of cases. A matematical formula that takes into consideration all known factors influencing risk of progression towards clinical AAD has been generated (Coco *et al*, 2006).

In subjects positive for 21OHAb and/or ACA, an ACTH stimulation test discriminates between a potentially reversible and an irreversible stage of the pre-clinical dysfunction. It has been shown that the high-dose 250 μg of synthetic ACTH used in the classical ACTH test (HDT) is supra-maximal and that as little as 1 μg of synthetic ACTH can determine a maximal stimulation of the adrenocortical cells (Arvat *et al*, 2000). The low-dose test with 1 μg of ACTH (LDT) has a high diagnostic sensitivity and specificity for pre-clinical adrenal dysfunction and can substitute the classical high-dose test (Laureti *et al*, 2000, 2002). An ongoing European multicenter study is currently testing the diagnostic accuracy and the predictive value of a 0.5 μg low-dose ACTH test.

Among the factors increasing significantly the risk of progression towards clinical adrenal insufficiency, in subjects with subclinical AAD, the most important are: male gender, presence of other concomitant autoimmune diseases, impaired response to the LDT and a high 21OHAb titer (Coco *et al*, 2006). HLA-DR3-DQ2, DR4-DQ8 and CTLA-4 gene polymorphism are significantly associated with appearance of 21OHAb, but do not influence the natural history of the disease and do not predict future clinical adrenal insufficiency (Coco *et al*, 2006; Falorni *et al*, unpublished data). Homozygosity for MICA5.1 was found to increase significantly the risk of progression towards clinical adrenal insufficiency (Barker *et al*, 2005), but larger, prospective studies are needed to confirm this finding. Because of the high predictive value of 21OHAb for future AAD, which is higher than 30% at 10 years of follow-up, screening of patients with ongoing endocrine autoimmune diseases with this marker is recommended to identify subjects with pre-clinical adrenal insufficiency.

3. Genetics of autoimmune adrenal insufficiency

Autoimmune Regulator (AIRE) is the causative gene for APS1 (The Finnish-German APECED Consortium, 1997). The AIRE gene product, Aire, acts as a strong activator of transcription (Pitkänen *et al*, 2000, 2001; Björses *et al*, 2000). Over 60 disease-associated

mutations of the AIRE gene have been so far identified. Many mutations are located in the homogeneously staining region (HSR) in the N-terminal end of the protein. Other mutations, such as C311Y, P326Q, L397fsX478 are located in the plant homeodomain zinc finger 1 and 2, while only a few mutations, such as G228W or R257X have been identified in the DNA binding motif (Bottomley *et al*, 2001). It has been reported that a kindred carrying the G228W variant presented with an autosomal dominant autoimmune phenotype manifesting predominantly with thyroidits and distinct from APS1 (Cetani *et al*, 2001). The generation of a G228W-knocking mouse model showed that this AIRE variant acts in a dominant-negative manner causing a unique autoimmune syndrome (Su *et al*, 2008).

Disease-associated AIRE mutations are highly conserved within defined geographical areas. The R257X is the most frequently found mutation in Northern Italy and in Finland, while 964del13 is the most common mutation in the UK. In Sardinia, R139X is the typical AIRE mutation found in APS 1 patients.

Outside APS1, AAD shares many predisposing genetic factors with other endocrine autoimmune diseases that are components of APS2, such as thyroid autoimmune diseases and T1DM, the major genetic markers associated with the diseases beeing located in the HLA region on chromosome 6. The disease is strongly and positively associated with both HLA-DRB1*0301-DQA1*0501-DQB1*0201 (DR3-DQ2) and DRB1*04-DQA1*0301-DQB1*0302 (DR4-DQ8) (Falorni *et al*, 2008). The HLA-DRB1*01-DQA1*01-DQB1*0501 and DRB1*13-DQA1*0103-DQB1*0603 haplotypes are negatively associated with genetic risk for AAD. Several studies have shown that the association of HLA class II haplotypes is still highly significant in patients with isolated AAD and does not depend on the coexistence of other autoimmune disorders in the same patient. DRB1-*0404 was more frequent among DRB1*04-positive AAD patients from US (Yu *et al*, 1999) and Norway (Myhre *et al*, 2002) as compared to both DRB1*04-positive healthy control subjects and DRB1*04-positive T1DM patients. The DRB1*0401 subtype was strongly and positively associated with T1DM, but not with AAD (Yu *et al*, 1999; Myhre *et al*, 2002). However, an Italian study from our group (Gambelunghe *et al*, 2005) did not confirm this hypothesis. In that study (Gambelunghe *et al*, 2005), it was observed that no statistically different distribution of DRB1*0401 and DRB1*0404 was detectable among T1DM patients and AAD patients. On the other hand, it was observed that the DRB1*0403 subtype, which was already known to confer strong protection for T1DM, was absent among 56 DRB1*04-positive AAD patients, but present in 27 % DRB1*04-positive healthy control subjects, thus conferring protection also for the development of AAD.

Other HLA genes contribute to the risk for AAD, among which the MHC class I chain-related A (MICA) gene polymorphism. The MICA gene encodes for a ligand for an activating receptor (NKG2D) present on γδ T cells, CD8+CD28- αβ T cells, natural killer cells and CD4+CD28- αβ T cells and is located within the HLA class III region. There are speculations that MICA gene polymorphism might influence affinity for the NKG2D receptor. AAD is strongly associated with the transmembrane MICA 5.1 allele, with odds ratio similar to those observed for HLA class II haplotypes (Gambelunghe *et al*, 1999; Park *et al*, 2002). MICA6 appears to be negatively associated with disease risk for AAD (Gambelunghe *et al*, 1999).

Though the association of MICA with AAD appears to be independent from that with HLA class II haplotypes, the strong *linkage disequilibrium* existing within the DRB1*03-DQA1*0501-DQB1*0201-MICA5.1-HLA-B extended haplotype has so far limited the possibility to discriminate the relative contribution of each gene marker and further studies on large populations are needed to provide an answer to this specific question.

Similarly to T1DM and other autoimmune diseases, the genetic background for AAD includes also other genes believed to modulate the function of the immune system. The CTLA-4 gene polymorphism and the PTPN22 gene polymorphism have been found to modulate genetic risk for AAD (Falorni *et al*, 2008; Skinningsrud *et al*, 2008a). A recent metanalysis of European studies has confirmed that the CTLA-4 Ala 17 polymorphism is strongly associated with genetic risk for AAD, independentenly from the well known association with the polymorphism of HLA class II genes (Brozzetti *et al*, 2010b).

Two independent European studies have shown the association of the class II transactivator (CIITA) (also denominated MHC2TA) gene with AAD (Ghaderi *et al*, 2006; Skinningsrud *et al*, 2008b). Modulation of the expression of HLA class II determinants in antigen-presenting cells may represent a critical factor in the activation and maintenance of the organ-specific autoimmune process and genetic factors that may influence such expression may have an important role on the pathogenesis of autoimmune diseases. The regulatory factor X complex (RFX) and CIITA are essential and specific for activation of class II promoters (Durand *et al*, 1997; Masternak *et al*, 1998; Reith & Mach, 2001; Nekrep *et al*, 2002). More specifically, the class II expression on antigen presenting cells is under the control of CIITA, that exhibits cell-specific, cytokine-inducible and differentiation-specific expression and is expressed in the same cells that express class II molecules, such as B cells, monocytes, dendritic cells and activated T cells. The genetic association of CIITA with AAD appears to be independent from that with HLA gene markers.

Other reported AAD genetic associations include the vitamin D receptor and the CYP27B1 (25-hydroxyvitamin D3-1α-hydroxylase) gene and the NACHT leucine-rich-repeat protein 1 (NALP1) gene (Magitta *et al*, 2009).

4. Pathophysiology of AAD

No model of spontaneous autoimmune primary adrenal insufficiency is yet available. Experimental autoimmune adrenalitis has been induced in mice, guinea pigs, rats and monkeys by injection of adrenal homogenates mixed with adjuvants (Betterle *et al*, 2002), with a resulting delayed type of hypersensitivity to adrenal antigens, rather than the induction of an adrenal autoimmune process. No conclusive data are yet available on the possibility of inducing an autoimmune reaction by injecting the major adrenal autoantigen 21-hydroxylase.

The current unavailability of a reliable animal model of spontaneous adrenal autoimmunity similar to the human disease, has so far limited the studies on the pathophysiology of AAD. Major efforts are being profused to develop an animal model of AAD that may prove useful for the understanding of the molecular mechanisms responsible for the human disease.

The effector cells of the autoimmune-mediated destruction of adrenocortical cells are thought to be T lymphocytes (Hayashi *et al*, 1989; Freeman & Weetman, 1992). Nerup *et al*., 1969, 1970, showed that patients with autoimmune adrenal insufficiency have T cells reactive against fetal adrenal extracts or adrenal mitochondrial fraction. Subsequently, it was shown that patients with recent-onset AAD have an increased expression of circulating Ia-positive T cells when compared to healthy control subjects (Rabinowe *et al*, 1984). Freeman & Weetman, 1992 were able to demonstrate T cell proliferation in response to stimulation with adrenal proteins fractionated according to molecular weight. Patients with autoimmune polyendocrine syndrome type II, but not individuals with isolated Addison's disease, seem to have $CD4^+CD25^+$regulatory T cells with defective suppressive capacity (Kriegel *et al*, 2004; Coles *et al*, 2005).

Although several studies support the hypothesis of a major role of cellular immunity in the autoimmune destruction of the adrenocortical cells in Addison's disease, little is known of the individual antigens recognised by autoreactive T cells. Husebye et al. immunized BALB/c and SJL mice with recombinant 21OH and showed a selective significant proliferative response of T cells from lymph nodes against the peptide 342-361 of 21OH that corresponds to the substrate binding site of the enzyme (Husebye *et al*, 2006).

Among Th cell subtypes, two main subtypes, denominated Th1 and Th2, are recognized. AAD is thougth to result from an unbalance of Th1/Th2 responses with a predominance of Th1 activity. Chemokines produced at the site of inflammation participate by recruiting T cells and by sustaining the immune reaction (Rotondi *et al*, 2007). Th1 cells mainly express CXCR3 and are recruited at the site of inflammation by CXCL9, CXCL10 and CXCL11. Th2 cells express different chemokine receptors, such as CCR4 and CCR8, thus being recruited in target tissues by CCL17, CCL22 and CCL1. The existence of an important IFN-γ-mediated pathogenetic loop has been proposed for endocrine autoimmunity, as release of CXCR3-binding chemokines is stimulated by IFN-γ and, in turn, these chemokines recruit Th-1 cells that produce IFN-γ (Rotondi *et al*, 2007).

In one report concerning the role of serum CXCL10 in AAD, serum levels of CXCL10 were significantly increased in patients with either clinically evident or subclinical adrenal insufficiency, as compared to healthy control subjects (Rotondi *et al*, 2005). The absence of a gender-related effect in AAD, either isolated or occurring within APS-2, suggested that autoimmune adrenalitis by itself was responsible for the high circulating levels of CXCL10. In the same study (Rotondi *et al*, 2005), it was also shown that release of CXCL10 by primary cell cultures of human zona fasciculata cells (hZFC) was undetectable basally, but significantly induced by stimulation with IFN-γ or IFN-γ plus TNF-α, while stimulation of hZFC with TNF-α alone was not able to induce chemokine secretion. Interestingly, increasing concentrations of hydrocortisone progressively and significantly inhibited IFN-γ-induced and IFN-γ- plus TNF-α-induced CXCL10 secretion (Rotondi *et al*, 2005).

The inhibitory effect of glucocorticoids on chemokine production is in line with their antiinflammatory and immunosuppressive actions. Glucocorticoids are able to suppress the production of several cytokines and chemokines by inhibiting the nuclear factor-κB and by activating protein-1 transcription factor families (Karin, 1998).

More recently, increased serum levels of Th1-related chemokines CXCL10 and macrophage inflammatory proteins 1alpha (CCL3/MIP-1alpha) and of the Th2- related chemokine macrophage inflammatory proteins 1 beta (CCL4/MIP-1beta) were observed in patients with AAD, which confirms the role of these chemokines in the autoimmune pathology of adrenal gland through the recruitment in loco of Th1 and Th2 cells (Bellastella *et al*, 2011).

Based on the above described body of evidence, we can hypothesize that Th1 cells play a major pathogenetic role, while autoantibody production is a side effect with no pathogenic relevance. The decrease in Treg activity is probably one of the main mechanisms at the basis of the unbalanced activity of Th1 cells. The active role of the adrenocortical cells in modulating the autoimmune inflammation, by producing Th1-attracting chemokines and cortisol, should not be underestimated. Based on knowledge derived from studies on ovarian autoimmunity (Samy *et al*, 2005), it is likely that the continuous drainage of autoantigens to regional lymph nodes be essential to maintain an adequate activity of antiinflammatory Treg cells and that the reduced activity of Tregs and the unbalance Th1/Th2 ratio observed in human endocrine autoimmunity occur at the site of regional lymph nodes.

5. Ovarian autoimmunity

Although it has been proposed that up to 30% of cases of primary ovarian insufficiency (POI) would have an autoimmune origin (Meskhi *et al*, 2006; Calongos *et al*, 2009), a more accurate estimate indicates that a documented ovarian autoimmune reaction is present in not more than 4-5% of women with ovarian insufficiency (Hoek *et al*, 1997; Bakalov *et al*, 2005).

Similarly to the study of adrenal autoimmunity, the unavailability of an animal model of spontaneous autoimmune oophoritis has limited the possibility to define the molecular mechanisms of immune-mediated ovarian insufficiency. The animal model of neonatal thymectomy has proven instrumental to unravel the critical role of $CD4^+CD25^+$ regulatory T (Treg) cells (Samy *et al*, 2006) in suppressing autoimmune processes in regional lymph nodes, under a continuous stimulation by autologous antigens (Samy *et al*, 2005). The ovarian autoantigen identified in the animal model of neonatal thymectomy is the ooplasm-specific MATER (Maternal Antigen That Embryo Requires) protein.

Immunization of mice with a peptide of inhibin alpha chain induced an initial increase in fertility, mediated by high serum levels of inhibin alpha neutralizing antibodies, that prevented inhibin-mediated downregulation of activin-induced FSH release (Altuntas *et al*, 2006). In a second, delayed phase, the activation of CD4+ T-cells resulted in a lymphocytic infiltration of the ovary that occurred in parallel with a progressive decrease in fertility and ovarian function (Altuntas *et al*, 2006).

However, it is still unclear to what extent these animal models are similar to human autoimmune POI.

Several lines of evidence support the autoimmune origin of a fraction of POI cases. Some autoimmune diseases are more frequent in women with POI than in the general population, and, conversely, POI occurs more frequently in women affected by some autoimmune diseases than in other women. Approximately 15% of women with POI present with autoimmune thyroiditis (Hoek *et al*, 1997) and the frequency of POI is higher in women with T1DM than in the general population (Hoek *et al*, 1997). However, it must be noted that biochemical and/or ultrasound signs of thyroiditis can be detected in 10-15% women in the general population and that the concomitant association of POI and thyroiditis or POI and T1DM in the same woman does not justify *per se* a diagnosis of "autoimmune POI". Surely more relevant is the association between POI and AAD (Betterle *et al*, 2002; Hoek *et al*, 1997; Falorni *et al*, 2002b). Approximately 4-8% of women with POI are positive for circulating adrenal autoantibodies, a frequency significantly higher than that expected in the general population (<0.5%) (Betterle *et al*, 2002; Falorni *et al*, 2002b). On the other hand, 10-20% of women with AAD develop POI before the age of 40 years (Betterle *et al*, 2002).

The existence of ovarian autoantibodies was first shown in the 1960's in studies that used indirect immunofluorescence on cryostatic sections of adrenal, ovary, testis and placenta (Blizzard *et al*, 1967; Irvine & Barnes, 1974). Subsequent studies have repeatedly confirmed the existence of ovarian autoantibodies in women with POI (Sotsiou *et al*, 1980; Elder *et al*, 1981; Ahonen *et al*, 1987; Betterle *et al*, 1993). Ovarian autoantibodies detected by indirect immunofluorescence cross-react with autoantigens expressed in other tissues, mainly the adrenal cortex, the testis and the placenta, which indicates that the autoantigen(s) recognized by these autoantibodies are not restricted to the ovary, but expressed also in other organs. Hence, a more correct definition of these autoantibodies is that of Steroid-Cell Autoantibodies (StCA). Interestingly, although all the tissue components are present in the

ovarian cryostatic sections used for the autoantibody assay, the immunofluorescence pattern of StCA is restricted to the theca cells of the growing follicle with no staining of primary follicles or granulosa cells in secondary and tertiary follicles. The same autoantibodies stain specifically Leydig cells of the testis.

Although indirect immunofluorescence is still the most widely used method to detect ovarian autoantibodies in clinical practice, the accuracy of this method has been questioned, mostly because of a low diagnostic specificity (Novosad *et al*, 2003). Some of the autoantigens recognised by StCA have been identified as the steroidogenic enzymes 17α-hydroxylase (17OH) and side-chain cleavage (P450scc) (Chen *et al*, 1996; Perniola *et al*, 2000; Falorni *et al*, 2002b). The development of immunoradiometric assays, using *in vitro* translated recombinant human 35S-radiolabelled autoantigens, have enabled the estimate of the diagnostic sensitivity and specificity of 17OHAb and P450sccAb for autoimmune POI (Chen *et al*, 1996; Perniola *et al*, 2000; Falorni *et al*, 2002b).

17OHAb and P450sccAb are each present in 50 to 80% of women positive for StCA (Chen *et al*, 1996; Perniola *et al*, 2000; Falorni *et al*, 2002b). Over 90% of StCA-positive women are positive for 17OHAb and/or P450sccAb, which is demonstrating that 17OH and P450scc are major targets of StCA, but other autoantigens may also be recognized by a subset of StCA.

StCA, 17OHAb and/or P450sccAb can be detected almost exclusively in women with POI who are also positive for adrenal autoantibodies, most specifically 21OHAb. The association of steroid-cell autoimmunity with adrenal autoimmunity is so strong that 21OHAb appear to be the marker at highest diagnostic sensitivity for autoimmune POI (Perniola *et al*, 2000; Falorni *et al*, 2002b, Bakalov *et al*, 2005). Since only less than 0.5% of women with POI can be found positive for StCA in the absence of 21OHAb, 17OHAb or P450sccAb, we can conclude that unequivocal biochemical signs of ovarian autoimmunity against steroidogenic enzymes are present almost exclusively in women with clinical or pre-clinical AAD.

Several studies have demonstrated that an autoimmune oophoritis can be found at ovarian biopsy only in women positive for ovarian and/or adrenal autoantibodies (Irvine, 1980; Gloor & Hurlimann, 1984; Sedmak *et al*, 1987; Bannatyne et al, 1990; Hoek *et al*, 1997; Bakalov *et al*, 2005), thus confirming that steroidogenic cell autoantibodies identify women with an ongoing ovarian autoimmune process. On the other hand, in the absence of ovarian and adrenal autoantibodies, no histological sign of autoimmune infiltration can typically be detected at ovarian biopsy, even in women who present with other autoimmune diseases, such as thyroiditis, T1DM, inflammatory bowel disease or systemic lupus erithematosus. Accordingly, a classification of autoimmune POI cannot be based only on the presence of other autoimmune manifestations, in the absence of specific autoantibodies in the serum.

Autoimmune oophoritis is characterized by a selective mononuclear cell infiltration into the theca layer of large, antral follicles, with earlier stage follicles consistently free of lymphocytic infiltration (Hoek *et al*, 1997; Bakalov *et al*, 2005). This finding is in line with the selective staining of theca cells at the indirect immunofluorescence assay and confirms that the steroidogenic cell ovarian autoimmune process is mainly directed against theca cells.

The absence of steroidogenic cell autoantibodies does not exclude the possibility that other autoantibodies may be present and other autoimmune mechanisms may be active. Many other potential autoantigens, such as LH receptor (Moncayo *et al*, 1989), FSH receptor (Ryan & Jones, 2004), zona pellucida (Kelkar *et al*, 2005), 82-86 kDa Ags and 52-63 kDa Ags (Wheatcroft *et al*, 1997) have been proposed as markers of ovarian autoimmunity, but the association of these autoantibodies with POI has not been confirmed (Anasti *et al*, 1995;

Tonacchera *et al*, 2004). Only detection of autoantibodies against steroidogenic enzymes can, accordingly, ensure, at present, an accurate identification of women with autoimmune oophoritis. Using these autoantibody markers, autoimmunity accounts for approximately 4-5% of all POI cases.

Along with the decline of serum concentrations of estradiol, POI is typically characterized with a reduction in synthesis and secretion of inhibins (Petraglia *et al*, 1998) and anti-müllerian hormone (AMH) (Méduri *et al*, 2007), as a result of the progressive decline in ovarian function. In an initial study of three women with autoimmune POI (Welt *et al*, 2005) this general paradigma was however questioned, as increased serum concentrations of inhibin B were detected. This initial finding was confirmed in a subsequent larger study (Tsigkou *et al*, 2008) that demonstrated increased levels of serum inhibin B and total inhibin in a group of 22 women with autoimmune POI, as compared to 71 women with idiopathic, non-autoimmune POI and 90 healthy fertile women. The results of these studies (Welt *et al*, 2005; Tsigkou *et al*, 2008) led to the formulation of a novel hypothesis of the pathophysiology of autoimmune POI. Differently from other forms of ovarian insufficiency, in which a general reduction of ovarian function can be observed, autoimmune POI is characterized by the selective autoimmune destruction of theca cells with preservation of granulosa cells that produce low amounts of estradiol because of lack of substrates. The subsequent increase in FSH levels stimulates viable granulosa cells that, in return, produce increased amounts of inhibins.

AMH levels have been found to be normal in women with hypogonadotrophic amenorrhea while they are very low or undetectable in women with physiological menopause or hypergonadotrophic amenorrhea (POI) (Méduri *et al*, 2007; La Marca *et al*, 2006; Knauff *et al*, 2009). AMH is exclusively produced by primary and pre-antral/small antral follicles, and the immunohistochemical findings of the absence of an inflammatory reaction around primary follicles (Hoek *et al*, 1997; Bakalov *et al*, 2005) provided a sound rationale to estimate AMH production in women with autoimmune POI. We recently documented normal serum AMH concentrations in two-thirds of women with recently diagnosed autoimmune POI (La Marca *et al*, 2009), which provides the first demonstration of the existence of a subgroup of women with POI with a preserved ovarian follicle pool for several years. Since AMH is the best biochemical marker of residual follicle pool, the results of our study (La Marca *et al*, 2009) are highly relevant for the future planning of clinical trials of immunotherapy aimed at preserving the residual functional tissue and/or delay the progression of the destructive ovarian autoimmune process in women with autoimmune POI.

6. Conclusions

Autoimmune Addison's disease is made evident by the apperance of circulating 21OHAb, the gold immune marker for the identification of subjects with an ongoing adrenal autoimmune process. The appearance of 21OHAb marks pre-clinical autoimmune Addison's disease, in asymptomatic subjects. In these individuals, the response to the ACTH-stimulation test enables the discrimination of an early, potentially reversible phase, from an irreversible phase of the disease.

POI affects around 1% of women below the age of 40 years and the detection of steroid cell autoantibodies enables the identification of autoimmune cases. At present, only the detection of 21OHAb and 17OHAb or P450sccAb may accurately identify autoimmune cases of POI.

Although autoantibodies are widely used in clinical practice, they have no pathogenic role for either AAD or POI and these diseases are thought to be the consequence of a T-cell mediated autoimmune destruction.

Substitutive therapy of either AAD or POI is not influenced by presence or absence of autoantibodies, but identification of autoimmune forms of these disease is nevertheless important. Approximately two thirds of AAD patients have at least another associated autoimmune disease, either clinical or pre-clinical. Accordingly, screening for other autoimmune disorders is mandatory in autoimmune AAD, but not strictly necessary in other forms of PAI. More importantly, autoimmune POI has a distinct pathophysiology which differs from that of other forms of ovarian insufficiency. Detection of steroid-cell autoantibodies enables the identification of subjects with an initially preserved follicle pool.

No animal models of spontaneous steroid-cell autoimmunity are currently available, and there is strong need for the generation of such models that may enable the improvement of our understanding of the molecular mechanisms of these diseases.

During the next few years large multicentric studies are expected to be performed to identify subjects at high-risk for developing AAD and clinical trials are being planned with the aim of immunomodulate the adrenal autoimmune response and delay progression towards clinical AAD. The availability of novel immunological technologies will prove instrumental in the molecular characterization of the steroid cell autoantigens and peptides recognised by human autoreactive T cells responsible for the adrenocortical cell destruction. In addition, epidemiological studies are needed to provide novel insights on the role of putative environmental factors.

7. References

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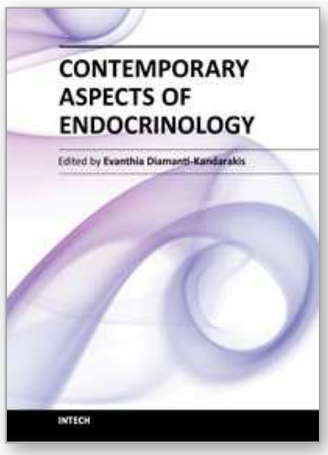
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This book aims to provide readers with a general as well as an advanced overview of the key trends in endocrine disorders. While covering a variety of topics ranging from thyroid carcinogenesis and pituitary adenomas to adrenal tumors and metabolic bone disease, this book also focuses on more specific issues not yet fully elucidated (e.g. the molecular pathways involved in thyrotropin beta gene regulation or monogenic phosphate balance disorders). Readers of different fields and background will have the opportunity to update their knowledge and more importantly to clarify areas of uncertainty and controversies in several topics of endocrine disorders.

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