

# Autoimmune Adrenal Insufficiency and Autoimmune Polyendocrine Syndromes: Autoantibodies, Autoantigens, and Their Applicability in Diagnosis and Disease Prediction

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Recent progress in the understanding of autoimmune adrenal disease, including a detailed analysis of a group of patients with Addison's disease (AD), has been reviewed. Criteria for defining an autoimmune disease and the main features of autoimmune AD (history, prevalence, etiology, histopathology, clinical and laboratory findings, cell-mediated and humoral immunity, autoantigens and their autoepitopes, genetics, animal models, associated autoimmune diseases, pathogenesis, natural history, therapy) have been described. Furthermore, the autoimmune polyglandular syndromes (APS) associated with AD (revised classification, animal models, genetics, natural history) have been discussed.

Of Italian patients with primary AD (n = 317), 83% had autoimmune AD. At the onset, all patients with autoimmune AD (100%) had detectable adrenal cortex and/or steroid 21-hydroxylase autoantibodies. In the course of natural history of autoimmune AD, the presence of adrenal cortex and/or steroid 21-hydroxylase autoantibodies identified patients at risk to develop AD. Different risks of progression to clinical AD were found in children and adults, and three stages of subclinical hypoadrenalism have been defined. Normal or atrophic adrenal glands have been dem-

onstrated by imaging in patients with clinical or subclinical AD.

Autoimmune AD presented in four forms: as APS type 1 (13% of the patients), APS type 2 (41%), APS type 4 (5%), and isolated AD (41%). There were differences in genetics, age at onset, prevalence of adrenal cortex/21-hydroxylase autoantibodies, and associated autoimmune diseases in these groups. "Incomplete" forms of APS have been identified demonstrating that APS are more prevalent than previously reported.

A varied prevalence of hypergonadotropic hypogonadism in patients with AD and value of steroid-producing cells autoantibodies reactive with steroid 17 $\alpha$ -hydroxylase or P450 side-chain cleavage enzyme as markers of this disease has been discussed. In addition, the prevalence, characteristic autoantigens, and autoantibodies of minor autoimmune diseases associated with AD have been described.

Imaging of adrenal glands, genetic tests, and biochemical analysis have been shown to contribute to early and correct diagnosis of primary non-autoimmune AD in the cases of hypoadrenalism with undetectable adrenal autoantibodies. An original flow chart for the diagnosis of AD has been proposed. (*Endocrine Reviews* 23: 327-364, 2002)

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Abbreviations: ACA, Adrenal cortex autoantibodies; AD, Addison's disease; APS, autoimmune polyglandular syndrome; CT, computerized tomography; CTLA-4, cytotoxic T lymphocyte antigen-4; H Abs, hydrocortisone autoantibodies; HLA, human leukocyte antigen; IIF, indirect immunofluorescence; IPA, immunoprecipitation assay; NMR, nuclear magnetic resonance; 17 $\alpha$ -OH Abs, 17 $\alpha$ -hydroxylase autoantibodies; 21-OH Abs, 21-hydroxylase autoantibodies; POF, premature ovarian failure; P450 scc, cytochrome P450 side chain cleavage enzyme; SF-1, steroidogenic factor 1; StCA, steroid-producing cell autoantibodies; TPH Abs, tryptophan hydroxylase autoantibodies.

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### I. Historical Introduction of Adrenocortical Insufficiency or Addison's Disease (AD)

In 1855, Thomas Addison (1), while working at the Guy's Hospital in London, described for the first time the signs and symptoms of: "a morbid state, the leading and characteristic features of which are anemia, general languor and debility, remarkable feebleness of the heart's action, irritability of the stomach and a peculiar change of color of the skin, occurring in connection with a diseased condition of the suprarenal capsules". On postmortem examination of 11 of his patients he had found: six cases with adrenal tuberculosis, three cases of adrenal malignancies, one case of adrenal hemorrhage, and one case of an adrenal fibrosis of unknown origin. The case of "idiopathic" adrenal fibrosis had been described by Addison as follows: "the two adrenals together weighted 49 grains, they appeared exceedingly small and atrophied, so that the diseased condition did not result as usual from a deposit either of a strumous or malignant character, but appears to have been occasioned by an actual inflammation, that inflammation having destroyed the integrity of the organs, and finally led to their contraction and atrophy" (1). Thus, this was the very first description of an autoimmune adrenalitis in literature. In addition, Dr. Addison observed that the patient affected by idiopathic adrenalitis showed also a vitiligo described as follows: "there were in the midst of this dark mottling certain insular portions of integument presenting a blanched or morbidly white appearance . . . from an actual defect of coloring matter in this part."

Subsequently, vitiligo has become a recognized and significant skin marker of autoimmune disorders and itself an autoimmune disease (2, 3). Taking all signs and symptoms described by Dr. Addison into consideration, this first case of autoimmune adrenalitis was most likely the very first described case of a patient with an autoimmune polyendocrine syndrome (APS). After this first report, in 1856 Trousseau (4) defined an adrenocortical insufficiency as an "Addison's disease," and this term has been in use ever since.

### II. Prevalence and Etiology of AD

Adrenocortical insufficiency or AD can be due to the destruction of the adrenal cortex itself (primary adrenocortical insufficiency), whereas the secondary forms may occur as a result of pituitary or hypothalamic diseases (5). The adrenocortical insufficiency can manifest as chronic or acute, and in both cases if diagnosis is missed, the patient will probably die (5). The different causes that can contribute to the development of primary and secondary AD are summarized in Table 1.

Primary adrenal insufficiency is a relatively rare disease with a prevalence ranging from 0.45 cases per 100,000 inhabitants in New Zealand (6) to 11.7 per 100,000 in Italy (7). A prevalence of 4–11 cases per 100,000 has been reported in

TABLE 1. Etiology of adrenocortical insufficiency or AD

|                                        |                                                                       |
|----------------------------------------|-----------------------------------------------------------------------|
| Primary adrenocortical insufficiency   |                                                                       |
| 1. Autoimmune adrenalitis              |                                                                       |
| 2. Infectious adrenalitis              |                                                                       |
|                                        | Tuberculosis                                                          |
|                                        | Fungal                                                                |
|                                        | Viral (HIV, CMV)                                                      |
| 3. Neoplastic diseases                 |                                                                       |
|                                        | Adrenal carcinomas                                                    |
|                                        | Metastasis: lung, breast, stomach, lymphomas                          |
| 4. Adrenal hemorrhage                  |                                                                       |
|                                        | Waterhouse-Friderichsen syndrome                                      |
|                                        | Anticoagulation therapy (dicumarol, heparin)                          |
|                                        | Traumas (external or by invasive procedures)                          |
| 5. Adrenal thrombosis                  |                                                                       |
|                                        | Systemic lupus erythematosus                                          |
|                                        | Panarteritis nodosa                                                   |
|                                        | Antiphospholipid syndrome                                             |
|                                        | Traumas                                                               |
| 6. Drug-induced                        |                                                                       |
|                                        | Adrenolitic therapy (mitotane, aminoglutetimide, trilostane)          |
|                                        | Other agents (ketoconazole, etomidate, rifampin, cyproterone acetate) |
|                                        | Anticoagulation                                                       |
| 7. Other causes                        |                                                                       |
|                                        | Sarcoidosis                                                           |
|                                        | Amyloidosis                                                           |
|                                        | Hemochromatosis                                                       |
|                                        | Histiocytosis                                                         |
| 8. Neonatal                            |                                                                       |
|                                        | Maternal Cushing's syndrome                                           |
|                                        | Traumas at birth                                                      |
| 9. Genetic                             |                                                                       |
|                                        | Adrenoleukodystrophy                                                  |
|                                        | Congenital adrenal hypoplasia                                         |
|                                        | Familial ACTH resistance syndromes                                    |
|                                        | Familial glucocorticoid deficiency                                    |
|                                        | Triple A syndrome                                                     |
|                                        | Kearns-Sayre syndrome                                                 |
|                                        | Congenital adrenal hyperplasia                                        |
|                                        | Smith-Lemli-Opitz syndrome                                            |
| Secondary adrenocortical insufficiency |                                                                       |
|                                        | 1. Pituitary or metastatic tumor                                      |
|                                        | 2. Craniopharyngioma                                                  |
|                                        | 3. Pituitary surgery or irradiation                                   |
|                                        | 4. Lymphocytic hypophysitis                                           |
|                                        | 5. Sarcoidosis                                                        |
|                                        | 6. Histiocytosis                                                      |
|                                        | 7. Empty sella syndrome                                               |
|                                        | 8. Hypothalamic tumors                                                |
|                                        | 9. Withdrawal from steroid therapy                                    |
|                                        | 10. Sheehan syndrome                                                  |
|                                        | 11. Head trauma, lesions of the pituitary stalk                       |
|                                        | 12. Pituitary surgery for adenoma                                     |

CMV, Cytomegalovirus.

Northern European countries (8–11) and of about 5 cases per 100,000 in the United States (12). Before the introduction of effective chemotherapy, tuberculosis was undoubtedly the most common cause of AD worldwide. For example, in 1930 Guttman (13) reported that 70% of adrenal glands examined during autopsy of patients with AD were affected by damage related to tuberculosis and only 17% showed signs of idiopathic adrenal atrophy. More recently, analysis of 1240 patients with AD in different European countries demonstrated that the autoimmune form of AD was the most common, ranging from 44.5–94% of all cases, compared with AD due to tuberculosis or other causes, which ranged from 0–33.3%

and 1–22.2%, respectively (see Table 2) (8, 10, 14–22). As a consequence of the reduction of prevalence of tuberculosis, the overall incidence of AD might be expected to decrease; however, current epidemiological data suggest that AD shows relatively stationary prevalence over the years (23–25).

In addition to autoimmunity and tuberculosis, infectious fungal diseases (coccidioidomycosis and histoplasmosis) or viral infections (cytomegalovirus and HIV) have been reported to be responsible for chronic adrenal damage leading to clinical AD (Table 1) (26). Primary tumors or metastases from malignant tumors elsewhere (lung, breast, stomach, lymphomas, and melanoma) are known to cause chronic adrenal insufficiency (24, 25). In addition, adrenal hemorrhage can lead to acute adrenal failure, *e.g.*, during anticoagulation therapy with dicumarol or heparin or in the course of the Waterhouse-Friderichsen syndrome. Waterhouse-Friderichsen syndrome describes an acute adrenal hemorrhage as a result of septicemic shock caused by infection with *Neisseria meningitidis* or by other microorganisms such as *Hemophilus influenzae*, *Pseudomonas aeruginosa*, *Escherichia coli*, pneumococci, and *dysgonic fermenter bacillus*. Adrenolytic drugs (mitotane, aminoglutethimide, metopyrone, trilostane) and other drugs (ketoconazole, rifampin, etomidate, cyproterone acetate) are known to cause adrenal insufficiency. External traumas, some invasive procedures (such as bilateral venography), systemic lupus erythematosus, panarteritis nodosa, or the primary antiphospholipid syndrome may induce adrenal thrombosis and, consequently, adrenal insufficiency (27). Chronic adrenal failure may also result from metabolic disorders, amyloidosis, hemochromatosis, and sarcoidosis. Rare congenital causes, such as hypoplasia of the adrenal gland, deficiencies of enzymes involved in the cortisol synthesis pathway, adrenal hemorrhage due to traumas at birth (23, 24, 28), or maternal Cushing's disease may all be responsible for adrenal insufficiency.

Rare genetic disorders associated with hypoadrenalism are listed in Table 1. Adrenoleukodystrophy is a hereditary disorder, also known as brown Schilder's disease, which is characterized by progressive demyelination within the central nervous system. This syndrome is caused by mutations of a gene located in the terminal segment of chromosome X coding for a structural protein of the peroxisomal membrane, which belongs to the ATP binding cassette su-

perfamily of transporters (29, 30). The disease is associated with elevated levels of circulating very-long-chain fatty acids, which are well recognized biochemical markers of adrenoleukodystrophy (30). Progressive accumulation of very-long-chain fatty acids leads to damage of the target organs. There are different forms of the disease, and in many cases the clinical signs of adrenal insufficiency precede the neurological signs.

The magnetic resonance of the brain reveals features that are often characteristic, with symmetrical demyelination in the parieto-occipital region. The imaging of the adrenal reveals that the adrenals are normal (30). Adrenoleukodystrophy is the most frequent etiological cause of AD not associated with autoimmunity or tuberculosis in males.

Congenital adrenal hypoplasia is an X-linked recessive disorder characterized by: 1) an adrenal insufficiency as a result of failure of the development of adrenal cortex, and 2) a delayed puberty with hypogonadotropic hypogonadism due to abnormal gonadotropin secretion at both hypothalamic and pituitary levels. This disease is associated with mutations of the dosage-sensitive sex reversal-adrenal hypoplasia congenita region on the X chromosome (DAX-1) gene located on the short arm of chromosome X coding for a nuclear receptor or with mutations of the steroidogenic factor (SF-1) gene on chromosome 9 controlling the synthesis of SF-1 (31). These two nuclear receptors (SF-1 and DAX-1) may act as coregulators and be components of a regulatory cascade required for normal gonadal, adrenal, and hypothalamic development.

A multisystem mitochondrial cytopathy known as a Kerns-Sayre syndrome may also be associated with adrenal insufficiency caused by various deletions of mitochondrial DNA and characterized by a wide range of clinical symptoms including progressive external ophthalmoplegia, retinal pigmentary degeneration, cardiac conduction defects, and deafness (32). In addition to adrenal insufficiency, several different endocrinopathies such as GH deficiency, diseases of the thyroid, hyperaldosteronism, hypogonadism, diabetes mellitus, and hypoparathyroidism have been observed to be associated with this syndrome (31).

Other genetic defects associated with adrenal insufficiency include familial ACTH resistance syndromes such as familial glucocorticoid deficiency and the triple A syndrome (31). Familial glucocorticoid deficiency is a rare autosomal disorder characterized by failure to thrive, re-

TABLE 2. Report of etiological forms of primary adrenocortical insufficiency in Europe from 1972–1996

| Authors                              | Year      | Country | No. of cases | Autoimmune (%) | Tuberculosis (%) | Other (%) |
|--------------------------------------|-----------|---------|--------------|----------------|------------------|-----------|
| McHardy-Young <i>et al.</i> (15)     | 1974      | UK      | 33           | 81.0           | 19.0             | n.d.      |
| Nerup (8)                            | 1974      | Denmark | 108          | 65.7           | 17.6             | 16.7      |
| Irvine <i>et al.</i> (14, 16)        | 1967/1979 | UK      | 434          | 83.8           | 15.1             | n.d.      |
| De Rosa <i>et al.</i> (17)           | 1987      | Italy   | 54           | 44.5           | 33.3             | 22.2      |
| Betterle <i>et al.</i> (18)          | 1989      | Italy   | 75           | 68.0           | 21.2             | 2.6       |
| Papadopoulos and Hallengren (19)     | 1990      | Sweden  | 62           | 71.0           | 19.4             | 9.7       |
| Kasperlik-Zaluska <i>et al.</i> (20) | 1991      | Poland  | 180          | 69.0           | 28.9             | 1.7       |
| Kong and Jeffcoate (10)              | 1994      | UK      | 86           | 94.0           | 0.0              | 6.0       |
| Zelissen <i>et al.</i> (21)          | 1995      | Holland | 91           | 91.2           | 6.6              | 2.2       |
| Söderbergh <i>et al.</i> (22)        | 1996      | Norway  | 117          | 83.0           | 2.6              | 14.5      |
| Total cases                          |           |         | 1240         | 44.5–94        | 0–33.3           | 1–22.2    |

n.d., Not determined.

current hypoglycemia, pigmentation, and recurrent infections. Biochemical tests show high levels of ACTH and low levels of cortisol. Mutations of the G protein-coupled ACTH receptor gene have been detected in about 40% of the patients; however, in about 60% of patients specific genetic mutations have not yet been found (31–34). The triple A syndrome, also known as Allgrove's syndrome, is an autosomal recessive disorder associated with mutations of a gene on chromosome 12, characterized by the triad of 1) adrenocortical failure due to ACTH resistance, 2) achalasia, and 3) alacrimia (31).

Congenital adrenal hyperplasia due to 21-hydroxylase deficiency is the most common cause of salt-wasting adrenal crisis in the first 2 wk of life. Affected females have ambiguous, virilized genitalia and are usually diagnosed at birth. Males, however, often go undiagnosed until they present with a salt-wasting crisis often 2–3 wk after birth. Deficiency of 3 $\beta$ -hydroxysteroid dehydrogenase or P450 scc enzyme also can present with adrenal insufficiency in the neonatal period, with affected boys presenting with ambiguous genitalia or phenotypically as females. Congenital adrenal hyperplasia due to defects in aldosterone synthetase leading to isolated aldosterone deficiency is not associated with sexual ambiguity (35). Nuclear magnetic resonance (NMR) can reveal a hyperplasia of the adrenals (Fig. 2H).

Finally, the Smith-Lemli-Opitz syndrome results from mutations in the sterol- $\delta$ -7 reductase gene, which catalyzes the final step in cholesterol biosynthesis leading to primary adrenal insufficiency. The syndrome can present with mental retardation, microcephaly, congenital cardiac abnormalities, syndactyly, and incomplete development of male genitalia in boys (35).

The causes of secondary adrenal insufficiency are also listed in Table 1. The disease is very rare. Among patients

with pituitary or hypothalamic disorders, especially space-occupying lesions, few patients have only adrenal insufficiency. Other hormonal axes are usually involved, and neurological or ophthalmological symptoms may accompany, precede, or follow adrenal insufficiency (5). A much more frequent type of isolated secondary adrenal insufficiency is that induced by suspension of glucocorticoid therapy, which is mainly due to prolonged suppression of the production of CRH (5).

From 1969 to 1999 we collected and studied 322 Italian patients with AD; 317 had primary and 5 had secondary adrenocortical insufficiency. The etiologies, the female/male ratio, children/adult ratio, and age at onset in the group of patients with primary disease are summarized in Fig. 1.

The majority of our cases (83%) were autoimmune; in this form the F/M ratio was 1.7, and the mean age at presentation was 30 yr. AD due to tuberculosis was relatively rare (12%) with a greater prevalence in males, with a mean age of presentation of 52 yr. There were no children in this group. Other minor causes, contributing to 4% of all cases, were more prevalent in males with the mean age at presentation of 28 yr. AD due to minor causes was sometimes found among children; adrenoleukodystrophy was the most frequent in this subgroup.

### III. Clinical Manifestations and Laboratory Diagnosis of AD

Most of the symptoms of primary and secondary adrenocortical insufficiency, ill-defined fatigue, weakness, listlessness, orthostatic dizziness, weight loss, and anorexia, are similar and nonspecific and usually occur insidiously (5, 35). Some patients initially present with gastrointestinal symp-

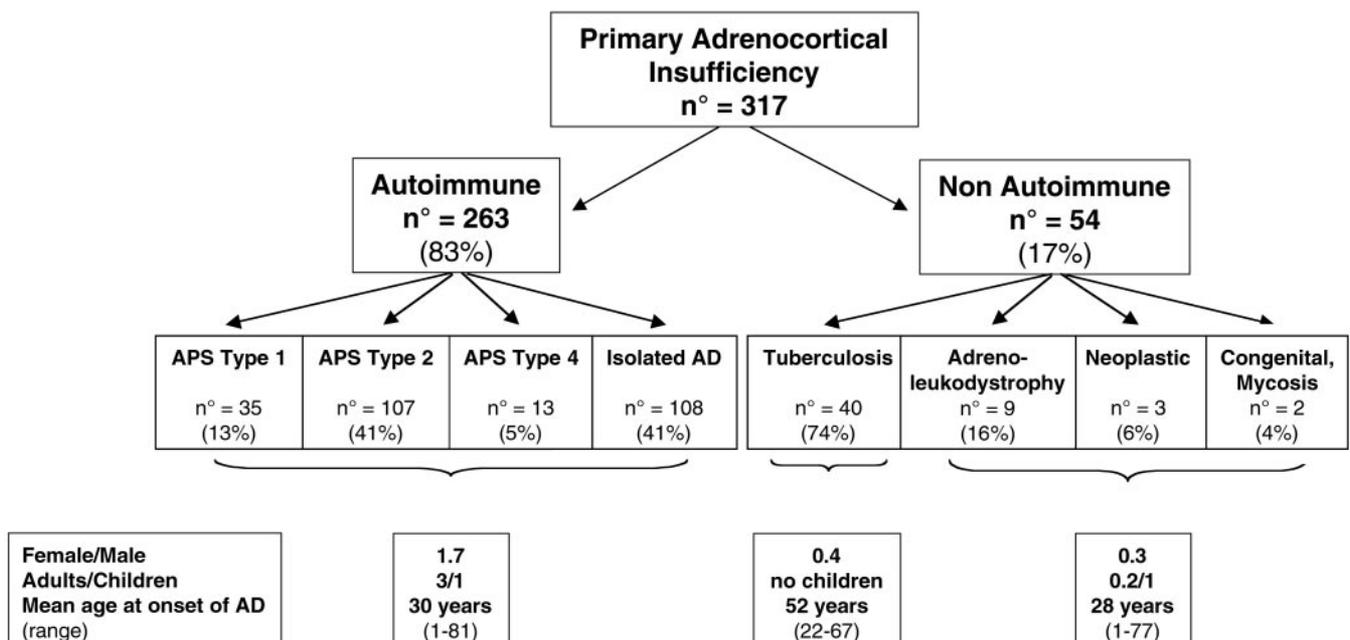


FIG. 1. Primary adrenocortical insufficiency: different clinical presentation in a group of Italian patients (n = 317) in the years 1969–1999.

toms such as abdominal cramps, nausea, vomiting, and diarrhea. The disease may be misdiagnosed sometimes as depression or anorexia nervosa. The most specific sign of primary adrenal insufficiency is hyperpigmentation of the skin and mucosal surfaces, which is due to the high plasma corticotropin concentrations that occur as a result of decreased cortisol feedback. On the other hand, pallor may occur in patients with corticotropin deficiency typical of secondary adrenocortical insufficiency (5, 35). Another specific symptom of primary adrenocortical insufficiency is a craving for salt. Thinning of axillary and pubic hair is common in patients with secondary disease, but it is not usually found in patients with isolated corticotropin deficiency. Decreased potency and libido as well as amenorrhea can be present in primary and secondary adrenal insufficiency. Orthostatic hypotension is more marked in primary than in secondary adrenal insufficiency because of aldosterone deficiency and hypovolemia.

In a patient with fatigue or other nonspecific symptoms, screening laboratory tests are often performed and the following abnormalities, encountered in a varying percentage of patients with adrenal insufficiency, can lead to the diagnosis: hyponatremia, hyperkalemia, acidosis, slightly elevated creatinine concentrations, hypoglycemia, hypercalcemia, mild normocytic anemia, lymphocytosis, and mild eosinophilia (5). Although hyponatremia occurs in both primary and secondary adrenal insufficiency, its pathophysiology in the two disorders differs. In the primary condition, adrenocortical insufficiency is mainly due to aldosterone deficiency and sodium wasting, whereas in the secondary form, adrenal insufficiency is due to cortisol deficiency, increased vasopressin secretion, and water retention (5).

In patients in whom adrenal insufficiency is merely to be ruled out, cortisol can be measured between 0800 and 0900 h. Hormonal pattern of morning plasma cortisol concentrations of less than 3  $\mu\text{g}/\text{dl}$  (83 nmol/liter) are indicative of clinical adrenal insufficiency whereas concentrations of more than 19  $\mu\text{g}/\text{dl}$  (525 nmol/liter) rule out the disorder.

Measurement of plasma corticotropin can be used to differentiate between primary and secondary adrenal insufficiency. In patients with primary adrenal insufficiency, plasma corticotropin concentrations invariably exceed 100 pg/ml (22 pmol/liter), even if the plasma cortisol levels are in the normal range. Normal plasma corticotropin values rule out primary, but not mild secondary, adrenal insufficiency. In primary adrenocortical insufficiency, basal plasma aldosterone concentrations are low or at the lower end of normal values, whereas the PRA or concentration is increased because of sodium wasting (5).

In patients with suspected hypoadrenalism in whom the previous measurements were normal, the short corticotropin stimulation test (ACTH test), which uses 250  $\mu\text{g}$  of synthetic ACTH, is the most commonly used test for the diagnosis of primary adrenal insufficiency (5) (see also potential AD).

In the diagnosis of AD, radiological procedures [computerized tomography (CT) or NMR] of the adrenals or of the pituitary gland should be carried out only after an endocrinological diagnosis has been established by hormonal tests.

#### IV. Idiopathic AD as an Autoimmune Disease

In 1957 Witebsky *et al.* (36) proposed the criteria for defining a disease as autoimmune, summarized in Table 3. Subsequently, the original postulates of Witebsky and associates have been revised, and now it is accepted that three types of evidences are necessary to establish that a human disease is autoimmune in origin: 1) *direct proof*, such as transfer of the disease by either pathogenic autoantibody or autoreactive T cells; 2) *indirect evidence* based on reproduction of the autoimmune disease in experimental animals, and 3) *circumstantial evidence* arising from destructive clinical clues, such as lymphocyte infiltration of the affected organs, association with other autoimmune diseases, correlation with particular major histocompatibility complex genes, and benefit from immunosuppressive therapy (37) (see Table 3).

In subsequent years, on the basis of these criteria, many diseases previously considered as idiopathic have been included in this group; consequently, the number of diseases classified as autoimmune has increased, and today more than 60 diseases (previously considered as idiopathic) are included in the group of autoimmune diseases, as recently reviewed by Betterle *et al.* (38).

In regard to idiopathic AD, circulating adrenal cortex autoantibodies (ACA) were discovered in 1957 (39). A number of subsequent reports indicated that idiopathic AD might be autoimmune in nature as reviewed by many authors (40–46). These findings include 1) the histopathological findings of a diffuse mononuclear cell infiltration progressing to atrophy of all the three layers of the adrenal cortex, 2) the demonstration of a cell-mediated immunity to adrenal cortex antigens, 3) the ability to induce the disease in animal models by immunization with adrenal cortex extracts, 4) the identification of steroidogenic enzymes expressed in adrenals as self-antigens, 5) the association with other organ-specific autoimmune diseases, and 6) the association with antigens of the major histocompatibility complex.

#### V. Histopathology of Adrenals in Autoimmune AD

The adrenal glands in patients with autoimmune AD are small (Fig. 2, A–E), in contrast to patients with tuberculosis or neoplasias when the adrenals are shown as a mass with or without calcifications (Fig. 2, F–G). In autoimmune AD the

TABLE 3. Criteria for defining a disease as autoimmune

|                                                                                                                                                                                                                          |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1. According to Witebsky <i>et al.</i> (36)                                                                                                                                                                              |
| Demonstration of circulating autoantibodies and/or cellular immuno-mediated events                                                                                                                                       |
| Demonstration of lymphocytic infiltration in the target organs                                                                                                                                                           |
| Identification and characterization of autoantigens                                                                                                                                                                      |
| Induction of the disease in animal models with the injection of autoantigens and passive transfer by serum or lymphocytes                                                                                                |
| 2. According to Rose and Bona (37)                                                                                                                                                                                       |
| Direct proof (such as transfer of the disease by either pathogenic autoantibody or autoreactive T cells)                                                                                                                 |
| Indirect evidence (based on reproduction of the autoimmune disease in experimental animals)                                                                                                                              |
| Circumstantial evidence (lymphocyte infiltration of the affected organs, association with other autoimmune diseases, correlation with major histocompatibility complex genes and benefit from immunosuppressive therapy) |

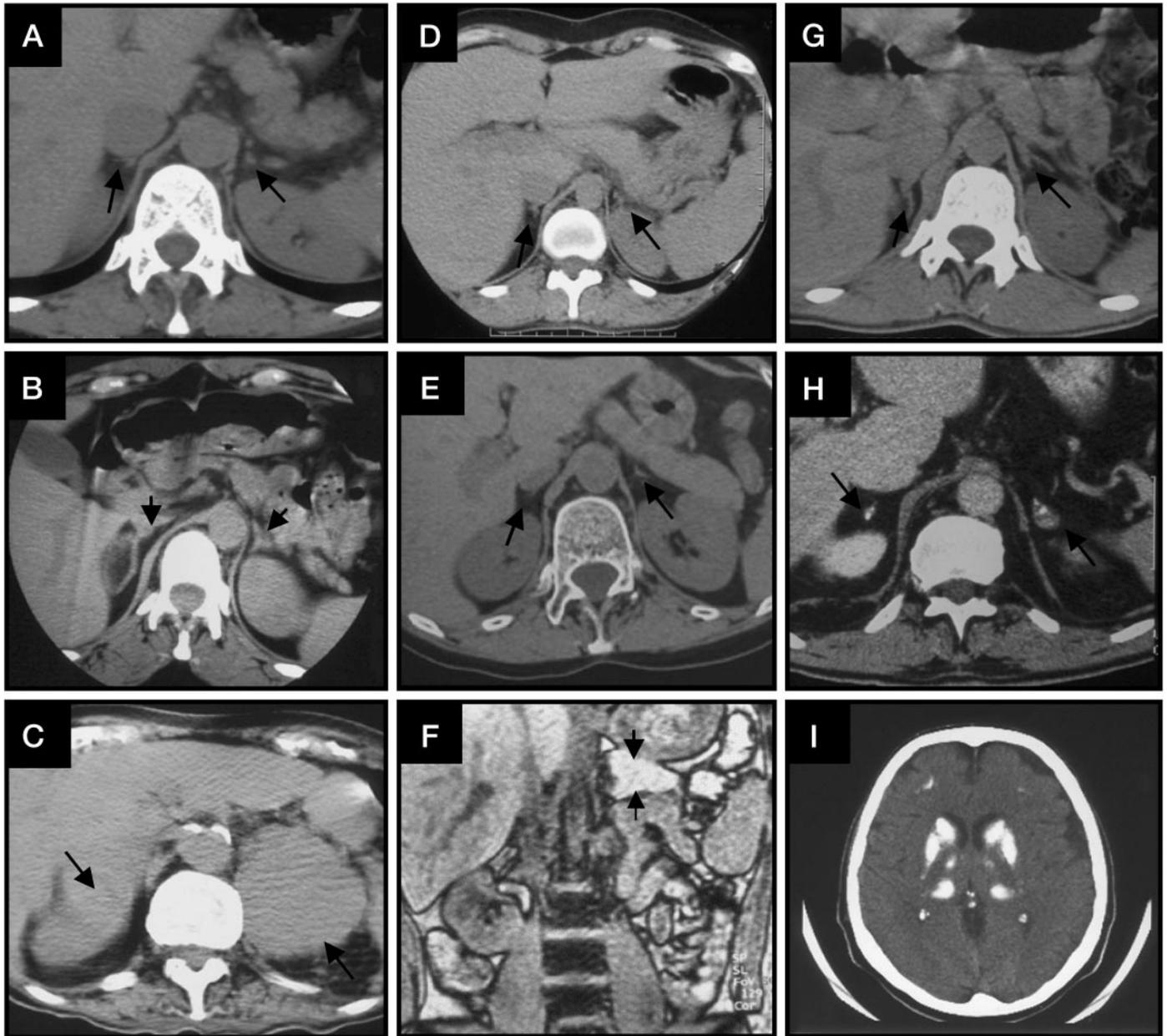


FIG. 2. CT scan (A–G) or NMR (H) of adrenal glands in patients with primary AD. Minuscule adrenal glands (*arrows*) in a patient with autoimmune AD in the context of APS type 1 (A) and in one with APS type 2 (B). Normal adrenal glands (*arrows*) in a patient with isolated autoimmune AD (C), and in a patient with potential AD (2 yr before the onset of clinical AD) (D). Minuscule adrenal glands in a patient with long standing AD (10 yr after diagnosis) in the context of APS type 2 (E). Adrenal bilateral calcifications with enlarged left adrenal gland (*arrow*) in a patient with AD caused by tuberculosis (F). Bilateral adrenal masses in a patient with AD caused by adrenal bilateral adenocarcinoma (G). [Courtesy of Dr. L. Benedetti from the Department of Imaging, Azienda Ospedaliera, Padova, Italy]. NMR of adrenal glands in a patient with AD showing a hyperplasia of left adrenal (*arrows*) due to congenital adrenal hyperplasia (H). [Courtesy of Dr. M. Cappa, Ospedale Pediatrico Bambin Gesù, Rome, Italy]. CT scan of the brain in a patient with APS type 1: symmetrical basal calcifications (I).

adrenals often weigh only about 1 g in end-stage disease, and it is often difficult to identify them at autopsy. In the active phase of the disease there is a widespread, but variable, mononuclear cell infiltrate consisting of lymphocytes, plasma cells, and macrophages. There is loss of normal three-layer structure of the adrenal cortex, and adrenocortical cells show necrosis and pleiomorphism. Residual cortical nodules may persist as the disease progresses, but these are eventually destroyed and the cortex is replaced by fibrous tissue. In

the end stage of adrenal cortex destruction, the remaining normal cellular components found within the adrenals are the cells in the medulla. At this stage there may be little or no signs of inflammation within the cortex, presumably because of the lack of cortical cells to elicit further immune response. Occasionally, complete absence of the adrenals in patients with AD have been reported, but this most likely reflected sampling error or possible damage to adrenal glands secondary to an ischemic episode (47). In contrast to

other autoimmune diseases, *e.g.*, thyroid autoimmune diseases (48), in AD there has been no description of the cellular components of the immune response in the affected tissue.

In the current literature there is only one report of an immunohistochemical study of the mononuclear cell infiltration of the adrenal cortex at autopsy in young and older individuals without AD or other autoimmune disease (49). This study showed various degrees of infiltration with mononuclear cells present in 63% of older and in 7.4% of younger subjects analyzed. The infiltration was mainly composed of CD3<sup>+</sup> T cells, with a considerable proportion of activated CD4<sup>+</sup>. The significance of these observations is not clear at present in view of the rarity of the ACA positivity as well as the rarity of autoimmune AD among the adult population in general.

### VI. Cellular Immunity in Autoimmune AD

Evidence for an antigen-specific T lymphocyte response in AD was suggested by early studies, by the migration inhibition assay, using adrenal cortex antigens obtained from pooled fetal (50), human adult glands (51), or monkey and porcine adrenals (52). However, similar antigens were unable to stimulate T cell proliferation in a blastogenesis assay (53). Furthermore, a nonspecific reduction of suppressor T lymphocyte function has been reported in patients with AD (54, 55). Another study suggested an increased percentage of activated T lymphocytes in the peripheral blood in patients with recent onset disease compared with those with longstanding autoimmune AD (56). More recently, a proliferative T cell response to an adrenal-specific protein fraction of 18–24 kDa molecular mass has been demonstrated in 6 of 10 patients with autoimmune AD (57).

### VII. Animal Models of Autoimmune AD

Experimental autoimmune adrenalitis has been produced in guinea pigs, rabbits, rats, monkeys, and mice by injection of autologous or heterologous adrenal homogenates mixed with various adjuvants. The histology of affected adrenals showed a mononuclear cell infiltration consisting mainly of lymphocytes and plasma cells grouped in foci of various sizes (for reviews see Refs. 41 and 58). The cortical cells were frequently abnormal with eosinophilia and vacuolization of the cytoplasm as well as with loss of nuclear definition. Reduced plasma corticosterone levels, fasting hypoglycemia, and increased excretion of salt and water during a salt-free diet in animals with adrenalitis were also observed. The adrenal lesions were more severe and the antibody titers higher when heterologous rather than homologous adrenal homogenates were used for immunization. Furthermore, the repeated immunization caused a delayed type hypersensitivity to adrenal antigens (58). It has not been possible to passively transfer adrenalitis from an affected animal to a healthy animal by means of serum. In some experiments, however, although the disease was transferred with lymph node (59, 60) or spleen cells (58), adrenal insufficiency has not developed, suggesting that the cell-mediated immunity may

have a critical role in the pathogenesis of autoimmune experimental adrenalitis.

### VIII. Autoimmunity to Nonadrenal Tissues in Autoimmune AD

After the first description by T. Addison of idiopathic AD with vitiligo, an association between autoimmune AD with other autoimmune manifestations was described in 1926 when Schmidt (61) described two patients with an association of a nontuberculous AD with chronic lymphocytic thyroiditis (named Schmidt's syndrome). In 1964, Carpenter *et al.* (62) reported that some patients with Schmidt's syndrome can also develop type 1 diabetes mellitus. In 1931 the first case of the association between AD, diabetes mellitus, and hyperthyroidism was described (63). In the following year, the first association of the triad AD, diabetes mellitus, and hypothyroidism was reported in one patient that died from diabetic ketoacidosis; at autopsy, the pancreatic islets of Langerhans were completely hyalinized, with a poor lymphocyte infiltration. In the adrenals, a few cortical cells were in a stroma of dense connective tissue and chronic inflammation, and in the thyroid an infiltration by lymphoid tissue compressing and displacing many of the glandular follicles was observed (64). In the following years, this cluster of autoimmune diseases was reported with increasing frequency: in 1959, 63 cases were reported (65), in 1964 more than 100 (62), and in 1981 there were 224 cases (66).

A child with chronic tetany due to hypoparathyroidism and chronic candidiasis was described for the first time in 1929 by Torpe and Handley (67). However, not until 1943, was a 12-yr-old girl with nontuberculous AD associated with idiopathic hypoparathyroidism, moniliasis, and phlyctenular keratoconjunctivitis described (68). In 1956, Whitaker *et al.* (69) added AD to the syndrome described earlier by Torpe and Handley. This observation was followed by two reports describing patients with variable combinations of chronic moniliasis, chronic hypoparathyroidism, and AD: 50 patients were described by Bronsky *et al.* in 1958 (70) and 71 patients were reported by Neufeld *et al.* (66) in 1981.

In addition, it has been reported that about 40% of the patients with autoimmune AD, compared with only 12% of patients with AD due to tuberculosis, were affected by other (nonadrenal) autoimmune diseases (40). The most frequently found organ-specific autoimmune diseases associated with autoimmune AD and their respective prevalences among European patients ( $n = 1240$ ) are summarized in Table 4. Autoimmune AD was associated, in order of frequency, with autoimmune thyroid diseases, chronic atrophic gastritis, type 1 diabetes mellitus, hypoparathyroidism, hypogonadism, vitiligo, alopecia, celiac disease, pernicious anemia, multiple sclerosis, inflammatory bowel diseases, Sjögren's syndrome, chronic hepatitis, and lymphocytic hypophysitis (8, 10, 14–22, 66). Furthermore, 4–17% of the patients with isolated autoimmune AD (*i.e.*, AD not associated with other clinical autoimmune diseases) showed evidence of autoimmunity to other organs at serological level and were positive for one or more nonadrenal autoantibodies (18, 21, 22). Autoantibody positivity to nonadrenal antigens in these pa-

TABLE 4. Prevalence of clinical autoimmune diseases in a cumulative population of 1240 patients with autoimmune AD<sup>a</sup>

| Diseases                       | Range (%) |
|--------------------------------|-----------|
| Hashimoto's thyroiditis        | 3.7–32    |
| Graves' disease                | 2.0–22.7  |
| Atrophic gastritis             | 25        |
| Chronic candidiasis            | 0.8–21    |
| Diabetes mellitus (type 1)     | 1.2–20.4  |
| Hypoparathyroidism             | 1.2–20    |
| Hypergonadotropic hypogonadism | 4.5–17.6  |
| Vitiligo                       | 0.8–16    |
| Alopecia                       | 0.8–12    |
| Celiac disease                 | 1.2–8     |
| Pernicious anemia              | 0.8–6     |
| Multiple sclerosis             | 3.7       |
| Inflammatory bowel diseases    | 2.4       |
| Sjögren's syndrome             | 2.4       |
| Chronic hepatitis              | 1.6–3     |
| Lymphocytic hypophysitis       | 0.8       |

<sup>a</sup> Data derived from Refs. 8, 10, 14–22, and 66.

tients could indicate a latent form of APS, suggesting that the prevalence of APS might be more frequent than previously estimated (see below).

### IX. Classification and Characterization of APS

Multiple endocrine gland insufficiencies sometimes associated with other autoimmune and non-autoimmune diseases may be observed in some patients with AD and their families. The associations between various autoimmune diseases were noted not to appear at random but in particular combinations (see above). Consequently, in 1980 Neufeld and Blizzard (71) organized and classified these clinical clusters in four main types defined as polyglandular autoimmune diseases, also termed autoimmune polyendocrine syndromes (APS) which are summarized in Table 5. According to this classification, autoimmune AD is one of the major components of APS type 1, type 2, and type 4.

In our series of Italian patients with AD ( $n = 322$ ), an autoimmune AD was diagnosed in 263 patients, and in this subgroup an APS, according to the Neufeld's classification (71), was found in 155/263 (59%) of patients. In particular, 35 cases (13%) could be classified as APS type 1, 107 cases (41%) as APS type 2, and 13 cases (5%) as APS type 4, but in 108 cases (41%) AD was apparently isolated (see Fig. 1).

### X. Animal Models of APS

To date, only a few animal models of experimentally induced or spontaneous APS have been documented. In particular, mice infected with reovirus type 1 developed an APS involving pancreatic islets, anterior pituitary, and gastric mucosa (72, 73). Organ-specific autoantibodies detected in this animal model, in contrast to main autoantibodies found in the human APS, reacted with the respective hormones produced by affected endocrine glands and did not recognize cytoplasmic or microsomal antigens. It has been reported that, after infection with mouse cytomegalovirus, some strains of mice may develop a type 2-like APS (74). Circulating autoantibodies to adrenal cortex, thyroid, stom-

TABLE 5. Classification of the APS according to Neufeld and Blizzard (71)

|            |                                                                                                                                     |
|------------|-------------------------------------------------------------------------------------------------------------------------------------|
| APS type 1 | Chronic candidiasis, chronic hypoparathyroidism, autoimmune AD ( <i>at least two present</i> )                                      |
| APS type 2 | Autoimmune AD + autoimmune thyroid diseases and/or type 1 diabetes mellitus ( <i>AD must always be present</i> )                    |
| APS type 3 | Thyroid autoimmune diseases + other autoimmune diseases ( <i>excluding autoimmune AD, hypoparathyroidism, chronic candidiasis</i> ) |
| APS type 4 | Two or more organ-specific autoimmune diseases ( <i>which do not fall into type 1, 2, or 3</i> )                                    |

ach, pancreatic islets, and ovary were detectable; in addition, lymphocytic infiltrations of adrenals, islets of Langerhans, liver, myocardium, and salivary glands have been found in these animals, but the disease remained at a subclinical level and did not progress to the overt disease (74).

The role of a depletion of regulatory T cells in the development of APS after immunomanipulation in some experimental animals has been also suggested. For example, athymic nude mice developed APS (autoimmunity toward thyroid, stomach, and ovaries/testis but not adrenals) by transfer of splenic cell suspensions depleted of  $\text{Lyt-1}^+ \text{,2,3}^-$  cells with a suppressive activity from a mice with APS. In contrast, the APS was prevented if  $\text{Lyt-1}^+ \text{,2,3}^-$  cells were included in the suspension of transferred cells (75). In another experiment, an APS (gastritis with parietal cell autoantibodies and oophoritis with oocyte autoantibodies) was induced in mice treated in the neonatal stage with cyclosporin A, which caused a selective deficiency of regulatory T cells. APS was prevented if cyclosporin-treated animals were inoculated with the spleen T cells from syngenic mice. However, removal of the thymus immediately after neonatal cyclosporin treatment induced an APS involving a wider spectrum of organs (adrenitis, oophoritis/orchitis, insulinitis, thyroiditis, and gastritis) (76).

The obese strain chicken develops a spontaneous autoimmune thyroiditis and sometimes has detectable autoantibodies to adrenals but also in this model the full spontaneous APS type 2 is not usually observed at the clinical level (77). The nonobese diabetic mouse is an animal model of spontaneous type 1 diabetes mellitus in which features of cell-mediated and humoral immunoreactions against thyroid, adrenal cortex, and salivary glands have been described (78). In this animal model, a lymphocytic parathyroiditis (79) was additionally described, but also this APS remains at a subclinical level. In 1995, Kooistra *et al.* (80) reported a spontaneous APS type 2 (AD and thyroiditis) in a boxer dog.

### XI. Pathogenesis of APS

In 1908, Claude and Gourgerot (81), in their review on polyglandular insufficiencies, suggested a common pathogenesis for these diseases. In 1912, Hashimoto (82) described a mononuclear leukocyte infiltration in some goitrous thyroid glands that was defined as "struma lymphomatosa". In 1940, similar lesions within pancreatic islets of patients with type 1 diabetes mellitus ("insulinitis") were described by Von Mayenburg (83). In 1954, Bloodworth *et al.* (84) suggested, for the first time, that the accumulation of antibodies in the

thyroid gland in patients with Schmidt's syndrome may be related to reduced levels of adrenal cortex hormones. In 1956, three independent groups demonstrated: 1) the presence of autoantibodies to thyroid autoantigens in sera from patients with Hashimoto's thyroiditis (85); 2) the induction of chronic thyroiditis in rabbits after immunization with autologous thyroid tissue in Freund's adjuvant (86); and 3) the presence of a long-acting thyroid stimulator in sera from patients with Graves' disease (87), which was later identified as an autoantibody to the TSH receptor (88, 89). In 1957 (39) it was discovered that idiopathic AD is autoimmune in nature.

These key observations heralded the rapid development of scientific interest and a continuous progress in studies on autoimmunity, including organ-specific autoimmune diseases. Various hypotheses have been proposed to explain the mechanisms of tolerance and autoimmunity in organ-specific autoimmunity (90). Autoimmune diseases can be due, in genetically susceptible individuals, to release of sequestered antigens, virus-induced alterations of host membrane proteins, cross-reactivity between environmental agents and host antigens, T cell bypass, or alteration of lymphoid cells and immune regulatory cells (90). All these theories, however, fail to explain the cascade of autoimmune aggression toward multiple organs in one individual, as in APS.

It has been suggested that development of multiple autoimmunity may be due to shared epitope(s) (one or more) between an environmental agent and a common antigen present in several endocrine tissues (90). Furthermore, it was also suggested that the organs derived from the same germ layer express common germ layer-specific antigens, and these could serve as targets for the autoimmune responses in APS (91). According to this theory, APS type 2 would be the result of both mesodermal (adrenal cortex) and endodermal (thyroid and pancreas) autoimmunity. Lack of spontaneous animal models of complete APS also contributes to our poor understanding of the pathogenesis of APS.

## XII. Features of Autoimmune AD (in APS and in Isolated Forms)

### A. APS type 1

1. *Main clinical features.* APS type 1 is characterized by the presence of three major component diseases: chronic candidiasis, chronic hypoparathyroidism, and autoimmune AD. This condition is sometimes referred to as *Candida* endocrinopathy syndrome (92) or autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (93, 94). To define APS type 1, at least two of the three major components need to be present (66, 71, 92, 94–101). World-wide prevalence of APS type 1 is very low; however, among the Iranian Jewish community, in Finland and in Sardinia, the estimated prevalence is 1/9,000, 1/14,400, and 1/25,000 inhabitants, respectively (94, 96, 97). In contrast, in other countries, *e.g.*, in Norway, the prevalence of APS type 1 is even lower, 1/80,000 (102). A higher prevalence of APS type 1 among some populations compared with the rest of the world could be related to a founder gene effect (see below).

The female-male ratio varies in different reports from 0.8–

2.4 (66, 71, 94, 95, 98). In general, the three major component diseases occur in a fairly precise chronological order (*Candida*, hypoparathyroidism, and AD), but they are present all together only in about half of the patients (94, 95, 98, 99). In most cases, APS type 1 starts at a young age, and the disease develops completely before the age of 20 yr (66, 71, 94, 95, 99).

*a. Chronic candidiasis and T cell defect.* In most cases of APS type 1, chronic candidiasis is the first manifestation of the disease, often occurring before the age of 5 yr. Candidiasis may affect the nails, the skin, the tongue, and the mucous membranes and may produce also angular cheilosis. Chronic candidiasis is considered to be the clinical expression of a selective immunological deficiency of T cells to *Candida albicans* (66, 71, 94, 95) combined with normal B cell responses to *Candida* antigens, which prevents the development of a systemic candidiasis (100). In some patients, chronic candidiasis leads to esophagitis with retrosternal pain and severe complications such as esophageal stricture or systemic candidiasis (94, 95, 98). Further, chronic candidiasis may lead in some patients to the development of epithelial carcinoma of the oral mucosa (95, 98). Abdominal pain, meteorism, and diarrhea were reported in patients with positive fecal cultures for *Candida* and symptoms subsided after systemic anticandidal therapy (94, 98). Anergy to candidal antigens is commonly found in patients with APS type 1 as well as anergy to tuberculin (98). According to the protocol of DePadova-Elder *et al.* (101), periodical antifungal treatment with itraconazole in patients with chronic candidiasis is often required, although this treatment gives good results in patients with nail infections but not in those with mucosal infections (95). Candidiasis is observed in 17–100% of patients and appears to be markedly less prevalent among the Iranian Jewish (17%) (96) compared with the Italian (83%) (95), Finnish, or Norwegian patients (100%) (99, 102). As the chronic *Candida* infection is a typical feature of APS type 1, this syndrome has been now classified by WHO as an immunodeficiency disease (103).

*b. Chronic hypoparathyroidism and parathyroid autoantibodies.* In the course of APS type 1, candidiasis is followed by chronic hypoparathyroidism, which usually appears before the age of 10 yr and affects 70–100% of patients. When chronic hypoparathyroidism develops during the neonatal period, it is important to differentiate this from genetic diseases such as Di George's syndrome (caused by a 22q11 deletion) (104, 105), Kenney-Caffey disease (locus mapped to chromosome 1q42-q43) (106), or the Barakat syndrome (caused by GATA3 haploinsufficiency) (105, 107). In particular, Di George's syndrome is characterized by defective development of organs dependent on cells of embryonic neural crest origin and includes congenital cardiac defects, mainly involving the great vessels, hypocalcemic tetany due to failure of development of parathyroid tissue, and isolated T cell defect due to the absence of a normal thymus (108). Finally, hypoparathyroidism not associated with APS type 1 occurs as an isolated familial disease with different patterns of inheritance (autosomal dominant, autosomal recessive, or X-linked recessive) (109–111).

The rare autopsy studies of parathyroid glands from patients with APS type 1 affected by chronic hypoparathyroidism showed atrophy and an infiltration of the parathyroids with mononuclear cells; in some cases parathyroid tissue was undetectable (69, 98, 112).

The history of the measurement of specific parathyroid cytoplasmic autoantibodies is rather complex. These autoantibodies, detected by indirect immunofluorescence (IIF), were initially described in 11–38% of patients with chronic hypoparathyroidism (113, 114), but subsequent studies in other laboratories were unable to confirm the presence of specific autoantibodies reacting with the chief cells of parathyroid glands (115). Some authors have reported that the autoantibody reactivity was not toward specific microsomal parathyroid antigens in the chief cells (116) but toward a human antigen of 46-kDa molecular mass present in mitochondria (117). These mitochondrial autoantibodies were different from the mitochondrial autoantibodies found in patients with primary biliary cirrhosis, which recognize non-organ- and non-species-specific mitochondrial antigens (118). In a later study, autoantibodies reacting with the surface of human parathyroid cells (or parathyroid sections) that had the ability to inhibit PTH secretion were described (119). Furthermore, cytotoxic autoantibodies reacting with cultured bovine parathyroid cells have been reported (120), but these autoantibodies lost their reactivity after absorption with endothelial cells (121). About half of the patients with chronic hypoparathyroidism in the context of APS type 1 were reported to have autoantibodies reacting with the extracellular domain of the calcium-sensing receptor (122). This observation suggested that the calcium-sensing receptor might be a specific autoantigen involved in autoimmune hypoparathyroidism. In a more recent study (98), however, calcium-sensing receptor autoantibodies were not detected in APS type 1 patients (n = 61), the majority of whom had hypoparathyroidism.

Although attempts to identify specific autoantibodies reactive with autoantigens within parathyroid glands have failed thus far, a role of autoimmunity in the pathogenesis of chronic hypoparathyroidism appears highly likely; however, to date this is the only organ-specific autoimmune disease without a defined serological marker. Further studies are necessary to identify specific autoantibodies and the trigger autoantigen(s) of this disease (123).

*c. AD and adrenal cortex autoimmunity.* In the course of APS type 1, AD tends to be the third disease to appear after chronic candidiasis and/or hypoparathyroidism, and it develops usually before 15 yr of age and affects 22–93% of patients. In most cases the disease is heralded by the presence of ACA, frequently found at the onset of the other main clinical manifestations of this type of APS (candidiasis and/or hypoparathyroidism).

The rare studies of adrenal glands obtained at autopsy of APS type 1 patients revealed adrenal atrophy with a lymphocytic infiltration (Ref. 112, and C. Betterle, personal observation). In patients with AD, CT or NMR of adrenals show normal or atrophic adrenal glands (see Fig. 2A). The majority of the patients with APS type 1 having AD were found to be positive for ACA (see Section XIV for further details). In our

group of 35 Italian patients with APS type 1 suffering from AD, ACA and/or 21-hydroxylase autoantibodies (21-OH Abs) were detected in 100% of the patients at the onset of AD (Table 6).

2. *Incomplete APS type 1.* ACA are frequently detectable in patients with chronic candidiasis and/or hypoparathyroidism without AD. These patients represent incomplete APS type 1 and have 100% risk of developing clinical AD (see Table 7, and Section XIV on potential AD).

3. *Other clinical features.* In addition to the major clinical manifestations, other immune- or not immune-mediated diseases often appear in patients with APS type 1 and they include: 1) *other endocrinopathies*: hypergonadotropic hypogonadism (24–60%), type 1 diabetes mellitus (0–12%), chronic thyroiditis (2–36%), and lymphocytic hypophysitis (7%); 2) *autoimmune gastrointestinal diseases*: chronic atrophic gastritis (13–27%), pernicious anemia (0–15%), and celiac disease; 3) *malabsorption* (6–22%) due to intestinal lymphangiectasia, exocrine pancreatic insufficiency, cystic fibrosis, intestinal infections, autoimmune gastrointestinal dysfunction, and deficiency of cholecystokinin (see below); 4) *liver diseases*: chronic active hepatitis (5–31%) and cholelithiasis (44%); 5) *autoimmune skin diseases*: vitiligo (8–25%) and alopecia (13–72%); 6) *autoimmune exocrinopathies*: Sjögren's syndrome (12–18%); 7) *rheumatic diseases*; 8) *ectodermal dystrophy* (10–52%) characterized by keratoconjunctivitis, nail dystrophy, defective dental enamel formation, faultless teeth; 9) *immunological defects*: T cell defect to *C. albicans*, IgA deficiency, polyclonal hypergammaglobulinemia; 10) *acquired asplenia* (suspected on the basis of a peripheral blood smear that shows Howell-Jolly bodies, thrombocytosis, anisocytes, poikilocytes, target cells, and burr cells); 11) *neoplasias* (epithelial carcinoma of the oral mucosa and of the esophagus and adenocarcinoma of the stomach); 12) *calcifications* of basal ganglia (see Fig. 2I), tympanic membranes, and subcapsular lens opacities; 13) *vasculitis* (3%); 14) *nephrocalcinosis* (complication related to vitamin D therapy due to hypocalcemia) (66, 71, 92, 94–96, 98, 102, 112, 124, 125).

It has been observed that the earlier the first APS type 1 component disease appears, the more likely it is that multiple components will develop (66, 94, 95). Furthermore, with increasing age, the number of component diseases increases and various neoplasias may develop (98).

A wide range of autoantibodies associated with these different autoimmune diseases have been found in patients with APS type 1, and in some cases these autoantibodies herald the development of the clinical disease.

For example, steroid-producing cell antibodies are associated with hypogonadism (see below).

Thyroid peroxidase and/or thyroglobulin autoantibodies are detectable in the majority of patients with chronic thyroiditis (95, 98, 125).

Chronic autoimmune hepatitis is associated with liver-kidney microsomal antibodies (126), reactive with cytochrome P450 (CYP IA2) (97) and CYP 2A6 antigens (127).

Antibodies to tyrosine hydroxylase are found in patients with alopecia areata (128), and complement-fixing melanocyte antibodies have been found in patients with vitiligo (3,

TABLE 6. Clinical features of 263 Italian patients with autoimmune AD in the context of APS or in isolated form

|                                       | APS             |                    |             | Isolated AD |
|---------------------------------------|-----------------|--------------------|-------------|-------------|
|                                       | Type 1          | Type 2             | Type 4      |             |
| Synonymous                            | APECED          | Schmidt's syndrome |             |             |
| No. of cases (% of total)             | 35 (13%)        | 107 (41%)          | 13 (5%)     | 108 (41%)   |
| Female/male ratio                     | 1.8             | 3.6                | 3.3         | 0.8         |
| Adults/children                       | 0.08            | 7.6                | 13          | 8.7         |
| Mean age at onset of AD (yr)          | 14              | 36                 | 36          | 30          |
| Deceased                              | 4               | 0                  | 0           | 0           |
| Familial APS (%)                      | 9 cases (26%)   | 0                  | 0           | 0           |
| Genetic                               | AIRE mutations  | HLA DR3            | HLA DR3 (?) | HLA DR3     |
| Autoantibodies at the onset of AD (%) |                 |                    |             |             |
| ACA                                   | 100             | 100                | 80          | 76          |
| 21-OH Abs                             | 100             | 100                | 100         | 80          |
| StCA                                  | 80              | 36                 | 38          | 4           |
| 17 $\alpha$ -OH and/or P450 scc Abs   | 80              | 40                 | 40          | 20          |
| Major components                      | Frequency (%)   |                    |             |             |
| Addison's disease                     | 100             | 100                | 100         | 100         |
| Chronic hypoparathyroidism            | 88              | Absent             | Absent      | Absent      |
| Chronic candidiasis                   | 79              | Absent             | Absent      | Absent      |
| Autoimmune thyroid diseases           | 13              | 83                 | Absent      | Absent      |
| Diabetes mellitus (type 1)            | 6               | 28                 | Absent      | Absent      |
| Other components                      | Frequency (%)   |                    |             |             |
| Hypergonadotropic hypogonadism        | 61 <sup>a</sup> | 9                  | 61          | 0           |
| Alopecia                              | 38              | 5                  | 8           | 0           |
| Vitiligo                              | 22              | 11                 | 31          | 0           |
| Chronic hepatitis                     | 19              | 4                  | 0           | 0           |
| Pernicious anemia                     | 19              | 1                  | 0           | 0           |
| Sjögren's syndrome                    | 16              | 1                  | 8           | 0           |
| Malabsorption                         | 15              | 0                  | 0           | 0           |
| Keratoconjunctivitis                  | 12              | 0                  | 0           | 0           |
| Neoplasias                            | 12              | 3                  | 0           | 1           |
| Chronic atrophic gastritis (isolated) | 6               | 11                 | 8           | 0           |
| Turner's syndrome                     | 3               | 0                  | 0           | 0           |

<sup>a</sup> Eight of 13 patients over the age of 14 yr.

95, 129). Recently, it has been reported that 63% of patients with APS type 1 and vitiligo had antibodies to transcription factors SOX9 and SOX10 (130). If this observation is confirmed, these factors could be considered as relevant autoantigens in autoimmune depigmentation.

Type 1 diabetes mellitus is rare in APS type 1 and is characterized by the presence of islet-cell antibodies (ICA) and autoantibodies to glutamic acid decarboxylase (GAD Abs), to second islet autoantigen (IA2 Abs), and to insulin as in the classical type 1 diabetes mellitus (95, 98, 131, 132).

In sera from patients with atrophic gastritis, parietal cell autoantibodies have been frequently found, and in those with pernicious anemia, intrinsic factor antibodies are additionally present (95, 125). Celiac disease has been associated with antibodies to reticulin and/or endomysium (95).

Since 1953, intestinal dysfunction, characterized by malabsorption, has been described in patients with APS type 1 (133, 134) and is observed in 18–22% of the patients (66, 94, 95). Malabsorption and/or steatorrhea can be due to a variety of causes such as celiac disease (95), cystic fibrosis (135), pancreatic insufficiency (136, 137), intestinal infections with *C. albicans* or *Giardia lamblia* (137), or intestinal lymphangiectasia (138). In some patients the malabsorption is well controlled by immunosuppression therapy, suggesting the possibility of an involvement of autoimmune mechanisms (139, 140). Recent findings may well confirm this hypothesis, *i.e.*, autoantibodies to tryptophan hydroxylase (TPH-Abs)

have been detected in 48% of APS type 1 patients, and the presence of these autoantibodies correlated significantly with gastrointestinal dysfunction. The sera from the patients positive for TPH-Abs caused cytoplasmic staining of enterochromaffin cells in normal human small intestine. Furthermore, the intestinal biopsy specimens obtained from patients with TPH-Abs showed no immunostaining of serotonin-containing enterochromaffin cells, which is usually observed in normal duodenum (141). TPH-Abs were not detected in any of the patients with gastrointestinal disorders not related to APS type 1; consequently, these autoantibodies may be considered markers of autoimmune gastrointestinal dysfunction in APS type 1 (141). Tryptophan hydroxylase and tyrosine hydroxylase are enzymes belonging to the group of pteridine-dependent hydroxylase enzymes involved in the biosynthesis of neurotransmitters (142). The complexity of intestinal dysfunction in APS type 1 has been demonstrated even further when an idiopathic deficiency of cholecystikinin was described in a patient with APS type 1 and malabsorption (143). Overall, these observations indicate that the gastrointestinal dysfunction in APS type 1 may have complex and different pathogeneses.

Antibodies to a novel 51-kDa antigen of the pancreatic islet  $\beta$ -cells (144), identified as aromatic L-amino acid decarboxylase (145), have been described in patients with APS type 1 in association with chronic active hepatitis, vitiligo, or type 1 diabetes mellitus (146).

TABLE 7. Incomplete APS type 1, 2, or 4

| Clinical disease                                         |   | Serology                                    |
|----------------------------------------------------------|---|---------------------------------------------|
| APS type 1                                               |   |                                             |
| Chronic hypoparathyroidism                               | + | ACA/21-OH Abs                               |
| Chronic candidiasis                                      | + | ACA/21-OH Abs                               |
| APS type 2                                               |   |                                             |
| Addison's disease                                        | + | Thyroid Abs and/or ICA and/or GAD Abs       |
| Thyroid autoimmune diseases                              | + | ACA/21-OH Abs                               |
| Type 1 diabetes mellitus                                 | + | ACA/21-OH Abs                               |
| Thyroid autoimmune diseases and type 1 diabetes mellitus | + | ACA/21-OH Abs                               |
| None                                                     | + | ACA + thyroid Abs and/or ICA and/or GAD Abs |
| APS type 4                                               |   |                                             |
| Addison's disease                                        | + | PCA and/or IFA                              |
| Addison's disease                                        | + | EmA and/or t-TGA                            |
| Addison's disease                                        | + | LKMA and/or AMA                             |
| Atrophic gastritis                                       | + | ACA/21-OH Abs                               |
| Alopecia                                                 | + | ACA/21-OH Abs                               |
| Vitiligo                                                 | + | ACA/21-OH Abs                               |
| Pernicious anemia                                        | + | ACA/21-OH Abs                               |
| Celiac disease                                           | + | ACA/21-OH Abs                               |
| Autoimmune hepatitis                                     | + | ACA/21-OH Abs                               |
| Primary biliary cirrhosis                                | + | ACA/21-OH Abs                               |
| Hypophysitis                                             | + | ACA/21-OH Abs                               |

ICA, Islet-cell autoantibodies; GAD Abs, glutamic acid decarboxylase autoantibodies; PCA, parietal cell autoantibodies; IFA, intrinsic factor autoantibodies; EmA, endomysium autoantibodies; t-TGA, tissue transglutaminase autoantibodies; LKMA, liver-kidney microsomal autoantibodies; AMA, antimitochondrial autoantibodies.

Other autoantibodies, for example PRL-secreting cell antibodies (147), have been described in APS type 1 patients but their clinical importance is not clear at present.

In many patients with APS type 1, autoantibodies to one or more of the above discussed antigens may be present also in the absence of the respective autoimmune clinical disease, and in some cases the presence of autoantibodies can precede the clinical disease (95, 98, 125, 131, 148, 149).

We have observed 35 patients with AD in the context of APS type 1, and the clinical, genetic, and serological features of these are summarized in Table 6. In addition to the main components of APS type 1, the most frequently observed disease was hypergonadotropic hypogonadism (61%) followed by alopecia (38%), vitiligo (22%), chronic hepatitis (19%), and Sjögren's syndrome (16%). Malabsorption was present in 15% and neoplasias in 12%. As mentioned above, APS type 1 is the autoimmune syndrome with the greatest simultaneous combination of autoimmune diseases and autoantibodies in an individual. This has been confirmed in our group of 35 patients with APS type 1 in whom we have observed a total of 150 clinical diseases.

**4. Genetic pattern.** APS type 1 is a condition occurring sporadically or among siblings (99, 112, 150–152) and is inherited in an autosomal recessive fashion (93, 153). Some studies reported an increased frequency of human leukocyte antigen (HLA)-A28 in patients with APS type 1 compared with normal controls, and of HLA-A3 in those with APS type 1 and ovarian failure compared with those with normal ovarian function (154). Furthermore, associations with HLA-DR5 both in Persian Jewish (155) and Italian patients (95) have been reported. No correlation between cytotoxic T lymphocyte antigen-4 (CTLA-4) gene polymorphism and APS type 1 of different ethnical provenance has been found to date (156). In 1994, a study of 14 Finnish families with APS type 1 identified a genetic linkage between the clinical presenta-

tion of this syndrome and genes located on the long arm of chromosome 21 (157). Subsequently, the gene responsible for this condition has been isolated, cloned, and defined as AIRE (autoimmune regulator) gene. AIRE gene consists of 14 exons and encodes a protein consisting of 545 amino acids that contains two plant homeodomain zinc finger motifs, three LXXLL motifs, and a proline-rich region, suggestive of its putative role as a nuclear transcriptional regulator (158, 159). To date, 42 separate mutations associated with APS type 1 in various racial groups have been identified in the AIRE gene. Of these 42 mutations, four appear to be the most important (160). The first described mutation was R257X in exon 6 (158–161) and was found in 82% of the Finnish APS type 1 alleles. This is also the most frequent mutation in patients with APS type 1 in other ethnic groups, such as Northern Italians, Swiss, British, Germans, New Zealanders, and American whites (162–164). The mutation *del13* present in exon 8 (158–161) has been detected in APS type 1 patients of various ethnic backgrounds, accounting for 5 of 18 of the North Italian alleles; it is also the most common in American Caucasian patients, particularly in those of Northern or Western European origin, or in British patients (102, 159, 162–166). The R139X mutation is present in exon 3 and represents the most common mutation in Sardinian patients with APS type 1 being present in 18 of 20 independent alleles (165). Only one mutation was detected in Iranian Jewish patients; it is a missense mutation in codon 85 within exon 2 defined as Y85C (167).

Other described mutations in the patients with APS type 1 are: *insA*, three different deletions of C (*delC*, *delG*, and *insC*), K83E, Q173X, R203X, X546C, L28P, and R15L (140, 161, 168, 169).

APS type 1 is the first autoimmune disease that has been shown to be caused by the mutations of a single gene. Mutation of AIRE gene in both alleles is usually associated with

the clinical expression of the syndrome. In contrast, the parents of patients with APS type 1 who carry only one mutant AIRE allele are not, in general, affected by the syndrome. Thus, the genetic mutations observed in APS type 1 may be responsible for the breakdown of immunotolerance in humans (161). Consequently, an understanding of the biological role of the AIRE protein should provide an insight into the mechanism of tolerance and autoimmunity (98).

The AIRE gene is expressed in relatively high levels in the thymus (in medullar epithelial cells and cells of the monocyte-dendritic cell lineage; both cell types representing a population of antigen-presenting cells) and in lower levels in the spleen, lymph nodes, pancreas, adrenal cortex, and in peripheral blood mononuclear cells (158, 159). Attention has already been drawn to its nuclear localization in a speckled pattern resembling nuclear dots and to its probable role in transcriptional regulation of the encoded proteins (167). AIRE interacts, *in vitro*, with the common transcriptional coactivator cAMP-response element-binding protein, and the transcriptional transactivation properties of AIRE together with its interaction with cAMP response element-binding protein might be involved in transcriptional regulation and, in consequence, in the negative selection or anergy induction of self-reactive thymocytes (170).

Among the 35 Italian patients with APS type 1 that we have studied, 9 patients were from 4 different family groups and the other 26 cases were sporadic. Of 35 patients, 17 were from Veneto, a region with 3.5 million inhabitants. The calculated prevalence of APS type 1 in this region was 0.46 cases per 100,000 inhabitants. Furthermore, it is interesting to note that nine of these 17 Venetian cases were all from Bassano del Grappa, a town of about 40,000 inhabitants near Vicenza city. This allowed us to calculate that, in this town, the prevalence of APS type 1 was 1.0 case per 4,400 inhabitants, which represents the highest concentrations of APS type 1 in the world. It is possible that this area is a “hot spot” for the mutations of the AIRE gene. The results of analysis of different mutations found in the AIRE gene in 10 of our 17 patients with complete APS type 1 from the Veneto region are summarized in Table 8.

In agreement with the previous report (162), in our population R257X was the most frequently found mutation (as in the Finnish patients), being present in seven of 10 individuals (homozygous in five patients and heterozygous with *del13* in two patients). Two individuals were

homozygous for *del13* (the most common in American and British patients). Interestingly, both patients with homozygous *del13* developed APS type 1 in adulthood. In one patient, the mutation typical of the Sardinian population (R139X) was found (Table 8).

Furthermore, among our 35 patients with APS type 1, a total of 150 clinical autoimmune and non-autoimmune diseases were observed. A similar accumulation of diseases among APS type 1 patients has been also described in other studies (94, 98). Thus, APS type 1 represents the syndrome with the highest concentration of autoimmune diseases in humans, and this may be consistent with the concept that a breakdown of immunotolerance as a consequence of a gene mutation is the main feature of APS type 1.

Consequently, the identification of AIRE gene mutations, particularly R257X, *del13*, R139X, and Y85C, occurring as the predominant mutations in different populations, should aid in the genetic diagnosis of APS type 1 in communities at high risk and in the screening of unaffected family members of APS type 1 patients.

## B. APS type 2

**1. Main clinical features.** APS type 2, also known as Schmidt's syndrome (61), is a rare condition occurring with a prevalence of 1.4–2.0 per 100,000 inhabitants (169). The female-male ratio ranges from 2–3.7. APS type 2 may occur at any age and in both sexes, but it is most common in middle-aged females and very rare in childhood (45, 66, 71, 171).

APS type 2 is characterized by the presence of autoimmune AD in association with either autoimmune thyroid diseases and/or type 1 diabetes mellitus. AD is present in 100% of the patients, autoimmune thyroid diseases in 69–82%, and type 1 diabetes mellitus in 30–52% of the patients (19, 61, 66, 72, 92, 171).

At the onset of AD, ACA and/or 21-OH Abs are detectable in the majority of the patients; in our patients these autoantibodies were present in 100% of the cases (see Table 6) (for further details on ACA see Section XIV). In patients with APS type 2, CT or NMR scans of the adrenals show normal or atrophic adrenal glands (see Fig. 2B), but in longstanding AD the adrenals are atrophic (Fig. 2E).

Patients with type 1 diabetes mellitus are frequently positive for ICA, GAD Abs, or IA2 Abs.

Patients with chronic thyroiditis are frequently positive for

TABLE 8. Haplotype analysis in 10 Italian patients with APS type 1

| Patients  | Mutations         | Sex | Major clinical diseases | Age at onset of AD |
|-----------|-------------------|-----|-------------------------|--------------------|
|           | Allele 1/Allele 2 |     |                         |                    |
| 1. F.T.   | R257X/R257X       | F   | CC + CHP + AD           | Adulthood          |
| 2. F.L.   | R257X/R257X       | F   | CC + CHP + AD           | Childhood          |
| 3. A.E.   | R257X/R257X       | M   | CC + CHP + AD           | Childhood          |
| 4. C.A.   | R257X/R257X       | M   | CC + CHP + AD           | Childhood          |
| 5. C.G.   | R257X/R257X       | M   | CC + CHP + AD           | Childhood          |
| 6. C.G.   | del 13/R257X      | F   | CC + CHP + AD           | Childhood          |
| 7. C.E.   | del 13/R257X      | F   | CC + CHP + AD           | Childhood          |
| 8. T.P.   | del 13/del 13     | F   | CC + CHP + AD           | Adulthood          |
| 9. D.G.F. | del 13/del 13     | M   | CC + CHP + AD           | Adulthood          |
| 10. S.M.  | R139X/R139X       | F   | CC + CHP + AD           | Childhood          |

Patients 4 and 5 are brothers, and patients 6 and 7 are sisters. CC, Chronic candidiasis; HP, chronic hypoparathyroidism.

thyroid microsomal (thyroid peroxidase) and/or thyroglobulin autoantibodies and usually show a thyroid gland with a hypoechogenic pattern at ultrasonography. In particular, patients with Graves' disease have thyroid-stimulating antibodies reactive with TSH receptor (171).

APS type 2 component diseases tend to develop in a specific sequence: type 1 diabetes mellitus develops in general before autoimmune AD, whereas autoimmune thyroid diseases develop before, contemporary with, or after AD (171). In terms of autoimmune thyroid diseases, Graves' disease tends to develop before, and Hashimoto's thyroiditis tends to develop contemporary or after, the onset of autoimmune AD (19, 65, 171). We have studied 107 patients with APS type 2 and the mean age at onset was 36 yr; 89% showed the presence of AD with another main component disease (50% AD + Hashimoto's thyroiditis, 21% AD + Graves' disease, and 18% AD + type 1 diabetes mellitus); only 11% showed the presence of the complete triad. The main clinical, genetic, and serological features of Italian patients with APS type 2 we have studied are summarized in Table 6.

**2. Incomplete APS type 2.** In the original report, Neufeld and Blizzard stated that a patient with type 1 diabetes mellitus and thyroid autoimmune disease should be categorized as having APS type 2 if a sibling had AD plus type 1 diabetes mellitus and/or thyroid autoimmune diseases, *i.e.*, if a sibling had complete APS type 2 (71).

In our view, a patient with type 1 diabetes mellitus and/or thyroid autoimmune disease showing the ACA in the serum or a patient with AD and thyroid and/or islet cell autoantibodies should be classified as incomplete APS type 2, irrespective of their family history. Although these patients cannot be classified as "complete" APS type 2, they are clearly "borderline" or they can develop the "complete" APS type 2 in the future.

We propose to split these incomplete APS type 2 into subclinical and potential. "Subclinical" APS type 2 is defined by the presence of one clinical disease characteristic of this syndrome with one or more serological marker(s) of the other components but in the presence of subclinical impairment of the target organ. For example, patients with subclinical APS type 2 are those with AD + thyroid autoantibodies and subclinical hyper- or hypothyroidism, or those with AD + ICA and/or GAD Abs and impaired oral glucose tolerance, or those with type 1 diabetes mellitus + ACA/21-OH Abs and subclinical hypoadrenalism, or those with thyroid autoimmune disease + ACA/21-OH Abs and subclinical hypoadrenalism, or those with thyroid autoimmune disease and type 1 diabetes mellitus + ACA/21-OH Abs and subclinical hypoadrenalism. In addition, patients not having any overt component of APS type 2 but with detectable ACA + thyroid autoantibodies and/or ICA and subclinical hypoadrenalism and/or subclinical thyroid dysfunction and/or impaired glucose tolerance could also be classified as "subclinical" APS type 2.

We propose to define as "potential" APS type 2 those patients showing one clinical autoimmune disease of the syndrome with autoantibody markers of another fundamental disease but with a normal function of the target organs.

A summary of different combinations of incomplete APS type 2 is shown in Table 7.

In view of the natural history of APS type 2 and its different forms, it appears that the autoantibody status is relevant for classification of the disease for the diagnosis of overt disease itself. Consequently, it would be appropriate that at the onset of type 1 diabetes mellitus, all patients are tested for ACA/21-OH Abs and at the onset of autoimmune AD, all patients are tested for ICA, GAD Abs, IA2 Abs, and for thyroid autoantibodies. Such autoantibody screening should not present difficulties as the reliable, sensitive, and relatively easy-to-use diagnostic tests are currently available. This approach should allow early and more extensive identification of patients with or at risk of complete APS type 2 in the population. Thus, patients with one autoimmune disease characteristic of APS would represent the "tip of the iceberg" and could well have other autoimmune diseases in the latent phase. Early diagnosis and therapy may be beneficial to such patients before the overt disease develops. Indeed, specific tests ( $fT_3$ ,  $fT_4$ , TSH, oral glucose tolerance test, ACTH test) in these patients often reveal a subclinical impairment of the thyroid, the pancreatic  $\beta$ -cells, or the adrenal cortex function and may identify patients already affected by a subclinical or potential APS who are at high future risk of developing the clinical APS type 2 (171–175).

**3. Other clinical features.** Other autoimmune diseases that are not the major components may be present in APS type 2: for example, hypergonadotropic hypogonadism (4–9% of patients), vitiligo (4.5–11% of patients), alopecia (1–4% of patients), chronic hepatitis (4% of patients), chronic atrophic gastritis with or without pernicious anemia (4.5–11% of patients), and hypophysitis. However, these autoimmune diseases are present with a lower frequency than in APS type 1 (92, 171). In general, these minor component diseases are associated with the presence of the respective serological markers, but sometimes the autoantibodies precede the development of the clinical disease itself (171).

In our group of 107 patients with APS type 2, 240 autoimmune diseases were cumulatively present, and this suggests that an important failure of the self-tolerance may be present also in patients with APS type 2, as observed for APS type 1. However, unlike APS type 1, the genetic susceptibility in APS type 2 is linked to different genes (see below).

**4. Genetic pattern.** APS type 2 often occurs in many generations of the same family in an autosomal dominant, with incomplete penetrance pattern of inheritance (176, 177). In addition, an increased frequency of autoimmune diseases in first-degree relatives of patients with APS type 2 has been observed (176). HLA play a key role in determining T cell responses to antigens, and various HLA alleles have been shown to be associated with many T cell-mediated autoimmune disorders (178, 179).

Conflicting results have been reported about the association of HLA-B8 and autoimmune AD. Thomsen *et al.* (180) first described the association of HLA-B8 and AD in Caucasians, and this report has been confirmed by Eisenbarth and associates (176) but not by others (181, 182). An association of autoimmune AD and HLA-DR3, which is in linkage

disequilibrium with HLA-B8, has been reported in the later study. An increased prevalence of HLA-DR3 and/or DR4 has been found in patients with autoimmune AD, except when the disease occurred as a component of APS type 1 (183). The calculated relative risk of autoimmune AD for Caucasian subjects carrying both HLA-DR3 and HLA-DR4 alleles was high at 46.8 (184).

Several subsequent studies have confirmed the association of autoimmune AD in APS type 2 patients with various alleles within the HLA-DR3-carrying haplotype including DRB1\*0301, DQA1\*0501, and DQB1\*0201 (171, 184–190). In contrast, the association of HLA-DR4 with autoimmune AD appeared less convincing (171, 185–187, 189). Huang *et al.* (189) demonstrated that the subtype HLA-DR3 DQB1\*0201 was increased in the US patients with APS type 2 and that HLA-DR4 DQB1\*0302 was increased in those with autoimmune AD and type 1 diabetes mellitus. Our own studies have shown that in Italian patients with autoimmune AD in APS type 2 patients in addition to HLA-DR3, the prevalence of HLA-DR5 was increased in patients with both autoimmune AD and thyroid autoimmunity (171).

Other genes within the HLA complex have also been studied for an association with autoimmune AD. However, due to the strong linkage disequilibrium of genes within this region, it is difficult to determine the independent role of a particular gene in conferring susceptibility to the disease. For example, it has been shown that the association of autoimmune AD with a polymorphism of the TNF gene located in the class III HLA region was due to linkage disequilibrium with the class II HLA genes (188). Similarly, it is likely that the recently reported association between autoimmune AD with a microsatellite polymorphism in major histocompatibility class I chain-related (MIC-A) gene is a result of linkage disequilibrium, rather than a primary association (190).

The CTLA-4 gene on chromosome 2q33 encodes a costimulatory molecule that is an important negative regulator for T cell activation (191). This locus is linked to type 1 diabetes mellitus and associated with autoimmune thyroid diseases (Graves' and Hashimoto's thyroiditis) (192–194). Studies of German patients with autoimmune AD (either isolated or in the context of APS type 2) suggested that CTLA-4 ala17 allele may be significantly associated with AD only in a subgroup of patients carrying the HLA-DQA1\*0501 allele (193). Furthermore, one study on patients from different European countries with either isolated AD or in the context of APS type 2 showed a significantly increased association between the CTLA-4 microsatellite gene polymorphism and AD either in isolated form or in APS type 2 in English, but not in Norwegian, Finnish, or Estonian patients (156). Recently, a study of 91 English patients with either isolated AD or with APS type 2 showed a significantly increased frequency of the G allele of CTLA-4 when the patients were analyzed as a group; however, when the patients were analyzed separately, this correlation could not be found (195). In the same study, patients with AD either isolated or in the context of APS type 2 were evaluated for the presence of *del13* on AIRE gene (typical of the British population with APS type 1). Only one patient was found to be positive in heterozygosis for this mutation, and this frequency was not different from the control population (195), indicating that

this mutation does not make a contribution to the etiology of AD when isolated or in the context of APS type 2.

In our studies, HLA-DR3 has been found with a statistically significant higher frequency among 38 patients with APS type 2 compared with normal controls ( $P$  corrected = 0.05) (149).

### C. APS type 3: autoimmune thyroid diseases and other autoimmune diseases excluding AD

In the original classification of Neufeld and Blizzard (71), APS type 3 was defined as the association between one of the clinical entities of the autoimmune thyroid diseases (Hashimoto's thyroiditis, idiopathic myxedema, symptomless autoimmune thyroiditis, Graves' disease, endocrine ophthalmopathy) and one or more of other autoimmune diseases [type 1 diabetes mellitus (type 3a), atrophic gastritis, pernicious anemia (type 3b), vitiligo, alopecia, myasthenia gravis (type 3c)]. Autoimmune AD and/or hypoparathyroidism were not included into the component diseases of APS type 3 according to this original classification.

Subsequently, it has been shown that different and multiple clinical combinations could be found in APS type 3 and that the classification of APS type 3 may be more complicated than initially reported. Furthermore, the genetic and immunological aspects of this syndrome have been recently reviewed (42). Consequently, we have recently proposed a new classification criteria for APS type 3 (see Table 9), but it is possible that these will require revision in the future as our understanding of the APS improves (196). However, APS type 3 will not be discussed in more detail in the present review.

### D. APS type 4: autoimmune AD associated with other autoimmune diseases

1. *Clinical features.* APS type 4 is a rare syndrome characterized by the association of autoimmune combinations not falling into the above categories (71). For example, AD with one or more minor component autoimmune diseases (hypogonadism, atrophic gastritis, pernicious anemia, celiac disease, myasthenia gravis, vitiligo, alopecia, hypophysitis, etc.) but excluding the major component disease characteristics of APS type 1 and type 2 (chronic candidiasis, hypoparathyroidism, thyroid autoimmune diseases, type 1 diabetes mellitus) can be included in this particular APS. In our studies, ACA and/or 21-OH Abs are present in 100% of patients at the onset of AD (see Table 6) (for further details on ACA, see Section XIV). The associated clinical autoimmune diseases are in general marked by the presence of the respective autoantibodies.

Patients with the clinical apparent APS type 4 should be tested for ICA, GAD Abs, and thyroid autoantibodies, because the presence of one or more of these autoantibodies helps to differentiate patients with "false" APS type 4 from patients with potential or latent APS type 2. In APS type 4 it is also important to exclude the presence of chronic candidiasis and/or signs of clinical or latent hypocalcaemia to exclude a subclinical APS type 1.

We have studied 13 APS type 4 patients, and their prin-

TABLE 9. Characteristics of APS type 3 (modified from Betterle, 2001) (196)

| Autoimmune thyroid diseases                                                          |                                                                                                                |                                                               |                                                                                                                                                                                                           |
|--------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Hashimoto's thyroiditis<br>Idiopathic myxedema<br>Symptomless autoimmune thyroiditis |                                                                                                                | Graves' disease                                               |                                                                                                                                                                                                           |
|                                                                                      |                                                                                                                | Endocrine ophthalmopathy                                      |                                                                                                                                                                                                           |
| +                                                                                    | +                                                                                                              | +                                                             | +                                                                                                                                                                                                         |
| Type 1 diabetes mellitus<br>Hirata's disease<br>Lymphocytic hypophysitis             | Chronic atrophic gastritis<br>Pernicious anemia                                                                | Vitiligo<br>Alopecia                                          | SLE o DLE<br>Mixed connective tissue disease<br>Rheumatoid arthritis<br>Seronegative arthritis<br>Systemic sclerosis<br>Sjögren's syndrome<br>Werlhof syndrome<br>Antiphospholipid syndrome<br>Vasculitis |
| POF                                                                                  | Celiac disease<br>Chronic inflammatory bowel diseases<br><br>Autoimmune hepatitis<br>Primary biliary cirrhosis | Myasthenia gravis<br>Stiff-man syndrome<br>Multiple sclerosis |                                                                                                                                                                                                           |
| 3A                                                                                   | 3B                                                                                                             | 3C                                                            | 3D                                                                                                                                                                                                        |

SLE, Systemic lupus erythematosus; DLE, discoid lupus erythematosus.

cial clinical, genetic, and serological features are summarized in the Table 6.

In these patients, similar to the patients with APS type 1 and type 2, CT or NMR imaging reveal normal or atrophic adrenal glands (C. Betterle, personal observation).

2. *Incomplete APS type 4.* Incomplete APS type 4 is more frequent, as previously reported. The summary of various conditions observed in patients classified as incomplete APS type 4 is shown in Table 7.

3. *Genetic pattern.* From the various group of patients with AD studied for genetic pattern, it is difficult to select the patients with APS type 4. We have assessed HLA-DR status in seven patients with APS type 4 and DR3 was found with a higher frequency compared with controls, but the low number of studied cases was a limiting factor for statistical evaluation (see Table 6).

#### E. Isolated autoimmune AD

1. *Clinical features.* Isolated AD represents the fourth clinical presentation of the disease, defined by the absence of any other clinical autoimmune disease. We have observed 108 cases with isolated AD representing 41% of our 263 autoimmune AD patients. The female-male ratio was 0.8, and mean age at onset was 30 yr (Table 6). As in the case of APS, in patients with isolated AD, NMR or CT imaging reveals normal or atrophic adrenal glands (see Fig. 2C).

ACA and/or 21-OH Abs were present at the onset of AD in 80% of our patients (for further details on ACA, see Section XIV and see Table 6). The patients with isolated AD, negative for ACA and with normal or atrophic adrenal glands at imaging, should be investigated further to identify evidence of possible autoimmune pathogenesis of AD (*i.e.*, analysis of the HLA-DR status; screening for other organ-specific, antiphospholipid, or antinuclear autoantibodies should be carried out); or to identify different pathogenesis (*i.e.*, by performing the determination of very-long-chain fatty acids or other genetic investigations). The cases when the etiology can not be clearly established should be considered as "apparently idiopathic".

2. *Isolated AD as incomplete APS.* After diagnosis of the clinically isolated AD, we suggest that periodic (at the onset and every 2–3 yr) autoantibody screening is carried out routinely. This strategy allows us to find, during the lifetime, one or more of the serological markers of other autoimmune diseases (thyroid, parietal cells, intrinsic factor, islet cell, glutamic acid decarboxylase, endomysium, tissue transglutaminase, steroid-producing cells, mitochondria, nuclear autoantibodies) in 48% of the patients with apparently isolated autoimmune AD. These cases represent incomplete APS (see Table 7). In these patients, specific function tests often show subclinical impairment of the thyroid gland, of the gastric mucosa, of the endocrine pancreas, and of the bowel and of hepatic or collagen disease, and may herald a future risk of developing clinical APS. Patients who are positive for autoantibodies, but do not demonstrate functional or biochemical impairment of the target organs, should have the tests repeated periodically as they are at risk of developing first a subclinical and later a clinical APS.

Due to the dynamic nature of both autoantibody positivity and the onset of different autoimmune diseases, the patients with isolated autoimmune AD may need to be reclassified during the follow-up (*e.g.*, a patient with isolated AD may become a patient with APS type 1, 2, or 4 during several years of observation) (C. Betterle, personal observation).

3. *Genetic pattern.* An increased frequency of HLA-DR3 was found in some patients with isolated AD (186, 188). In addition, in English patients with isolated AD, the G allele of CTLA-4 was found to be increased but without a significant correlation (195).

In 15 of our patients with isolated autoimmune AD, the prevalence of HLA-DR3 was significantly ( $P = 0.05$ ) increased compared with normal controls. The main immunological, clinical, and genetic data of our patients with isolated AD are summarized in Table 6.

#### XIII. Autoimmune AD: Four Well Defined Clinical Entities with the Same Serological Marker

As discussed in previous paragraphs, the main criteria for identification of the autoimmune nature of adrenal insuffi-

ciency are primarily based on the presence of circulating ACA and/or 21-OH Abs and on the evidence of normal/atrophic adrenal cortex by morphological studies. In addition, other clinical (age at onset, type of autoimmune-associated diseases, presence of candidiasis, presence of hypogonadism) and/or genetic findings (mutations of AIRE gene, HLA-DR, CTLA-4) contribute to a spectrum of factors to be considered in the context of autoimmune AD. Taken together, four main distinct clinical forms of autoimmune AD (summarized in Table 10) can be identified; all are characterized by a common denominator: the presence of ACA or 21-OH Abs and/or normal or atrophic gland.

#### XIV. Serological Markers of Autoimmune AD

One of the hallmarks of autoimmune diseases is the presence of antibodies recognizing self-antigens (36, 37) (see Table 3). The main autoantibodies involved in adrenal autoimmunity are 1) ACA or steroid 21-OH Abs, and 2) steroid-producing cell autoantibodies (StCA). Autoantibodies of minor clinical importance directed to the other components of adrenal cortex are 1) adrenal surface autoantibodies, 2) ACTH-receptor antibodies, and 3) anticorticosteroid hormone autoantibodies.

##### A. ACA/21-OH Abs and autoantigens

1. *In patients with clinical AD.* ACA were discovered in 1957 by Anderson *et al.* (39) using a complement-fixation test. In the initial studies ACA were detected in 36% (range, 25–43) of patients with idiopathic AD but also in 9% (range, 0–40) of the patients with adrenal insufficiency due to tuberculosis (14, 39, 197, 198). Subsequently, Blizzard and Kyle (197) introduced the IIF test for ACA. In the years from 1963 to 1990, ACA were cumulatively assessed by IIF test in 1178 patients with idiopathic AD and in 214 patients with adrenal insufficiency due to tuberculosis. Overall, ACA were detected by IIF test in 60% (range, 38–73) of patients with idiopathic AD and in 7% (range, 0–60) of AD due to tuberculosis (8, 19, 40, 197–208). These results suggested that the prevalence of ACA varied considerably between laboratories probably due to the differences in IIF technique such as different substrates used (animal or human tissues), time of incubation of samples with substrates, and/or differences in geographical or racial origins of the patients, in patients' gender, age at onset, duration of the disease, and type of associated autoimmune disorders (8, 19, 40, 197–208). Furthermore, difficulties in correctly identifying the true nature of AD in the past may explain some of the conflicting data of earlier studies. However, despite these differences, the IIF test using unfixed cryostat sections of human or animal adrenal glands has been the most widely used method until the recent years for measuring ACA (209).

ACA are organ-specific autoantibodies that react with all three layers of the adrenal cortex, producing a homogeneous cytoplasm-staining pattern. Some rare sera react exclusively with one or two of the three cortical layers (40, 198, 206). Reported titers of ACA varied greatly from 1:1 to 1:2560 in different studies (8, 197, 201, 203). ACA are usually of IgG<sub>1</sub>, IgG<sub>2</sub>, and IgG<sub>4</sub> subclasses (210).

In our studies of 165 patients with different forms and duration of AD, ACA using the IIF test were found in 81% of patients with autoimmune AD (overall in isolated AD and AD associated with APS) and in none of the patients with non-autoimmune AD (Fig. 3A). The prevalence of ACA in patients with autoimmune AD was higher (90%) in those with recent onset disease ( $\leq 2$  yr of disease duration) than in those with longstanding disease (79%) ( $> 2$  yr of disease duration) (Fig. 3C). Furthermore, the prevalence of ACA varied in relation to the clinical presentation of the disease, with ACA being present in 86%, 89%, and 73% of patients with APS type 1, type 2, and isolated AD, respectively (see Fig. 3B) (211). Finally, when the ACA test was performed in patients close to the clinical onset of the AD, the antibodies were present in 100% of cases with APS type 1, type 2, or type 4 and in 76% of patients with isolated AD (C. Betterle, personal observation).

These results indicate that the ACA test at the onset of clinical manifestation is very useful in identifying autoimmune AD. Furthermore, ACA positivity in patients with autoimmune AD tends to persist longer after the disease onset (Fig. 3C) compared with, for example, ICA positivity in type 1 diabetes mellitus (212).

Other methods have been described to measure ACA, *e.g.*, ELISA or RIA based on human adrenal microsome preparations (207, 208), but none of these assays showed specificity or sensitivity comparable to the IIF test (209).

In 1988, a specific 55-kDa protein reactive with ACA was identified in human adrenal microsomes (213). Subsequently, in 1992, the screening of a human fetal adrenal cDNA library with the sera from children with autoimmune AD in the context of APS type 1 allowed isolation of clones with high homology to steroid 17 $\alpha$ -hydroxylase (17 $\alpha$ -OH) (214). This study concluded that 17 $\alpha$ -OH was the autoantigen associated with autoimmune AD in children with APS type 1 (214). Reactivity of the sera from patients with APS type 1 with P450 side chain cleavage (scc) was reported soon afterward (215). In the same year, steroid 21-OH was identified as a major adrenal autoantigen in two independent studies of patients with autoimmune AD excluding those with APS type 1 (215–217). Reports on the identification of 21-OH as a major adrenal autoantigen were confirmed by studies in several laboratories using different methods, including Western blotting or immunoprecipitation based on native or recombinant 21-OH expressed in bacteria, yeast, and mammalian cells, or in an *in vitro* transcription/translation system irrespective of whether AD presented as isolated, in the context of APS, or in ACA-positive patients without overt AD (22, 218–225). Direct evidence that 21-OH is the major autoantigen recognized by ACA is now emerging from absorption studies carried out recently in our laboratories. Sera from six patients with different types of autoimmune AD positive for ACA (with titers ranging from 1:16 to 1:64) and 21-OH Abs (2.6–1311 U/ml) were used in the study. In addition, one of the six sera was positive for StCA, 17 $\alpha$ -OH Abs, and P450 scc Abs, one serum for StCA and P450 scc Abs, and one serum for StCA and 17 $\alpha$ -OH Abs. After incubation with purified human recombinant 21-OH, all six sera lost their ACA positivity and 21-OH Abs activity. In contrast, reactivities of sera to StCA, 17 $\alpha$ -OH, and/or P450 scc (when

TABLE 10. Main features of the four clinical presentations of autoimmune AD in humans

|                                                                | APS type 1                                                                                                                                                                | APS type 2                                                                                                                        | APS type 4                                                                                                                        | Isolated AD                                       |
|----------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------|
| Gender                                                         | F>M<br>13                                                                                                                                                                 | F>M<br>36                                                                                                                         | F>M<br>36                                                                                                                         | M>F<br>30                                         |
| Mean age at onset (yr)                                         | APS type 1 (25%)                                                                                                                                                          | AD (rare)<br>Other autoimmune diseases (frequent)                                                                                 | AD (rare)<br>Other autoimmune diseases (frequent)                                                                                 | AD (rare)<br>Other autoimmune diseases (frequent) |
| Family history for                                             | AIRE gene mutations<br>Candidiasis,<br>hypoparathyroidism, AD                                                                                                             | HLA-DR3, DR4, <sup>a</sup> DR5 <sup>a</sup><br>AD, thyroid autoimmune diseases, type 1 DM                                         | HLA-DR3<br>AD                                                                                                                     | HLA-DR3<br>AD                                     |
| Genetic                                                        | Present                                                                                                                                                                   | Absent                                                                                                                            | Absent                                                                                                                            | Absent                                            |
| Major component diseases                                       | 15%                                                                                                                                                                       | 3%                                                                                                                                | Rare                                                                                                                              | Rare                                              |
| Ectodermal dystrophy                                           | 100%                                                                                                                                                                      | 100%                                                                                                                              | 100%                                                                                                                              | 80%                                               |
| Cancer                                                         | 62%                                                                                                                                                                       | 30%                                                                                                                               | 38%                                                                                                                               | 13%                                               |
| ACA and/or 21-OH Abs (at onset of AD)                          | In general associated with clinical hypogonadism or preceding it                                                                                                          | In general associated with clinical hypogonadism or preceding it                                                                  | In general associated with clinical hypogonadism or preceding it                                                                  | In general preceding clinical hypogonadism        |
| StCA and/or 17 $\alpha$ -OH Abs and/or P450 scc Abs            | 11–60%                                                                                                                                                                    | 0–11%                                                                                                                             | 100%                                                                                                                              | 0%                                                |
| Minor autoimmune diseases                                      | Gonadal failure, vitiligo, alopecia, atrophic gastritis, pernicious anemia, celiac disease, chronic hepatitis, hypophysitis, malabsorption, cholelithiasis asplenia, etc. | Gonadal failure, vitiligo, alopecia, atrophic gastritis, pernicious anemia, celiac disease, chronic hepatitis, hypophysitis, etc. | Gonadal failure, vitiligo, alopecia, atrophic gastritis, pernicious anemia, celiac disease, chronic hepatitis, hypophysitis, etc. |                                                   |
| Presence of Abs in the absence of respective clinical diseases | About 65%                                                                                                                                                                 | About 50%                                                                                                                         | About 50%                                                                                                                         | About 50%                                         |
| NMR or CT of adrenals                                          | Normal or atrophic glands                                                                                                                                                 | Normal or atrophic glands                                                                                                         | Normal or atrophic glands                                                                                                         | Normal or atrophic glands                         |
| Histopathology                                                 | Lymphocytic adrenalitis                                                                                                                                                   | Lymphocytic adrenalitis                                                                                                           | Lymphocytic adrenalitis                                                                                                           | Lymphocytic adrenalitis                           |

<sup>a</sup> Depending on main associated autoimmune diseases. DM, Diabetes mellitus; F, female; M, male.

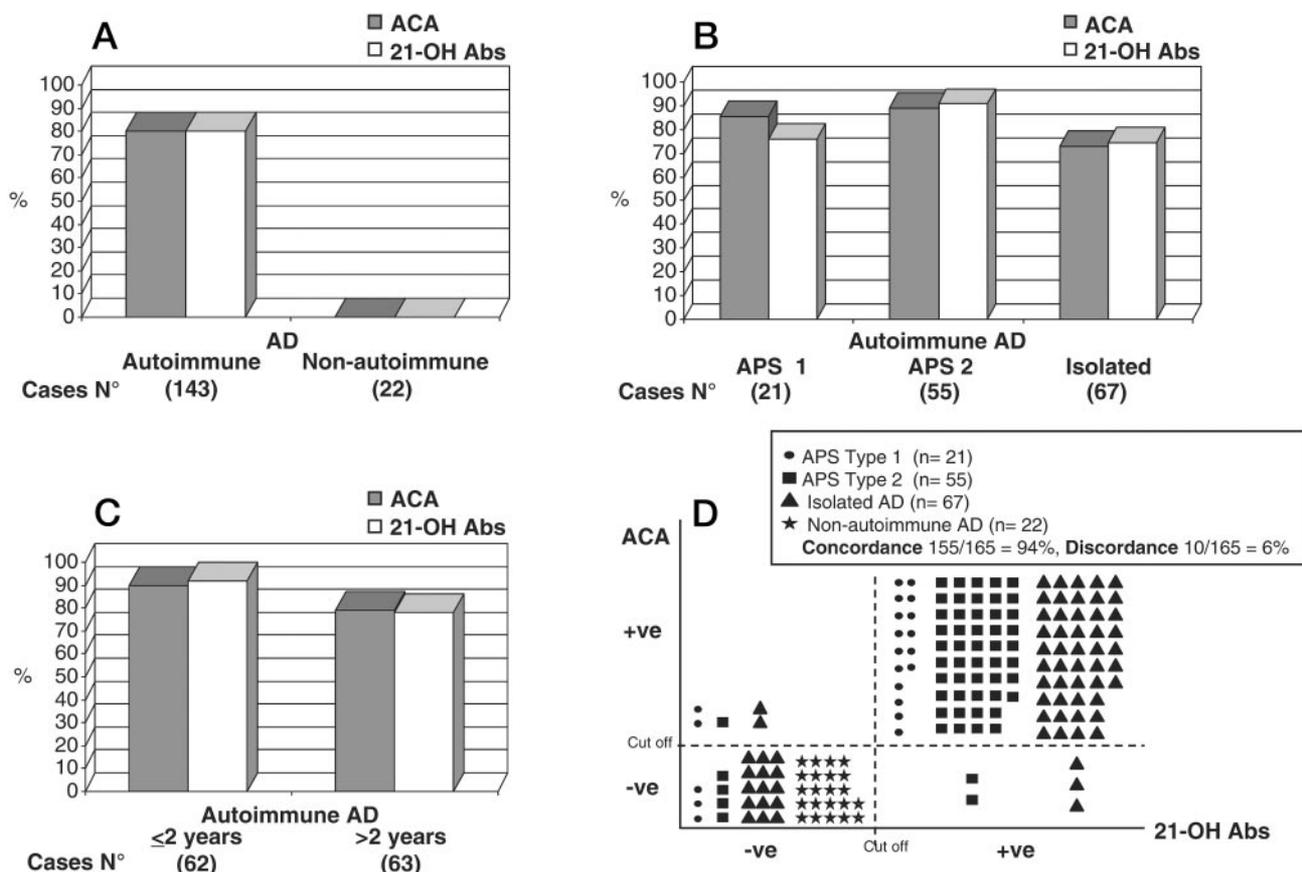


FIG. 3. Frequency of ACA and 21-OH Abs in patients with autoimmune and non-autoimmune AD (A); with APS or isolated AD (B); with different duration of AD (C); relationship between ACA and 21-OH Abs (D). [Reproduced from C. Betterle *et al.*: *J Clin Endocrinol Metab* 84:618–622, 1999 (211). © The Endocrine Society.]

present) were unaffected by preadsorption with purified 21-OH (226). Extended adsorption studies using all three purified recombinant adrenal autoantigens (21-OH, 17 $\alpha$ -OH, and P450 scc) and a larger number of sera are currently under way and are likely to further our understanding of the specificity of the immune responses in autoimmune adrenal disease.

21-OH Abs can be measured by Western blotting using native or recombinant proteins (22, 215–217, 220) or by more convenient immunoprecipitation assays (IPA) (218, 219, 222, 224). IPA for 21-OH Abs can be carried out using <sup>35</sup>S-labeled 21-OH expressed in an *in vitro* transcription/translation system based on rabbit reticulocytes; this assay is characterized by good sensitivity and specificity (218, 222, 224, 227). Recently, a highly sensitive, specific, and convenient-to-use assay to measure 21-OH Abs based on <sup>125</sup>I-labeled recombinant human 21-OH produced in yeast has also been developed (219). There is an overall good agreement between results of ACA by the IIF test and 21-OH Abs measured by the IPA (218, 219, 225, 227). However, greater sensitivity of measurement of 21-OH Abs by IPA compared with measurement of ACA by IIF test in patients with long standing AD has been reported in one study (225). The reasons for the discrepant results in this one study are not clear at present; future standardization of ACA and 21-OH Abs measurements should clarify some of these differences.

Reactivity toward other steroidogenic enzymes such as 11 $\alpha$ -hydroxylase (220, 223, 228), aromatase, and adrenodoxin (223) has not been found in sera from patients with autoimmune AD. Antibodies to 3 $\alpha$ -hydroxysteroid dehydrogenase have been reported in patients with premature ovarian failure (POF) associated with AD and APS type 1 (228, 229); however, these observations are not consistent with the reports from other laboratories (220, 223).

In the previously mentioned 165 Italian patients with AD we have studied not only ACA by the IIF test but also 21-OH Abs by IPA based on <sup>35</sup>S-labeled human 21-OH produced in an *in vitro* translation/transcription system (211). 21-OH Abs were detected in 81% of patients with autoimmune AD, and in none of the patients with non-autoimmune AD (Fig. 3A). The prevalence of 21-OH Abs varied from 92% in patients with recent onset (<2 yr from diagnosis) of autoimmune AD to 78% in patients with longer disease duration (>2 yr) (Fig. 3C). 21-OH Abs were detected in 78%, 91%, and 75% of patients with APS type 1, type 2, and isolated AD, respectively (Fig. 3B) (211). Furthermore, as mentioned above, 21-OH Abs in our own group of patients were present at the onset of autoimmune AD in 100% of cases with type 1, type 2, and type 4 APS and in 80% of those with isolated AD (see Table 6). The relationship between 21-OH Abs and ACA in this group of patients is shown in Fig. 3D. Sera from 155 of 165 (94%) patients were concordant in the two assays, and 10

sera showed discrepant results (211). There were five samples positive for ACA but negative for 21-OH Abs; this may reflect a lower specificity of the IIF test on the presence of a different autoantigen from 21-OH. In contrast, five samples negative for ACA showed low levels of 21-OH Abs in the IPA. This may reflect a greater sensitivity of the IPA compared with the IIF test (Fig. 3D).

In addition, we have assessed the relationship between 21-OH Abs measured by IPA based on  $^{125}\text{I}$ -labeled recombinant human 21-OH produced in yeast and ACA determined by IIF test in 100 sera from patients with autoimmune AD and found a good agreement between the two measurements, with a Pearson correlation coefficient of 0.69 ( $n = 100$ ) (Fig. 4).

Overall, ACA and 21-OH Abs are good markers of adrenal cortex autoimmunity, and the measurement of adrenal autoantibodies by either the IIF test or by the IPA is essentially equivalent. However, international standardization and proficiency programs for adrenal autoantibody measurements should be performed in the near future, similar to the successfully established programs for  $\beta$ -cell autoantibodies (230–232).

**2. In patients without clinical AD: markers of potential autoimmune AD.** After the first discovery of ACA in patients with clinical autoimmune AD, ACA have been reported to be present occasionally in patients without clinical AD (13, 39). Subsequently, ACA were reported to be present in 0–48% of the patients with nonadrenal autoimmune diseases (6, 174, 175, 205, 206, 233–239). Patients with idiopathic hypoparathyroidism and POF appear to be positive for ACA at the highest prevalence among the nonadrenal autoimmune group (48% and 9%, respectively) (40, 174, 175, 197, 233, 235, 237). ACA can be found in 4% of first-degree relatives of patients with AD (152, 203) and in identical twins discordant for AD (234). Furthermore, ACA were reported in 4% of hospitalized patients (6, 39) and in 0–0.6% of the normal population (8, 14, 174, 175, 201, 204, 206, 236).

The significance of ACA positivity in patients without AD remained unclear until the 1980s. In this period, two different studies (6, 240) failed to show any dysfunction of the adrenal cortex or progression toward AD in ACA-positive patients.

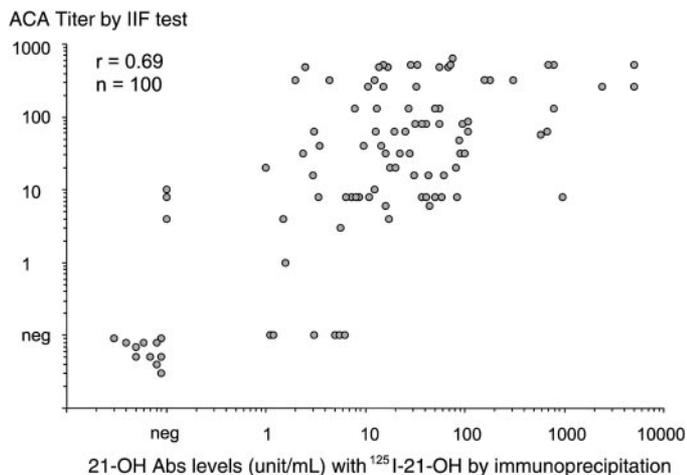


FIG. 4. Relationship between ACA titers and 21-OH Abs levels.

However, four different studies revealed that a proportion of ACA-positive patients with nonadrenal organ-specific autoimmune diseases had or later developed impaired adrenocortical reserve during an ACTH test (234, 236, 241, 242). Early introduction of a replacement therapy in a proportion of these patients (234) and lack of longitudinal observation (236) did not allow workers to assess whether this type of patient would have otherwise progressed to the overt disease. In 1983, we observed the progression toward clinical AD after 1–41 months of follow-up in four of nine ACA-positive patients with one or more organ-specific autoimmune diseases but without clinical AD (235).

All four patients who progressed to overt AD were positive for complement-fixing ACA (235). This observation was later confirmed in a larger cohort of 24 patients together with the demonstration that an increased risk of AD was related to the higher titers of ACA and to the presence of HLA-B8 and HLA-DR3 (237). Further, five distinct stages of adrenal cortex function revealed by an ACTH test in the course of the natural history of AD have been defined (237) (see Table 11). The initial stage (stage 0) is characterized by the presence of ACA only, without any biochemical signs of adrenal dysfunction (potential AD). The first biochemical evidence of adrenal subclinical failure (stage 1) is indicated by an increase in PRA in the presence of normal or low levels of aldosterone, suggesting that the zona glomerulosa is initially affected or may be the most sensitive to autoimmune aggression. After several months or years, dysfunction of the zona fasciculata becomes evident as shown by a decrease in plasma cortisol response to ACTH (stage 2), and later, by a discrete increase in the plasma ACTH level (stage 3). Finally, an evident decrease in basal plasma cortisol levels associated with a clear increase of ACTH levels occurs, along with the onset of overt symptoms of adrenal insufficiency (stage 4) (237). We have observed that the clinical signs of AD, in particular the skin hyperpigmentation, tend to appear late, usually many months after the increase of ACTH levels (stage 3) (see Table 11). The morphological study by CT scans in these patients revealed normal adrenal glands (Fig. 2D).

It has been reported that some ACA-positive patients at different stages of subclinical hypoadrenalism can become ACA negative with full recovery of adrenal dysfunction either after immunosuppressive therapy for active Graves' ophthalmopathy or without any therapy (243–245). In our study of 58 ACA-positive patients with one or more autoimmune diseases but without AD during the mean period of follow-up of 45 months, all patients maintained their ACA positivity, and none of those with ongoing hypoadrenalism showed a recovery of normal adrenal function either spontaneously or after immunosuppressive therapy (174, 175).

The prevention of autoimmune diseases by immunosuppression or by immunomodulation with self-antigens attracts a great deal of attention (90). At present, the value of such strategies in autoimmune AD is not clear. The controlled clinical studies similar to those carried out in the case of other organ-specific autoimmune diseases may help in our understanding of the possible preventive measures for autoimmune AD (246–250). Until then, the observations of a spontaneous recovery or prevention of the development of

TABLE 11. Stages of adrenal function in patients with ACA during rapid ACTH test

| Time                  | ACTH |     | Cortisol |     | PRA | Aldosterone | Clinical Signs of AD |
|-----------------------|------|-----|----------|-----|-----|-------------|----------------------|
|                       | 0'   | 60' | 0'       | 60' | 0'  | 0'          |                      |
| Stage 0 (potential)   | N    | N   | N        | N   | N   | N           | Absent               |
| Stage 1 (subclinical) | N    | N   | N        | N   | ↑   | N/↓         | Absent               |
| Stage 2 (subclinical) | N    | N   | ↓        | ↓   | ↑   | N/↓         | Absent               |
| Stage 3 (subclinical) | N/↑  | ↓   | ↓        | ↓   | ↑   | ↓           | Absent               |
| Stage 4 (clinical)    | ↑    | ↓   | ↓        | ↓   | ↑   | ↓           | Present              |

PRA, Plasma renin activity; N, normal value; time 0', basal; time 60', 60 min after iv injection of 0.25 mg of synthetic ACTH. [Modified from C. Betterle *et al.*: *J Endocrinol* 117:467–475, 1988 (237).]

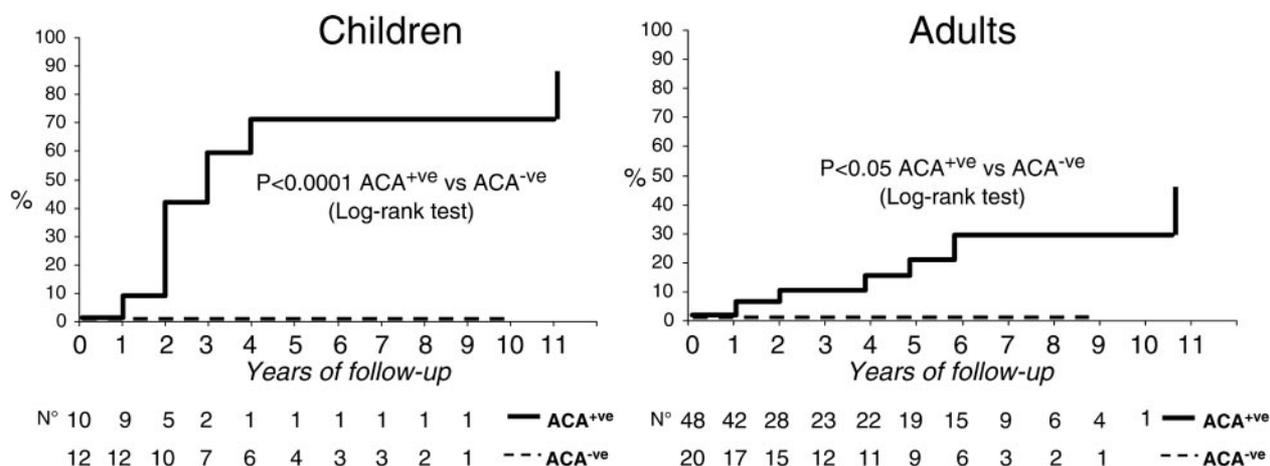


FIG. 5. Cumulative risk for AD in 58 ACA<sup>+</sup> and 32 ACA<sup>-</sup> patients with organ-specific autoimmune disorders. *Right*, Cumulative risk in adults [Reproduced from C. Betterle *et al.*: *J Clin Endocrinol Metab* 82:932–938, 1997 (174). © The Endocrine Society.] *Left*, Cumulative risk in children. [Reproduced from C. Betterle *et al.*: *J Clin Endocrinol Metab* 82:939–942, 1997 (175). © The Endocrine Society.]

overt AD with immunosuppression, although of some preliminary value, should be interpreted with caution.

Of the 58 ACA-positive patients mentioned above, 54 were also positive for 21-OH Abs, and 21 developed overt clinical AD (all 21 patients were positive for both ACA and 21-OH Abs). In our study, we have also observed a different rate in the progression toward clinical disease between children and adults. In children, the annual incidence of AD was 34.6%/yr with a cumulative risk of developing AD of 100% at 11 yr of age (174). In contrast, in adults AD developed with an annual incidence of 4.9%/yr and with a cumulative risk of 31.6% (175) (See Fig. 5).

These observations can be compared with the cases of type 1 diabetes mellitus where the detection of ICA in unaffected first-degree relatives of patients indicates a higher risk for the development of overt diabetes in young people than in adults (251). A tendency for children with ICA or ACA to develop type 1 diabetes mellitus or AD at a higher rate than adults could be related to the more aggressive cell-mediated autoimmune responses occurring in childhood. This hypothesis, however, should be investigated further.

Recent observations from other laboratories on the prevalence of 21-OH Abs in individuals susceptible to AD have confirmed our earlier reports that 21-OH Abs are good markers of potential AD (225, 252–254). Furthermore, studies from other laboratories have confirmed that measurement of 21-OH Abs in these patients correlated well with ACA in IIF test (223, 239, 244).

The rate of progression to overt AD in ACA-positive pa-

tients appeared to be related to the nature of the preexisting autoimmune disease, being highest in patients with hypoparathyroidism and lowest in those with autoimmune thyroid disease or type 1 diabetes mellitus (174, 175, 254). Overall, several factors involved in the assessment of risk of development of AD include high titers of ACA, ability of ACA to fix complement, young age of patients, presence of hypoparathyroidism or autoimmune thyroid diseases or type 1 diabetes mellitus, HLA-B8 and HLA-DR3 (174, 175).

The availability of accurate measurements of ACA by the IIF test or 21-OH Abs by IPAs has had the following impact on the diagnosis and management of adrenal diseases: 1) it helps to establish the etiology of adrenal failure, 2) it reveals the prevalence of autoimmune markers of adrenal disease in non-Addisonian individuals, 3) it identifies patients with potential or subclinical autoimmune AD, 4) it allows for early treatment and prevention of AD, and 5) it leads to a better understanding of the natural history of AD.

Prediction and early detection of AD based on autoantibody screening should allow prevention of the adverse effects of hypoadrenalism such as water-salt imbalance, hypoglycemia, or cardiovascular crisis due to loss of fluids. In addition, although it occurs rarely, life-threatening overt adrenal failure, which may present with either atypical or non-specific symptoms and signs, might be prevented (255). Furthermore, as some of the events associated with hypoadrenalism may be aggravated by the preexisting endocrine defects (*i.e.*, type 1 diabetes mellitus, thyroid dysfunction,

and/or hypoparathyroidism), screening for ACA/21-OH Abs in patients at risk (see above) should be recommended.

3. *In patients with Cushing's syndrome.* The presence of ACA in a patient with Cushing's syndrome was first demonstrated by Wegienka and associates (256) and subsequently reported in 2.7% of patients with Cushing's syndrome (257). However, the reactivities of ACA found in these patients with specific adrenal antigens have not been determined. Subsequently, it has been reported that ACTH receptor antibodies were present in some patients with Cushing's syndrome due to pigmented adrenocortical micronodular dysplasia. These antibodies were reported to be able to stimulate cortisol production and the DNA synthesis by adrenal cortex cells *in vitro* (258). After these observations, it was hypothesized that some cases of Cushing's syndrome may result from an autoimmune stimulation of the ACTH receptor and that the ACA found in these particular cases are as thyroid microsomal autoantibodies found in sera from patients with Graves' disease (258, 259). However, the specificity of the ACTH receptor-stimulating antibodies is questionable; it has been found that the IgG preparations from the majority of patients with Cushing's syndrome due to an adrenal adenoma show effects similar to those attributed to the ACTH receptor antibodies (259).

#### B. Steroid-producing cell antibodies (StCA) and autoantigens

1. *In patients with AD and clinical hypergonadotropic hypogonadism.* In patients with autoimmune AD, the characteristic reactivity of ACA by the IIF test is limited to cytoplasm antigens of adrenal cortical cells. In addition, some patients have autoantibodies reactive with other steroid-producing cells such as Leydig cells of the testis, theca cells of the ovary, and syncytiotrophoblasts of the placenta, and these autoantibodies are defined as steroid-producing cell autoantibodies (StCA) (260). ACA and StCA both react with the adrenal cortex and show an identical immunofluorescent pattern. Consequently, it is not possible to distinguish the presence of one, the other, or both reactivities (ACA, StCA, or both) by IIF tests on the adrenal cortex sections only, whereas such distinction can be made using, first, adrenal and then gonadal tissues (206). ACA can be present in the absence of StCA, but StCA are always associated with ACA (see Table 12). StCA are polyclonal IgG antibodies and can be distinguished from ACA by preadsorption tests with homogenates of steroid-producing target organs (adrenal or gonads) that remove StCA, whereas exclusive reactivity of ACA with the gonadal tissue remains unaffected (205). A pathogenic role of StCA has been suggested after it was demonstrated that sera from StCA-positive patients with AD were able to induce a complement-dependent cytotoxicity against granulosa cells of the ovary *in vitro* (261).

StCA were first described in two ACA-positive males with autoimmune AD (260). Subsequently, StCA were found in 28% of unselected patients with autoimmune AD (206, 209), but their prevalence varied depending on the gender. In one study, 26% of the women and 4% of the men with autoimmune AD were reported to be positive for StCA (40). The

TABLE 12. Immunological combinations in 13 patients with autoimmune AD and POF

| Patients                    | ACA   | 21-OH Abs | StCA  | 17 $\alpha$ -OH Abs | P450 scc Abs |
|-----------------------------|-------|-----------|-------|---------------------|--------------|
| 1. APS 1 + POF              | +     | +         | +     | +                   | +            |
| 2. APS 1 + POF              | +     | +         | +     | +                   | +            |
| 3. APS 1 + POF              | +     | +         | +     | +                   | +            |
| 4. APS 1 + POF              | +     | +         | +     | +                   | +            |
| 5. APS 1 + POF <sup>a</sup> | +     | +         | –     | –                   | –            |
| 6. APS 2 + POF              | +     | +         | +     | +                   | –            |
| 7. APS 2 + POF              | +     | +         | +     | –                   | +            |
| 8. APS 2 + POF              | +     | +         | +     | –                   | +            |
| 9. APS 2 + POF              | +     | +         | +     | –                   | +            |
| 10. APS 2 + POF             | +     | +         | –     | –                   | +            |
| 11. APS 2 + POF             | +     | +         | +     | +                   | –            |
| 12. APS 2 + POF             | +     | +         | +     | +                   | +            |
| 13. AD + POF (APS 4)        | +     | +         | +     | +                   | +            |
|                             | 13/13 | 13/13     | 11/13 | 8/13                | 10/13        |

[Modified from C. Betterle *et al.*: *J Clin Endocrinol Metab* 84:618–622, 1999 (211). © The Endocrine Society.]

<sup>a</sup> Turner's syndrome.

presence of StCA generally correlated with the presence of a primary gonadal failure (hypergonadotropic hypogonadism) in the presence of a normal chromosomal pattern. In a study of 77 patients with autoimmune AD, a POF was found in six patients, five of whom were StCA positive (262). The relationship between StCA and POF has been later confirmed in several studies (43, 171, 206).

Histological descriptions of the ovaries from patients with AD associated with POF and StCA are rare; however, available reports revealed a close correlation between this form of hypogonadism and lymphocytic oophoritis (reviewed in Refs. 46 and 263). The presence of a lymphocytic oophoritis at biopsy was documented in 18 of 18 patients with POF, StCA, and autoimmune AD (262, 264–273). Furthermore, 78% of all patients with evidence of lymphocytic oophoritis at biopsy were found to be StCA positive (263). In the majority of described cases, the pattern of microscopic infiltration of the ovary was similar. The primordial follicles were unaffected as well as the cortex of the ovary; however, the developing follicle was predominantly infiltrated by mononuclear inflammatory cells showing a clear pattern of increasing density within the more mature follicles. Preantral follicles were surrounded by small rims of lymphocytes and plasma cells, whereas larger follicles showed progressive, more dense infiltrates usually in the external and internal theca. The granulosa layer was usually spared in this process until luteinization of the degenerating follicle occurred. Atretic follicles and, when present, corpora lutea or corpora albicantia were infiltrated as well (263). Immunohistochemical analysis of the lymphocytic oophoritis revealed that the infiltrating cells are mainly from T lymphocytes (CD4<sup>+</sup> and CD8<sup>+</sup>) with a few B lymphocytes together with a large number of plasma cells. Macrophages and natural killer cells could also be found. The plasma cells secreted mainly IgG, but also IgM and IgA, suggesting that ovarian autoantibodies were produced *in situ*. Studies on animal models of autoimmune oophoritis suggested the important role of T cells in the immune destructive process of the ovary (263).

StCA are uncommon in males but, if present, they can be

considered markers of primary gonadal insufficiency. In one study, three males in a group of 79 patients with autoimmune AD were positive for StCA, and one of them showed autoimmune testicular failure (40).

The prevalence of StCA differs in patients with different forms of autoimmune AD, being present in 60–80% of the patients with APS type 1, in 25–40% of those with APS type 2, and in 18% of patients with isolated autoimmune AD (45, 171, 206, 209, 263). The high prevalence of StCA in patients with APS type 1, compared with lower StCA prevalence in APS type 2 and in APS type 4, reflects different prevalences of gonadal failure in these different groups of patients (263, 274). In the majority of patients with APS type 1, hypogonadism appears after the onset of autoimmune AD, whereas in general it precedes AD in those with APS type 2 and 4 (275).

After identification of the 21-OH as the major adrenal autoantigen (22, 218–225), the possibility that other steroidogenic enzymes may be involved in the autoimmune responses in autoimmune adrenal disease has been investigated. Reactivities of autoantibodies present in patients' sera toward steroid 17 $\alpha$ -hydroxylase (17 $\alpha$ -OH Abs) and to P450 scc (P450 scc Abs) have indeed been found (see above). However, the reports on the prevalence of 17 $\alpha$ -OH Abs and P450 scc Abs in patients with isolated AD or AD in the context of APS type 1 and type 2 varied significantly in different studies (214, 215, 218, 221, 223, 227, 276).

In particular, of 15 sera from patients with POF in the context of APS type 1 studied by immunoblotting, nine sera (60%) reacted with P450 scc, six sera (40%) with 17 $\alpha$ -OH, and only five (33%) with 21-OH (221). These early observations were followed by immunoprecipitation studies that showed that all the patients with AD and POF in the context of APS type 1, were positive for 17 $\alpha$ -OH and/or P450 scc Abs; however, some of these patients were negative for 21-OH Abs (223). Winqvist *et al.* (277) reported that StCA reactive with Leydig cells present in sera from patients with autoimmune AD were directed mainly toward P450 scc (80% of the sera), and a 51-kDa protein of unknown function (60% of the sera) present in granulosa cells and placenta. Only 40% of these sera reacted with 21-OH. In contrast, in our studies the presence of 17 $\alpha$ -OH Abs and P450 scc Abs in patients with APS type 1 and APS type 2, isolated and potential AD was found to be associated closely with the presence of 21-OH Abs (218). For example, 32 of 33 sera (97%) positive for 17 $\alpha$ -OH and/or P450 scc Abs were also positive for 21-OH Abs. Furthermore, the comparison of StCA positivity with measurements of 17 $\alpha$ -OH Abs and/or P450 scc Abs indicates that 17 $\alpha$ -OH and/or P450 scc are the major targets of StCA measured by IIF test (211, 218, 219).

Of the 143 Italian patients with autoimmune AD we have studied, 37 (26%) were StCA positive, whereas none of 22 patients with non-autoimmune AD was positive (Fig. 6A). In particular, StCA were found in 62% of APS type 1, in 29% of type 2, and in 12% of isolated AD (Fig. 6B). Of 143 of our patients with autoimmune AD, 13 were affected by POF, and 11 (85%) had detectable StCA (Fig. 6C). The correlation between StCA and 17 $\alpha$ -OH Abs and P450 scc Abs in different patients is summarized in Fig. 6D (211). Consequently, these studies suggest that 17 $\alpha$ -OH Abs and P450 scc Abs are the

major components of StCA measured by the IIF test (211, 218, 278). The autoantibody combinations in these 13 cases with POF associated with autoimmune AD are summarized in Table 12. All 13 patients were positive for ACA and 21-OH Abs; 12 patients had autoimmune AD associated with an "idiopathic" POF, and all were additionally positive for at least one of the following: StCA, 17 $\alpha$ -OH Abs, or P450 scc Abs. The patient with autoimmune AD with POF due to Turner's syndrome was negative for all these three autoantibodies (211). Further, these studies have shown that StCA and 17 $\alpha$ -OH and/or P450 scc Abs are good markers for identifying autoimmune POF associated with autoimmune AD (211).

In conclusion, the differences in reactivity to steroidogenic enzymes among patients with hypergonadotropic hypogonadism reported in different studies could be due, at least in part, to different groups of patients studied, particularly in terms of the etiology of gonadal failure and to different methods used to detect autoantibodies (IIF test, Western blotting, or IPAs) and to differences in the origin of autoantigens (native or recombinant expressed in different systems; full-length antigens or fragments) used for the tests. Consequently, standardization and proficiency programs for StCA, 17 $\alpha$ -OH Abs, and P450 scc Abs similar to those suggested in the case of 21-OH Abs (279) appear to be urgently needed.

2. *In patients with AD without clinical hypergonadotropic hypogonadism.* StCA have also been reported in 10–43% of patients with autoimmune AD in the absence of gonadal failure (40, 171, 206, 263). In our own recent study, StCA were found in 26 of 130 (20%) of patients with autoimmune AD without clinical hypogonadism (Fig. 6C). In particular, 43% of patients with APS type 1, 18% of patients with APS type 2, and 11% of patients with isolated AD were positive for StCA (209). In this study, StCA were highly associated with 17 $\alpha$ -OH Abs and/or P450 scc Abs (209). The follow up of StCA-positive patients with autoimmune AD without POF showed a high risk of developing gonadal failure in females but not in males (275, 280).

3. *In patients with clinical hypergonadotropic hypogonadism without AD.* A proportion (10–39%) of POF patients without autoimmune AD is affected by one or more autoimmune diseases, mainly at subclinical level (263, 275, 281–284). Thyroid autoimmunity is the most prevalent (14%), followed by gastric autoimmunity (4%), type 1 diabetes mellitus (2%), and myasthenia gravis (2%) (263). In these patients with POF, isolated or associated with other autoimmune diseases but without AD, StCA were found with a low frequency (7%) and a lymphocytic oophoritis was present in biopsy specimens from these StCA-positive patients (263). Furthermore, all the StCA-positive POF patients without AD were also positive for ACA/21-OH Abs; these patients may have a high risk of developing clinical autoimmune AD in the future (275).

However, the majority (93%) of the patients with POF isolated or associated with other autoimmune diseases excluding AD were StCA negative, and lymphocytic oophoritis is an exceptional finding being described in only six of 198 of these patients (46, 263). These observations indicate that POF due to lymphocytic oophoritis is closely related to the

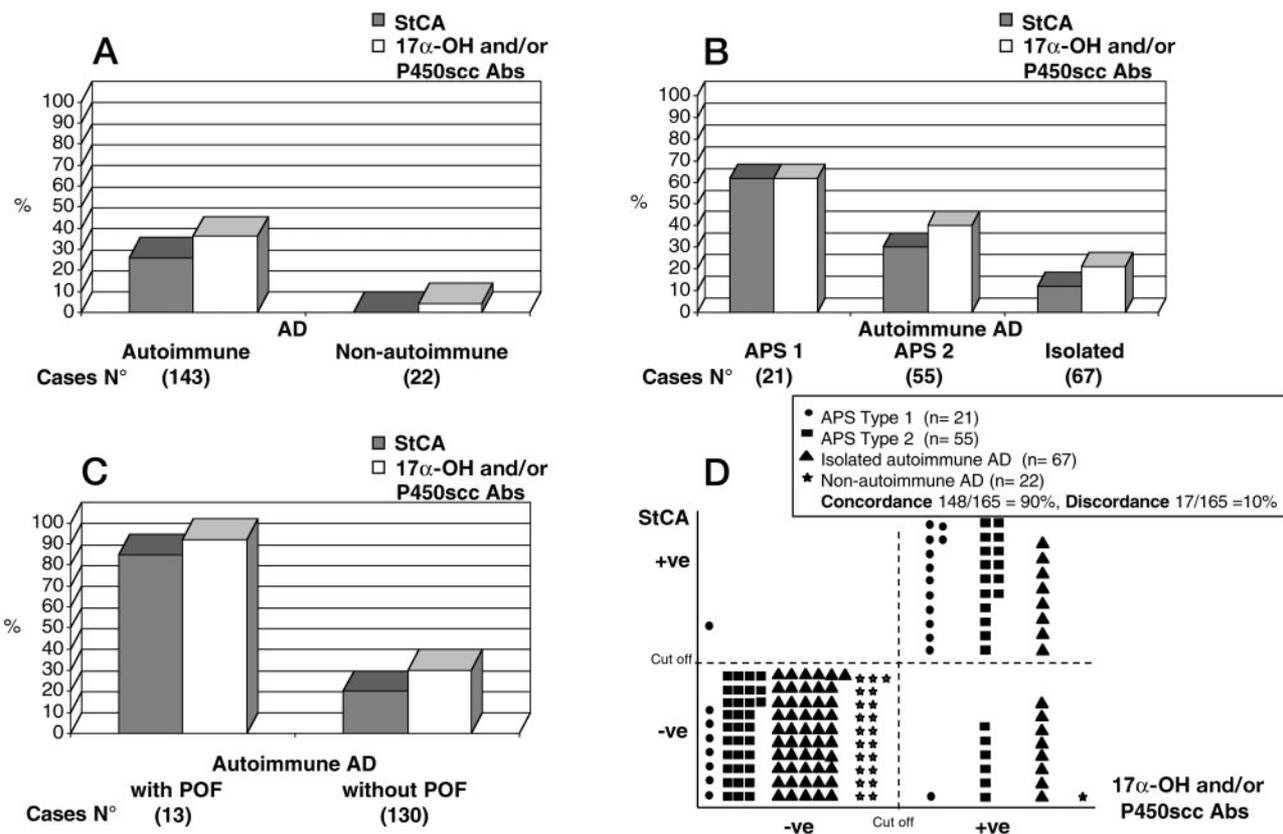


FIG. 6. Frequency of StCA, 17 $\alpha$ -OH Abs, and P450 scc Abs in patients with different autoimmune and non-autoimmune forms of AD (A); with APS (B); with or without POF (C); relationship between these markers (D). [Reproduced from C. Betterle *et al.*: *J Clin Endocrinol Metab* 84:618–622, 1999 (211). © The Endocrine Society.]

presence of ACA and StCA; however, T cell-mediated cytotoxic mechanisms are believed to be involved in the damage of the ovaries (263).

In the absence of StCA, other autoimmune mechanisms may be responsible for POF. For example, Savage's syndrome (named after the first patient described with this disease), in patients with primary or secondary amenorrhea, is characterized by the presence of numerous primordial follicles in the ovaries, hypergonadotropic hypoestrogenic hormone profile, and a poor response to therapy with high doses of exogenous gonadotropins used for ovulation induction (285). The presence of autoantibodies with the ability to block the gonadotropin receptor in patients with the clinical picture of Savage's disease has been reported in some studies (263, 286–288). These studies suggested that the autoantibodies were responsible for an "immunological block" at the level of gonadotropin receptors in the ovaries in the absence of a lymphocytic oophoritis. However, the existence of these autoantibodies has not been confirmed in further studies (289).

It has also been reported that sera from patients with POF without StCA may react with different preparations of ovarian antigens (263, 290–296). However, sera from control subjects, postmenopausal women, and patients with iatrogenic ovarian failure were also found to be reactive with various ovarian preparations (263). This suggests that the reactivity to ovarian proteins may be secondary to ovarian damage rather than to a primary autoimmune response (263).

Clearly, POF is a complex disease that may be related to autoimmunity or to various other causes such as infections, environmental or iatrogenic exposure, and genetic factors (297). For example, deletions or translocation of X chromosome or mutations of gonadotropins or gonadotropin receptors have been recently identified in some patients with POF (298–300). However, the pathogenesis of the gonadal failure in patients with POF without StCA and without chromosomal abnormalities remains uncertain.

### C. Autoepitopes in autoimmune AD

Studies on the localization of 21-OH autoepitopes recognized by autoantibodies in sera from patients with autoimmune AD have been carried out using different methods. These include: Western blotting analysis and/or IPA using 21-OH expressed in an *in vitro* transcription/translation system, in bacteria or yeast. In these experiments, the reactivity of 21-OH Abs with intact 21-OH was compared with reactivity with 21-OH containing N-terminal, internal, and C-terminal deletions or 21-OH containing amino acid mutations. It has been determined that the central and the C-terminal regions of the 21-OH sequence (amino-acids 241–494) were involved in forming 21-OH Abs binding sites (220, 301–303). The amino acid sequences within the C-terminal part of the 21-OH molecule interact with the heme group and form a steroid-binding site and, consequently, are important for 21-OH enzyme activity (278). Amino acid mutations

within this region (*e.g.*, Pro453 to Ser) are associated with impaired 21-OH enzyme activity, and 21-OH proteins containing single amino acid mutations have shown markedly reduced ability to bind autoantibodies (219, 302). Extensive stretches of 21-OH sequences have been found important for 21-OH Abs binding (see above); also, 21-OH Abs in sera from different patients have shown different reactivities with mutated 21-OH or 21-OH fragments. These observations suggested that 21-OH Abs in patients' sera were heterogeneous. However, no significant differences have been found between the epitopes recognized by 21-OH Abs in patients with different forms of autoimmune AD, either isolated or in the context of APS type 1 and 2, or with subclinical or potential autoimmune AD (303, 304).

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) (278). More detailed analysis of autoantibody binding epitopes has been carried out using mouse monoclonal antibodies to 21-OH directed to the epitopes within the C-terminal part of the molecule (305). Mixtures of Fab or F(ab')<sub>2</sub> fragments isolated from the mouse IgG caused almost complete inhibition (80–90%) of binding of 21-OH Abs in patients' sera. The 21-OH amino acid sequences reactive with these mouse monoclonal antibodies have been identified and, consequently, three different amino acid sequences (amino acids 335–339; 391–405; 406–411) in the C-terminal part of 21-OH were determined to be important for 21-OH Abs binding (305). No major differences in the recognition of these epitopes were observed when 21-OH Abs in sera from patients with different forms of autoimmune AD were studied (305). Two of the three identified sequences important for 21-OH Abs binding appeared to be human 21-OH specific, and one was identical in human and bovine 21-OH. This emphasizes the importance of using human rather than bovine adrenal tissue sections in the IIF test for ACA (305). Furthermore, analysis of amino acid sequence homologies of human, porcine, and mouse 21-OH tends to suggest that neither porcine nor mouse would be useful substitutes for human adrenal material (305).

#### D. Autoantibodies to adrenal enzymes in the pathophysiology of autoimmune AD

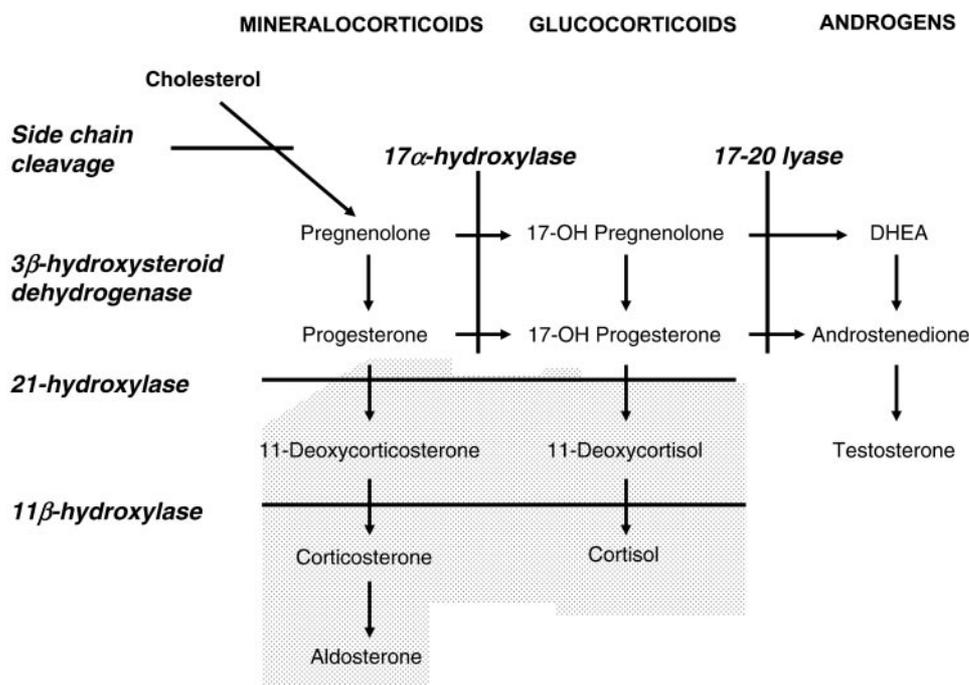
The three main enzymes recognized as target autoantigens in autoimmune AD are members of the cytochrome P450 family of enzymes located in the endoplasmic reticulum or mitochondria; their activity depends on nicotinamide adenine dinucleotide phosphate (reduced) cytochrome P450 reductase, and these enzymes are not expressed on the cell surface (306, 307).

Of the three enzymes, 21-OH is adrenal specific (it converts 17-OH-progesterone into 11-deoxycortisol and progesterone into 11-deoxycorticosterone), 17 $\alpha$ -OH is expressed in adrenals and in gonads (it converts pregnenolone to 17-OH-

pregnenolone and dehydroepiandrosterone). P450 scc is the first rate-limiting enzyme present in adrenals, gonads, and placenta (it converts cholesterol to pregnenolone) (see Fig. 7). In the adrenal cortex, the three enzymes are ubiquitous. However, 21-OH and P450 scc are mainly located in the zona glomerulosa, fasciculata, and reticularis, while 17 $\alpha$ -OH is located in the zona fasciculata and reticularis (307, 308). These enzymes are involved in the synthetic pathway of the four main steroid hormones derived from cholesterol: 1) cortisol is a glucocorticoid involved in the regulation of metabolic changes in response to stress; it modifies gene expression in a large number of cells, including lymphoid cells; 2) aldosterone is the principal mineralocorticoid, which together with the peptides, renin and angiotensin, is responsible for the control of blood pressure through Na<sup>+</sup> and K<sup>+</sup> excretion by the kidney; 3) androsterone and 4) dehydroepiandrosterone are androgenic hormones (44, 307, 309). Zona glomerulosa is the major source of mineralocorticoids, whereas the zona fasciculata and zona reticularis are thought to act as a functional unit in the production of cortisol and androgens (309).

The relationship between the amino acid residues important for 21-OH enzyme activity and for 21-OH Abs binding described above has led to studies on the effect of 21-OH Abs on 21-OH enzyme activity. IgGs from autoimmune AD sera positive for 21-OH Abs caused a marked, dose-dependent inhibition of 21-OH enzyme activity *in vitro* (310). Could the inhibiting effect of 21-OH Abs on 21-OH enzyme activity be involved in the pathogenesis of the adrenal insufficiency in affected individuals? In an attempt to clarify this question, steroid synthesis markers have been studied in 21-OH Abs-positive patients with clinical or subclinical autoimmune AD after stimulation with ACTH (311). However, no increased levels of 17-OH progesterone were found, which would have been consistent with inhibition of 21-OH enzyme activity. This study indicated that the inhibiting effect of 21-OH Abs on 21-OH enzyme activity is not evident *in vivo* (311) (see Fig. 7). Furthermore, to date there is no evidence of passive transient neonatal hypoadrenalism in babies from ACA-positive pregnant women with autoimmune AD, although ACA have been found to cross the placenta and to be transiently detectable in the blood of newborns (8, 44, 312). Recently, we studied the change over time and the effects on adrenal function of ACA/21-OH Abs in a child of a mother with AD who was positive for ACA and 21-OH Abs. ACA and 21-OH Abs were present at birth in the baby's serum at higher levels than in the mother's circulation; after 3 and 6 months ACA and 21-OH Abs were still present, with a decrease of titers. ACTH, cortisol, and 17-OH progesterone levels were at normal range at birth and after 3 and 6 months (C. Betterle, personal observation). This pattern is different from that observed for pathogenic autoantibodies such as TSH receptor autoantibodies, which cross placenta from a mother to a baby and are responsible for neonatal hypothyroidism or hyperthyroidism (313–315) or Ach receptor antibodies that may induce a transient neonatal myasthenia (259). At present, a pathogenic role for ACA/21-OH Abs has not yet been demonstrated *in vivo*.

FIG. 7. Enzymes involved in the synthetic pathways of mineralocorticoids, glucocorticoids, and androgens (see text for details). The levels of hormones in the gray area are low in AD; in the case of reduction of 21-OH enzyme activity by human 21-OH Abs, increased levels of accumulated precursor hormones (above the gray area) would be expected.



#### E. Adrenal surface autoantibodies

Adrenal surface autoantibodies reactive to adrenal antigens on the cell surface have been demonstrated in 86% of ACA-positive patients with idiopathic AD in an IIF test using viable human adrenal cells (316). This study suggested that surface adrenal antibodies reacted with a microsomal antigen expressed on both the membrane and the cytoplasm of adrenal cortex cells. The fact that cytoplasm antigens may also be expressed on the cell surface could be relevant to the pathogenesis of autoimmune AD. It can be postulated that ACA have a direct cytotoxic effect on adrenal cells, by means of opsonization, complement involvement, and activation of monocytes or killer cells (317).

#### F. ACTH receptor autoantibodies

It has been reported that some autoantibodies have the ability to bind to the cell receptors and to affect the receptor's function either through mimicking normal ligand action or blocking the ligand-binding site on the receptor as reviewed by Wilkin (259). The serum IgG fraction from a woman with autoimmune AD was reported to block ACTH-induced release of cortisol from guinea pig adrenal cells *in vitro* (318), suggesting that the autoantibody that bound to the ACTH receptor was able to inhibit both ACTH-induced adrenal DNA synthesis and cortisol production by guinea pig adrenal segments. Initially, this effect was reported in more than 90% of patients with clinical autoimmune AD (317). However, a subsequent study did not confirm these observations, and the earlier described inhibiting effects appeared to be related to nonspecific components of IgG preparations (319). At present, the existence of ACTH receptor-blocking autoantibodies is still under discussion and needs further investigations.

#### G. Hydrocortisone autoantibodies (H Abs)

In organ-specific autoimmune diseases, hormones or pro-hormones can become the targets of autoimmune reactions. The main examples are anti-T<sub>3</sub>, anti-T<sub>4</sub>, anti-TSH, and anti-thyroglobulin autoantibodies in autoimmune thyroid diseases and anti-insulin antibodies in type 1 diabetes mellitus (320). Using an ELISA method, H Abs were found in 45% of patients with AIDS (321). In addition, H Abs have been reported in patients with cytomegalovirus or Epstein-Barr virus infections, but in none of the patients with autoimmune AD or in normal controls (321). In patients with AIDS, H Abs may inactivate the cortisol in the adrenal cells, which would be consistent with the reported elevated serum ACTH levels in these patients (26). Autoantibodies staining the periphery of adrenocortical cells were found by the IIF test in the sera of patients with AIDS when adrenal glands from another patient with AIDS were used as a substrate (321). The specificity of H Abs is not clear at present. It might be that a specific immune reaction against viruses on the infected glands is responsible for the observed immunofluorescence. Adrenal cortical insufficiency is the most serious, commonly occurring endocrine disease in AIDS patients; however, the pathogenesis of this form of adrenal failure and its possible relationship to the autoimmune response has yet to be demonstrated (26).

### XV. Pathogenesis of Autoimmune AD

The sequence of pathogenic events involved in autoimmune destruction of the adrenal cortex is not clear at present. It can be postulated, at least in the case of APS type 1, that the genetic susceptibility related to homozygous AIRE gene mutations (see above) may be an essential element in inducing lack of tolerance to self-antigens. This may be related to

a defect of T cell suppressor activity at a very young age, probably aggravated by chronic *Candida* infection, and these may have an implication for development of multiple autoimmunity at an young age and neoplastic disease in adult age.

In the case of autoimmune AD other than APS type 1, particular HLA genes may be necessary but not sufficient for the development of autoimmune AD (90). Environmental agents such as infections, drugs, food products, or stress are highly suspected to act as cofactors (Fig. 8).

Activated T lymphocytes are likely to recognize self-adrenocortical antigens in the context of class II HLA molecules; however, the specific autoantigens reactive with autoreactive T cells in autoimmune AD have not yet been identified. The activation of T helper (Th) lymphocytes may lead to the production of IL-2 and other lymphokines that induce an activation of both T-cytotoxic (Tc) lymphocytes and B lymphocytes able to secrete specific ACA/21-OH Abs (Fig. 8). As a consequence of the lymphocytic infiltration, ACA/21-OH Abs become detectable in serum, and this could be considered the marker of a silent T cell-mediated aggression in the adrenals. In the case of adrenal cortex autoimmunity, unlike thyroid autoimmunity, this proposed sequence of events has been only hypothetical and not studied experimentally. For example, Yoshida *et al.* (322) studied the postmortem histology of the thyroid of 70 patients without overt thyroid disease and demonstrated that 83% of the subjects positive for lymphocytic thyroid infiltration had thyroid autoantibodies, whereas 91% of subjects positive for thyroid autoantibodies showed lymphocytic infiltration of the thyroid. This study showed that a highly significant correlation between morphological infiltration of the target organ and

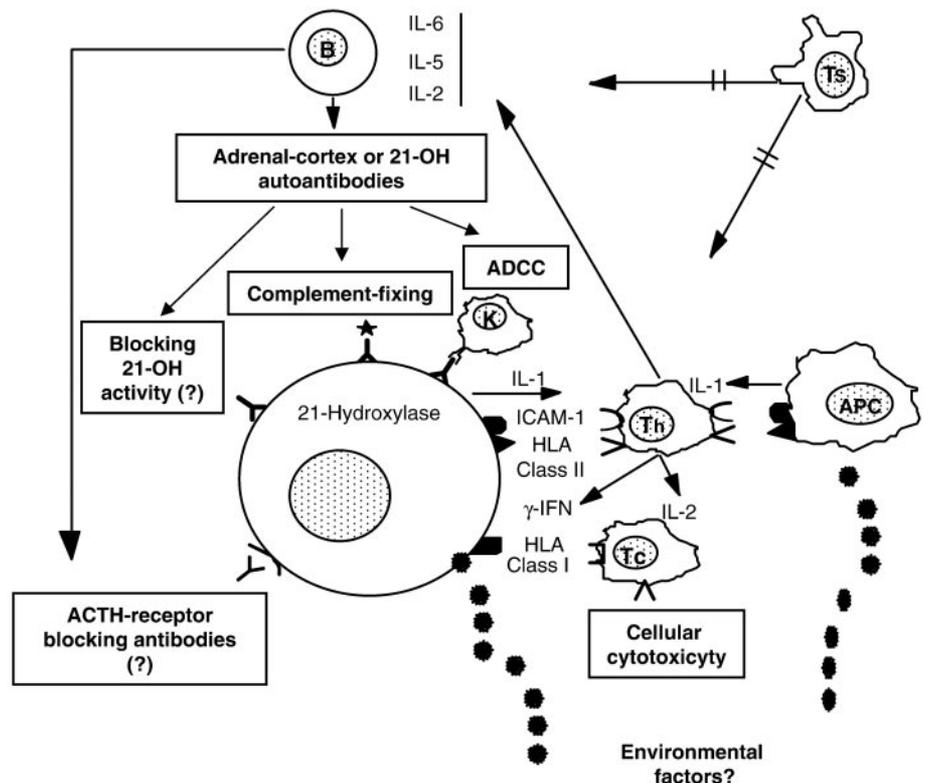
serological findings can be found in patients with thyroid autoimmunity.

As for thyroid microsomal autoantibodies, there is no evidence that ACA/21-OH Abs are responsible for the development of autoimmune AD; however, they are surely good serological markers of adrenal autoimmunity and are also valuable in the prediction of adrenal insufficiency. The progression of autoimmune adrenal disease is significantly related to the titers of ACA/21-OH Abs, to the age of patients, and to preexisting chronic candidiasis, chronic hypoparathyroidism, or type 1 diabetes mellitus.

However, the role of immune processes involved in the progressive and inevitable deterioration of adrenal cortex function are not clear at present. Damage resulting from local cytokine release from infiltrating T cells seems to be the most probable perpetuating cause of the adrenal cortex destruction (Fig. 8).

The mechanisms involved in the development and progression of autoimmune AD remain elusive at present, and the current major obstacles to a better understanding of these mechanisms are the difficulties in obtaining adrenal tissue specimens with the infiltrating cells from patients with sub-clinical or clinical autoimmune AD at onset. The absence of spontaneous animal models of autoimmune adrenalitis is another obstacle to the study of initial events in autoimmune AD. In contrast, the ability to study spontaneous animal models in other autoimmune diseases (323), and the experiments carried out with the target organ tissues from patients at the onset or before the clinical onset of the disease, have led to a better understanding of autoimmune phenomena in other autoimmune diseases such as thyroid diseases (324, 325) and type 1 diabetes mellitus (326–328).

FIG. 8. Pathogenic mechanisms in autoimmune AD (see text for details). [Modified from C. Betterle *et al.* *Malattie Autoimmuni del Surrene*. In: C. Betterle, ed. *Le Malattie Autoimmuni*. Padova, Italy: Piccin; 135–154, 2001 (329)].



**XVI. Natural History of Autoimmune AD**

Although the events leading to adrenal autoimmunity have not yet been clearly determined, there has been progress in the study of the natural history of autoimmune AD. It is now well understood that, similarly to other organ-specific autoimmune disorders, AD is a chronic disease, with a long silent preclinical period. Three main phases can be identified in the course of the natural history of the disease: 1) potential, 2) latent or subclinical, and 3) clinical (see Fig. 9). The potential phase (stage 0) is characterized by the presence of a genetic susceptibility and/or by the presence of ACA and/or 21-OH Abs in the absence of any detectable impairment of adrenocortical function (Fig. 9 and Table 11). In the next phase, adrenal impairment may develop after a typical sequence of three subclinical dysfunctional stages (stages 1-3), first affecting the zona glomerulosa and subsequently the zona fasciculata. This characteristic sequence of events in the natural history of autoimmune AD is difficult to explain, but it may be related either to a greater sensitivity of the zona glomerulosa to the lymphocytic attack and/or to the protection of the zona fasciculata from lymphocytic infiltration for a longer period by locally produced high concentrations of corticosteroid hormones, and/or to a greater ability of a zona fasciculata to regenerate. In this phase, life events requiring an increase in cortisol secretion (such as traumas, infections, surgery, pregnancy, or other stress-related events) may easily precipitate adrenocortical failure (Fig. 9). However, when adrenal autoimmune destruction is more advanced, the zona fasciculata may also become infiltrated and irreversibly damaged by autoreactive T lymphocytes, and in this phase clinical signs of adrenocortical insufficiency are manifest.

Measurement and follow-up of ACA/21-OH Abs in patients without AD have resulted in great progress in the study of the natural history of AD. This has enabled us to

predict the development of autoimmune AD in susceptible individuals and to aid in early diagnosis and therapy of the disease. Studies on the development of the strategies leading to prevention of AD have now become a real opportunity.

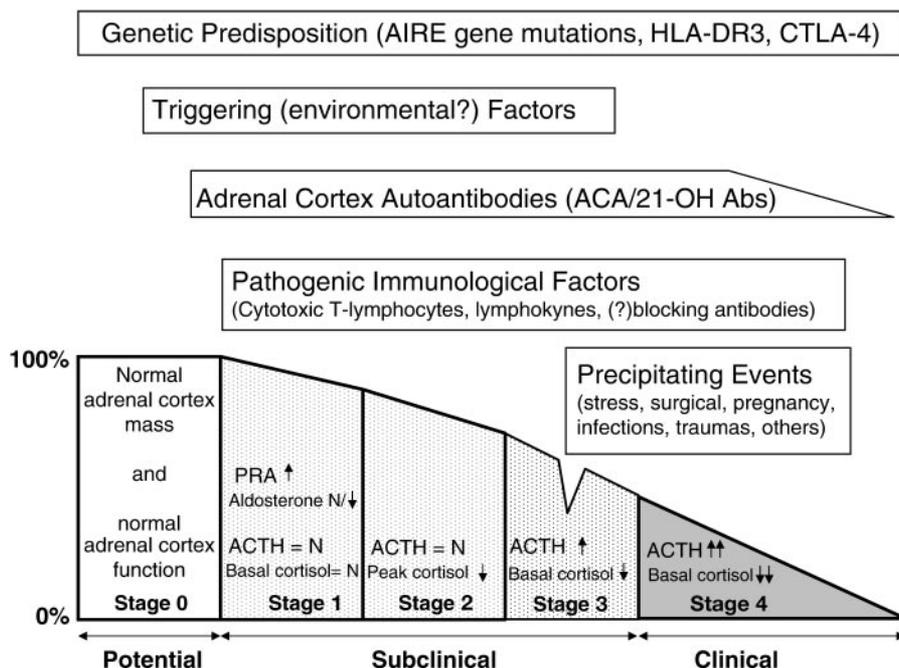
**XVII. Therapy of AD**

Patients with symptomatic adrenal insufficiency should be treated with hydrocortisone or cortisone in the early morning and afternoon. The usual initial dose is 25 mg of hydrocortisone (divided into doses of 15 and 10 mg) or 37.5 mg of cortisone (divided into doses of 25 and 12.5 mg), but the daily dose may be decreased to 20 or 15 mg of hydrocortisone as long as the patient's well being and physical strength are not reduced. In order to prevent weight gain and osteoporosis, the goal should be to use the smallest dose that relieves the patient's symptoms. Measurement of urinary cortisol may help determine the appropriate dose of hydrocortisone (5).

Patients with primary adrenal insufficiency should also receive fludrocortisone, in a single daily dose of 50-200 µg, as a substitute for aldosterone. The dose can be guided by measurement of blood pressure, serum potassium, and PRA, which should be in the normal-upper range (5).

All patients with adrenal insufficiency should carry a card containing information on current therapy and recommendation for treatment in emergency situations, and they should also wear some type of warning bracelet or necklace, such as those issued by medic alert (5). Patients must be advised to double or triple the dose of hydrocortisone temporarily whenever they have any febrile illness or injury and should be given ampoules of glucocorticoid for self-injection or glucocorticoid suppositories to be used in the case of vomiting (5).

FIG. 9. Natural history of autoimmune adrenalitis with the various stages of potential, subclinical, and clinical hypoadrenalism (see text for details). N, Normal.



**XVIII. Flowchart for the Etiological Diagnosis of AD**

Today, in the presence of ACA and/or 21-OH Abs, the etiological diagnosis of AD is quite easy to perform, and the study by imaging of adrenal glands may not be necessary. In all other cases, a CT or a NMR scan of the adrenal glands should be performed for the differential diagnosis.

These techniques of imaging have greatly improved the identification of the morphological pattern of non-autoimmune AD. In fact, in the presence of a normal adrenal morphology, very long chain fatty acids (VLCFA) are highly recommended (mainly in males) for performing the differential diagnosis of adrenoleukodystrophy.

The finding of small dense adrenal glands is typical of hemochromatosis.

The marked enlargement of adrenal glands with or without calcifications is usually a sign of tuberculosis, fungal or viral infections, histiocytosis, amyloidosis, other granulomatosis, or primary or metastatic cancer. A CT-guided fine-needle biopsy of adrenal masses can be helpful in the differential diagnosis.

In the remaining cases, in the presence of an enlarged, normal, or atrophic adrenal gland, the etiology of the adrenal failure should be carried out using further clinical, biochemical, and also genetic tests.

In the presence of adrenal hemorrhage or infarction, sepsis, systemic lupus erythematosus, antiphospholipid, or dis-coagulation syndromes have to be investigated.

After the etiological diagnosis of AD is done, further in-

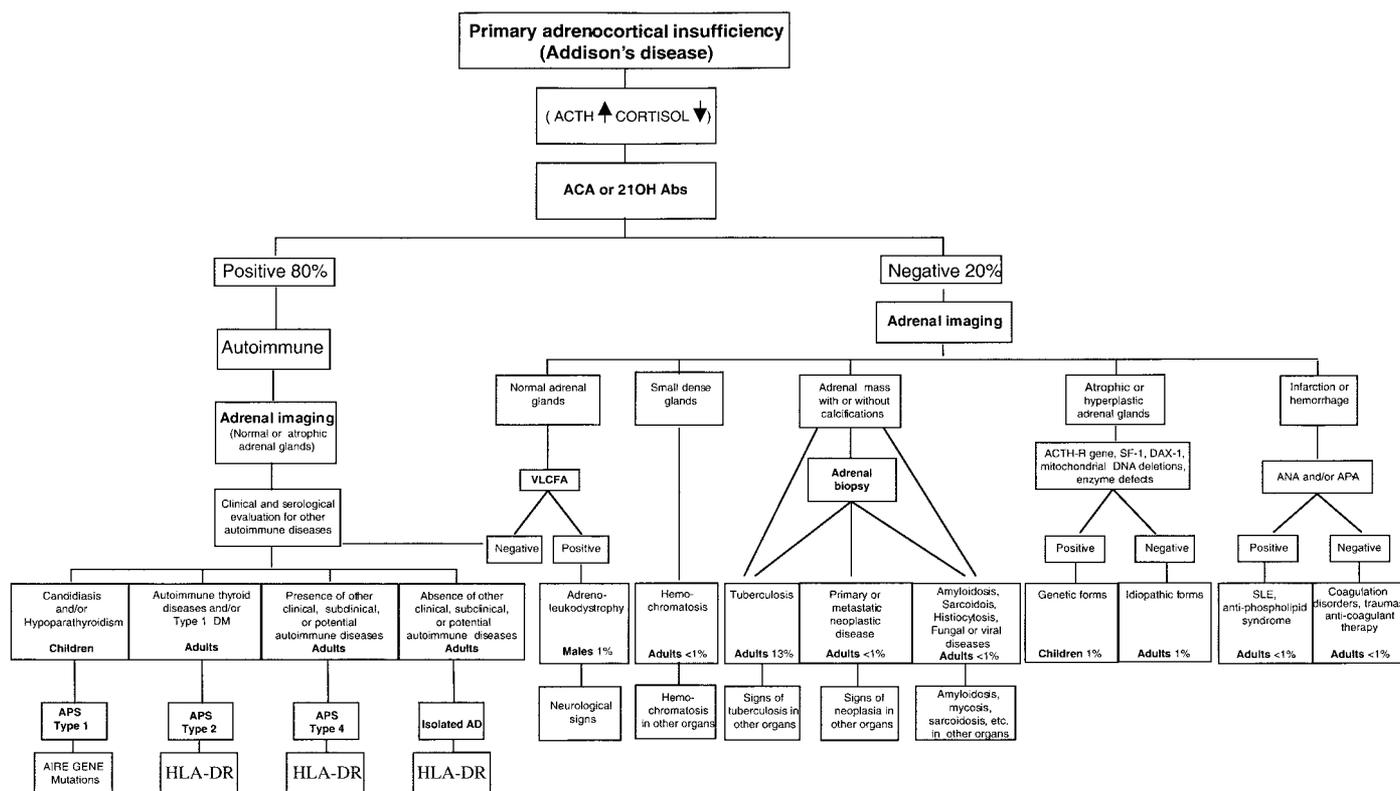
vestigations should be performed. An original flowchart of the etiological diagnostic procedures in the diagnosis of AD is presented in Fig. 10.

**XIX. Concluding Remarks**

Recent years have brought considerable advances in our understanding of the autoimmune AD and its natural history.

Analysis of the molecular interaction between autoantibodies and autoantigens should allow better understanding of the relationship between the specificity of the autoimmune response and the functional activity of the autoantigens. The identification of specific adrenal and gonadal autoantigens and respective autoepitopes and the development of new, sensitive assays to measure adrenal cortex and gonadal autoantibodies could improve the diagnosis and monitoring of both autoimmune AD and POF. The characterization of self-antigens and their respective autoantibodies should be helpful in the prediction of autoimmune AD by identification of the subjects at high risk (first-degree relatives, children, or adults with autoimmune diseases).

Further, introduction of an early replacement therapy in those with ongoing AD could prevent adrenal crisis, and future progress in studies of the role of T lymphocytes and the identification of autoantigens recognized by their receptors might lead to the development of an effective vaccine. The identification of environmental factors involved in the



APA = Anti-phospholipid antibodies; VLCFA = Very long chain fatty acids;  
 ACA = Adrenal-cortex autoantibodies; 21-OH Abs = 21-Hydroxylase autoantibodies  
 ANA = Anti-nuclear antibodies; APS = Autoimmune Polyendocrine Syndrome

FIG. 10. Etiological flowchart of AD.

pathogenesis of the disease may enable effective intervention in the early stages of autoimmune AD.

Furthermore, the progress in morphological studies by CT or NMR revolutionized the imaging of the adrenal glands, helping the evaluation of the morphology and the characteristics of adrenal glands in more detail in primary adrenal insufficiency (330).

The recent advances in the molecular pathogenesis of both congenital and acquired adrenocortical failure have great clinical implications for both children and adult patients with these disorders. The genetic analysis of the mutations in the AIRE gene is likely to aid in diagnosis of APS type 1 both in communities at high risk and in the screening of unaffected family members of APS type 1 patients. Furthermore, future progress in the studies of the role of AIRE genes in the immune response should allow better understanding of the general mechanisms of tolerance and organ-specific autoimmunity. Finally, the identification of the genes in rare non-autoimmune forms of adrenal insufficiency has prognostic and therapeutic implications for the patients and their families.

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### Erratum

In the article by A. Slominski and J. Wortsman entitled “Neuroendocrinology of the skin” (*Endocr Rev* 21:457–487, 2000), an error appears in Fig. 7. In that figure, carbon 25 has an extra line that appears to represent an additional methyl group (CH<sub>3</sub>). Carbon 25 should have two instead of three methyl groups.