

The immunofluorescence techniques in the diagnosis of endocrine autoimmune diseases

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Received: 3 April 2012 / Accepted: 5 May 2012 / Published online: 6 June 2012
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Abstract In the study of autoimmune diseases, the laboratory plays a very important role. We describe the immunofluorescence techniques (direct, indirect, complement-fixing, double) for determining the presence of autoantibodies and their role in the autoimmune endocrine diseases.

Keywords Immunofluorescence techniques · Autoimmune diseases · Thyroid · Gastric · Adrenal · Parathyroid · Gonadal · Pancreatic · Pituitary

Introduction

In the study of autoimmune diseases, the laboratory plays a very important role in predicting or detecting, by immunological studies, the reactions between antigens (Ags) and antibodies (Abs).

Many researchers applied these techniques, year after year more sophisticated and accurate as reported by Betterle [1], for detecting the presence of autoantibodies in patients affected by the autoimmune diseases.

The main analytical techniques used in clinical immunology's laboratories may be summarized in Table 1.

Immuno-histochemical and immuno-enzymatic techniques

Antigen–antibody interactions

The key reactions of immunology and immune defense are the interaction of Abs and Ags forming large irreversible

macromolecule complexes. These interactions, analogous to those observed in enzyme-substrate interactions, involve non-covalent binding of antigenic determinants.

In research, purified antibodies (monoclonal or polyclonal) are most commonly used to identify and locate intracellular and extracellular proteins or autoantibodies present in body fluids or attached to some cellular or tissue antigens. Antibodies are used in flow cytometry, immunoprecipitation [2], Western blot analyses [3] and in immunohistochemistry or immunofluorescence (IF) [1] to examine the protein expression in tissue sections or to locate proteins within cells.

Some general characteristics of the laboratory's techniques (accuracy, precision, linearity, sensitivity, specificity and plausibility) must be applied also to the immunofluorescence techniques. This is an important point, because sometimes it is not easy to find all these parameters simultaneously present in the same test because for e.g., the sensitivity is not always in accord with the specificity. It is certainly difficult to find equilibrium between these parameters but this must be the purpose both the implementation of the various tests and international standardization studies.

Some other parameters (preparation of substrates, solutions buffers, fixation and others) [4] may play an important role in the immunofluorescence studies: in the past, the laboratory's techniques were entrusted to the skill and dexterity of the technicians, today there are many companies specialized in the preparation of substrates for immunohistochemistry.

Today, the technical procedures have changed significantly because the previous manual techniques have been replaced by automated systems. But today only the visual check by microscope is left to the experience of the technician who reads the reaction.

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Table 1 Analytical techniques used in the autoimmunity laboratory

Agglutination
Immunoprecipitation
Turbidimetry
Nephelometry
Double immunodiffusion
Counterimmunoelectrophoresis
Immunofluorescence
Immunoassays
ELISA
IRMA
Immunoblot
Western blot
Dot blot

Immunofluorescence techniques

The immunofluorescence is a histochemical laboratory staining technique that uses the specificity of Abs to their antigen. It is a widely used in immunohistochemistry based on the use of some fluorochromes [5] to visualize the location of the Abs.

The immunofluorescence technique was firstly described in 1942 and refined by Coons in 1950, which used a fluorescence microscope able to read the specific immunological reaction on tissue or cellular prepared on slides. Subsequently, in 1963, granular deposits of IgG and C3 were described along the dermo-epidermal junction in lesions of lupus erythematosus. In 1964, Beutner [6] used the indirect immunofluorescence (IIF) technique to demonstrate antibodies in the sera of patients affected by pemphigus and, in the same years, this technique was used to detect the most important autoantibodies in endocrine autoimmune diseases (see below).

There are four major IF techniques:

1. Direct immunofluorescence technique: it is a one-step histological staining procedure for identifying in vivo antibodies that are bound to tissue antigens, using a single antibody labeled with a fluorophore [5] for staining the tissues or cells. The antibody recognizes the target molecule and binds to it.
2. Indirect immunofluorescence technique: it is a two-step serological technique for the detection of circulating antibodies in body fluids, using two antibodies. The unlabeled first (primary) antibody specifically binds the target molecule, and the secondary antibody, which carries the fluorophore, recognizes the primary antibody and binds to it.
3. Indirect immunofluorescence complement-fixation (IIF-CF) technique: Ags and Abs bind to one another to generate many molecules of C3. This amplification

principle is used in complement IIF, which is more sensitive than IIF.

4. Double immunofluorescence technique: this technique allows identifying the presence of two different antigens on a cell or for the identification of specific cell in a tissue by two antibodies labeled with different fluorophores (for es. FITC or rhodamine). Double staining can be used as a direct/indirect method. The indirect method has very high sensitivity.

Historical features in autoimmune diseases of the endocrine glands

The *ante litteram* description of the autoimmune nature of the endocrine glands goes back to 1855, when Addison [7] described a case with adrenal insufficiency due to an idiopathic (lymphocytic) infiltration of the adrenals; and to 1912, when Hashimoto [8] described mononuclear leukocyte infiltration with an enlarged thyroid gland in patients, defining it as “lymphomatous goiter” and to 1940, when Von Mayerburg [9] described a lymphocytic infiltration of pancreatic islets (insulinitis) in patients with type 1 diabetes mellitus. In 1956, three important discoveries were reported permitting the start of the era of autoimmune diseases: (a) the demonstration of autoantibodies against thyroglobulin in chronic thyroiditis [10], (b) the presence of an agent stimulating the thyroid in patients with Graves’ disease [11] and (c) the demonstration that chronic thyroiditis was reproducible in rabbits by immunization with homogenates of autologous thyroid tissue [12]. In 1957, Witebsky [13] established the criteria for defining an autoimmune disease summarized in Table 2.

In the 1957, it was discovered that “idiopathic” Addison’s disease (AD) had complement fixing autoantibodies against adrenal cortex extracts [14]. In 1962, antibodies to parietal cells were identified by complement fixation test in patients with pernicious anemia [15]. In the 1968, antibodies to steroid-producing cells were described by IIF technique in patients with gonadal failure [16]. In 1974, the autoimmune nature of type 1 diabetes mellitus was firstly described by IIF when islet cells autoantibodies were demonstrated [17].

On the basis of these discoveries from 1956 to 2006, a progressively increased number of diseases previously

Table 2 Criteria for defining a disease as autoimmune (modified from Witebsky [13])

Presence of autoantibodies and/or cell-mediated events in the serum
Presence of lympho-monocyte infiltration in the target organs
Possibility of identification and isolation of autoantigens
Possibility of experimentally induction of the disease in animals by immunization with autoantigens and the transfer the disease passively by serum or lymphocytes

considered as idiopathic, entered in this new group of disorders, so that in 2006 in the preface of the book that celebrated the 50th Anniversary of the discovery of autoimmunity, Rose and Mackey affirmed that “more than 80 diseases are attributable to autoimmunity and one or another affect some 7 % of the population” [18].

Natural history of endocrine autoimmune diseases

The autoimmune endocrine diseases are chronic disorders characterized by genetic predisposition, presence of circulating autoantibodies and lymphocytic infiltration in the target organs and the natural history of these diseases develops in three separate phases: (a) potential, (b) sub-clinical or latent, and (c) clinical. Autoantibodies are the circulating markers that encompassed all the three phases. On the basis of their ability to induce or not a damage of target organ, the autoantibodies can be subdivided as pathogenetic and non-pathogenetic [19].

Autoantibodies in autoimmune endocrine diseases

The endocrine system is one of the systems more affected by autoimmunity. The main autoimmune diseases of the endocrine system are summarized in the Table 3.

Table 3 Autoimmune endocrine diseases

Thyroid diseases
Chronic thyroiditis
Graves' disease
Endocrine ophthalmopathy
Gastric diseases
Autoimmune gastritis
Pernicious anemia
Adrenal diseases
Addison's disease
Adrenal medullitis
Gonadal diseases
Ovarian insufficiency
Male infertility
Chronic hypoparathyroidism
Pancreatic diseases
Type I diabetes mellitus
Flier syndrome
Hirata syndrome
Pituitary diseases
Lymphocytic adenohypophysitis
Infundibuloneurohypophysitis
Polyendocrine syndromes Type I, II, III, IV

Thyroid autoimmune diseases

Thyroid autoimmune diseases (TAD) are summarized in Table 3. They are marked by the presence of thyroglobulin autoantibodies (TGABs) and thyroid microsomal autoantibodies (TMABs).

TGAB were initially detected by immunoprecipitation in agar [10] and then by IIF technique on thyroid sections fixed in methanol [20]. On 1961, they were detected by passive hemoagglutination using sheep red cells [21] and on 1978 using turkey-nucleated red cells [22]. In 1974, TGABs were tested by RIA [23, 24] and, in 1980, by ELISA [25, 26], nevertheless for a long period TGABs were tested using IIF on unfixed thyroid tissue (Fig. 1).

TMABs were detected, since 1967, by IIF on unfixed thyroid tissue [20], and from 1973 also by passive hemoagglutination using sheep red cells [27], and from 1976 using or turkey-nucleated red cells [28]. They were tested by RIA or ELISA using microsomal antigens [29, 30].

By IIF or hemoagglutination techniques the TGABs and TMABs were found in 85–95 % of patients with chronic thyroiditis and in 85 % of patients with Graves' disease [26–28].

After the discovery on 1985, thyroperoxidase (TPO) is the target antigen [31] recognized by TMABs, antibodies to TPO (TPOABs) were detected by RIA [32, 33] or ELISA [34] using recombinant antigens.

These methods are a slightly more sensitive and specific than the previous tests [35], nevertheless, the IIF test on unfixed thyroid tissue was used for many years after.

TGABs and/or TMABs/TPOABs were also present in 50 % of first-degree relatives of patients with autoimmune thyroid diseases, in 15–40 % of patients with other autoimmune diseases and they can also be found in 20 % of patients with thyroid carcinoma [26]. These auto-Abs can be found in 12–26 % of euthyroid females and in 2.8–14.4 % of euthyroid males [36]. The presence of these autoAbs was considered a marker of future thyroid dysfunction [37, 38].

The IIF test on thyroid tissue in addition to the detection of TMABs and TGABs permitted also to individuate others autoantibodies (such as mitochondria antibodies AMA and anti-nuclear antibodies ANA) (Fig. 1e, f).

Gastric autoimmune diseases

The gastric autoimmune diseases are summarized in Table 3. The diseases are marked by the presence of parietal cells autoantibodies (PCA). Initially, PCA were detected by complement fixation test using homogenates of stomach [39, 40] and were found in 62 % of patients with pernicious anemia and in 4 % of normal controls. Subsequently they were detected by IIF using human or animal gastric mucosa;

AUTOANTIBODIES TO THYROID TISSUE

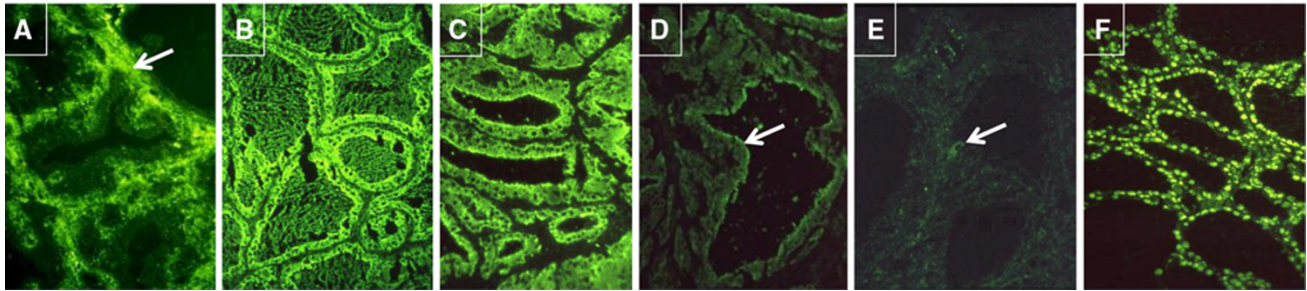


Fig. 1 Indirect IF on unfixed thyroid tissue. **a** Pattern produced by a normal serum: the cytoplasm of thyrocytes and the thyroglobulin inside the follicles are negative, the fluorescence is concentrated in the connective tissue between the thyroid follicles (*arrow*). **b** Pattern produced by a serum of a patient with high titers of TMA and TgAbs: the cytoplasm of the thyrocytes is diffusely and intensively stained and the substance inside the follicles is also intensively stained. **c** Pattern produced by a serum of a patient with high titers of TMA: the cytoplasm of the thyrocytes is diffusely and intensively stained and the substance inside the follicles is negative. **d** Pattern produced by a

serum of a patient with low titers of TMA: a weak positivity is present in the cytoplasm of thyrocytes with reaction in the apical rim of thyrocytes where the microsomal antigen is concentrated (*arrow*). **e** Pattern produced by a serum of a patient with high titers of mitochondria Abs: the serum shows a granular pattern of cytoplasm of thyrocytes and the reaction is concentrated in scattered cells rich in mitochondria (see *arrow*). **f** Pattern produced by a serum of a patient with high titers of anti-nuclear Abs: the reaction is positive on the nuclei of thyrocytes

AUTOANTIBODIES TO GASTRIC TISSUE

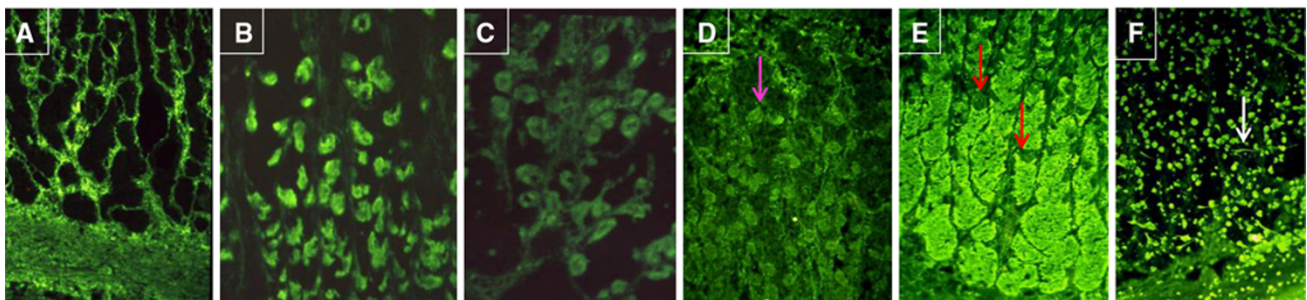


Fig. 2 Indirect IF on human unfixed gastric mucosa. **a** Pattern produced by a normal serum: the cells in the glands of the stomach are negative, the fluorescence is concentrated in the connective tissue. **b** Pattern produced by a serum of a patient with high titers of PCA: the reaction is positive with a diffuse pattern of cytoplasm of the cells scattered in the glands (parietal cells), while the chief cells are negative. **c** Pattern produced by a serum of a patient with low titers of PCA: the serum reacts as a diffuse pattern on the cytoplasm of the cells scattered in the glands (parietal cells), while the chief cells are negative. **d** Pattern produced by a serum of a patient with high titers of mitochondria Abs: the serum reacts to the parietal cells (rich in

mitochondria) (*pink arrow*) with a major intensity and weakly the cytoplasm of chief cells having a minor concentration of mitochondria. **e** Pattern produced by a serum of a patient with high titers of anti-ribosomal Abs: the serum stains intensively the cytoplasm of the chief cells but it does not react with the cytoplasm of parietal cells (*red arrow*). **f** Pattern produced by a serum of a patient with high titers of anti-nuclear Abs: the serum stains the nuclei of all the cells of the gastric mucosa. In this case some nuclei show an aspect of comet DNA (*white arrow*) (this pattern is indicative of antibody to double stranded DNA)

with this technique, PCA were found in 86 % of patients with pernicious anemia and in 11 % of controls [40]. PCA are organ, but not strictly species-specific auto-Abs of IgG class, reacting with the microsomal fraction of parietal cells [40] (Fig. 2). On 1984, a RIA was introduced using microsomal gastric antigen in order to detect PCA [41].

On 1987, ATPase H^+/K^+ was identified as the target antigen detected by PCA and an ELISA was produced to detect ATPase H^+/K^+ Abs and with this method were found in 88 % of patients with chronic gastritis [42]. PCA can be found in normal population with a frequency that increases with the age, in fact they are present in 4.8 % in subjects

under 50 years but reached 9.6 % in those with more than 80 years [43]. PCA are also present in 5–25 % of the patients with other autoimmune diseases, particularly in those with thyroid autoimmune diseases, Addison's disease, type 1 diabetes or vitiligo [42, 44]. PCA can be found in asymptomatic patients and they can be considered markers of latent autoimmune gastritis [45] or predictors of future autoimmune gastritis [46]. Until today, the IIF test on gastric tissue is already the most used to detect PCA.

The IIF test on gastric mucosa is able to detect also other auto-Abs such as ANA, AMA, ribosomal antibodies (ARA) and smooth muscle antibodies (SMA) (Fig. 2d–f).

Autoimmune diseases of the endocrine pancreas

The autoimmune diseases of the endocrine pancreas are summarized in Table 3. Islet-cell autoantibodies (ICA) were discovered on 1974 by IIF test using normal human

group 0 pancreas [17]. They react with an unidentified antigen common to all the cells of the pancreatic islets (Fig. 3b). They are of IgG class, able to fix the complement, organ- but not strictly species-specific because they can react also with primate pancreas. On 1985 under the aegis

AUTOANTIBODIES TO PANCREATIC TISSUE

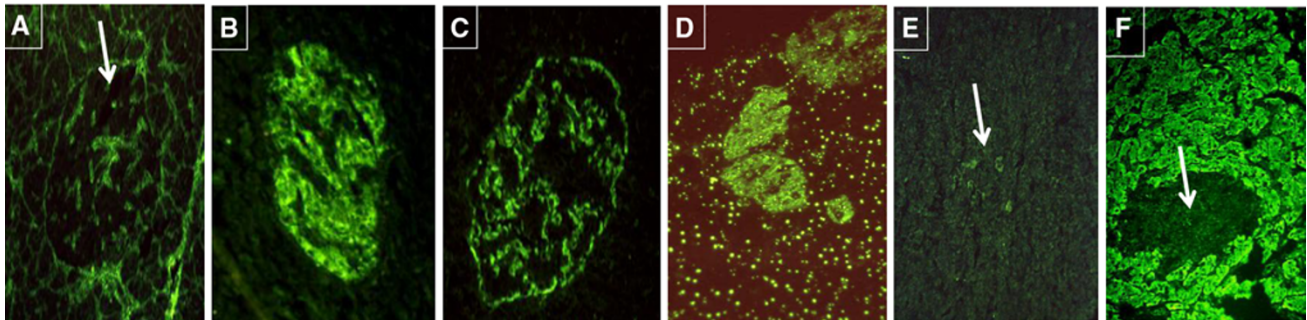


Fig. 3 Indirect IF on human unfixed pancreas. **a** Pattern produced by a normal serum: the cells of the islet are negative (*arrow*), the fluorescence is concentrated in the connective tissue inside and outside the islet. **b** Pattern produced by a serum of a patient with high titers of ICA: the positivity is present on cytoplasm of all the islet-cells, while the capillaries inside the islets are negative. **c** Pattern produced by a serum of a patient with antibodies to separate cells of the islet: the serum stains with a great intensity the cytoplasm of cells scattered in the islet. **d** Pattern produced by the serum of a patient

with high titers of ICA and of anti-nuclear Abs: the serum stains intensively the cytoplasm of the islet-cell and the nucleoli of all the cells inside and outside the islet (nucleolus pattern). **e** Pattern produced by a serum of a patient with high titers of mitochondria Abs: the serum stains weakly the cytoplasm of all the cells in the exocrine tissue, with a major intensity against some cells (rich in mitochondria) (*arrow*). **f** Pattern produced by a serum of a patient with ribosomal autoantibodies: the cytoplasm of the cells of the exocrine tissue is diffusely stained, on the contrary the islet-cells are negative (*arrow*)

AUTOANTIBODIES TO PANCREATIC TISSUE

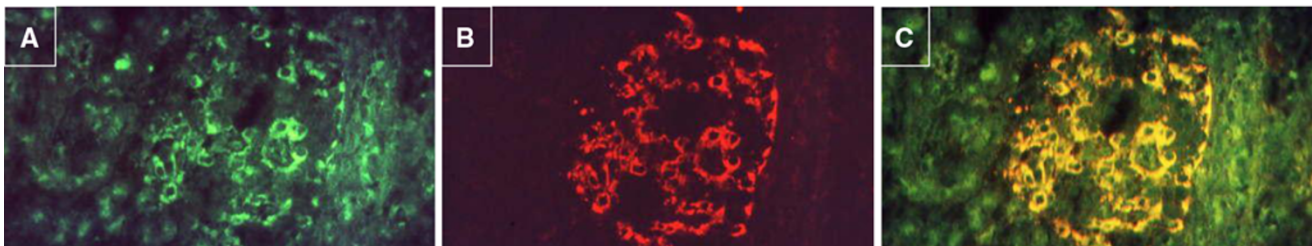


Fig. 4 Double IF on human unfixed pancreas. **a** *Green* pattern produced by a serum of a patient counterstained by FITC-labeled goat anti-human serum, the fluorescence is concentrated on the cytoplasm of scattered cells of the islet. **b** *Red* pattern produced by an anti-glucagon rabbit serum counterstained with a rhodamine-labeled anti-

rabbit serum: the rabbit serum stains in *red* the same cells as the human serum. **c** The double microphotographic exposure of the images **a** and **b**, produces a *yellow* colour (sum of the *green* and the *yellow*) confirming that the human serum reacts against glucagon-producing cells

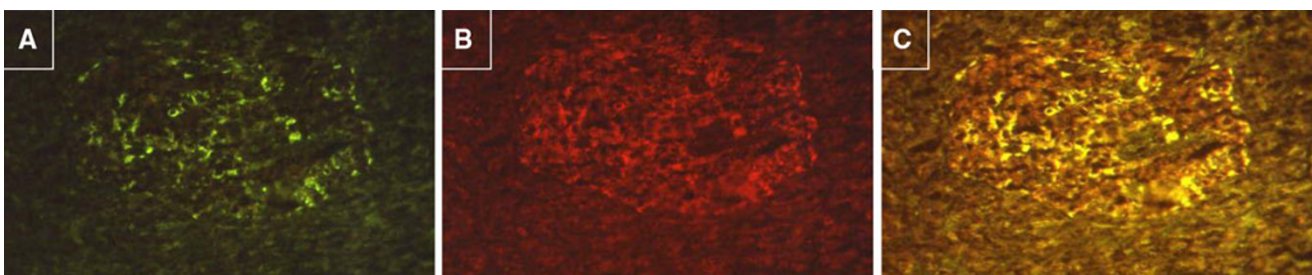


Fig. 5 Double IF on human unfixed pancreas. **a** *Green* pattern produced by a serum of a patient counterstained by FITC-labeled goat anti-human serum, the fluorescence is concentrated in the cytoplasm of scattered cells of the islet. **b** *Red* pattern produced by an anti-somatostatin rabbit serum counterstained with a rhodamine-labelled

anti-rabbit serum the rabbit serum stains in *red* the same cells as the human serum. **c** The double microphotographic exposure of the images **a** and **b**, produced a *yellow* colour (sum of *green* and *red*) confirming that the human serum reacts against somatostatin-producing cells

of the Juvenile Diabetes Foundation, a standard positive serum with a value a 80 U/JDF was identified and the distribution worldwide of this standard serum permitted to improve the sensitivity and specificity of the various laboratories detecting ICA by IIF [47–49]. Subsequently, other pancreatic autoantibodies were identified against insulin (IAA) [50], GAD (GADAb) [51], second islet autoantigen (IA2Ab) also called ICA 512 [52]. The IIF test identified ICA in 48–60 % of newly onset type 1 DM [53, 54]. ICA can be also present in about 9 % of the patients with type 2 DM [54] and they are able to identify, in combination with GADAb, the latent autoimmune diabetes of the adults (LADA) [55]. ICA associated to other pancreatic Abs can also be identified in individuals without diabetes (first-degree relatives of patients with type 1 DM, schoolchildren, or patients with other autoimmune diseases) and they can be markers of future disease with an increased risk directly correlated with the number of pancreatic auto-Abs [56, 57].

Using IIF technique on human pancreas, other reactivities against different cells of the islets were also discovered (Fig. 3c) and identified by the double IF technique as autoantibodies to glucagon-producing cells (GPCAb) (Fig. 4) or to somatostatin-producing cells (SPCAb) (Fig. 5) [58]. But, so far these antibodies were not correlated with a clinical deficiency of these hormones [59].

The determination of ICA in combination with other pancreatic Abs remains, today, the best method for the identification of patients with autoimmune type 1 DM, LADA or the subjects at risk of type 1 DM.

The IIF test on pancreas identified other reactivities such as AMA, ANA, ARA (Fig. 3d, e) and Abs against cyokeratin.

Autoimmune diseases of the adrenal

The adrenal cortex autoimmune diseases are summarized in Table 3. Adrenal cortex autoantibodies (ACA) are markers of autoimmune adrenal insufficiency and were discovered on 1957 by complement fixation using adrenal cortex extracts [14]. From 1963, they were tested by IIF technique using human or animal tissues [60]. ACA react with the cytoplasm of all the three cells layers of adrenal cortex (Fig. 6b), they are of IgG class, organ, but not strictly species, specific and fix the complement. Using the IIF technique on human or animal adrenal tissues, many patients affected by autoimmune and tuberculous AD were studied, and ACA were found positive in 61 and 6.7 %, respectively [61].

In 1992, it was discovered that the enzyme 21-hydroxylase (21-OH) is the major autoantigen recognized by ACA [62, 63]. After this discovery, a RIA test using ^{35}S -21-OH [64] was used to detect 21-OHAbs. Subsequently, a more convenient assay to measure 21-OHAbs has been developed using ^{125}I -21-OH expressed in yeast [65].

ACA by IIF on human adrenal cortex or 21-OHAbs by IPA are present in 89 and 91 %, respectively, of patients at the onset of autoimmune Addison's disease, but absent in patients with non-autoimmune forms. There is a strict correlation between the two methods even if 21-OHAbs are able to detect very low titers of antibodies and ACA can detect some autoantibodies different from 21-OHAbs [66].

They can be present also in individuals without hypoadrenalism and they can predict the future adrenal insufficiency [67]. On 2012, under the aegis of Euradrenal Committee, a program for the standardization of 21-OH

AUTOANTIBODIES TO ADRENAL TISSUE

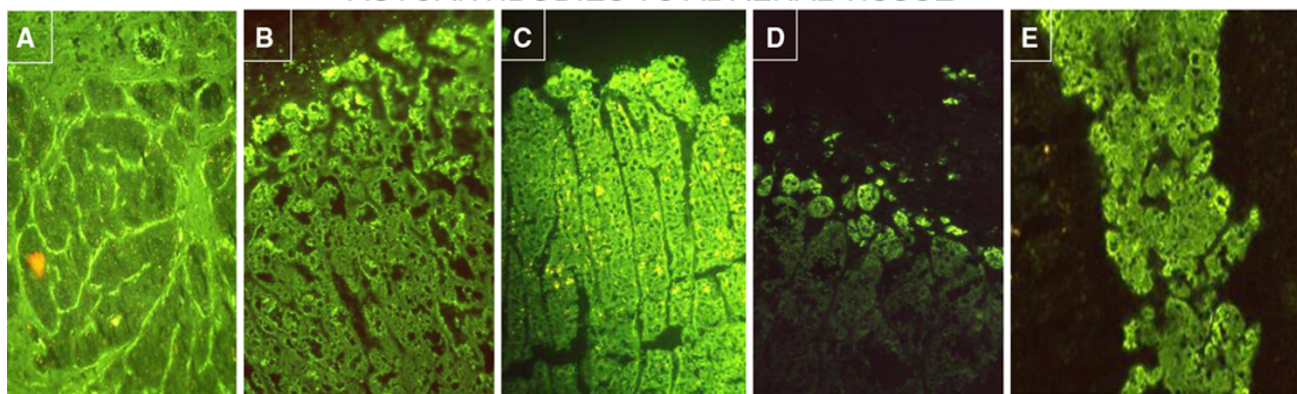


Fig. 6 Indirect IF on human unfixed adrenal tissue. **a** Pattern produced by a normal serum: the cells of the adrenal cortex are negative, the fluorescence is concentrated in the connective tissue. **b** Pattern produced by a serum of a patient with high titers of ACA: the reaction is positive on cytoplasm of the cells of all the three layers of the adrenal cortex with a diffuse pattern. **c** Pattern produced by a serum of a patient with high titers of ACA: the serum stains the

cytoplasm of the cells of the glomerulosa with a diffuse pattern. **d** Pattern produced by a serum of a patient with low titers of ACA: the serum stains the cytoplasm of the cells of the glomerulosa. **e** Pattern produced by a serum of a patient with high titers of adrenal-medullary-cell antibody: the serum diffusely stains the cells of the adrenal medulla

AUTOANTIBODIES TO STEROID-PRODUCING TISSUES

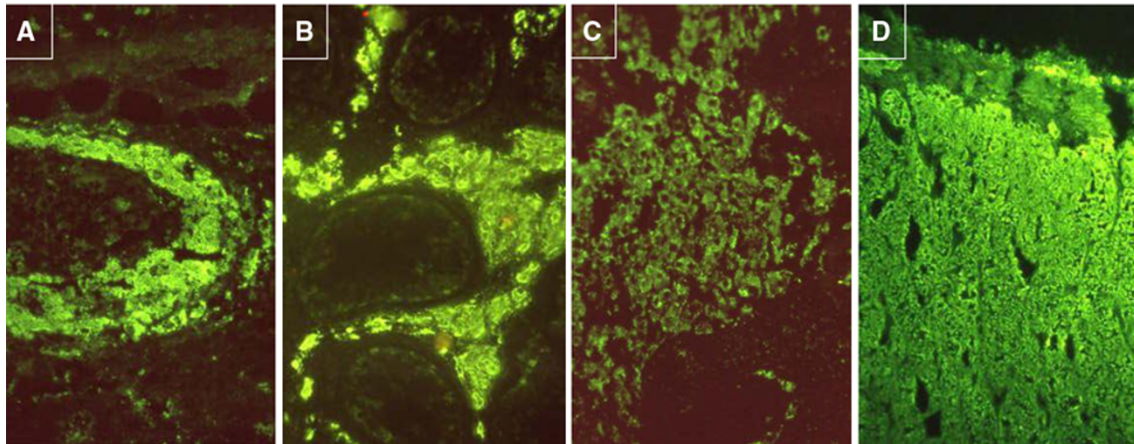


Fig. 7 Indirect IF on human unfixed ovary, testis, placenta and adrenal. Pattern produced by a serum with antibodies to steroid-producing cell: this serum diffusely stains the cytoplasm of the theca

cells of the ovary (a), of the Leydig cells of the testis (b), of the syncytiotrophoblast of placenta (c), and of the adrenal cortex cells (d)

Abs involving multiple international laboratories has been initiated and the results of this study will be published (Betterle personal observation).

Adrenal medulla autoimmunity

Autoimmune diseases of the adrenal medulla are summarized in Table 3. In 1984, it was described an adrenal-medullary-cell antibody (AMCA) of IgG class, reacting to an unidentified cytoplasmic antigen of adrenal medulla [68]. The AMCA can produce a “diffuse” (Fig. 6) or a “granular” pattern. AMCA are detected in 30 % of ICA-positive and 4 % of ICA-negative patients with type 1 DM, and in 32 % of ICA-positive patients without type 1 DM [69].

The clinical significance of this Abs is not clear. But it is important to remember that: (a) a deficit of catecholamines has been described in type 1 DM patients with AMCA [70], (b) it has been demonstrated an adrenal medulla fibrosis in type 1 DM with longstanding disease [69], (c) some patients with type 1 DM revealed an insufficient adrenergic response to insulin-induced hypoglycaemia [69]. On the basis of all these data it has been hypothesized that these Abs mark, in type 1 DM, a lymphocytic medullitis inducing a deficit of catecholamines [69, 71].

Autoimmune diseases of the gonads

Autoimmune diseases of the gonads are summarized in Table 3. In 1968, an antibody to steroid-producing cells (StCA) was detected by IIF test [16], reacting to the cells of the ovarian theca, to Leydig cells of the testis, syncytiotrophoblast of the placenta and to adrenal cortex cells (Fig. 7).

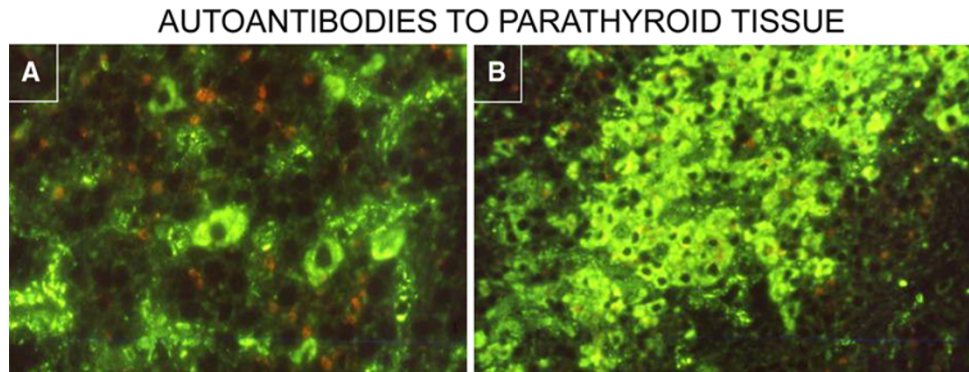
In females affected by Addison’s disease, they are markers of premature ovarian failure (POF) [72] characterized by a lymphocytic oophoritis [73]. After the discovery that StCA reacted with steroidogenic enzymes 17 α -hydroxylase (17 α -OH) or side chain cleavage (SCC) [74], autoantibodies to 17 α -OH (17 α -OHAbs) or to SCC (SCCAbs) were tested by a RIA technique, using recombinant antigens [75]. These Abs are present with high frequency (92 %) of Addisonian patients with POF but they are also present in female patients affected by Addison’s disease with normal menses and can be predictive markers of future POF [76].

Despite the StCA were initially identified in two male patients affected by Addison’s disease [16], it is not clear if in those patients they can be markers of autoimmune testicular insufficiency.

Autoimmune parathyroid diseases

Parathyroid autoimmune diseases are shown in Table 3. The detection of parathyroid autoantibodies represents one of the most problematic and not so far defined cases in autoimmunity. Initially, they were detected in 1966 by IIF using unspecified parathyroid tissue [77], subsequently confirmed with the same method [78]. These autoantibodies reacted to the cytoplasm of the cells of parathyroid tissue (Fig. 8), but they were present with low frequency in chronic hypoparathyroidism (CH), in patients with other autoimmune diseases and also in normal controls [78]. Subsequently, by IIF, autoantibodies reacting with the surface of human parathyroid cells and able to inhibit the PTH secretion or to mediate a complement-dependent cytotoxicity in cultured bovine parathyroid cells were reported in patients with CH [79, 80] but different studies

Fig. 8 Indirect IF on human unfixed parathyroid tissue. Pattern produced by a serum of a patient with chronic hypoparathyroidism: this serum stains the cytoplasm of cells of parathyroid (a, b). This reaction is not against chief cells of the parathyroid gland but against oxyphil cells rich in mitochondria and it recognizes a human mitochondria antigen of 46 kD



AUTOANTIBODIES TO PITUITARY TISSUE

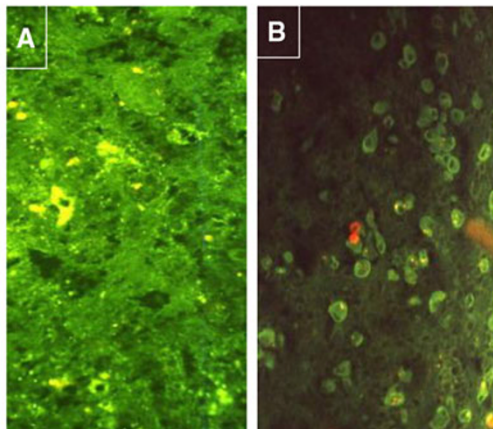


Fig. 9 Indirect IF on human unfixed pituitary. a, b The serum of this patient intensively stains the cytoplasm of some pituitary cells

did not confirm this report [81]. Other studies reported that the parathyroid cytoplasmic antibodies, initially detected by IIF, were reactive only with oxyphil parathyroid cells [82] recognizing a human mitochondrial antigen of 46 kDa molecular weight [83]. Subsequently, autoantibodies against a human parathyroid membrane-antigen of 120–40 kDa, identified as the calcium sensing receptor (Ca-SR) by immunoblot and Western blotting analysis were identified in 20 % of the patients with CH (isolated or in the context of autoimmune polyendocrine syndrome type 1) and in 0 % of controls [84]. Subsequently, Ca-SRABs detected by Western blot, were found in 49 % of the patients with CH but also in 13 % of the normal controls [85]. In contrast, by immunoprecipitation assay using ^{35}S labelled recombinant Ca-SR antigen, Ca-SRABs have not been detected in any of 90 patients with CH [86].

Recently, an antibody to a parathyroid antigen defined NACHT leucine-rich-repeat protein 5 (NALP5Abs) was identified in 48 % of the patients with CH in the context of

autoimmune polyendocrine syndrome type 1 and not in the other forms of CH or in other autoimmune diseases [87].

Autoimmune pituitary diseases

Autoimmune pituitary diseases are summarized in Table 3. Using human pituitary, the autoantibodies against some pituitary cells permitted to demonstrate, by a double IIF technique, a reaction to prolactin-secreting cells [88]. These Abs were described in patients with autoimmune endocrine diseases but in none affected by hypopituitarism [57].

Subsequently, pituitary antibodies were investigated in patients with lymphocytic adenohypophysitis (LAH) by indirect IIF (Fig. 9). Using as substrates human, primate or rodent pituitary specimens, human fetuses, or animal pituitary cell lines, pituitary Abs were found in only 36 % of patients with documented LAH as reviewed by Caturegli [89]. The specificity of pituitary Abs is poor and they were found in various other non-autoimmune diseases as Cushing's syndrome, pituitary adenomas, empty sella syndrome and Sheehan syndrome, as in other autoimmune diseases such as type 1 diabetes, Hashimoto's thyroiditis, and Graves' disease as reviewed by Caturegli [89]. Autoantibodies to a not specified anterior pituitary components were also described in patients with autoimmune diseases and able to predict future hypopituitarism [90]. The biggest problem about the evaluation of these autoantibodies is that do not exist an international standardization of this procedure, furthermore it was documented that the Fc fragment of immunoglobulins bind to a receptor of ACTH producing cells of the pituitary [91].

As regard to Abs to diencephalon or hypothalamus, initially they were tested by IIF using baboon diencephalon, and antibodies to vasopressin-producing cells were described in 37 % of patients with idiopathic diabetes insipidus [92] (Fig. 10). The specificity of these antibodies is poor being described also in 54 % of patients with DI secondary to histiocytosis X and in other autoimmune disorders [93].

AUTOANTIBODIES TO DIENCEPHALON TISSUE

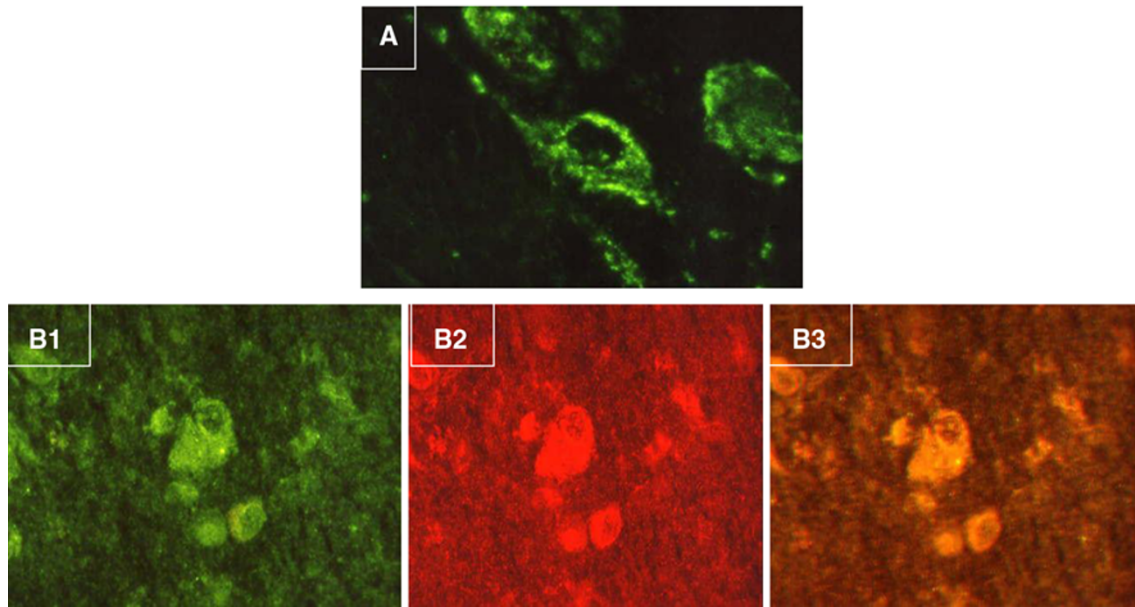


Fig. 10 Double IF on baboon unfixed diencephalon. **a** Pattern produced by a serum of a patient with diabetes insipidus: some cells of the diencephalon are stained. (from Ref. [1], p. 207, Fig. 7.7). **b1** The serum of this patient counterstained by a goat anti-human FITC serum produced a *green* pattern concentrated in the cytoplasm of some cells. (from Ref. [1], p. 207, Fig. 7.8). **b2** A rabbit serum anti-

vasopressin counterstained with an anti-rabbit rhodaminated serum stains in *red* the same cells stained by the patient's serum. **b3** The double microphotographic exposure of the imaging **a** and **b**, produced a *yellow* colour (sum of *green* and *red*) of the cytoplasm of the cells confirming that the human serum reacts against vasopressin-producing cells

General conclusion

Concluding our study and considering that, in these recent years, IF was considered as obsolete and outdated, we think important to report these Mackay's considerations in a recent review on the history of autoimmunity [94]: "in the early 1960s', the immunofluorescence led to the confident nomination of diverse diseases as autoimmune and the technique soon became the laboratory "workhorse" for routine laboratory diagnosis". These words may be valid also today because this technique may be considered very useful in the diagnosis of various organ- and non-organ specific autoimmune diseases.

Acknowledgments This study was supported in part by a grant from the European Union Seventh Framework Programme, the Euradrenal project: Pathophysiology and Natural Course of Autoimmune Adrenal Failure in Europe. Grant Agreement No. 2008-201167 and from Grant ex 60 % of University of Padova. We like to thank Mr. Stefano Masiero for his technical assistance.

Conflict of interest None.

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