SYMPOSIUM

VI WORKSHOP ON AUTOIMMUNITY

AUTOMATED READING IN INDIRECT IMMUNOFLUORESCENCE

MARKERS IN DIABETES MELLITUS TYPE 1

October 19th, 2016
Grifols Museum
(Barcelona, Spain)

GRIFOLS

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VI WORKSHOP ON AUTOIMMUNITY:

AUTOMATED READING IN INDIRECT IMMUNOFLUORESCENCE

MARKERS IN DIABETES MELLITUS TYPE 1

CONTENTS

Automated reading of indirect immunofluorescence. Present and future of the technique

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INTRODUCTION

Indirect immunofluorescence (IIF) is a technique for detecting autoantibodies in which patients' serum is incubated on a substrate (cells or unfixed tissue) that expresses the antigens against the desired autoantibodies. This is followed by incubation (after washing the non-specific immunoglobulins (Ig) with a human anti-Ig serum labelled with a fluorochrome (generally fluorescein isothiocyanate: FITC in this use). After a second washing, the preparation is read in a microscope under ultraviolet light (UV), which, by exciting the fluorochrome, produces an energy emission (due to electron orbital jump), which appears as green fluorescent staining in the tissue. This is one of the classic methods for detecting autoantibodies, others being counterimmunoelectrophoresis, the Ouchterlony technique, immunodiffusion, immunoblotting, immunoprecipitation, gel precipitation, haemagglutination and radioimmunoassay. Several of these methods are no longer used in healthcare while others still are, often with different nuances, especially in their routine application. Some of the methods are very time- and labour-consuming, while others, such as IIF, can be automated.

Any discussion of IIF should begin with the substrates, a crucial issue with regard to automation systems. The substrates most readily transferred to automation and most frequently used are HEp-2 cells, described as early as the 1950s. At that time, a number of procedures were tested that allowed tumours to be maintained *in vitro* to obtain cell lines or for their transplantation into animals[1,2]. Thus, at least three lines derived from a human larynx epidermoid carcinoma were developed: HEp-1, HEp-2 and HEp-3[3]. After a few years, these cell lines, especially Hep-2, were used for immunofluorescence in diagnosis, a practice that became widespread in the following decades. At the same time, in the 1960s, immunofluorescence in tissues was first applied for this use[4,5]. Later, in the mid-1980s, after the first antibodies against elements of neutrophil cytoplasm had been identified, neutrophils were introduced for the diagnosis of certain types of vasculitis. Their use soon became routine and eventually led to the differentiation of a vasculitis group associated with antineutrophil cytoplasmic antibodies (ANCA)[6].

Today, there are a wide variety of autoantibody detection methods (**Table 1**). However, their profile has changed so much over the years that some are now hardly used in routine practice. IIF, on the other hand, has not only gained subtlety, but has also incorporated techniques to extend it, in addition to classical immunofluorescence and immunofluorescence on mosaics in the same well with different cell types. More recently, immunofluorescence has also been incorporated into transfected cells (not just HEp-2 cells), thereby providing a means of introducing the gene that expresses a given protein, a very useful and practical method for detecting rare autoantibodies against the neo-expressed protein. Immunofluorescence is also available on combinations in the same well and specific antigens, either in the imprint of the purified antigen and even, more recently, with microparticle beads

Table 1. Methods for detection of autoantibodies

• Immunofluorescence: "classical" IIF, mosaics, transfected cells, specific combination of tissues / antigens on the slide or in particles, etc. • **Immunoenzymatic methods (EIA)** • **Immunoblot** • **ALBIA** • **Chemiluminescence** • Immunoprecipitation • Radioimmunoassay (RIA) • Western blot • In development: microarrays ...	RECOMBINANT ANTIGENS • Purity and stability • Reproducibility ↓ AUTOMATION

with different antigens attached that generate different images and can only be read with any degree of reliability by automated systems.

The early 1990s saw the start of mass production of recombinant antigens, characterised by their high purity and stability. They could also be obtained from different cell types, thus permitting constant production. The mass availability of pure and stable antigens greatly facilitated automation, with reproducibility fully guaranteed for some techniques. This, in turn, led to a decline in use of immunofluorescence as a key technique in autoantibody serology laboratories, as use of antigen mixtures (and even cell extracts) to detect antibodies increased. At the same time, however, a number of studies were published showing a discrepancy between clinical symptoms and what were traditionally interpreted as 'antibodies associated with those clinic symptoms'. Consequently, the American College of Rheumatology sponsored a review of a large number of publications in which various autoantibody detection techniques had been used, resulting in the publication in 2010 of the Society's positioning in this regard[7]. This in turn led to the creation of a working group that published recommendations on the subject in 2011, in which immunofluorescence remained the gold standard for testing antinuclear antibodies[8].

ADVANTAGES AND DISADVANTAGES OF IMMUNOFLUORESCENCE

Immunofluorescence has several advantages, but also certain drawbacks (**Table 2**).

One of the key advantages is its high sensitivity, which over the years has enabled an increasing number of antibody patterns associated with specific pathologically-significant antigens to be detected (more than 50 IIF patterns identified with associated specificities). In the case of the HEp-2 substrate, a major advantage is that it enables detection of autoantibodies which recognise antigens that are not in the nucleus, other than the classic antinuclear antibodies (ANA). This is because this cell line includes intact cells, so antibodies directed against a great diversity of cellu-

Table 2. Advantages and disadvantages of IIF

Advantages
• Very high sensitivity
• Information on non-antinuclear autoantibodies (ANA)
• Detection of possible new autoantibodies
• Cost / efficiency
Disadvantages
• Need for expert readers
• Quality highly dependent on equipment and reagents
• Occupancy time in reading
• Personnel and time requirements in large laboratories
• Difficult automation (?)

lar structures can be detected. It should also be borne in mind that when the cells divide, this leads to the expression of proteins that are not in the resting phase; in many cases antibodies directed against them have pathological significance. A third advantage is the detection of possible new, i.e. previously unidentified, autoantibodies. This is very important because although some have no clinical significance, others do and eventually become related to a particular pathology. In practice, established antibody patterns are so diverse that committees have been set up to produce a consensus in ANA (the International Consensus on ANA Patterns: ICAP)[9-13] and to standardise interpretation of autoantibodies in general (International Autoantibody Standardization: IAS). And last but not least, immunofluorescence has the advantage of good cost efficiency.

One of the most significant disadvantages of immunofluorescence is that it requires expert readers who are capable of identifying artefacts, knowledgeable of different patterns to guide further study and who can provide information for the clinician. A further drawback is that the quality of the procedure is highly dependent on the equipment and reagents used, which, as shown below, does not completely disappear with automation. It should also be borne in mind that the technique requires occupancy time for reading, which in large laboratories may require more personnel in charge of the task or even staff working exclusively on it.

AUTOMATED IIF SYSTEMS

To counteract some of the aforementioned drawbacks, there has been a trend towards developing different automated devices. Automation frees up personnel for other activities, since reading is limited to negative or positive checks and only requires greater attention for dubious and positive results, either to confirm them or ratify a pattern. Logically, the degree of autonomy provided by automation depends on the equipment used, but results are still dependent on the quality of the reagents, substrates and equipment.

Different types of equipment can be broadly classified by certain characteristics. Thus, there are automated systems that only perform readings, requiring another apparatus to mount slides, while other devices combine assembly and reading. However, on the date of this presentation, there was only one such machine on the market, although the launch of at least two more has been announced, one that integrates both functions and another that incorporates a mounting system.

Some devices provide prior identification of the cells, with the advantage of eliminating, or at least reducing, the likelihood of interpreting certain artefacts, such as specific types of fluorescence signals. Other devices add the fluorescent marker DAPI (4', 6-diami-

no-2-phenylindole) as an identifier, which binds to DNA by staining cell nuclei. These devices also have other advantages, but their drawbacks include delay in focus.

With regard to image capture, some devices select a few images and identify positivity or negativity in them, or even suggest patterns in some cases, while others carry out a sweep with the whole mosaic of the well. There are also instruments that integrate both systems, as they can select images or allow the operator to do so, or they can perform a complete mosaic of the well.

A number of devices can only distinguish positives and negatives and suggest results that, obviously, have to be ratified and validated. Other instruments perform a mathematical calculation based on the fluorescence signal of the final titre, which may be relevant, depending on whether the laboratory reports titres. In the author's opinion, establishing the final titre in fluorescence is not currently worthwhile, given the possibility that specific antibodies can later be identified using quantitative methods. There are also instruments that suggest a pattern result; in other words, they identify or supposedly identify a pattern and then include it in the report. Finally, there are devices that permit identification of the specificity, as they include small particles with purified antigens attached in the same well.

The author's laboratory has had a year's experience using Helios, an automatic analyser that performs complete mounting and reading. Clearly the inclusion of both activities means it incorporates a high number of processes.

CRITICAL POINTS

There are also a number of critical points in the use of automated IIF equipment. Fortunately, they can generally be solved by applying certain simple procedures that are worth mentioning here.

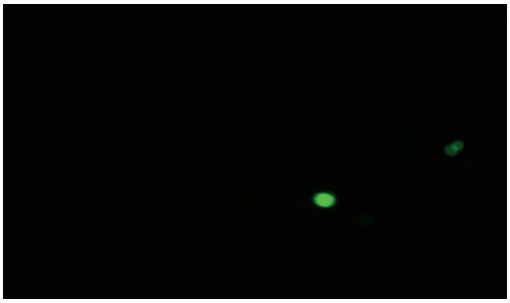

Figure 1. Non-focused anti-neutrophil cytoplasmic antibody and possible practical solution.

Problem: contaminant particles that capture fluorescence. Practical solution: previous washing (reagent quality control). *Material from this publication has been used with the permission of Dr. Aresio Plaza López.*

One such point is focus, which can be disturbed in certain circumstances. For instance, in the case of ANCAs (anti-neutrophil cytoplasmic antibodies), as there are not as many cells in the well as when working with HEp-2, the fluorescence signal is often not as high, and contaminating particles may be present that can pick up the fluorescence and cause blurring (**Figure 1**). However, this problem can generally be corrected by a simple procedure such as pre-washing with ethanol, a step that is not included in the equipment operating protocols.

More rarely, a blur may occur in HEp-2 or, more often, in triple tissue, which can either be a total blur, or, if a mosaic reading option is selected, a partial blur or blurring in only some areas (**Figure 2**). This is due to system settings, such as those resulting from the meniscus that forms after mounting is complete when adding the glycerol-containing solution without putting a cover slip over it. This can alter the light signal and influence the focus. The meniscus should

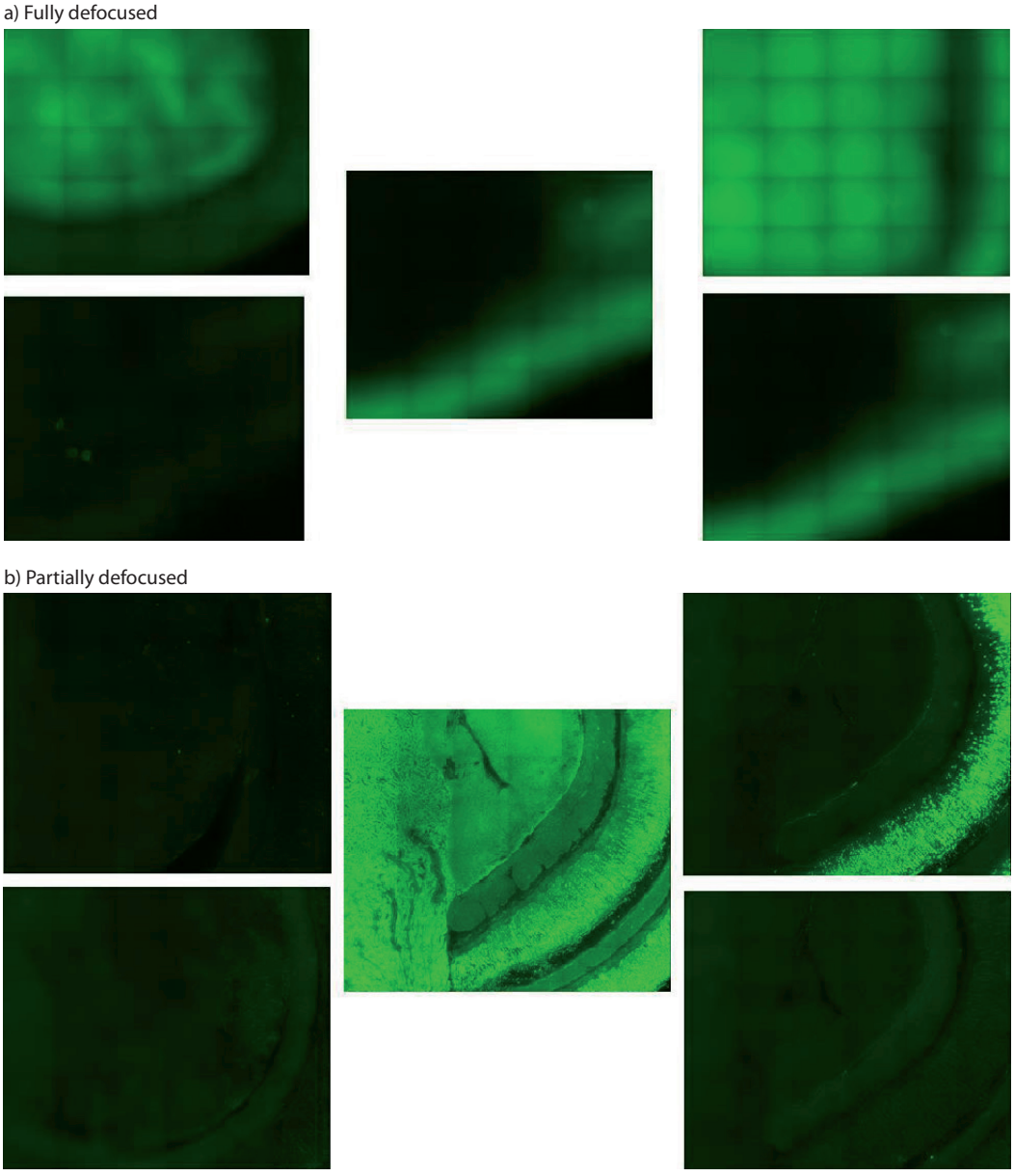

Figure 2. Defocused triple tissue and possible solutions.

Possible problems: meniscus in the solution during the assembly, adjustments of the slide.
Solutions: fine tuning reagent dispensing, calibration or adjusting the slide position.
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therefore not be too large and should be in the right point of the slide, centred and evenly distributed, to avoid its shape becoming too convex. Because the focus is in micra, it is important to ensure the slides are

well fitted and that the lens moves without slack, all of which is achieved through a series of fine adjustments that obviously require the appropriate technical service.

Another possible critical point is image selection. With regard to cells, the most suitable and practical approach is to select a number of images, usually two or three, distributed throughout the well, which is usually sufficient and does not require too much time. Indeed, almost all positive patterns can be viewed with three images. In the case of triple tissue, a small number of images can also be selected in the chosen areas. This is an advantage, as the time it takes depends on the number of images selected. The disadvantage of selecting a small number of images is that the block of tissue may not be in exactly the same position, even in the different wells on a slide, being further up, down or to the right or left, so some of the selected images might not be clear, making it difficult to identify a particular pattern (**Figure 3**). In such cases, a possible solution is trial and error, checking the microscope for images that suggest a positive. Another option is to use mosaics, since the equipment provides the option of selecting grids from 2×2 to 8×8. It should be noted that in such cases, if a small grid is selected and the tissue is displaced, only one part of the tissue may be visible, which might not be the representative tissue. While it is true that a larger grid can always be chosen, this involves a longer reading time, as 54 seconds are required for a 2×2 grid whereas 14 minutes are required in each well for an 8×8 grid. Clearly, the time equipment takes to complete the readings is not that important, but if for some reason a later check under the microscope is required, the tissue will already be 'burnt'. Thus, a balance is required between the two extremes, either by first selecting one part followed by another, or alternatively, if available, using a lens with a lower magnification to produce a larger image

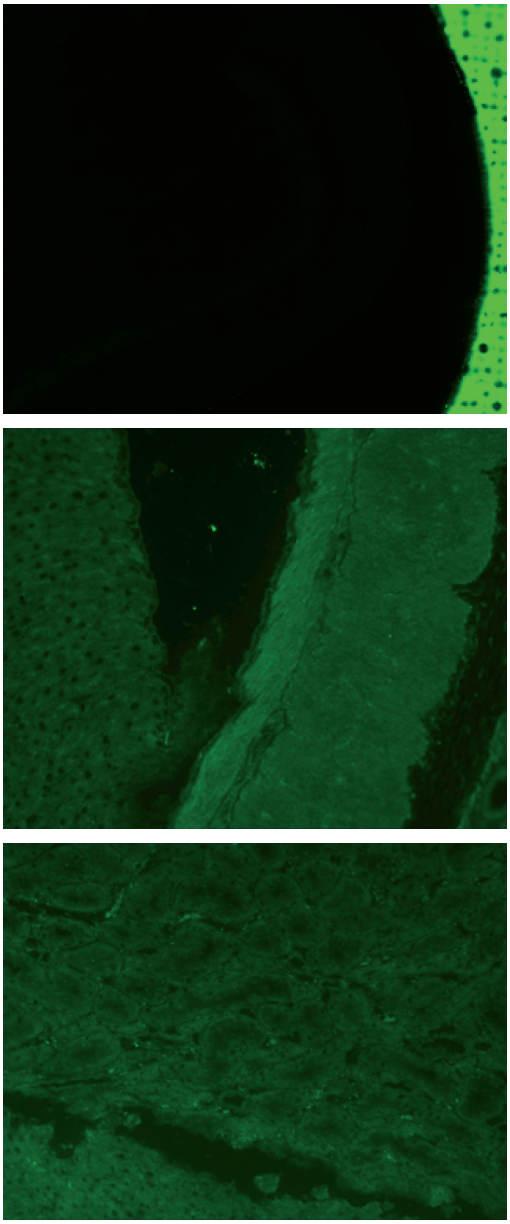

Figure 3. Possible problems and solutions in the selection of images of a triple tissue.

Possible problems: position of the tissue block, insufficient images.

Solutions: trial and error, with review under the microscope.

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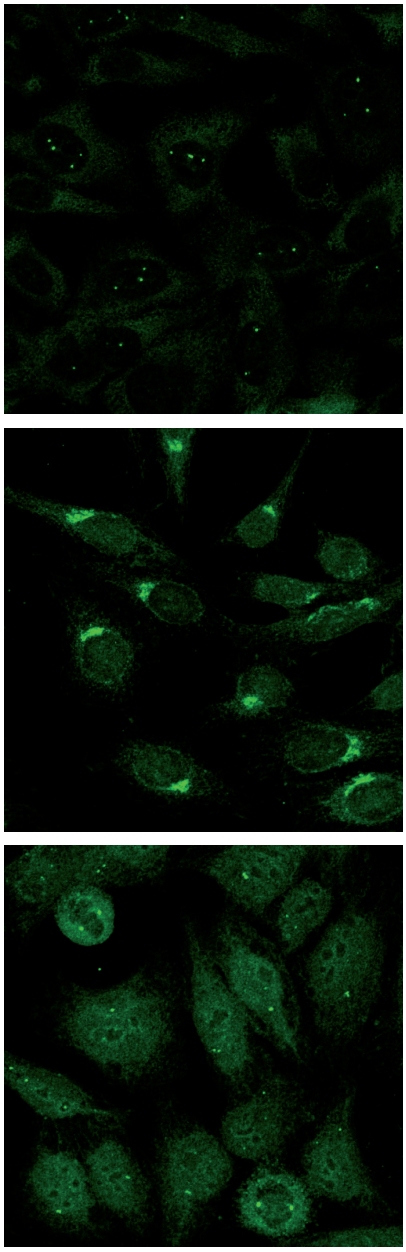

Figure 4. Low signal intensity in HEp-2 cells.

Possible problems: 'discrete' patterns in HEp-2 cells interpreted as negative.
Solution: review 'negative' images.
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of the whole well and then, if necessary, checking the area of most interest. In these cases, using a lens with a lower magnification than the one fitted in the microscope (×20 magnification) could generally solve the problem.

Another possible drawback is signal intensity, especially in HEp-2 cells, in the case of patterns with a very 'discrete' signal, due to autoantibodies directed against antigenic structures that are poorly represented or have a discrete fluorescence signal, as with nuclear dots or centrioles (**Figure 4**). The instrument can interpret such cases as 'negative', although a review of the images is likely to detect something to suspect otherwise, thereby requiring confirmation under a microscope. Thus it is essential to review negative images. A further possibility is to modify the signal threshold at which the apparatus indicates negative and positive; however this is very risky for clinical interpretation, since a very low signal detection level will produce many false positives, whereas too large an increase in the detection level will produce the opposite effect.

Finally, another important point, which does not depend on the user so much as on the supplier, is the quality of the substrate. This should contain no (or a minimal amount of) elements capable of generating artefacts, such as particles, dirt, etc. Furthermore, in the case of HEp-2, cell distribution should be uniform, with a high enough mitosis index to view the different phases of the cell cycle and not miss possible specificities; in the case of tissues, folded or broken tissue should be avoided, requiring thorough quality control to do so.

FINAL COMMENTS

- Automated systems can be considered very useful for routine work. While the results need to be re-

viewed, automation frees up a lot of the workload and allows staff to make better use of their time.

- In the author's opinion, the most useful application of these instruments is screening negatives, which represents a large portion of the work.
- Equipment settings should be suitable and finely tuned, based on experience and results. This requires technical support, fluid dialogue and rapid response from the supplier, which is not always given the importance it deserves, even though it is crucial.
- Before acquiring such equipment, the real needs of the laboratory in terms of activity should be analysed, thereby permitting assessment and selection of the most suitable apparatus.
- It is important to have realistic expectations and not think that an automated computer can read and solve samples in the same way as an experienced professional, because good practice still requires use of a microscope.
- In the future, the trend will be to expand and improve existing methods for detecting serological markers, but this could well disappear in the long term, due to the rise of personalised medicine, with increasing importance given to different profiles types: genomic, metabolic, microbiotic and possibly even what might be termed 'antibodynomic' or 'autoantibodynomic'.
- One cannot rule out that in the future, currently undeveloped algorithms could give rise to fully automated interpretation and diagnoses; however today's automated immunofluorescence equipment represents an essential contribution to daily lab practice.

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Diabetes mellitus type 1. Main markers, preclinical markers and risk assessment markers

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DIAGNOSTIC CRITERIA

Diabetes mellitus (DM) is defined as a disruption to the metabolism of carbohydrates, characterised by persistent hyperglycaemia, caused by abnormal secretion or synthesis of insulin. The diagnostic criteria are[1]:

- In asymptomatic patients, positives in the following tests on at least two separate occasions: 1) fasting blood sugar ≥ 126 g/dL, 2) blood sugar two hours after an oral overload of 75 g glucose ≥ 200 mg/dL or 3) a glycated hemoglobin (HbA_{1c}) level ≥ 6.5%.
- In symptomatic patients, diagnosis is based on a single random glycaemia ≥ 200 mg/dL along with the cardinal symptoms (polyuria, polydipsia, and polyphagia and weight loss).

CLASSIFICATION

The American Diabetes Association (ADA) defines four major groups of diabetes[1]:

Type 1 Diabetes (DM1). This is due to a destruction of pancreatic beta cells, leading to an absolute insulin deficiency which, in some conditions, can also lead to ketoacidosis.

Type 2 diabetes (DM2). This usually involves an increase in insulin resistance, although in its final stages it may also be associated with a partial insulin deficiency.

Gestational diabetes. This type includes all diabetes diagnosed in the second or third trimester of pregnancy that does not clearly correspond to DM1 or DM2.

Other types of diabetes. This group encompasses different types of diabetes with different causes, such as genetic diabetes (e.g., neonatal diabetes, mitochondrial diabetes or monogenic diabetes, formerly known as maturity onset diabetes of the young or MODY), diabetes caused by exocrine pancreas diseases (e.g. cystic fibrosis, chronic pancreatitis or diseases such as iron overload) and, finally, toxin or drug- associated diabetes (e.g. steroid diabetes and diabetes associated with protease inhibitors or immunosuppressive drugs).

The most prevalent types are DM1 and DM2, which cover between 95% and almost 100% of cases.

DIABETES MELLITUS TYPE 1

This represents 5-10% of all cases of diabetes. Its incidence varies greatly with geographical region, with over 350-fold variability, from 0.1 cases per 100,000 inhabitants in countries such as China and Venezuela, to 36.5 cases per 100,000 inhabitants in Sardinia and Finland[2]. The rapid rise in the incidence of the disease in recent times is probably related to higher survival rates and lower patient morbidity.

The development of DM1 (**Figure 1**) is influenced by genetic factors, while environmental factors act as triggers. The genetic combination with the greatest risk

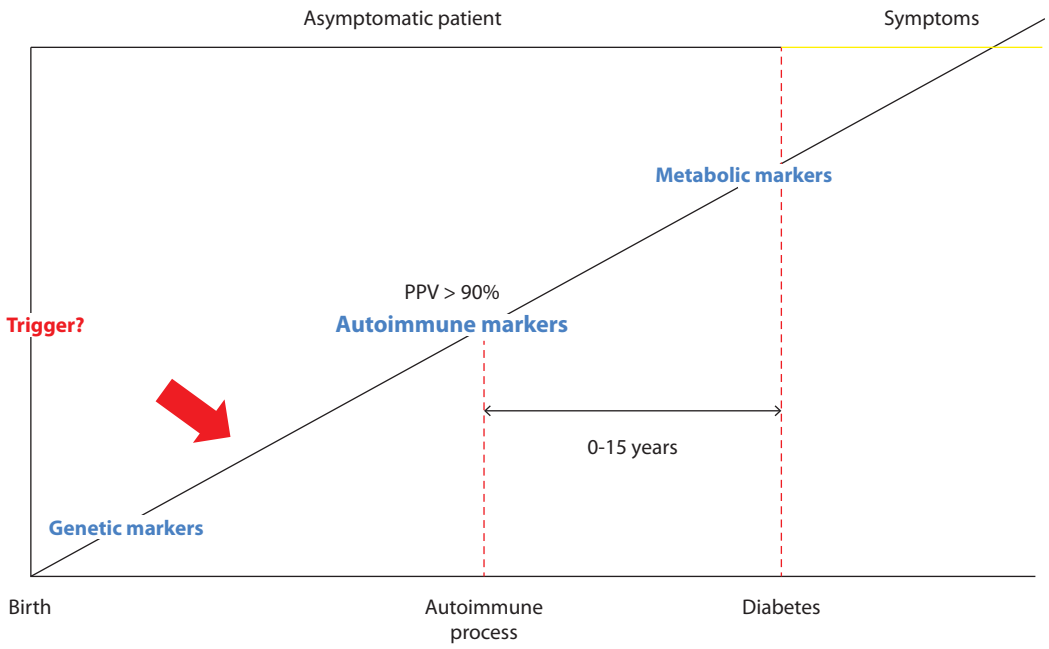

Figure 1. Natural history of diabetes type 1.

PPV: positive predictive value.

of DM1 is HLA-DR3-DR4, which represents a risk of developing antibodies to pancreatic islets of over 80%, thus eventually producing diabetes.

Asymptomatic stage. The patient has no symptoms but antibodies are already detectable in serum. Presence of these antibodies has a very high predictive value, as it means the chances of developing diabetes are over 90%.

Symptomatic stage. The ongoing autoimmune process leads to progressive destruction of the mass of beta cells and the onset of hyperglycaemia and the classic symptoms of diabetes, such as polydipsia, polyuria and weight loss.

Previously, DM1 almost always debuted with diabetic ketoacidosis (DKA) decompensation, but currently only one-third of patients present with this metabolic complication at onset.

Type 1B or idiopathic diabetes

Currently, 15% of patients with DM1 are considered to have this subtype of diabetes, the cause of which is still unknown. Family aggregation has been observed and some studies associate it with the same HLA antigens that pose a risk of DM1. Patients have negative autoimmunity, which raises certain doubts about whether it is another type of non-autoimmune diabetes or whether it is the same autoimmune diabetes in which the autoimmunity could not be detected. Notable, among its clinical characteristics are the higher frequency in people of African or Asian origin and a course that tends to ketoacidosis, like classic DM1,

although insulin deficiency varies between episodes of ketoacidosis, so that some patients need insulin therapy while others need little or no insulin at all.

LADA Type Diabetes

Three clinical criteria are required for the diagnosis of latent autoimmune diabetes in adults (LADA): 1) presentation after 30 years, 2) presence of positive antibodies and 3) slow progression of the insulin deficiency. There should be at least six months from the onset of symptoms to insulin being required for this type of diabetes to be diagnosed.

It is estimated that 10% of patients diagnosed with DM2 show signs of autoimmunity. Indeed, the UK Prospective Diabetes Study (UKPDS) found that 11.6% of 4,545 patients clinically diagnosed with DM2 tested positive to antibodies[3] and that the chances of positive autoimmunity were greater when patients were younger, so that 25% of cases were patients under 35 years of age. Clinically, patients with LADA are generally younger and thinner than patients with DM2, with higher baseline glycaemia and higher HbA_{1c} levels, as well as lower insulin resistance.

The diagnosis and identification of LADA requires testing for antibodies in all patients where autoimmune causes are suspected. A positive antibody test helps identify patients who are likely to have a worse response to oral antidiabetics, a higher risk of ketoacidosis, and an earlier need for insulin. There is no established therapeutic strategy in patients with LADA, so the treatment should always be individualised, taking into account the high probability of requiring insulin early. Insulin requirement is not determined solely by the antibody count, but also by glycaemia, HbA_{1c} levels and presence of cardinal clinical symptoms.

DIAGNOSTIC CLUES

Classifying a patient's type of diabetes requires consideration of a number of parameters that serve as diagnostic clues (**Table 1**).

Clinical history is of paramount importance, because it permits the analysis of the patient's background, suggesting a given type of diabetes (such as the case of a drinker who may have suffered chronic pancre-

Table 1. Diagnostic factors for determining a patient's type of diabetes

Clinical history
• Personal/family history
• Age
• Cardinal symptoms: polyuria, polydipsia and weight loss
• Medicinal products/toxins
• Response to treatment
Physical examination
• Body mass index
• Waist circumference
• Acanthosis nigricans
Tests
• Hyperglycaemia development time
• Triglycerides/lipoproteins
• Pancreatic reserve
– Diabetic ketoacidosis
– C-peptide at baseline and after glucagon test
• Faecal elastase/fat in faeces
• Ferritin/ceruloplasmin
• Autoimmunity
Imaging tests
Genetic test

atitis or a patient with a personal or family history of other diseases such as iron overload or deafness) associated with mitochondrial diabetes. Age is also a key factor, although it is not considered as pathognomonic. The cardinal symptoms show whether or not the patient has insulin reserve. It is also very important to know whether the patient has taken drugs with possible toxic effects. Furthermore, the response to treatment after debut is sometimes highly indicative, because there is usually underlying autoimmunity in patients for whom antidiabetic therapies fail.

In the **physical examination**, the body mass index (BMI) and waist circumference are basic data. Furthermore, if acanthosis nigricans is detected, this is revealing as it is a marker for insulin resistance and tends to tip the scales towards DM2.

With regard to **tests**, it is important to establish the time it takes for hyperglycaemia to develop. Sometimes checking historical blood sugar levels reveals that although the patient claims to have been diabetic for only a few months, values have in fact been in the diabetic range for years, which would suggest DM2. The triglyceride/high density lipoprotein (HDL) ratio (an important marker for insulin resistance when > 3) may also be useful, in this case pointing to DM2. Pancreatic reserve data are important, as patients with significant cardinal symptoms or who have had ketoacidosis usually do not have pancreatic reserve. Since insulin production is related to C-peptide blood levels, these data can provided by the lab by requesting a glucagon stimulation test to measure baseline C-peptide and at six minutes after administration of glucagon: the lower the C-peptide values, the lower the pancreatic reserve. In patients who have had pancreatitis or who are drinkers, a measurement of faecal elastase or fat in faeces may be required. Ferritin, transferrin saturation and ceruloplasmin can be ordered if chronic pancreatitis is suspected or if there is a history of hereditary haemochromatosis or Wilson's disease. Logically, antibodies will be tested when there is reasonable suspicion of autoimmunity, as discussed in depth below.

Imaging tests are sometimes useful, as when major involvement of the pancreas is suspected. It should be noted that there are cases, even if uncommon, where diabetes unmasks pancreatic cancer.

Finally, **genetic testing** should be ordered if mitochondrial, MODY or neonatal diabetes is suspected.

AUTOIMMUNITY

Types and prevalence of antibodies

Among the diabetes-related antibodies, islet cell cytoplasmic antibodies (ICA) have been used the longest and require little discussion as they are well known to specialists treating patients with diabetes. Attention is better focussed on certain specific antibodies, all related to the beta cell secretion apparatus, indicating their prevalence at the onset of diabetes (**Table 2**).

First of all, there are the anti-insulin and anti-proinsulin antibodies, known as IAA, for which measurement is recommended in the first two weeks after starting insulin therapy, as there may be cross-reactivity. These are more frequently positive if the patient is under 12 years of age, but rarely so in adult patients. Furthermore, an inverse association with the age at onset of the disease has been observed.

The most frequently used antibodies in clinical practice are the glutamic acid decarboxylase antibodies, known as GADA, whose prevalence is 70-80% at diagnosis, and those that react against insulinoma-associated proteins with tyrosine kinase activity, called

Table 2. Main antibodies in autoimmune diabetes

Name	Antigen	Characteristics
IAA	Insulin and proinsulin	• First two weeks • Very high on debut in patients < 12 years and frequently negative in patients > 12 years • Inversely related to age at debut
GADA	Glutamic acid decarboxylase (GAD)-65	• 70-80% at diagnosis
IA-2A/ICA512 IA-2B	Insulinoma-associated protein 2	• Tyrosine phosphatase activity • 60-70% at diagnosis • Later disappearance than GADA or IAA
ZnT8A	Zinc channel	• 60-80% at diagnosis

IA-2A or ICA512 and IA-2B, with a prevalence at diagnosis of 60-70% and which usually disappear from serum later than the GADA or IAA. Other antibodies began to be used after the above and are therefore considered 'new': known as ZnT8A, they act against the zinc channel and are positive at diagnosis in 60-80% of cases.

The Diabetes Antibody Standardization Program (DASP)[4] was created to standardise antibody levels and assess the implementation of test methods for their determination. This group has shown that the reproducibility of diabetes-related antibodies is very high and argues that their detection is the best established and validated predictive marker. DASP found that the most commonly used antibodies in participating centres were GADA and IA-2A, which have much higher sensitivity and specificity than IAA, which are generally quite difficult to detect at low concentrations. DASP also states that the enzyme immunoassay kits (ELISAs) for GADA and IA-2A are practically equivalent to radioimmunoassay (RIA), whereas the ELISA for IAA is less recommendable than the RIA.

It is important to note that the presence or absence of antibodies is a continuous variable; in other words, although the terms 'positive' or 'negative' for antibodies are used, this is a simplification. In fact, in practice a cut-off point at the 99th percentile of the population is used, and it should be borne in mind that very high or very low titres are also relevant.

The prevalence of antibodies that reflect autoimmunity at diagnosis of DM1 can be considered as very high, but not total. For instance, a study on 256 children with DM1 to investigate the prevalence of autoimmunity at diagnosis[5] measured ICA, GADA, IA-2A and IAA antibodies, finding that more than 90% of the patients were positive for two or more antibodies while only 2-4% of patients were negative. In adults, the prevalence of patients testing negative for antibodies is generally much higher.

ZnT8A, considered new antibodies and which are tested for less frequently than classic antibodies, have characteristics that also make them useful. They appear later than GADA or IA-2A and also tend to become negative early, although this is not always the case. One

study found that in a cohort of children under 17, only 2% were positive for ZnT8A, while 13% tested positive in another cohort with patients between 15 and 34[6]. Another trial concluded that the inclusion of ZnT8A in the DM1 autoimmunity study reduced the percentage of patients testing negative for antibodies from 5.8% to 1.8%[7]. This justifies routine testing for ZnT8A antibodies and their consideration as important markers in these patients.

Diagnostic value of antibodies

It has already been pointed out that detecting antibodies is useful for confirming a diagnosis of DM1, but obviously testing for this purpose is not indicated in all patients, only those in which this type of DM is suspected. In general, antibodies studies are recommended in the following cases: 1) all patients where the age at onset of diabetes is < 50 years; 2) patients presenting with acute symptoms (cardinal or ketoacidosis); 3) patients with a BMI < 25 kg/m^2; 4) patients with a personal or family history of autoimmune diseases. It is estimated that in subjects who meet two of these four criteria sensitivity is 90% and specificity is 71% for predicting GADA positivity.

Prognostic value of antibodies

Positive testing for these antibodies in people who do not have diabetes is also useful for predicting the future development of the disease, as shown in a number of studies. In one interesting study, GADA and IA-2A antibodies were screened in 755 siblings of patients with DM1 and almost 3,500 children in the general population, with 15 years' follow-up[8]. The screening found more positives for antibodies among the relatives of diabetic patients than among the general population: 7% versus 1% for GADA, 5.4% versus 0.4% for IA-2A and 3.8% versus 0.2% for both. More importantly, during the follow-up, the risk of diabetes was very similar in subjects who were positive for GADA and IA-2A, in both diabetic relatives and the general population. Indeed, after 15 years, 80% of such subjects in both groups had developed diabetes; hence it may be deduced that, had the follow-up continued for 20 or 30 years, the disease would have developed in practically all the subjects. Thus, it can be inferred that the presence of antibodies has a predictive value of practically 100%.

Antibodies also have a predictive value in adults with LADA, as it has been proven that the more positive antibodies there are, the more likely it is that beta cell damage will occur. A study in which C-peptide levels corresponding to the insulin reserve were tested as a parameter to determine beta-cell deterioration found that the risk of deterioration of pancreatic function was much lower when there was only IA-2A positivity than when positivity was also detected for GADA or ICA[9]. Thus, in these cases, IA-2A appears to be generally less predictive of impaired beta-cell function.

Antibody positivity has also been shown to relate to development of the disease in children. In a study of children under 15 years of age with newly diagnosed diabetes, the four classical antibodies (GADA, IA-2A, IAA and ICA) were measured, and positives for a greater number of antibodies were associated over time with lower beta cell function (determined by C-peptide values) and, consequently, a greater need for insulin[10]. Thus, it can be concluded that more positives for antibodies predict a much faster development of diabetes.

ANTIBODY SCREENING: INDICATIONS AND OBJECTIVES

All of the above raises questions about which subjects should be screened for diabetes-related antibodies

and, in particular, whether such screening should be indicated only for relatives of patients with DM1 or extended to the general population. There is no doubt that such screening has major implications, because the positive predictive value of the antibodies is > 90%; thus virtually everyone in whom their presence is detected will develop the disease at some point in the future. What cannot be determined is when the disease will develop, although it is something that is likely to occur over the subsequent 15 to 20 years, or earlier when there are more positive antibodies, higher titres, greater persistence and also positives for GADA antibodies. It is known that about 90% of patients with DM1 do not have a family history of the disease, so limiting screening to family members would mean missing the chance of preventing the likely occurrence of diabetes in a significant percentage of the general population. However, there are those who question whether screening the general population is really worthwhile, given that there is currently no preventative treatment. For many people, screening would cause frustration by knowing they are bound to develop the disease at some point but can do nothing to avoid or even delay it.

In relation to prevention, there have been two clinical trials on preventative treatment for DM1. One of them[11] was based on the use of nicotinamide or vitamin B3, a drug that had been shown to delay the onset of autoimmune diabetes in mice. A total of 552 relatives of antibody-positive diabetic patients were randomised to this agent or placebo, finding no difference in the risk of developing the disease between the two groups. In the other study[12], a total of 339 antibody-positive patients were either randomised for observation or receiving a slow insulin dose of 0.25 U/kg/day for an average of 3.7 years. Again, no difference in the incidence of diabetes was detected between both groups.

At present, no preventative treatment is available. Yet despite this, it could be argued that warning about the chances of developing disease could favour early diagnosis. Illustrative in this context is a trial comparing the development among 21 children with DM1 diagnosed by screening in the DAISY study and another 21 diagnosed in the general population by the onset of the disease[13]. It was found that, at the time of diagnosis, patients in the screening group had lower HbA_{1c} levels, better C-peptide values and required lower doses of insulin. However, after six months there was no difference in HbA_{1c} and after one year there were practically no differences in any of the endpoints. Thus, knowledge of future development of diabetes through antibody screening was not found to have any use worthy of consideration.

In summary, the detection of antibodies for predictive or prognostic purposes can only be considered useful in the context of research, whereas in clinical practice it is used in patients with diabetes to characterise the type of disease. In other words, antibody detection is useful, but not essential.

CONCLUSIONS

- Antibodies associated with diabetes have a predictive, diagnostic and prognostic value.
- Antibodies are useful, but not essential.
- The cost-effectiveness of measuring antibodies has not yet been established.
- There is still no preventative treatment for diabetes.
- Measuring antibodies does not change how diabetes is treated.
- Studying antibodies can help identify other associated pathologies.

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