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Hook Effect in Indirect Immunofluorescence Assay: Always on Alert

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KEYWORDS

Prozone effect
Indirect immune-
fluorescence assay
anti-mitochondrial
antibodies
anti-liver cytosol
antibodies

Abstract

Introduction: Indirect immunofluorescence assay (IIFA) represents the most effective method for screening autoantibodies, it has been known to hold the standard over other techniques for its low report of false negatives, although observed rarely in IIFA a marked prozone effect occurred leading to erroneous results. by reporting these observations, we aim to put this phenomenon in the spotlight mainly in AMA2 and anti-LC1 antibodies detection.

Methods: This retrospective study includes four patient's laboratory findings that indicate the prevalence of false-negative anti-mitochondrial (AMA) and anti-liver cytosol (anti-LC1) antibodies resulting from excess antibodies in serum.

Results: Absence of immunofluorescence at certain dilutions, its appearance at others highly suggest the presence of prozone phenomenon in these cases

Conclusion: All laboratories providing IIF assays should be alert to the continuing possibility of hook effect, excellent communication between laboratory and clinical staff is key to minimize the risk due to analytical errors.

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Introduction:

Indirect immunofluorescence assay (IIFA) represents the most effective method for screening autoantibodies since it allows the detection of most antibodies that are useful for diagnosis, prognosis and monitoring of organ-specific and non-specific autoimmune diseases, The observed fluorescence images provides initial information on the identity of the target, Other methods will allow the precise identification of the molecular nature of the

antigen against which the detected antibody is directed. [1]

The technique has been known to hold the standard over other methods for its low report of false negatives. A prominent explanation of false negative reports has been the occurrence of the hook effect, also known as the prozone effect. a phenomenon where a decreased or absent fluorescent signal are observed, even when the antigen is present because of the interference of high antibody concentration with the labeled antibody's binding (secondary antibody) to the primary antibody, which is essential for visualizing the target. [2]

This retrospective study includes four patient's laboratory findings that indicate the prevalence of false-negative anti-mitochondrial (AMA) and anti-liver cytosol (anti-LC1) antibodies resulting from excess antibodies in serum.

Materials and methods

The samples were delivered to the immunology laboratory of Constantine's university hospital center from three separate divisions: Hepato-gastro-enterology, Internal medicine and pediatrics for testing. The brief medical history with the patient's personal information were received along with the Four samples and were specifically ordered to test for autoimmune liver diseases panel.

All case samples were in the form of blood sera; all samples were stored at 4°Celsius until use.

The samples were tested with the following techniques: indirect immunofluorescence assay (IIFA) and Immunoblot using the kits provided by Euroimmun, Germany. The assays were performed following the manufacturer's instructions.

The semi-quantitative detection of human antibodies of immunoglobulin class IgG against mitochondria (AMA), smooth muscles (ASMA), liver kidney microsomal type 1 (LKM 1) and liver cytosol antigen type 1 (LC-1) using slides containing a combination of three rat tissue sections: Liver, Kidney and stomach while slides coated with HEP-2 cells acted as substrate for the determination of those against cell nuclei.

For preparation, each sample was incubated with its respective BIOCHIPs slides for 30 minutes at room temperature before applying its conjugate, if the reaction is positive, the bound antibodies are stained with FITC-labelled anti-human antibodies and made visible with the fluorescence microscope. Positive and negative controls were used on each slide.

For monospecific antibody detection, The EUROLINE test kit provided a qualitative determination of human autoantibodies of immunoglobulin class IgG to 9 different antigens: **AMA-M2**(pyruvate dehydrogenase complex),**M2-3E** (BPO,fusion protein of the E2 subunits of the alpha-2-oxoacid dehydrogenases of the inner mitochondrial membrane),**sp100** (nuclear granula protein,), **PML** (promyelocytic leukaemia protein,),**gp210**(integral protein of nuclear membrane,),**LKM-1**(liver-kidney microsomes,), **LC-1**(cytosolic liver antigen type 1),**SLA/LP** (soluble liver antigen /liver-pancreas antigen) and **Ro-52**.

The test kit contains immunoblot strips coated with parallel lines of purified antigens, which are incubated with diluted patient sample (1/101 dilution), in case of positive samples, the specific IgG antibodies will bind to the corresponding antigenic site, the bound is detected using an

enzyme-labelled anti-human IgG catalyzing a color reaction.

It is important to mention that in our laboratory we follow the recommendations issued in 2004 by the Committee for Autoimmune Serology of the International Autoimmune Hepatitis Group (IAIHG) regarding serum dilutions on rat tissue sections substrate.

IAIHG states that the conventional starting serum dilution is 1/10. In adults, the positivity cut-off is 1/40, whereas in children and adolescents titers from 1/10 for anti-LKM1 and for anti-LC1 are considered positive, since autoantibodies are rare in healthy subjects of these age groups [3]. Whereas initial serum dilution on HEP-2 cells is 1/80. [4]

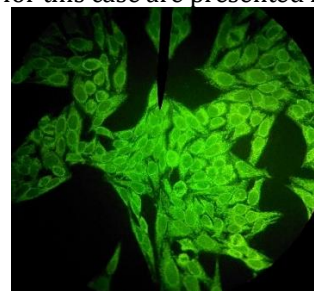
Nevertheless, the kit manufacturer instructs an initial dilution of 1/100 on both substrates.

In these four cases both dilutions were tested, those recommended by IAIHG and those instructed by the manufacturer.

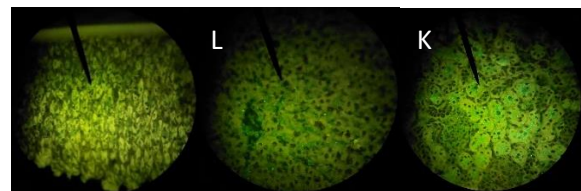
Résultats

Case 1:

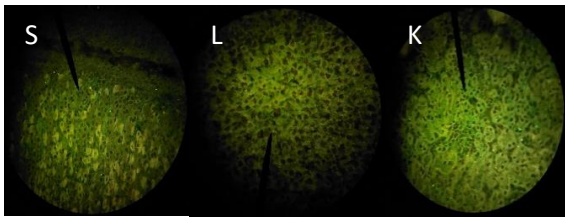
A 47 years old female with a confirmed Primary biliary cirrhosis (PBC) since 2023, treated with AUDC(acideursodésoxycholique,cholestyramine,a vlocardyl), she presents an important biological liver cytolysis (Alanine transaminase (ALT) and Aspartate transaminase (AST) levels were 5 times the standard upper limit) and cholestasis (alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (GGT) levels were 5 and 3 times the standard upper limit, respectively). along with xerostomia and xerophthalmia. sent from hepato-gastro-enterology division for a suspicion of an association of Sjögren's disease and autoimmune hepatitis. the staining patterns obtained on both substrates for this case are presented in Fig.1.



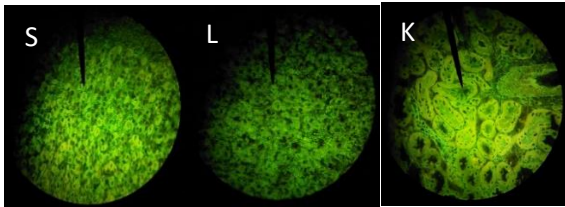
(A): 1/80 dilution 40X



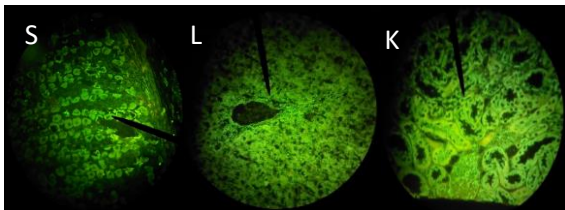
(B): 1/40 dilution 40X



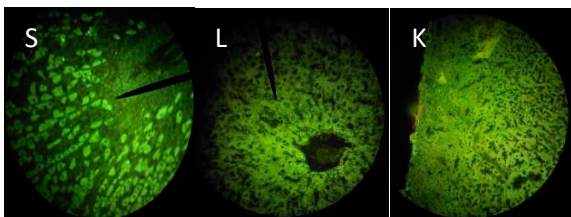
(C) :1/100 dilution 40X



(D) : 1/160 dilution 40X



(E):1/320 dilution 40X



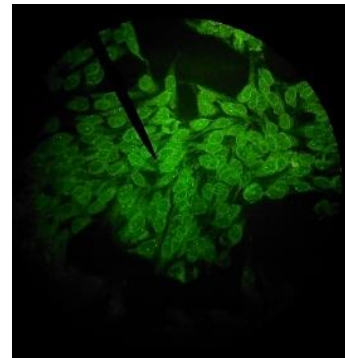
(F) : 1/640 dilution 40X

Fig. 1. Indirect Immunofluorescence staining of anti-mitochondria antibodies on HEp-2 cells (A: distinctive cytoplasmic reticular or granular pattern) and on rat tissue sections: stomach (S), Liver (L) and Kidney (K) showing: With initial dilutions, there is no staining: B (1/40), C (1/100) and with subsequent dilutions staining becomes evident: D (1/160), E (1/320), F (1/640) (Hook effect in case one).

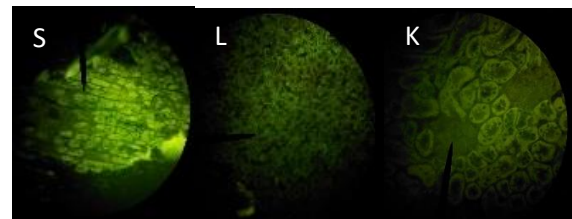
Case 2

A 55 years old female, with history of PBC and rheumatoid arthritis (RA) 4 years ago, high blood pressure 6 years ago and hypercholesterolemia, she began reporting symptoms for 2 months followed the previous diagnosis for which patient was receiving treatment (AUDC 1200 mg /j, CTC 40 mg/j, Imurel 100 mg/j, Exforge , crestatine, Aspegic), she complained of nausea ,pruritus ,anorexia and general malaise.

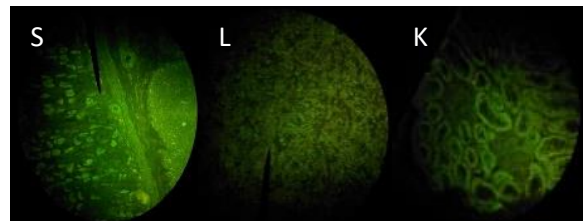
On consideration of an overlap syndrome, serum sample was sent from hepato-gastro-enterology division to our laboratory for testing. the staining patterns obtained on both substrates for this case are presented in **Fig.2**.



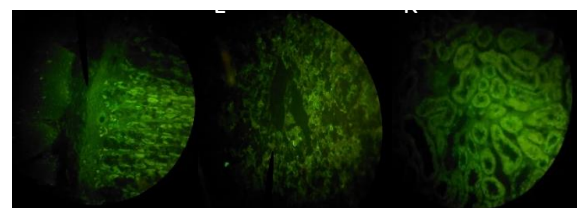
A : 1/80 dilution 40X



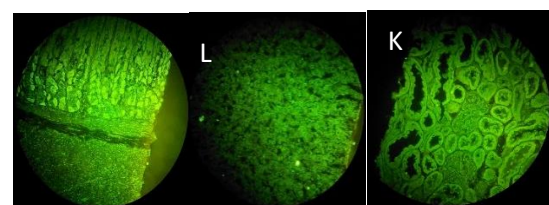
B :1/40 dilution 40X



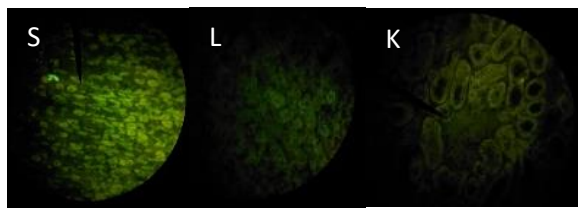
C :1/100 dilution 40X



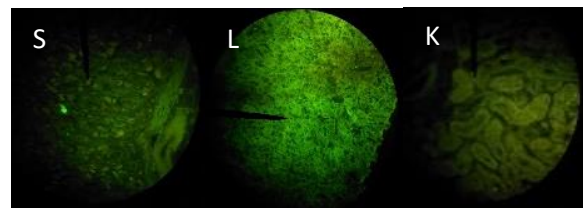
D : 1/160 dilution 40X



E: 1/320 dilution 40X

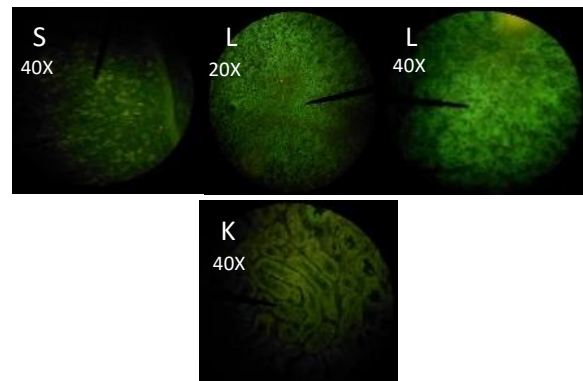


F: 1/640 dilution 40X



D : 1/100 dilution 40X

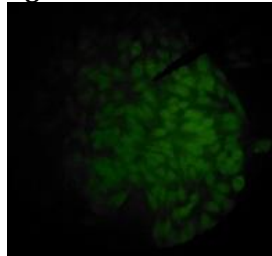
Figure. 2. Indirect Immunofluorescence staining of anti-mitochondria antibodies on HEp-2 cells (A: distinctive cytoplasmic reticular or granular pattern) and on rat tissue sections: stomach (S), Liver (L) and Kidney (K) showing: With initial dilutions, there is no staining: B (1/40), C (1/100) and with subsequent dilutions staining becomes evident: D (1/160) and E (1/320), and then disappears at F (1/640) (Prozone phenomenon in case 2).



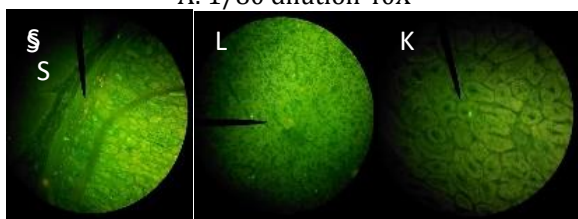
E: 1/160 dilution

Case 3

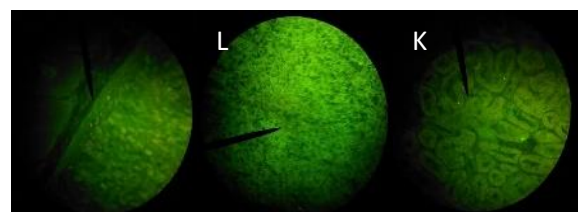
A three years old female child presented with a 3 months history of progressive jaundice and pruritus. Laboratory examinations revealed elevated levels of aspartate aminotransferase and alanine aminotransferase, sent from pediatrics division for further tests due to a suspicion of autoimmune hepatitis. the staining patterns obtained on both substrates for this case are presented in Fig.3.



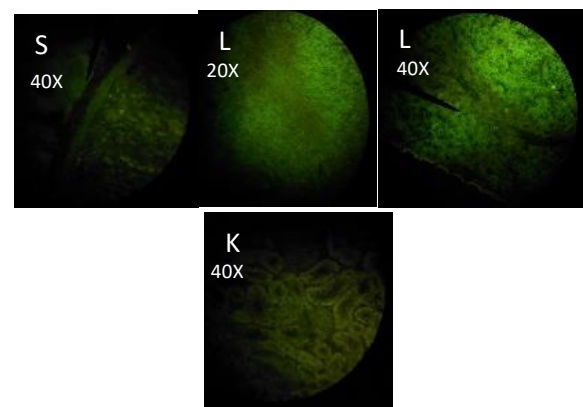
A: 1/80 dilution 40X



B: 1/10 dilution 40X



C: 1/40 dilution 40X



F: 1/320 dilution

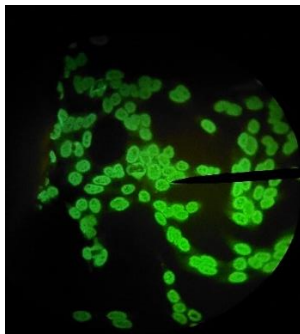


G: 1/640 dilution 40X

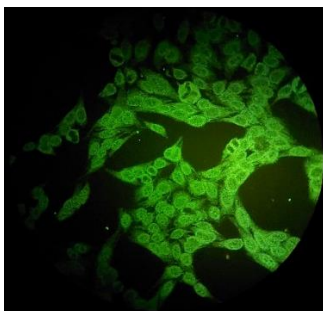
Figure. 3 Direct Immunofluorescence staining of anti-LC1 antibodies on rat tissue sections: stomach (S), Liver (L) and Kidney (K) showing: With initial dilutions, there is no LC-1 specific staining: B (1/10), a suspicious one on C (1/40) and with subsequent dilutions staining becomes evident: D (1/100), E (1/160), F (1/320) and G (1/640), the test on HEp-2 cells was negative (A: 1/80) (Prozone phenomenon in case 3).

Case 4

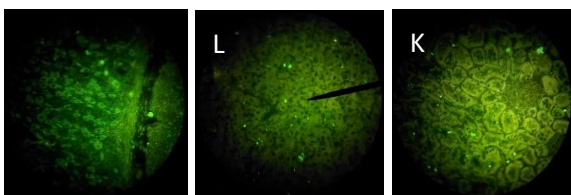
The patient is a 52 years old female sent from internal medicine division known to have Systemic lupus erythematosus, diagnosed approximately 10 years ago. She presented with complaints of fatigue, dry skin, and pruritus that started almost three years prior, she had a liver biopsy due to chronic cholestasis, the alkaline phosphatase and gamma-glutamyl transpeptidase levels were 3 and 7 times the standard upper limit, respectively. Autoantibody testing results are showed in **Fig.4**.



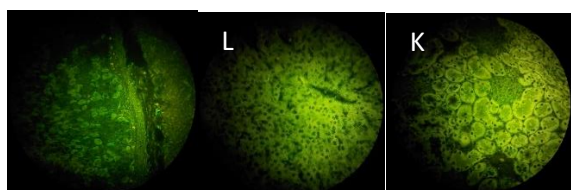
A : 1/80 dilution (40X)



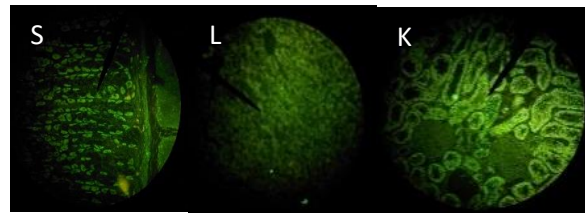
B : 1/320 dilution (40X)



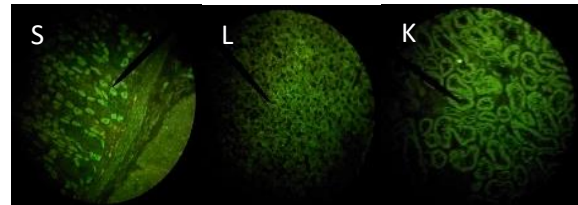
C : 1/40 dilution (40X)



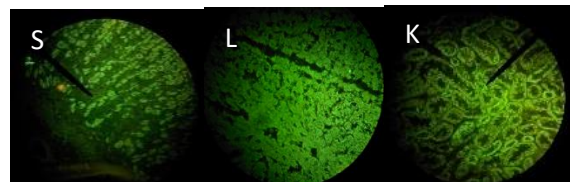
D : 1/100 dilution (40X)



E: 1/160 dilution (40X)



F: 1/320 dilution (40X)



G: 1/640 dilution (40X)

Figure. 4. Indirect Immunofluorescence staining on HEp-2 cells (A: absence of cytoplasmic granular pattern, B: appearance of a distinctive cytoplasmic granular pattern), Indirect Immunofluorescence staining of AMA on rat tissue sections: stomach (S), Liver (L) and Kidney (K) showing: With initial dilutions, a suspicious staining: C (1/40), D (1/100) and with subsequent dilutions staining becomes evident: E (1/160), F (1/320), and G (1/640), (Prozone phenomenon on both substrates in case 4).



Overall, the initial investigation of case 1 and case 2 sera with the standard 1:100 dilution as per the diagnostic kit manufacturer's instructions resulted as negative for anti-mitochondria antibodies on rat tissue sections (stomach, Liver and Kidney) and positive on HEp-2 cells at a dilution rate of 1/80 showing a diffuse granular filamentous staining extending throughout the cytoplasm.

These two patients are already known for PBC so we were strongly expecting a positive AMA2 antibody, we decided to perform the immunoblot assay that tests for antibodies against 9 antigens related to auto immune liver diseases. The immunoblot test returned positive for both cases, the first one was positive for anti- AMA-M2+++, M2-3 E+++, gp210+++ and Ro-52+++, the second one tested positive also for AMA-M2+++, M2-3

E+++ , gp210+++ , Ro52+and also Sp100+++ PML+++ at a serum dilution of 1:101.

Indirect immunofluorescence test was repeated serially with doubling dilution, increasing from 1/40 to 1/640 dilution on liver, kidney, and stomach tissue sections (Fig.1 and Fig.2) and from 1/80 to 1/1000 dilution on HEp-2 cells.

The first strong fluorescence was observed at a dilution of 1:160 for both cases with staining pattern indicating AMA antibodies. (cytoplasmic granular fluorescence in liver hepatocytes, distal and poorly proximal tubules of the kidney and stomach gastric parietal cells).

In addition of the characteristic cytoplasmic pattern of AMA antibodies on HEp-2, punctate staining of the nuclear envelope in interphase cells (with accentuation of fluorescence at the points where adjacent cells touch each other, no staining of the metaphase and anaphase chromatin plates) was observed in both cases suggesting the presence of anti-gp210 antibodies, countable discrete nuclear speckles (6 to 20 nuclear dots/cell) were also detected in case 2 which usually indicates the presence of anti-sp100 antibodies.

Case 3 was a delicate one due to the age of the patient and the strong suspicion of HAI, taking in consideration her critical state an immunoblot was performed immediately which came back highly positive (+++) for anti-LC1 antibodies.

the starting dilution recommended for children on rat tissues :1/10 was negative, but, multiple serum dilutions were performed on rat tissues increasing from 1/40 to 1/640 dilution, an anti LC1 antibody pattern with a brightly staining of the hepatocyte cytoplasm, sparing the centrilobular zone appeared clearly at a serum dilution of 1/100 and sustained until 1/640 dilution.

Case 4 followed a similar procedure. this time no cytoplasmic staining was observed on the standard screening dilution on HEp-2 cells (dilution 1/80) and a suspicious pattern of AMA antibodies on rat tissues was detected. Again in this case subject was already diagnosed for PBC, sample was retested for AMA2 antibodies based on suspicion of a false negative result. The serum was tittered serially using the doubling dilution method starting at 1/40 to 1/640 dilution on rat tissues and from 1/80 to 1/1000 dilution on HEp-2 cells.

A remarkable staining with characteristic recognizable patterns of AMA antibody specificity on both substrates was detected, starting from 1/320 dilution on HEp-2 cells and from 1/160 on rat tissues. The presence of AMA2 antibodies was confirmed by an immunoblot that returned highly positive (+++) for AMA-M2 and M2-3E.

The absence of immunofluorescence at certain dilutions, its appearance at others highly suggest the presence of prozone phenomenon in these cases which is usually believed to be seen less in the indirect immunofluorescence technique.

Discussion

The suspicion of a prozone effect was embarked upon due to the prior knowledge from laboratory investigators of patient's medical history in case 1,2 and 4 , and the insistence of an urgent testing from clinicians in case 3 , The lack of staining when using the standard dilution (both, those recommended by IAIHG and those as prescribed by the manufacturer) is accounted by the presence of excess antibodies, a falsely negative result is then seen with the starting dilution.

The occurrence of false negatives was observed in 1/40 and 1/100 dilutions in case 1 and 2 for AMA antibodies, in 1/10 and 1/40 dilutions in case 3 for anti-LC-1 antibodies.

Case 4 showed a false negative AMA in 1/80 dilution on HEp-2 cells and a suspicious pattern of AMA in 1/40 and 1/100 dilutions on rat tissue sections: Stomach, Liver and Kidney.

On high-titrated sera (1/160,1/320 and 1/640 dilutions on rat tissue sections) and 1/320 dilution (on HEp-2 cells), it was evident that the conjugate binding fluorescence confirms the concentration of antibodies bound to target antigens. The immunoblot assay procedure was ideal for confirming the antibody specificity, and the prozone effect. However, it cannot replace IIFA as a primary evaluation [5].

Prozone phenomena are seen in many secondary manifestations of antigen-antibody interactions. The precipitation reaction is the classical example [6], it can also be demonstrated in other systems, notably in bacterial agglutination and hemagglutination assays.

Prozone phenomena in IIFA have received little attention, even though the phenomenon has occasionally been noted in tests for antinuclear antibodies [7].

Immunofluorescence visualizes a primary antibody-antigen interaction. The absence of fluorescence in IIFA staining indicates that the conjugate did not bind. Such a lack of binding is usually due to failure of the primary antibody to bind to the target antigen. In the prozone effect observed in our cases, absence of binding of conjugate molecules occurred despite the binding of primary antibodies to target antigens.

E. UNDER & A. MIETTINEN observed in their study a marked prozone effect in indirect immunofluorescence with rabbit antisera against rat renal proximal tubular epithelial brush border (BB) antigens, they concluded that the antigenic sites of the attached antibodies somehow become inaccessible to the anti-immunoglobulin conjugates and that diluting the specific tissue-reactive antibodies involved exposure of these sites to the conjugate. The observed concentration-dependent inaccessibility may depend on tight packing of antibody molecules attached to the tissue and the space between bound

immunoglobulin molecules could become insufficient to allow interposition of anti-immunoglobulin conjugates [8].

Sundqvist, in an extensive microfluorometric study of factors influencing cell surface membrane staining in indirect and direct immunofluorescence, also observed a prozone phenomenon using IIFA. In discussing the mechanism of the prozone effect, he concludes that membrane-attached antibodies in concentrations corresponding to the prozone do not disappear from the cell surface but become inaccessible to the conjugate molecules [9][10].

As it has been mentioned earlier, our tests were performed on diagnosis kits of the same manufacturer: EUROIMMUN, Germany, there is a possibility that the prozone effect observed in these cases is due to the quality of its substrates or conjugates, it would be interesting to test the same serum dilutions of the same patients on other substrates proposed by other manufacturers.

Owing to the fact that only these kits are available in our laboratory, we were unable to investigate this possibility.

As we can see, the mechanisms of prozone effect in general has been well investigated in multiple studies: [11], [12] and [13]. But the occurrence of this phenomenon in IIFA have not been given much attention, by reporting these observations we managed to put this phenomenon in the spotlight mainly in AMA2 and anti-LC1 antibodies detection. These cases illustrate that all laboratories providing IIF assays should be alert to the continuing possibility of hook effect. Indirect immunofluorescence is generally touted for its higher sensitivity, reliable pattern recognition and simplicity in routine use. However, it is imperative that testing involves caution of possible false-negative results. Serial dilutions of these samples is recommended in order to ensure that the reported result is accurate. These cases also reinforce the critical importance of exchange of information and excellent communication between clinical and laboratory staff in order to minimize the risk of clinical errors arising from erroneous analytical results.

Déclaration d'intérêts

None.

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