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SMUFD D/A ltr, 15 Feb 1972

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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland
SEROLOGICAL DIAGNOSIS OF SYPHILIS

RECENT ACHIEVEMENTS: REITER'S TREPONEMIC ANTIGEN, IMMUNOFUORESCENCE.

Canadian Journal of Medical Technology
Vol. 25, pages 33-41, 1963

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In 1901 Wassermann, Neisser and Bruck applied the complement fixation method to syphilis. This method was discovered in 1901 by Bordet and Gengou for the tracking down of specific sensitivities which appear in the serum of patients with infectious diseases. From this moment on, serological reactions relating to syphilis have not ceased to multiply and improve in a nevertheless incomplete manner because the principal element of the reaction, the antigen, was always an alcohol extract of an organ, under these circumstances beef heart, and because of this, was not specific.

Research continued and finished in 1941 with Mary Pangborn's isolation of a phosphatide, cardiolipin, which is connected with serological activity. Thus, great progress was made in the direction of antigen standardization, but the nonspecificity of the reaction still persisted. Presently, the antigen used for complement fixation reactions, Bordet-Wasserman type, is constituted by an alcoholic solution of cholesterol.
and purified phosphatides: phosphatidic acids of the cardiac muscle (cardiolipin) and lecithine (phosphatidyl choline). But the principal turning point in the serology of syphilis came in 1949, with the perfection of the treponema immobilization test or T. P. I. (see note) by Nelson and Mayer.

Note: Henceforth for Treponema immobilization test.

At last these authors developed a serodiagnostic test for syphilis which was both specific and sensitive but, unfortunately, delicate to perform and limited to only a few specialized laboratories. Then appeared the complement fixation method with Reiter's treponema antigen and the immunofluorescence method.

All the techniques of classical serology are based on the same principle: the serum of syphilis subjects has the ability to form complexes with colloidal suspensions of lipids which act as antigens; only the exterior manifestations of the reactions differ according to the techniques and the working conditions. The antigen with a base of alcoholic extract of beef heart or cardiolipin now in wide usage, is a great handicap for the specificity of the classic syphilis reactions. On the other hand, with Nelson's and Mayer's test, a specific antigen, the pathogenic treponema, Treponema pallidum itself, reacts with an immobilizing or immobilisin antibody which is a different substance than the reagin. Because of its specificity and specificity, this test has often been considered as a reference method by serologists. But, in spite of the solution it has given to the specificity of syphilis serodiagnosis, this method has not reached the amplitude of development hoped for; its application remained too delicate and impractical for the majority of nonspecialized laboratories.

Many techniques using treponema antigen bases have been proposed to remedy this complexity of Nelson's and Mayer's test. Briefly, let us note a few here:

1. Methylene blue reaction of B. J. Roseman and J. F. Kent to track down the immobilisins: the positivity of the reaction is characterized by a lack of coloration, thus coloration appears with a negative serum.

2. Reactions using a dead pale pathogenic treponema:
   a. adherence-disappearance phenomena demonstrated in 1952 by R. A. Nelson, the treponemes, in contact with syphilitic serum and heparinized whole blood from a healthy subject, disappear by adhering to the red cells. 20 This phenomenon is due to other antibodies than the reagin.
b. various agglutination techniques with the chemically killed antigen not spontaneously agglutinable.

c. complement reaction with a pale delipidized treponeme (H. J. Magnuson and J. Portnoy): this is a Kolmer type serological reaction using as antigen a suspension of pathogenic treponemes of the Nichols' strain, successively delipidized by acetone or ether extraction.

d. immunofluorescence reaction.

3. Reactions using Reiter's nonpathogenic treponeme.

With the exception of the immunofluorescence reactions and the complement fixation reactions using Reiter's treponeme antigen, most of these techniques are not used in the current serodiagnosis of syphilis.

I. Reiter's treponeme antigen in complement fixation.

With the goal of attaining the specificity and sensitivity of Nelson's test, many serologists returned again to the research started in 1929 by Gaethgens who recommended a complement fixation reaction using a culture of Reiter's treponeme strain as antigen. The origin of this strain remains very obscure; according to Muster, it was isolated by Wassermann and Ficker in 1922, then recaptured by Reiter who adapted it to a relatively simple culture media and it is thus that it took Reiter's name. The origin of isolation is unknown.

The cultures of this treponeme, having a morphology distinctly different from that of Treponema pallidum, are deprived of all pathogenicity for animals receptive to syphilis. The culture media used are liquid anaerobic media, or Brewer's thioglycolate media with 10% serum, or simply a 2% peptone, 0.1% glucose broth with 10% human or rabbit serum. The spirochetes of an eight day old culture are mechanically separated then homogenized by ultra-son; they are then suspended in physiological phenolized serum. This treponema antigen, having a durable stability, is then used, undiluted or slightly diluted, to the limit of its anticomplementary ability to preserve its major quality which is sensitivity.

This last condition requires a minute titrating of the hemolytic system to avoid leaving the optimal activity zone. The reaction is then performed according to Kolmer's classical technique, particularly watching the serum hemolysis, the latter being less stable than in reactions with the lipid antigen. Much research has been undertaken with this
Reiter’s antigen; it was first used by the Italian school with D’Allessandro, Pucinelli, De Blasi, Oddo and Dardanelli\textsuperscript{7,8,9,10} who since 1941 has studied this treponeme antigen which she used under the name of "Pallignost" (see note). These authors attributed the specificity of this antigen to its protein fraction (thermolabile) rather than to the other lipid fractions (assimilable to the alcoholic extracts of organs and to cardiolipin) and polysaccharide fractions (thermostable). These functions of the different antigenic fractions are confirmed by the majority of authors (Gelperiti in particular).\textsuperscript{16} In Germany, this antigen is sold under the name of "Palliged". In France, many immunologists are interested in this question: Pautrizel and collaborators; Schier, Benazet, Brotes and Thivolet; Borel and Durel; Daguet, Pillet and Faure; Muttermilch and Delaville; Hamelin and Vaisman...to only cite the principal ones.

Note: "Pallignost": phenolated Reiter’s suspension, prepared by Institut serotherapie milanais (Milan therapeutic institute), S. Belfanti.

Before comparing the results obtained with classical serology and T.P.I. on one hand and complement fixation with treponema suspensions on the other hand, it seems opportune to reveal the conclusions put down by Pucinelli\textsuperscript{24} on the birth, duration and the disappearance of antibodies in syphilitic serum. These various serological methods do not reveal the same antibodies because the latter have a different evolution during the infection.

**Primary syphilis:** The protide antibody appears first (from the third to the fifth day) followed by the polysaccharide antibody (seventh to tenth day) then the reagin. The immobilizing antibody is the last to manifest itself and can only occur in the following period.

**Secondary Syphilis:** All the antibodies are present at an elevated titer.

**Latent and tertiary syphilis:** The two lipid and polysaccharide antibodies can be absent, but the protide and immobilising antibodies are always present. The same phenomenon is produced in syphilis of the nervous system where the protidic and immobilizing antibodies are constant.

**Treated syphilis:** According to the period given to treatment, the disappearance order of the antibodies can vary.

In recent syphilis with apparent clinical signs, the lipid antibody ordinarily disappears before the protide antibody while in old syphilis, without clinical manifestations, the opposite occurs with a lipid antibody.
lasting a long time or indefinitely. In both cases, the polysaccharide antibody is the first to disappear, while the immobilizing antibodies are the last survivors. Note that on this subject, Pautrizel, Bonnardot and Szernovicz reported that these antiprotein antibodies of birth, more precocious than reagin, disappeared more rapidly than reagin in early treated syphilis and, after it in late treated syphilis.

Thus, let us examine these various results:

Gaethgens (1937): study of 16500 syphilitic sera. Positive and nonspecific reactions were obtained respectively in 50% and 1.17% for B.W., 62% and 1.7% for flocculation and 71% and 1.4% for R.P.C.F. (see note).

Note: R.P.C.F. henceforth should read Reiter Protein Complement Fixation.

Erickson and Eagle, in an examination of 1032 sera, reported the following figures for the 490 syphilitic sera: B.W. +: 65%; flocculation +: (Eagle's reaction) 75.5%; R.P.C.F. +: 84.8%.

Gastinel, Valsman and Hamelin obtained a negative "Pallida -reaction" with a positive Nelson's test in 4.90% of the cases (study of 511 sera) and the opposite was produced in 2.70% of the cases while the "falsely" positive reactions of classical serology have an 6.65% incidence titer. Lastly, the cases of positive TPI with a negative BW occur in 9.40% of the cases.

Vogelsang, Pillot and Faure, in a comparative study between VDRL and RPCF (677 syphilitic sera), found a RPCF sensitivity comparable to that of the other test and a superior specificity.

Benazet, Brottes, Thivolet and Sohier, in a comparison study of the immobilization reaction of Nelson and Mayer and the complement fixation study of the immobilization reaction of Nelson and Mayer and the complement fixation reaction carried out with the treponema antigen, Reiter's strain (Italian "Pallignost" antigen), and the cardiolipid antigens, obtained the following results.

--for 464 sera studied, 177 agreed between the three reactions (TPI, RPCF, BW): 93 were positive, 84 were negative or 38.1% of the cases.

--269 sera were studied with TPI and RPCF. Let us examine these results.
TPI: 108 positive, 40.1%; 161 negative, 26.9%.

RPCF: 152 positive, 56.5%; 117 negative, 43.5%.

Of the 108 positive TPI sera, only 104 were positive for RPCF and 4 were negative. For the 161 negative TPI sera, RPCF gave the following results: 117 negative reactions and 44 positive reactions.

For the latter case, it seemed interesting to find which categories these 44 positive RPCF and negative TPI sera belonged to: they were divided thus:

- 7 gave doubtful reactions in classical serology and were considered as "falsely" positive reactions;
- 3 came from patients having recent syphilis, during serological change;
- 6 came from patients having known syphilitic antecedents;
- 28 had negative cardiolipid serology but 26 of these came from patients considered as old syphilitic patients.

In another study, the authors remarked that for 120 subjects having positive RPCF and BW reactions, TPI gave only 93 positive reactions for 16 negatives (for 11 sera TPI could not be performed); among these 16 negative sera, there were 3 primo-secondary syphilitics undergoing serological change.

Daguet, Pillot and Faure\(^6\) studied the various treponeme antigen preparations of Reiter from the Pasteur Institute in Paris and they compared these reactions to those of lipid serology: complement fixation with cardiolipid antigen, flocculation reaction. TPI was taken as the reference reaction. We considered the work of these authors in the form of a table giving four crosses (++++) to the strongest sensitivity, three crosses (+++) for the next, two (++) for the third and one (+) for the last. We considered this sensitivity at different stages of the disease.

<table>
<thead>
<tr>
<th>Category</th>
<th>T. P. I.</th>
<th>Kolmer Reiter</th>
<th>Kolmer Cardio</th>
<th>Kline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary S.</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++++</td>
</tr>
<tr>
<td>Secondary S.</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>Latent S.</td>
<td>**</td>
<td>+++</td>
<td>++</td>
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<tr>
<td>Tardy S.</td>
<td>**</td>
<td>++</td>
<td>++</td>
<td>+++</td>
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<tr>
<td>Treated S. still positive</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
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</tbody>
</table>
* In primary syphilis, TPI is definitely later than the other reactions.
** TPI is positive for all cases.

The "falsely" positive biological reactions: here, the sensitivity of RPCF is very close to that of TPI.

From the data given in this table, we have:

—in the case of recent syphilis, the sensitivity of Reiter's antigen is inferior to that of the cardiolipid antigens but more precocious than TPI. On this subject, note the disagreement with Pucinelli which demonstrates that the protein antigen appears first in the serum of the syphilitic subject and well before reagin:

—in latent, tardy and treated syphilis, Reiter's antigen seems superior to those of classical serology and has a sensitivity inferior to that of TPI.

In that which concerns the reproducibility of the reaction, these authors note a difference bearing on one or two serum dilutions, sometimes three but, in spite of everything, it is superior to that observed with the flocculation reactions whose constancy is remarkable.

According to these same authors, the specificity of this antigen is situated between TPI and lipid serology.

Rousseau and Desbier, attributing 100% sensitivity to TPI obtained a 101.2% incidence with RPCF against 92.2% with lipid serology (Kolmer) and 92% with Kahn's reaction. Elsewhere these same authors performing a RPCF on 443 sera having given different Kolmer and Kahn reactions, deduced a sensitivity agreement of 95.2% between RPCF and Kolmer cardiolipid and 54.1% between RPCF and Kahn.

In the course of treated syphilis, Thivollet, Rolland and Sehier reported that with seven sera whose TPI and cardiolipid reactions were negative, there were two reactions with Reiter's protein antigen.

With this outline of a few results obtained by different immunologists, it seems opportune to try to draw a few conclusions concerning the sensitivity and the specificity of this Reiter's treponema antigen, two qualities which seem definitely superior to those of the lipid extracts but, in spite of everything slightly inferior to those of Nelson's test.
From the sensitivity point of view:

-- in the course of treated syphilis, the value of the reaction of complement deviation with the help of Reiter's antigen is greatly debated. For some, this reaction will be positive about one week earlier than with lipid serology and the flocculation reactions (Gathgens, Fühner, Lauber, Maustardier, Ducinelli). For others, the sensitivity at this period of the infection will be less than that of the lipid antigens.

Lauber reported that, in 422 sera studied, the RPCF was ordinarily positive eight to ten days earlier than the other reactions.

-- often, it appeared as the only index of tertiary syphilis which it regularly revealed even when the lipid reactions were negative.

-- in the case of old latent syphilis, neuropsyphilis, this reaction is precocious and endowed with a great sensitivity. It thus assumes a very definite superiority over all the other classical tests.

From the specificity point of view:

If we take the results obtained by Gastinel, Valsman and Hamelin, we verify that the reaction defaults in only 2.7% of the cases as opposed to 6.65% with classical serology.

-- if suspicion exists with "falsely" positive lipid reactions, that is positives in the absence of syphilis (virus pneumonia, infectious mononucleosis, erythematous lumpus, acute malaria, smallpox vaccination reaction... diseases where the possible formation of nonspecific lipid antibodies can give very positive results to the Wassermann reactions), if we have a dissociated flocculation reaction and a complement fixation reaction where one hesitates to affirm the diagnosis of syphilis, if we are working with an insufficiently or tardily treated syphilis which is badly negative to the lipid antigens, if we are in the presence of a child born of a syphilitic mother whose serum contains antibodies from placenta infiltration, the use of complement fixation with the protein extract of Treponema pallidum, Reiter's strain, will be of great use in serodiagnosis.

II. Immunofluorescence

The second technique which seems to have much importance today is the immunofluorescence test in the presence of pale treponemes which demonstrates the presence of both the specific antibodies of a single pathogenic treponeme and the group antiprotein. It is due to an observation
of Marrack in 1934\textsuperscript{10} the chemical binding of a coloring to an antibody can occur without changing the immunological properties of the latter—that A. H. Coons\textsuperscript{4} in 1942 completed the immunofluorescence method which was the applied in 1957 by Deacon to the research of antibodies appearing during syphilitic infection. In 1959, Borel and Durel,\textsuperscript{2} also proposed a technique which was slightly different in that it used a fluorescent coloring. The antigens used are either pale treponemes, Nichol’s strain, cultivated in rabbit testicle or Reiter’s treponemes from a young culture, fixed on slides by heating one half hour at 60° C.\textsuperscript{23} The serum and the cerebrospinal fluid used in this study should be clear, not hemolyzed and preserved at -20° C if the test is not performed immediately. The revealing of the antigen-antibody complex by a fluorescent coloring, fluorescein isocyanurate, or better fluorescein isothiocyanate which requires no acetone to bind to the globulins, is made under ultra violet light: it is the direct process which still assumes some difficulty in that which concerns the need to make as many antibodies fluorescent as there are antigenic systems to examine.\textsuperscript{2} Also, the process most currently used, is it the indirect process which in the first phase consists in placing the antigen—(T. pallidum), fixed on a slide, in the presence of possible specific antibodies present in the serum, and in a second stage, the revealing of the first complex by the fixation of human antiglobulin antibodies marked by fluorescein isothiocyanate.\textsuperscript{2} The reading of the reaction is very simple: in the case of syphilitic serum, the treponemes are colored yellow-green, brilliant, very characteristic; in case of no syphilitic antibodies in the serum, the distinction of even the treponemes, dark green, is very difficult. Note briefly that the serum titer is expressed by the inverse of the highest dilution still giving a definite fluorescence.\textsuperscript{21}

After this brief recount of the principle of the immunofluorescence method applied to syphilis serodiagnosis, we will consider the results found by various serologists.

Borel and Durel\textsuperscript{2} reported that with 153 sera divided in the following manner: 41 nonsyphilitic, 112 taken from different stages of the disease; the 41 sera of nonsyphilitic subjects gave negative TPI and IF (see note) reactions, while for the 112 other sera, the results are thus: 16 negative; 6 doubtful and 90 positive for TPI as opposed to 5 negative and 100 positive with the immunofluorescence. Elsewhere, it must be reported that of 6 subjects having wrongly positive reactions with classical serology, all were negative with IF and TPI, which is in favor of good specificity for this method. As to the sensitivity, these same authors reveal a seven times negative TPI in treated subjects while I. F. remained positive. In seven cases of untreated primary syphilis, Borel obtained a positive IF while all were TPI negative.
Thivolet, Grospiron and Murat obtained 88 positive responses with IF as opposed to 73 with Nelson's test. In the case of treated syphilis, authors arrived at the following results: TPI: 57 positive; idoubtful; 38 negative, IF: 72 positive; 15 negative. This favors a very sensitive reaction in subjects affected with syphilis and treated for it.

Censusales and Garofalo noted, while comparing the types of reactions (TPI, IF, lipid serology and complement deviation reaction with the use of treponeme antigen) that they agreed on 100 sera, 38 times in positive cases, while only TPI and IF were positive in 16 other cases.

Daguet and Pillo determined that the 11 sera classified as "falsely" positive with lipid serology in 250,000 examined were all TPI negative while 3 were IF positive and one fixed complement with Reiter's treponeme antigen.

But, of all the compiled results, the most convincing seem to be those reported by Miel and Frribourh-Blanc. Their study was made on more than 5,000 sera and cerebrospinal fluids, and they drew the following conclusions:

--All the positive TPI sera were also IF positive and at almost always higher titers;

--the fluorescent antibodies appeared very early during the development of syphilis. Their appearance generally preceded that of the immobilizing antibodies by 15 to 30 days and the reagins by 2 to three days;

--in untreated secondary syphilis, the titer rise is rapid and very high;

--the study of cerebrospinal fluid is of great interest; the weak nonspecific serum positives are never found again. Very high fluorescent titers are noted in developed treponema nervous syphilis;

--in treated syphilis, the stage where classical serology is often at fault, IF has great sensitivity. The authors note that only primary or recent primo-secondary syphilis gives a total negative. In this last case, IF is even superior to TPI if we rely on the experiments of the authors.

--the fluorescent antibodies, as immobilisin, do not eliminate the possibility of a treponematosis (in particular yaws).
The thus exposed results permit us to state that the immunofluorescence test offers us appreciable and researched qualities, which have good sensitivity and specificity.

The specificity of this test, according to the principle of the immunofluorescence itself which consists in making the antibody visible on the antigen, without the intervention of any element other than this Treponema pallidum and the patient's serum, is not hypothetical but, to the contrary, confirmed. It is a test which would be interesting to use when we suspect "false" positive reactions and, in the possibility where the studied serum only contains reagin, the IF would be negative, while the presence in the serum of the antiprotein of the group demonstrated by a Reiter type treponeme antigen, for example, would also yield a positive reaction. Elsewhere, if the serum to be tested has reagin and specific antibodies for the pathogenic treponemes, IF would offer a result comparable to that of Nelson's and Mayer's test, which is a good reference for the technique. Borel and Durel qualify this specificity as "analogous to that of a TPI of known value".

As to sensitivity, these same authors classify it above that of TPI: an opinion which seems confirmed, however plus or minus, by all the authors (Deacon, Grosbiron and Murat, Falcone, Harris, Niel and Fribourg-Blanc...), moreover the results revealed above do not invalidate this interpretation. Borel and Durel added a third property to this technique, reproducibility. These authors, using 35 different protocols, found no variation in the dilutions of positive serum, only the intensity in the heart of the same dilution varies "one or two crosses". Moreover, this reproducibility is judged excellent by Niel and Fribourg-Blanc.

CONCLUSION

The classical serological reactions should not be abandoned for the benefit of the treponema antigen reactions alone. The latter are still evolving and their too brief experimentation does not authorize them to claim an exclusive place. In spite of everything compared to the reference reaction of the treponema immobilization test, they establish that they have two primordial qualities: sensitivity and specificity, properties which allowed TPI to be worth something. If, in precocious syphilis, the value of RPCF is still debatable, the immunofluorescence method is definitely more sensitive than any other test, TPI included. Note that on this subject that Niel and Fribourg-Blanc found titers of 90 to 150 (doubtful figures) with IF on the third day of a chancre while the BW, well understood, was negative. Also it is important to consider the reason the sample is taken: tracking down (blood donors), precocious diagnosis, latent syphilis.
therapeutic control... to determine the choice of the suitable technique.
For the diagnosis of tardy syphilis, the two reactions effectively
supplement the classical antilipid serological reactions.

These two treponema tests, and more particularly still, the
immunofluorescence, show a more marked specificity than the non-
treponema proofs. Their use with "biological false positives" found in
classical serology is of first importance.

In treated syphilis, IF has a sensitivity equal to that of Nelson's
test; as to RPCF, its indication in this case is superior to that obtained
with classical cardiolipid serology. These two methods have not reached
their full maturity; improvements are necessary to obtain a sure and
irreproachable technique (elimination of nonspecific antibodies: globulins
of indeterminate nature in the serum of some subjects; in IF standardization
of material and techniques; very limited activity zone for RPCF...). Also
today, it would not be a question of substituting these techniques for the
hemolysis and flocculation reactions on one hand, and for Nelson's test on
the other hand; they must collaborate between themselves. And in the
absence of a TPI, these two reactions can be performed in a current
analysis laboratory and give the Biologist and Clinician appreciable results
which they could not expect before the coming of these treponema reactions.
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