Syphilis Serology with Lipoidal Antigen: The Meaning of Positive Reactions*

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THE basis of positive reactions given by tests for syphilis has puzzled syphilologists and clinicians for many years. The reason is that the blood serum of the person suspected of having syphilis is tested with antigen that is unrelated to syphilis. The antigen consists of lipoidal material extracted from normal tissue. This lipoidal antigen reacts with antibodies in the blood serum of the syphilitic patient. The end result is a positive flocculation or precipitation test or a positive complement fixation (Wassermann) test, depend-in on the technique used.

The concentration of antibodies in syphilitic serum which react with lipoidal antigen must be unusually high. This is indicated by the excellent results physicians obtain with test for syphilis which employ lipoidal antigen. The occasional positive reactions with the same tests in malaria, leprosy, and in other diseases would indicate a low concentration of antibodies in those diseases. So low indeed, that the reactions are referred to as false positives. False positives are also encountered following the injection of various substances, such as foreign protein and vaccines.

As is well known, tests for syphilis which employ treponemal antigen are also available. But tests with lipoidal antigen are the more widely used routinely. Some of the reasons are the reduced cost of lipoidal antigen tests, the early positive results these tests give in primary syphilis, and the fact that these tests serve as a check on the therapy of syphilis. The questions we shall attempt to answer are why do lipoidal antigen tests work so well in syphilis? Also, if there is a close relationship between lipoidal antigen tests and syphilis, why are false positives encountered?

Our studies indicate that lipoidal antigen tests represent a distinctive system in immunity; that the antigen used in lipoidal tests actually consists of a multiplicity of antigenic lipids some of which react in syphilis, some in leprosy, some in malaria, some in other situations. The basis for this assumption is that, under appropriate technical conditions which we shall consider presently, different serologic patterns are encountered in those diseases and in certain other situations. The time will undoubtedly come when the different antigenic lipids in lipoidal antigen will be identified chemically. The identification herein reported is based on differences in serologic patterns.

AN EXPERIMENTAL SEROLOGIC TECHNIQUE WHICH HELPS TO CLARIFY REACTIONS IN SYPHILIS AND IN OTHER SITUATIONS

The determinations of different antigenic lipids in serodiagnostic antigens cannot be made with serodiagnostic tests for syphilis. These tests are too restricted technically to show differences in serologic patterns in different situations. To observe these patterns, we developed an experimental serologic technique with which to examine human and animal serums. The serums of animals free from syphilis are known to react with lipoidal antigen and this aspect also needs clarification.

Kahn antigen was used in the experimental technique. Although we also used Kolmer and cardiolipin antigens, we shall report our results with Kahn antigen because our major studies were carried out with that antigen.

The first step in the experimental serologic technique was to incubate the tests overnight at 5°C instead of completing them after three minutes' agitation. Precipitates resulting from mix-

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tures of lipoidal antigen with serum tend to dissolve at warm temperature and to become stabilized at cold temperature. Cold incubation was thus essential for the study of serum reactions with our experimental serologic technique.

The second step was to vary the NaCl concentration used in the experimental technique. We observed that serum reactions with lipoidal antigen are greatly affected by NaCl concentration. Syphilitic serums with lipoidal antigen react well with 0.9% NaCl solution, but animal serums and some nonsyphilitic human serums often react best at salt concentrations either higher or lower than 0.9 per cent NaCl.

Accordingly, we devised an experimental quantitative serologic system with Kahn antigen in which the salt concentration was lower than 0.9% in some quantitative tests and higher in others. In practice, we lined up seven quantitative tests of serial dilutions of serum, starting with distilled water and ending with 2.1% NaCl solution. Each quantitative test consisted of undiluted serum and the following six serial dilutions: 1:2, 1:4, 1:8, 1:16, 1:64, and 1:256 with the appropriate salt solution.

We thus had seven quantitative setups of seven serum dilutions each, ranging from distilled water and 0.15% to 2.1% NaCl solution. These dilutions were then tested with Kahn antigen suspension. The amounts used were 0.15 ml of each serum dilution and 0.025 ml of antigen suspension. The mixtures were agitated for three minutes in the Kahn shaker. To the quantiative set-ups with water and 0.15% NaCl solution, 0.2 ml of the respective diluent were added to each tube; to the other setups 0.5 ml of diluent were added, of the same concentration of NaCl solution as that used in preparing the serial dilutions of serum. The results were then read after three minutes' agitation and again after four hours and 24 hours' incubation at 5°C.

The reading before incubation of the tests was made especially to determine whether the serial dilutions of serum with 0.9% NaCl solution gave positive flocculation results. These serial dilutions make up the Kahn quantitative test used in diagnosis, and if positive, would indicate that the serum came from a person with syphilis. In nonsyphilitic situations, this test with 0.9% NaCl solution is commonly negative.

The readings after four and 24 hours' cold incubation were made to determine whether the results if positive, are essentially the same after these two periods of incubation or if after 24 hours' incubation they are stronger than after four hours. Syphilitic serums generally give the same results after these two periods of incubation. Nonsyphilitic serums give stronger reactions after 24 hours than after four hours' incubation.

RESULTS

When examining normal human and animal serums with this seven quantitative experimental method, some degree of positive reactivity, indicated by a flocculent precipitate, was obtained in each instance after cold incubation. Most human serums showed some flocculation only in the quantitative setup in which the NaCl concentration was either higher or lower than 0.9%.

An outstanding feature of the reactivity in normal persons was that each person examined showed repeatedly the same type of serologic pattern. A healthy person who gave weak flocculation, gave it repeatedly, and a person who gave strong flocculation, gave that pattern repeatedly. The repeat examinations were made monthly on a group of 30 volunteers who were inmates of the State Prison of Southern Michigan. Some were tested daily and some weekly, with essentially the same result.

The animal serums studied for flocculation were from mice, pigs, cows, dogs, rabbits, guinea pigs, horses and monkeys. The strongest reactions were shown by horses, cows, and pigs. The weakest reactions were shown by mice. But all animals tested showed some degree of flocculation. The serologic patterns in the different animals showed considerable variations. When the same rabbits were tested monthly, they showed constancy in their serologic pattern.

Because of the biologically universal reactivity of serum reactions with lipoidal antigen, we have referred to our experimental method as "Universal Serologic Reaction." In diseases tested thus far, flocculation in the Universal Reaction became increased. In a study of experimental syphilis in rabbits, the normal flocculation reactions became increased during the disease and reverted to the normal level following recovery due to therapy. The same was true in human malaria. A group of

volunteers were inoculated with *Plasmodium vivax*. Two weeks after the febrile attack, the flocculation was increased and in several months, reverted to the normal level.

In tuberculosis we have only one instance in which a laboratory worker normally showed a low degree of flocculation; on the development of tuberculosis, she showed increased flocculation, with a return to the normal level after a year in a TB sanitarium. In leprosy, it was observed that the strongest flocculation reactions were obtained in the lepromatous stage. We had no instance of lipoidal antigen reactions before leprosy had been developed.

The injection of various substances also led to an increase in reactions with lipoidal antigen with our biologically universal experimental technique. We have seen increased flocculation over the normal level in rabbits to the injection of horse serum, tissue lipids, paraffin oil, killed tubercle bacilli, Freunds' adjuvant and of vaccines. When we injected lanolin, no increase in flocculation was observed. The reason may be that lanolin is utilized by the body. In the case of the injection of horse serum, both precipitins to the horse serum and increased flocculation to lipoidal antigen over the normal level were obtained. In due time the strong lipoidal antigen reactions reverted to normal.

SERUM-LIPOIDAL ANTIGEN REACTIVITY AND TISSUE BREAKDOWN

The increase in reactivity of the universal reaction in disease would indicate that reactions with lipodal antigen are the result of tissue breakdown. The slight tissue breakdown associated with normal meabolism in health leads to weak reactions in most instances. The increased tissue breakdown resulting from disease leads to stronger reactions. On recovery, the tissue breakdown becomes reduced and the reaction returns to the normal level.

It is believed that, as in disease, the injection of foreign substances also leads to some tissue breakdown which in turn leads to increased reactivity in lipoidal antigen reactions with the universal technique and, in due time, to the return to the level of reactivity before the injection.

As a further indication that serum-lipoidal antigen reactions are the result of tissue breakdown, we found that in malaria, during the febrile state, 10 days after inoculation of *Plasmodium vivax* in volunteer human beings, there was no increase in serum-lipoidal antigen reactivity over the normal level. But 10 days to 2 weeks after the febrile attack, there was a marked increase in such reactivity. Apparently, the period of lag started with the febrile period. In syphilis also, serodiagnostic tests do not begin to become positive until 10 days or more after the appearance of the primary lesion, suggesting that the reactivity with lipoidal antigen is associated with tissue breakdown.

BIOLOGIC MECHANISM LEADING TO TEST-TUBE REACTIONS WITH LIPOIDAL ANTIGEN

The biologic mechanism of lipoidal antigen-serum reactivity in health, in disease and following the injection of foreign substances is undoubtedly of the same nature. Tissue breakdown appears to be a common factor in such reactivity. Accordingly, we believe that:

1. Tissue breakdown causes the liberation of both protein plus lipids from the injured body cells.

2. These lipids, in combination with the liberated and undoubtedly transformed protein, are rendered antigenic.

3. Antibodies are formed to these antigenic lipids, after an incubation period of about two weeks.

4. These antibodies are then detected *in vitro* by the biologically universal experimental technique with lipoidal antigen and to a limited degree by serodiagnostic tests for syphilis.

Lipids are haptens, according to Landsteiner, and are not capable of calling forth the formation of antibodies. But lipids in combination with liberated protein may undergo such chemical changes as to become foreign to the body and call forth antibody formation to lipoidal antigen.

WHY DO SERODIAGNOSTIC TESTS WITH LIPOIDAL ANTIGEN GIVE BEST RESULTS IN SYPHILIS?

If syphilis is compared with three other chronic infectious diseases, such as tuberculosis, malaria and leprosy, one can understand why serodiagnostic tests work so well in syphilis and not in the three other diseases.

Indications are that a person who is ill is generally not a good antibody producer to lipoidal antigen. If a person is overwhelmed by a disease,

he may not produce any antibodies to that antigen. For example, when we first developed the biologically universal experimental technique, we obtained reactions in febrile patients with early tuberculosis that did not seem to us strong. We believed, at that time, that if we obtained blood specimens from patients with far-advanced tuberculosis, we would obtain stronger reactions. Accordingly, we obtained 50 specimens from such patients (at the Howell Sanitarium, Howell, Michigan). We found that the reactions were much weaker in far advanced tuberculosis instead of stronger. When we obtained specimens from miliary tuberculosis, the reactions were still weaker—just as the tuberculin test is weak or negative in miliary tuberculosis. Tuberculosis is thus not a strong antibody producer.

It is true that in early tuberculosis, the universal reaction with lipoidal antigen become stronger than normal. But the reactions do not become as strong as in syphilis or as in lepromatous leprosy. In our experience, the universal reaction in tuberculosis rarely reached the serodiagnostic zone. The result was that tuberculosis rarely gave a false positive reaction with a serodiagnostic test, such as the Kahn test. A possible explanation for the low level of reactivity with lipoidal antigen in tuberculosis is that the immunity in this disease is largely cell mediated.

In malaria, the reactions with lipoidal antigen present another picture. A person with malaria might have a febrile period, followed by a period of quiescence which may last for years. Some two weeks after the febrile period this person is likely to give a positive serodiagnostic reaction for some weeks, after which he will become seronegative.

In leprosy also, patients might show a wide variation in the severity of the disease. One person might show only a small area of anesthesia and another might be overwhelmed by the disease.

In syphilis, during World War I, I saw young men with a secondary rash at work in factories. They were not ill enough to stay home. This might be one of the reasons why syphilis is an excellent antibody producer, because the patients are generally well enough to work.

In 1928, when I became a member of the faculty of the University of Michigan, Dr. Udo Wile, the syphilologist, would call my attention to patients with malignant syphilis, which was a fatal disease and the methods of therapy used at that time could not save the patients. I would see two or three such patients a year. Dr. Wile spoke of the disease as precocious malignant syphilis. The patients were overwhelmed with the disease and the Wassermann and Kahn tests were negative.

We did not have the opportunity to determine the type of universal reactions obtained in malignant syphilis. Such patients were rare 40 years ago and they may not exist any more. The fact that these persons gave negative serodiagnostic reactions fits in with our findings in far advanced and miliary tuberculosis. Briefly, when a person is overwhelmed by an infection, his serum reaction with lipoidal antigen is likely to be weak or negative.

We have expressed the belief that, because the person with syphilis is generally well enough to carry on his work, he is a good antibody producer with lipoidal antigen. Being a good antibody producer, he gives good results with serodiagnostic tests. Another reason for the good results is that the lipoidal antigen which reacts with syphilitic serum shows a distinctive characteristic which favors the reaction. This characteristic is that, in the universal reaction, the lipoidal antigen with syphilitic serum gives a serologic pattern in which maximal flocculation is obtained rapidly after the 3-minute shaking period with no increase in flocculation following overnight cold incubation. The rapidity of syphilitic serum-lipoidal antigen reactions indicates that they are optimal for flocculation. The chararacteristic pattern of the universal reaction in syphilis, in turn, suggests that distinctive lipids are involved in syphilitic reactions.

The behavior of syphilitic reactions in not becoming stronger on cold incubation, we believed at one time, might be employed to differentiate "true" from "false" positives given by serodiagnostic tests. The difficulty was that the syphilitic serologic pattern of a person in the universal reaction is superimposed over the normal serologic pattern of that person. Therefore, in instances in which the normal reaction is strong and the syphilitic reaction is weak, the syphilitic pattern would show characteristics of a nonsyphilitic pattern with a stronger reaction on cold incubation. Such syphilitic reactions might then be misinterpreted as false positive reactions.

WHY FALSE POSITIVES?

By the use of our experimental universal technique, we showed that serum reactions with lipoidal antigen are not restricted to syphilis, but are biologically universal in human beings and in animals. Therefore the occurrence of nonsyphilitic (false positive) reactions with serodiagnostic tests becomes understandable. It was also observed that only exceptionally strong universal reactions spilled over into the sero-diagnostic zone, leading to false positive reactions. This finding explains why, in any given situation in which false positives may be obtained, only a small percentage of persons give such reactions.

In smallpox vaccination, for example, we observed that only about 5 per cent of vaccinated persons, clinically free from syphilis, gave positive serodiagnostic reactions for syphilis. The remaining 95% gave negative serodiagnostic reactions.

Those persons who gave negative reactions showed weak universal reactions previous to the vaccination. Their universal reactions became stronger as a result of the vaccination. But the increase in flocculation was not strong enough to reach the 0.9% NaCl concentration—which is the setup for the serodiagnostic zone. The result was negative serodiagnostic reactions.

Five per cent of vaccinated persons showed strong universal reactions previous to the vaccination. In these reactions flocculation was close to the serodiagnostic zone. When these persons were vaccinated, they showed an increase in flocculation. This increase caused flocculation to spill over into the serodiagnostic zone, leading to positive serodiagnostic reactions. This finding in smallpox vaccination is likely applicable to other situations in which false positives are obtained.

The universal reaction also indicates why most false positives given by serodiagnostic tests are weak reactions. They are weak because they are partial or incomplete reactions. After the 3-minute shaking period, they need overnight incubation to render them complete reactions. We have already seen that syphilis reactions do not require overnight incubation to render them complete reactions.

Of importance clinically is that false positive reactions given by serodiagnostic tests are false only in relation to syphilis; they are believed to be the result of tissue breakdown in disease or following the injection of various substances. Stated differently, tissue breakdown in syphilis leads to true positives; tissue breakdown in nonsyphilitc situations may lead to false positives. It is well that persons giving false positives be kept under clinical observation to determine the cause of these positives. In most instances false positives are of short duration, lasting only several weeks. Persistent false positives may be indicative of disease. In our opinion any nonsyphilitic disease may lead to a false positive.

AUTOIMMUNE NATURE OF REACTIONS BETWEEN SERUM AND LIPOIDAL ANTIGEN SUMMARY

The findings with our experimental biologically universal reaction with lipoidal antigen support the view that positive serodiagnostic reactions in syphilis and in other situations are autoimmune reactions.

We showed that our biologically universal reaction occurs in health, is increased in diseases studied thus far and reverts to the normal level of reactivity on recovery. The reaction in health is also increased on the injection of various substances. The reaction then also reverts to the normal level after some weeks.

The basis of the universal reaction with lipoidal antigen in health is believed to be the tissue breakdown associated with normal metabolism. This tissue breakdown liberates lipids and proteins leading to antibody formulation against lipids. These antilipid antibodies are then detected *in vitro* with the use of lipoidal antigen.

In disease and following injections of various substances there is an increase in tissue breakdown with a corresponding increase in the formation of antilipid antibodies, leading to stronger universal reactions.

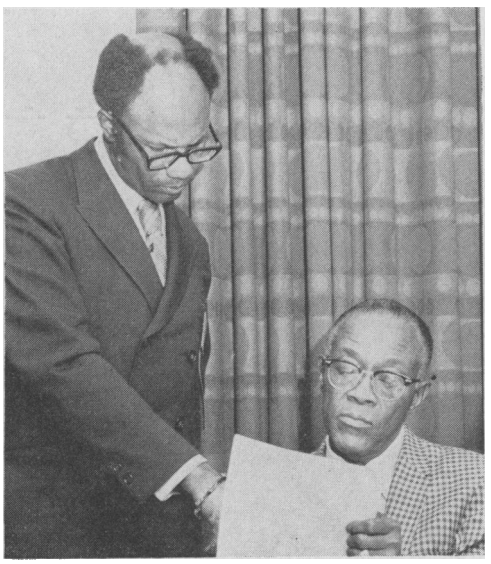
Based on these results of universal reactions with lipoidal antigen, serodiagnostic reactions with lipoidal antigen must also be due to tissue breakdown. It is assumed therefore that positive serodiagnostic reactions in syphilis are due to tissue breakdown in this disease, and that positive reactions in the absence of syphilis are due to tissue breakdown in other situations.

If tissue breakdown is the basis for positive serodiagnostic reactions, it is clear that such reac-

(*Concluded on p.* 183)

Charles C. Morchand, retired president of Charles C. Morchand Co., Inc., New York, printer and business manager of the *Journal*, receives an Honorary Membership Plaque from Dr. Edmund C. Casey, chairman, NMA Board of Trustees. (For previous citation of Mr. Morchand, see this *Journal*, v. 59, pp. 493-494, 1967.)

Lt. Col. Frank Berry Jr. (left) receives from Col. Vance H. Marchbanks, Jr., the "Colonel Vance H. Marchbanks, Jr. Award." Looking on is Maj. Gen. Daniel Thomas Crouch, Deputy Surgeon General, USAF.



Dr. Louis C. Brown, president of the Georgia State Medical Association (left) discusses program at the NMA Preconvention Postgraduate Workshop with Dr. John B. Johnson.

(*Kahn, from p.* 121)

tions in the absence of syphilis are not false positives; that the reason for the reactions should be determined and that persons giving these reactions should be under clinical observation until they become seronegative.

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