A Serological Reaction in Tuberculosis

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As is well known, the Mantoux (1) reaction with tuberculin is quite useful in determining the presence of tuberculous infection, but offers no information as to the presence of disease. When we stop to consider the findings of Ash (2), who ascertained by post mortem examinations that the clinical diagnosis of tuberculosis was only 11.5% accurate, we are brought face to face with the great need for an accurate means of sero-diagnosis. In our zealous attempt to produce this diagnostic aid to medical science, however, we are often prone to rely too strongly on laboratory methods, almost to the exclusion of clinical diagnosis. Obviously, the laboratory cannot supplant the clinician, and laboratory results must be considered as correlary evidence to be accepted in light of clinical findings. In our fight against disease one might well say that the sword of laboratory diagnosis must be tempered with the fire of clinical wisdom.

Perhaps the first serological manifestation of tuberculosis was that recorded by Arlong (3) in 1898, describing specific agglutination of the tubercle bacillus by serum of patients suffering from the White Plague. This work was repeated with the same general results by Koch (4) in 1901. Despite wide and varied experiments by competent workers this agglutination phenomenon has not yet produced a reliable means of serological diagnosis of tuberculosis.

Marzagalli, (5) in 1904 described " ... un nuovo metodo per la sierodiagnosi della tuberculosi", which was the earliest experiment we found recorded on precipitin reactions in tuberculosis. Thus far, no precipitin reaction has stood the test of clinical application, but the most promising method in this class is probably

that described by Doan, (6) in 1929.

As early as 1906 Wassermann and Bruck (7) described a complement fixation reaction for the sero-diagnosis of tuberculosis, but without much success. This work was elaborated on by Besredka, (8) in 1914, Stimson, (9) in 1915, and Wadsworth (10) in 1925. Though the rational of this type of reaction is well grounded, a suitable technique has not yet been developed.

Vernes (11) in 1928, described a non-specific reaction which he thought might solve the problem. This technique embraced the use of resorcin and has been rather thoroughly exploited, but found unreliable. As Hinton (12) points out, the lack of specificity of this reaction limits its use to the determination of activity in a known case of tuberculosis. Other methods following the same general principles have been outlined, but none has received universal recognition due to lack of dependability.

Hinton (12) in 1930, proposed a cholesterol agglutination reaction which he considered very promising. In a series of 18 known positive cases of tuberculosis, he obtained sixteen frank positive serological reactions and two doubtful. On the other hand, in a series of one hundred and seven presumably negative cases, from Boston Lying-In Hospital, he found only two that presented positive serological reactions. One of these two carried a tentative diagnosis of suspected tuberculosis, but no subsequent report has been available on either of the two cases.

Following the lead offered us by Hinton in his work on tuberculosis we have attempted to clarify in our own minds some of the problems entering into the intricacy of sero-diagnosis of tuberculosis.

We obtained stock cultures of living strains of H_{37} and R_1 from Dr. Hinton, and an unknown strain from Phipps Institute. All three strains were cultured on both Sauton's medium and on Long's Synthetic Liquid medium. While Hinton records that neither of these media showed any superiority over the other, it was our experience that the Long's Synthetic Liquid Medium was preferable, in that it produced more rapid and more luxurious growth. Following is the composition of each of the media:

Sauton's Medium:	Grams
Asparagine	4.0
Glycerin	60.0
Citric acid	2.0
Dipotassium phosphate	0.5
Magnesium sulphate	0.5
Ammoniacal iron citrate	0.05
Distilled water	1000.0

Long's Synthetic Liquid Medium	
Asparagine	5.0
Ammonium citrate	5.0
Potassium acid phosphate	3.0
Sodium carbonate (Anhy)	3.0
Sodium chloride	2.0
Magnesium sulphate	1.0
Ferric ammonium citrate	0.05
Glycerol	50.0
Distilled water	1000.0

The three strains of organisms were grown on the two media for a period of seven weeks, after which the liquid was filtered off through fine paper at room temperature, under atmospheric pressure. The residue of live bacilli was washed with sterile distilled water for a total of three washings, each equal in volume to that of the medium filtered off. The moist bacilli were then placed in a 55° incubator where they were allowed to remain for four days to thoroughly dry and kill the organisms.

At the end of four days the bacilli were weighed, and extractions made after the method described by Hinton, consisting of

extracting each of the three strains with four separate portions of ether (Squibb, for anesthesia). Each extraction involved the use of eight volumes of ether for each part by weight of bacilli. During each extraction the flask was vigorously shaken for ten minutes. After the fourth ether extraction, the residue was dried at room temperature and weighed. The ether-insoluble residue was then extracted with five volumes of 95% ethyl alcohol for each part by weight of residue.

It was our experience that this method resulted in an antigen not quite as promising as some derived from other methods of extraction, the most satisfactory of which in our experiments was as follows: Seven volumes of ether (Squibb, for anesthesia) were added for each part by weight of dried bacilli. The suspension was shaken for fifteen minutes, after which time the bacilli were allowed to settle out, leaving a rather milky supernatant fluid. The ether was filtered off and saved for evaporation. This procedure was repeated until a total of five extractions had been made, giving a grand total of thirty-five volumes of ether for each original part by weight of bacilli. The last extract was practically clear. Then the alcoholic extraction was performed according to Hinton's method. This final antigen was pale, straw colored, clear and sparkling.

For the actual serological tests that followed, we believe that the best results were obtained with a mixture of nine parts of 0.6% solution of cholesterol in absolute ethyl alcohol to one part of bacterial antigen.

Similar methods of extraction followed in which we used liver obtained from a patient who had died of miliary tuberculosis.

(The parenchyma of this liver was studded with miliary tubercles.) It was ground fine, dried on a glass plate at 55° for 4 days and weighed. The extractions were made according to those previously described by us. The resulting antigen was entirely unsatisfactory.

In the production of the cholesterinized antigen, which we called antigen, modified, we added nine parts of the 0.6% solution of cholesterol in absolute ethyl alcohol to one part of antigen derived from dried tubercle bacilli, and from this point we followed Hinton's outline for the preparation of what he elects to call 'glycerinated indicator'. The procedure was to add two parts of 5% solution of salt, (Merck, C.P.) to one part of antigen, modified. This mixture was vigorously shaken for one minute, and then twelve additional parts of salt solution were added with a second shaking for one minute. This mixture was allowed to stand ten minutes, and fifteen parts of 50% glycerine (Arthur H. Thomas, re-distilled, neutral) were added, with a third shaking for five minutes. This glycerinated indicator proved quite unstable, and was useless twenty-four hours after it had been mixed.

The sera were inactivated at various temperatures and for different periods of time, but the most promising results were obtained with sera inactivated at 55° for fifteen minutes. Wassermann tubes were set up in series of three rows. Inactivated sera were pipetted into the tubes as follows: 0.1 cc in the front row, 0.3 cc in the second row, and 0.5 cc in the third row. To each of these tubes 0.5 cc of glycerinated indicator was added. The tubes were shaken for three minutes at 250 oscillations per minute by machine. The tubes were incubated at 37.5° for sixteen hours, and then read.

The reading of these reactions, like the reading of the Hinton glycerol cholesterol reaction for syphilis, is among the least difficult of serological interpretations. In cases of known tuberculosis there was marked clearing of the fluid with massive clumping of the particles of cholesterol, producing the effect of a small coagulum of egg albumen in hot water. In the less strongly positive sera clearing was not so marked, and the particles appeared as large granules, similar to the result of a moderately positive Kahn reaction. The negative reactions were opalescent, with no evidence of clearing or clumping. In two series of presumably negative cases a white ring appeared at the meniscus, but in each tube the ring was quickly dispelled by gentle agitation. The following table gives a summary of our results:

Table I

# of Sera	Wassermann	Hinton	Kahn	Clin Diag	X-ray	Sputum	TB Serology .1cc	.3cc	.5cc
109	Neg	Neg	Neg	Pos	Pos	Pos	Positive		
16	Pos	Pos	Pos	Pos	Pos	Pos	Positive		
24	Neg	Neg	Neg	Pos	Pos	Neg	Positive		
3	Pos	Pos	Pos	Pos	Pos	Pos	Doubtful		
9	Neg	Neg	Neg	Neg	Pos	Neg	Positive		
14	Neg	Neg	Neg	Pos	Pos	Neg	Negative		

Table II

# of Sera	Wassermann	Hinton	Kahn	Clin	X-ray	Sputum	TB Serology .1cc	.3cc	.5cc
210	Neg	Neg	Neg	Neg	Neg	Neg	Negative		
116	Pos	Pos	Pos	Neg	Neg	Neg	Negative		
1	Pos	Pos	Pos	Neg	Neg	Neg	Positive		
1	Neg	Neg	Neg	Neg	Neg	Neg	Positive		

From table I it will be observed that in a series of 162 tests of cases diagnosed clinically, by X-ray or by sputum, or some combination

of the three, fourteen gave a negative reaction to tuberculosis antigen and three were doubtful. In this connection it is interesting to note that in Hinton's series of eighteen cases of known tuberculous disease only two were doubtful and none was negative.

Table II shows that in a series of 338 cases of sera drawn from presumably non-tuberculous patients, only two showed positive reactions with tuberculosis antigen. One of these cases (Reg. #9765) also presented positive luetic serology. This patient carried an X-ray interpretation of moderate calcification and fibrosis in the region of the hilus. The other, (Reg. #9845) was admitted with a clinical diagnosis of extensive trychophyton infection and pellagra. A diagnosis of tuberculosis had not been made. Subsequent examination failed to reveal clinical, X-ray or laboratory evidence of tuberculosis.

In the two series there was no apparent correlation between tuberculous and luetic serological reactions, while the clinical and serological manifestations were in accord in more than 90% of the known tuberculous cases, and in less than one per cent of the 338 presumably negative cases were false positive reactions obtained.

As was the case in Hinton's experiments, we found that antigens made from different cultural strains gave different reactions with certain sera. For instance, in one case the antigens from H_{37} and R_1 gave negative reactions, while the antigen from the unknown strain obtained from Phipps Institute gave a strongly positive reaction.

Lest an incorrect interpretation of our findings be made, we feel we should note here that among our 500 serological reactions, often two or three were performed on the same patient, so that, although our number of examinations totals 500, only two hundred and sixty

individual patients were included in the experiments.

The authors feel that this small series but serves as an indication of what may be expected in a larger series, and that standardization of the antigen and of the technique will result in greater correlation of clinical and serological findings. With that ultimate goal in mind, we hope to continue our work in the future.

We pause before concluding to extend our sincere appreciation to Dr. William A. Hinton for providing us with stock cultures of bacilli and for his interest and assistance in carrying on this work, and to Phipps Institute for their contribution of seed cultures without which our progress would have been greatly impeded. We wish, too, to thank the Veterans Administration and Colonel J. H. Ward for providing us with laboratory facilities and for their permission to use the sera of patients hospitalized at the Veterans Administration Facility at Tuskegee, Alabama.

Conclusion: A brief report of a series of experiments with a serological manifestation of tuberculosis has been presented.

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