CARDIOLIPIN ANTIGEN

I. A QUALITATIVE EXAMINATION OF SENSITIVITY AND SPECIFICITY

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Since Pangborn (1941a,b) succeeded in isolating cardiolipin and purified lecithin from beef hearts, these two substances, in conjunction with purified cholesterol, have been the subject of numerous investigations with regard to their suitability as antigens in serological tests for syphilis.

Cardiolipin is a complex phosphatide, free from nitrogen, soluble in ether, chloroform, and alcohol, but insoluble in acetone. When used alone, cardiolipin has an anticomplementary effect (Maltaner and Maltaner, 1945). Purified lecithin alone has no antigenic effect (Pangborn, 1948); according to other writers (Brown, 1946), lecithin was found to produce weak reactions with syphilitic sera from rabbits and human beings, but positive reactions were also found with sera from non-syphilitic patients. A mixture of cardiolipin and lecithin is not anticomplementary and acts as an antigen (Brown, 1944); the addition of cholesterol to a non-optimal mixture of cardiolipin and lecithin increases the antigenic effect. The addition of increasing amounts of cholesterol to an optimal adjustment of cardiolipin and lecithin results in a gradual decrease in activity, and addition of cholesterol to non-optimal adjustments increases the antigenic reactivity of the mixture up to a certain optimum, after which any further addition of cholesterol will cause a decline in activity (Brown, 1944). There are several reports on the suitability of the cardiolipin antigen for the sero-diagnosis of syphilis (Harris and Portnoy, 1944; Rein and Bossak, 1946; Hume and Guthe, 1947; Andajar and others, 1948; Levine and others, 1948; Vogelsang, 1948; Lundbäck, 1949; Price and Wilkinson, 1950). With a few exceptions (such as Kahn and McDermott, 1948; Boas, 1950), the authors agree that the cardiolipin antigen is an important improvement, in that it is more sensitive, more specific, and comparatively easy to reproduce. Since cardiolipin and lecithin are chemically quite well defined, and cholesterol is a chemically pure substance, it would seem possible by investigation of the optimal adjustments to develop once and for all a mixture which could be dispensed by weight, thus obviating the time-consuming adjustment experiments required with unpurified antigens.

The cardiolipin investigations in Denmark are of fairly recent origin, partly on account of the war, and this work includes a report on the first Danish investigations of the suitability of the cardiolipin antigen for complement-fixation experiments and for a slide test according to the method suggested by the Venereal Disease Research Laboratory (VDRL), Staten Island, New York (Harris and others, 1946).

The above-mentioned tests have been compared with the routine tests used by the Sero-diagnostic Department of the State Serum Institute, Copenhagen, viz. Wassermann’s complement-fixation test as modified by Mørch (WR-M), Kahn’s standard test (KR) and Meinicke’s clarification test (MR).

The material examined is to some extent influenced by the fact that the incidence of fresh syphilis cases in Denmark is low: according to the latest statistics only 3 to 4 cases annually per 10,000 inhabitants. It has been possible, however, to obtain from the Central Register of Syphilitic Patients, adequate data on the great majority of the patients. The Central Register of Syphilitic Patients was established in 1920 on the initiative of the late Physician-in-Chief Olaf Jersild (Jersild, 1919; Madsen and Krag, 1937). The register now includes practically all patients with syphilis diagnosed after 1920, and also a number of older cases diagnosed previously. In addition, it includes a number of patients with positive sero-reactions who present neither clinical evidence nor history of syphilis, i.e. mostly reactions which must be considered non-specific in the light of information obtained through questionnaires on such patients.
MATERIAL AND METHODS

The experiments were performed during 20 days, viz. the first five weekdays of each week from October 8 to November 4, 1949, inclusive. Qualitative complement-fixation tests with cardiolipin antigen, VDRL and MR, were performed only on specimens that had reacted in WR–M and/or KR screen-tests. These examinations were carried out concurrently with routine quantitative determinations of titres with WR–M. The examination in C–WR–M was generally only evaluated qualitatively on the basis of the haemolysis per cent. in one tube containing the maximum amount of serum used for the quantitative determination of WR–M. In cases where there were appreciable discrepancies between the WR–M and the C–WR–M reactions, such sera were examined quantitatively with both antigens on the following day. VDRL-slide tests and MR were carried out on nearly all the sera in the material where sufficient serum was left after doing the routine tests.*

All samples which showed discrepancies between WR–M, KR, and MR were subjected to repeated routine examinations; in such cases the samples were again examined with cardiolipin antigen. For sera which have thus been examined more than once, only the last result has been taken into account.

In order to achieve the best possible differentiation between the different categories of sera, questionnaires intended to elicit clinical evidence or history of syphilis were sent to the senders of positive samples from patients not included in the Central Register of Syphilitic Patients. In the case of positive samples from non-syphilitic patients, enquiries were also made about their symptoms and diagnoses. Hospitals and doctors who failed to answer the questionnaires received reminders; in the great majority of cases these efforts to collect the desired details were successful.

During the 20 days, 1,322 blood samples from 1,197 patients (502 men and 695 women) were examined. Two samples were received from a number of patients, and in a few instances three samples were submitted. Only in a very few cases was such duplication of samples the result of a request by this Institute; normally, the duplicate samples were sent in by doctors wishing to follow up the results of their treatment; hence, the double (or triple) samples come rather more often from fresh cases than from older ones.

Under the given conditions, each patient’s status could not be evaluated until the Central Register had collected the preliminary data (which generally takes about 3 months); collection of supplementary details required another 3 months with the result that complete data on the material were not available until about 6 months after the 20-day period.

The material was divided into five groups (Table I). The criteria employed for the individual groups were as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Patients</th>
<th>Number of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&quot;Fresh&quot; 154</td>
<td>186</td>
</tr>
<tr>
<td>2</td>
<td>&quot;Old&quot; 706</td>
<td>768</td>
</tr>
<tr>
<td>3</td>
<td>206</td>
<td>221</td>
</tr>
<tr>
<td>4</td>
<td>69</td>
<td>77</td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td>1,197</td>
<td>1,322</td>
</tr>
</tbody>
</table>

* MR is not performed as a routine test on all sera, but only for verification of positive results from WR–M and/or KR, by special request, and when a specimen has already been submitted for serological examination within the preceding six months.

into fresh cases (less than 12 months after infection), and old cases (more than 12 months after infection).

Group II: Sera from patients in whom the possibility of syphilis could be excluded with almost complete certainty, i.e. negative sera from patients for whom no registration card was to be found, and in whom clinical or anamnestic information on syphilis was absent, and positive sera from patients with no clinical or anamnestic evidence of syphilis but in whom the sero-reactions had become negative without antisypilic treatment. Also in such cases, information usually indicated that the patient suffered from a disease shown by experience to be capable of producing a positive non-specific sero-reaction (Reyn, 1941; Wiingaard, 1948).

Group III: Strongly positive sera from patients without clinical or anamnestic evidence of syphilis. These sera had been strongly positive for a long period and reacted less positively or not at all after antisypilic treatment.

Group IV: Sera from patients without clinical or anamnestic evidence of syphilis, but regarding whom the information obtained suggested that the possibility of syphilis could not be definitely excluded.

Group V: Sera from patients with incomplete data, either they could not be identified or because completed questionnaires were not returned by the hospital or doctor concerned.

TECHNIQUE AND READING OF RESULTS

ANTIGENS

Kahn Standard Reaction (KR).—This was performed with an antigen prepared at the State Serum Institute according to the method and technique described by Kahn (1928 and 1946); The titre of the antigen was 1–1.2. The results are recorded in degrees of potency according to the system adopted for the WR–M, see p. 26.
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Meinicke Reaction (MR).—This was performed with an antigen prepared at the State Serum Institute according to the method described by Meinicke (Meinicke, 1932; Josephson, 1936); the technique adopted for the preparation of the antigen was that used by the latter author. The MR was read both as a precipitation test and a clarification test. The results are recorded as: strong positive (+++) positive (+), weak positive (±) and negative (−).

Slide Test (VDRL).—This was the Standard Slide test (Harris and others, 1946), but slides of Thomas’ type with concave glasses were employed instead of the plane Permaslides used by the V.D. Research Laboratory. VDRL Flocculation Antigen Lot No. 4910 was used for the whole of this series of experiments. This antigen contains 0.3 per cent. cardiolipin, 0.9 per cent. cholesterol, and has a content of lecithin “adjusted in such a way that, compared with previous preparations, this antigen produces standard activity”. The readings were recorded as: positive (+), weak positive (±), negative (−), the designation (±) comprising both strong positive and positive reactions.

Wassermann–Morch Complement-Fixation Test (WR-M).—The antigen was prepared by alcoholic extraction of acetone-treatedveal-heart powder, fortified by the addition of equal parts of cholesterol in alcohol, generally from 2.5 to 3.3 per thousand (Krag, 1938). Before use, the alcoholic antigen was mixed with physiological saline in almost equal proportions (at present 1:1.1) and left for 10 minutes. The next dilution was made in a large volume of physiological saline (at present 190 ml.) according to a titre predetermined for each lot of antigen. The results were expressed in degrees of potency, see p. 26.

Cardiolipin Complement-Fixation Test (C-WR-M). This was performed with an alcoholic solution of American cardiolipin lot C35–37 and American lecithin lot L3/45, both received from Dr. Mary C. Pangborn, Albany, N.Y. The ratio was: 5 parts lecithin to 1 part cardiolipin, and 3.4 parts cholesterol to 1 part lecithin. The dilution used was 1:133 in physiological saline. Apart from the fixation times, the technique was the same as in WR-M.

SERA.—The sera examined were inactivated by heating at 56°C for 30 minutes, and re-inactivated for 10 minutes at 56°C. before the VDRL slide test and KR if these tests were performed more than 4 hours after the first inactivation.

DETAILS OF PROCEDURE.—Only the complement-fixation experiments will be dealt with in detail, the other tests having been carried out according to the standard technique. The same technique was also followed for the C-WR-M and the WR-M, apart from the fixation times. In the WR-M test the serum-antigen-complement mixture “fixes” for 45 minutes at room temperature and also for 45 minutes at 37°C. In the C-WR-M the primary fixation time is 2 hours at 4°C. and 30 minutes at 37°C. After addition of the blood, the haemolysis time for both C-WR-M and WR-M is 1 hour at 37°C.

Quantitative Technique.—The quantity of serum in the first tube was 0.025 ml., in the second 0.025, diluted 1 in 3, in the third 0.025, diluted 1 in 9, and in the fourth 0.025 diluted 1 in 27 ml. In accordance with the predetermined titre the antigen was diluted with physiological saline (WR-M 1 + 1:1 – 190, C-WR-M 1:133), and to two-thirds by volume of these antigen-saline dilutions was added one-third by volume of buffer saline in which complement had been dissolved so that 0.3 ml. of the mixture contained one haemolytic unit, determined by the presence of antigen in preliminary experiments. 0.2 ml. of a 2.5 per cent. sensitized suspension of sheep-blood cells was added. The total volume was thus about 0.5 ml., and the serum dilution in the first tube was 1:12 before the blood was added.

Haemolysin.—The haemolysin used was the serum from rabbits immunized either with washed sheep-blood cells or with guinea-pig-kidney emulsion (Forssman, 1928). After the collection the serum was diluted with equal parts of purified glycerine. The haemolysin titre was determined in the presence of excess complement, one haemolytic unit being determined as the minimum volume of rabbit-immune serum sufficient to haemolyse completely 0.2 ml. of 2.5 per cent. sheep-blood cells suspended in a total volume of 0.5 ml., corresponding to the total volume used in the complement-fixation test. Titration was done with every change of complement or sensitized sheep blood in the main experiment; three units of haemolysin were used for complement titration.

Blood.—Sheep-blood cells were washed three times and stored at +4°C. From the material treated in this manner a suspension of sheep-blood cells in buffer saline was prepared daily; the pH was adjusted to 7.38 and the concentration of cells was 5 per cent. Immediately before being used in the complement-fixation test this 5 per cent. suspension was diluted with an equal volume of physiological saline to which had been added sufficient haemolysin for 0.1 ml. to contain three haemolytic units.

Complement.—Fresh guinea-pig serum was obtained several times a week from at least twelve to fourteen healthy guinea-pigs. The complement was stored at –8 to –10°C.; it was generally titrated on the day before the main experiment. The haemolytic power was determined in the presence of antigen. One haemo-
lytic dose of complement was defined as the amount required to haemolysed completely 0.2 ml of a sensitized sheep-blood-cell suspension (2.5 per cent. sheep-blood cells).

Readings.—In the qualitative test (screen test) the results were read immediately the negative sera were completely haemolysed. In the quantitative test the tubes were left to haemolysed for 1 hour at 37°C, and the results were read the next morning when the tubes had been left overnight at 4°C. The degree of haemolysis in the individual tubes was evaluated by direct comparison with a haemolytic scale prepared on the basis of the completely haemolysed negative samples, the scale having the values 0 per cent., 20 per cent., 40 per cent., 60 per cent., and 100 per cent. The haemolytic values obtained correspond to the percentage of haemolysis in four tubes with decreasing concentrations of serum; they were converted from haemolytic percentages into "degrees of potency" by means of a specially designed "slide rule" (see Figure). The degree of potency is defined as the value for \( n \), rounded to a whole number in the formula \( a = 0.025 \times 3^n \) required to make a exactly the volume of serum containing just enough reagin to produce a minimum reaction, i.e., 60 per cent. haemolysis in the tube with the highest serum concentration. Since new complement and new sheep blood was received several times every week, adjustments had to be made for variations in the sensitivity of these components; this was done by preliminary examination of six "standard sera", the average potency of which had been determined by previous experiments. The average value of those sera was found by examining them on 10 separate days. The numerical average of these readings is described as the "standard value" (Kristensen, 1930). As mentioned above (p. 24) the degree of potency was calculated for the KR in more or less the same way. Here, too, the serum was diluted in the quantitative test, according to series of quotients with a quotient of 1/3. The weakest positive reaction is the presence of floccules of the order 1 in the first tube which contains 0.15 ml undiluted serum and no visible floccules in the other three tubes.

**Author's Investigations**

The sensitivity of an antigen may be defined as its capacity to produce a positive reaction with a serum from a patient with definite clinical and/or anamnestic syphilis. The diagnostic application of sero-reactions is based on the fact that sera from syphilitic patients who have either not been treated, or whose treatment has not yet been completed, contain reagin. This may not always be absolutely correct for the following reasons:

(i) Some patients with syphilis who have been clinically cured may continue to have reagin in their blood for a considerable time;
(ii) Some patients form no reagin;
(iii) Some reactions are non-specific.

Sera from definite cases of syphilis are known to react differently: some react negatively with all serological tests, some react positively to one test and negatively to others, and some react positively with nearly all antigens.

However, two methods which each give 60 per cent. of positive reactions with a given group of sera for example will not necessarily react with the same sera; in other words, a serum may react positively with one technique and negatively with another. Qualitative difference in the reagin or in the antigens may account for these discrepancies. For example, it is well known that the KR is more sensitive in older cases of syphilis than in fresh cases, whereas the reverse is true for the WR—M (Jersild, 1936).

A comparison of the sensitivity of the individual antigens is only possible as a comparison between the specific positive reactions with the different antigens.

In the present material the sensitivity is considered exclusively as a qualitative property without taking the degree of potency of the serum into account. A haemolytic percentage in the C—WR—M tube showing ≤ 50 per cent. haemolysis is counted as a positive reaction because when performed quantitatively as a rule such a reaction will show a degree of potency of ≥ 1.

**Sensitivity.**—As a first step, using the five different tests, the number of positive reactions was determined among sera from patients with syphilis, both fresh and old (Group I, Table II, overleaf). Some samples of serum were insufficient to permit examination by more than the routine tests. This is of practical significance only for the MR and the VDRL. In the case of the MR, 27 sera were not examined, and four were from patients with fresh syphilis. In eighty cases including ten of fresh syphilis there was insufficient serum for the VDRL slide test. These cases not examined for the MR and the VDRL form only a small proportion of the fresh cases which make up the smallest part of the material. Naturally, adjustments have been
made for the missing tests in the computation of the sensitivity within the individual groups, but it has not been considered necessary to disregard all these tests performed, merely because one or two had not been carried out on account of insufficient serum.

"Non-Readable" Reactions.—This description refers to reactions where the difference in haemolytic percentage between two consecutive titrations was \( \leq 30 \) per cent., since such sera react differently from the normal sera on which the graduation of the "slide rule" is based. Non-readable reaction in KR means that tubes containing decreasing amounts of serum show atypical flocculation. "Non-readable" reactions were always repeated, and if they were still "non-readable" after renewed examination, they were listed as such in a special group of the material, but were included among the positive reactions.

The highest degrees and relative uniformity of sensitivity were found for the MR and VDRL, viz. about 90 per cent. for the entire material if the weak-positive reactions are taken into account in the sensitivity survey. The two complement-fixation tests and the KR showed almost the same degree of sensitivity, viz. from 73 to 76 per cent. It should be noted, however, that in the autumn of 1949 the KR-antigen prepared at this Institute according to the method prescribed by Kahn, was found to be slightly weaker than one received from Dr. R. L. Kahn himself for comparison.

However, these summary tabulations of sensitivity comprise the whole material of Group I (patients with definite syphilis). It is also interesting to compare differences in the sensitivity of the different antigens between fresh and old cases in Group I. The fresh cases include those diagnosed within the last twelve months, and all sero-positive patients whose anamneses showed possibilities of infection within the last year. Sero-positive patients without symptoms of fresh infection or any definite anamnesis for potential infection within the last twelve months were included under old cases. Table III shows the results of the sensitivity examinations for fresh and old syphilis.

According to the overall survey of fresh cases, the WR-M, and perhaps also the KR, appear to be more sensitive than the C-WR-M, viz. 85 per cent., 76 per cent., and 70 per cent., respectively.

### Table II

<table>
<thead>
<tr>
<th>Tests</th>
<th>WR-M</th>
<th>C-WR-M</th>
<th>KR</th>
<th>MR</th>
<th>VDRL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>++/+</td>
<td>±</td>
</tr>
<tr>
<td>Samples examined</td>
<td>952</td>
<td>951</td>
<td>953</td>
<td>927</td>
<td>874</td>
</tr>
<tr>
<td>Positive reactions</td>
<td>723</td>
<td>699</td>
<td>695</td>
<td>486</td>
<td>348</td>
</tr>
<tr>
<td>&quot;non-readable&quot;</td>
<td>8</td>
<td>3</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Percentages of Sensitivity</td>
<td>75·9</td>
<td>73·5</td>
<td>72·9</td>
<td>52·4</td>
<td>37·5</td>
</tr>
</tbody>
</table>

### Table III

<table>
<thead>
<tr>
<th>Patients</th>
<th>With &quot;Fresh&quot; Syphilis</th>
<th>With &quot;Old&quot; Syphilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tests</td>
<td>WR-M</td>
<td>C-WR-M</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Samples examined</td>
<td>186</td>
<td>186</td>
</tr>
<tr>
<td>Positive reactions</td>
<td>159</td>
<td>130</td>
</tr>
<tr>
<td>&quot;non-readable&quot;</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Percentages of Sensitivity</td>
<td>85·5</td>
<td>69·9</td>
</tr>
</tbody>
</table>
The sensitivity of MR and VDRL is almost equal for the fresh cases and of approximately the same degree as that of the WR–M, about 80 per cent. when all degrees of reaction (strong-positive, positive, weak-positive) are included.

For older cases of syphilis in this material the C–WR–M was found to be slightly more sensitive than the WR–M and the KR, viz. about 74 per cent. against 73 per cent. and 72 per cent. respectively. The MR and VDRL are also very sensitive for older syphilitic cases, and far more sensitive than any of the other tests when all degrees of potency are included, viz. about 90 per cent. sensitivity.

The sensitivity of the WR–M and the C–WR–M was further compared by dividing fresh syphilitic cases into three categories: primary (SI), secondary (SII), and “fresh latent” syphilis. The “fresh latent” category comprises patients infected within the last year, but presenting no clinical signs of syphilis when the sample was submitted for examination. The results in these three stages are also compared with those for the older cases.

The two sensitivity percentages are not directly comparable, because some of the sera are identical and the usual significance-tests assume that the two materials to be compared are different. Comparison of two sets of sensitivity percentages is only possible by establishing how many of the negative reactions obtained from one method produce negative and positive reactions with the other method, and by accounting for the positive sero-reactions from the first method in the same manner. The reactions can now be tabulated in pairs thus:

\[
\begin{array}{c|c|c|c}
 & SI & C–WR–M \\
\hline
- & + & \\
- & 9 & 0 & 9 = 13\% \\
WR–M & + & 18 & 40 & 58 = 87\% \\
\hline
27 & 40 & 67
\end{array}
\]

The sensitivity percentages for WR–M and C–WR–M in this first example (SI) are 58/67 (= 87 per cent.) and 40/67 (= 60 per cent.) respectively. These sensitivity percentages deviate significantly from each other, in as much as the 40 deviates more than accidentally from the 58. These two figures originate partly from the same material, since 40 reactions were positive by both methods. Ultimately, therefore, we arrive at the question whether the deviation between 0 -/+ and 18 +/− is more than accidental, or whether the concurrent reactions are distributed equally between +/− and −/+.

If that is so the probability for both +/− and −/+ will be one-half, in which case the distribution over the two groups must conform to the binomial theorem according to which the probable distribution of \( n \) reactions with \( a \) in one group and

\[
\left(\frac{n}{a}\right)^a
\]

with \((n - a)\) in the other is. The hypothesis of equal distribution may be tested by statistical criteria described in ordinary manuals (Fisher, 1946; Hald, 1948). In the above example it will be found that on the assumption of equal distribution the probability of distribution, which is at least as extreme as the one established, will be \( P = \text{about } 10^{-5} \). Since this probability is very remote, the hypothesis of equal distribution must be discarded, and we must accept that C–WR–M is less sensitive than WR–M in the material in question (SI).

The reactions are listed opposite for each stage in four categories according to a qualitative evaluation. More than 50 per cent. haemolysis in the C–WR–M has been counted as a negative reaction. Calculations made on this basis show that:

(i) the sensitivity of the WR–M is significantly greater than that of the C–WR–M \( (P=10^{-5}) \) for the SI (primary syphilis) group.

(ii) there is no significant difference between the sensitivity of the WR–M and the C–WR–M in the SII (secondary syphilis) group.

(iii) the WR–M is significantly more sensitive than the C–WR–M \( (P=0.5 \text{ per cent.}) \) in the “fresh latent” group.

(iv) there is no statistically demonstrable difference between the sensitivities of the two tests in the “old” cases.

(v) the difference between the numbers of the combination WR–M plus/C–WR–M minus in SI and SII is significant \( (P = 0.1 \text{ per cent.}) \).

The quantitative difference in sensitivity between the WR–M and the C–WR–M has not been calculated because the material from titrated sera in the C–WR–M was selected. A comprehensive study on the quantitative reactions in the two complement-fixation tests performed under analogous conditions will be published later.

The diagrams show that a number of fresh cases would be lost if the C–WR–M was used instead of the WR–M, but as experience shows that the reactions of the individual syphilitic sero-reactions...
CARDIOLIPIN ANTIGEN—I

Out of 38 cases (23 old and 15 fresh) fulfilling the requirements of the above combination of complement-fixation tests, the WR-M gave the only positive reaction in five cases. This means that about 2 per cent. of the 275 titrated syphilitic sera would have remained unnoticed if the WR-M had not been performed. This loss of a few fresh cases should be viewed in the light of the number of non-specific reactions which may be avoided by exclusion of the WR-M: see the paragraph on specificity examinations. Among these 38 cases, 31 were MR positive or weak-positive, 26 were VDRL positive or weak-positive, and 24 were KR positive. Hence, the MR, VDRL, or KR react positively with the majority of sera from cases which react negatively with the C-WR-M.

SPECIFICITY.—Before proceeding to the sensitivity examinations for the various combinations of reactions, an attempt will be made to evaluate the specificity on the basis of Group II (patients in whom the possibility of syphilis may be disregarded with almost complete certainty). This examination of the specificity must be treated with great prudence, and considered as a preliminary orientation only, because the number of patients included in the material is relatively small (Table IV).

<table>
<thead>
<tr>
<th>Test</th>
<th>C-WR-M</th>
<th>WR-M</th>
<th>KR</th>
</tr>
</thead>
<tbody>
<tr>
<td>“non-readable”</td>
<td>8</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Degrees of Potency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>14</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>3-4</td>
<td>4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5-5</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Positive Reactions</td>
<td>30</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Percentage Patients</td>
<td>15.3</td>
<td>2.6</td>
<td>10.2</td>
</tr>
</tbody>
</table>

The number of non-specific positive reactions is numerically much lower for C-WR-M than for WR-M, and the difference in specificity was found

are qualitatively different, it is improbable that a single test will be considered sufficient. At least a precipitation test should be performed as well as a complement-fixation test.

Among 275 definitely syphilitic sera examined quantitatively in C-WR-M and concurrently subjected to the five tests reported in this paper, the other reactions were compared in cases where the complement-fixation tests showed WR-Mplus/C-WR-Mminus, to ascertain how many syphilitic sera would escape notice by excluding the WR-M.

<table>
<thead>
<tr>
<th>Test</th>
<th>C-WR-M</th>
<th>WR-M</th>
<th>KR</th>
</tr>
</thead>
<tbody>
<tr>
<td>“non-readable”</td>
<td>8</td>
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<td>8</td>
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<tr>
<td>Degrees of Potency</td>
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<td>1-2</td>
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<td>1</td>
</tr>
<tr>
<td>Positive Reactions</td>
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<td>20</td>
</tr>
<tr>
<td>Percentage Patients</td>
<td>15.3</td>
<td>2.6</td>
<td>10.2</td>
</tr>
</tbody>
</table>

The number of non-specific positive reactions is numerically much lower for C-WR-M than for WR-M, and the difference in specificity was found
to be significant, \((P = 10^{-5})\). There was no demonstrable difference between the specificity of WR–M and KR.

Since this material was selected (in the sense that it was determined by means of WR–M and KR) these two tests are placed in a position which is more unfavourable than it should be.

Finally, the MR was found to be very specific, and significantly more specific than VDRL \((P = 1\) per cent.). In previous reports, notably from Scandinavia (Krag and Lønberg, 1938; Vogelsang, 1941; Lindau, 1945; Wiingaard, 1948), the MR was accepted as being both specific and sensitive. If the reactions are considered not only qualitatively, but also in the light of the strengths, it is conspicuous that the MR is represented by the weakest degree of reaction; to some extent this also applies to the VDRL. The weaker reactions show the highest figures also for the other three tests. The “non-readable” reactions were included as positive; the designation “non-readable” means that the course of the haemolysis percentage with decreasing serum concentrations is atypical, so that a degree of potency cannot be assigned to the serum concerned according to our system, but such a serum does not react negatively.

The non-specific reactions which were positive in the VDRL were not positive in the C–WR–M at the same time; this might suggest that the non-specific reactions were due to the serum concerned or to the technique used, rather than to the antigen, since the antigens in both C–WR–M and VDRL contain the same purified components, although in slightly different amounts.

Groups III, IV, and V will not be considered in detail; according to their definition they are unsuitable for the evaluation of sensitivity and specificity. An attempt will however be made in a later report to shed more light on these groups, especially Group IV.

**Determination of the Optimal Combination of Three Different Methods**

In selecting a combination of different methods, the uses for which it is intended should be clearly defined. Generally, the object is to combine high sensitivity with high specificity, but which of these is the more important depends to some extent on the material to be examined. Thus, the ratio between fresh and old cases, and the incidence of diseases with a known tendency to produce non-specific reactions, may influence the selection. At the Sero-diagnostic Department of the State Serum Institute, the practice so far has been to use the WR–M and KR as routine tests, partly for screening and partly for quantitative determination, and the MR in special cases. This preliminary investigation of the individual tests shows:

For all cases of syphilis taken as a whole, the MR and the VDRL were the most sensitive, the complement-fixation tests came next, and the KR was the least sensitive. If fresh and old cases were examined separately, the WR–M was significantly the most sensitive in fresh cases, and the C–WR–M and the WR–M showed equal sensitivity in the older cases.

In regard to specificity, the WR–M was significantly more than the C–WR–M; the MR and the C–WR–M as a whole, and the VDRL for non-specific positive reactions (i.e. weak-positive reactions not included), produced the best results. If, therefore, it was found expedient to use a complement-fixation test and a flocculation or precipitation test which met our original requirements (high sensitivity and high specificity), the C–WR–M and the VDRL slide-test could be used, knowing that this would involve the risk of missing a few fresh cases. In addition to these two tests, it would be desirable to apply a third as an extra control, for which purpose the MR would probably be suitable.

However, the isolated examinations on which this judgment is based are not sufficiently exhaustive and the results of the different combinations must be investigated. The five different tests are capable of being combined in ten different ways, in sets of three. Only five of these have been examined here, the KR being the least sensitive having been omitted, except in our routine combination WR–M/KR/MR. The results have been tabulated for “old” and “fresh” syphilis according to the criteria previously laid down (Table V, overleaf).

As was to be expected, the overall sensitivity of the five combinations examined is fairly uniform, with a total sensitivity for all combinations >96 per cent.

The combination WR–M/MR/VDRL has the highest percentage of sensitivity, but there is no significant difference in the sensitivity of this combination and that of MR/C–WR–M/VDRL which comes second \((P=6\) per cent.). The differences between the combination WR–M/MR/VDRL and the others (WR–M/KR/MR, WR–M/MR/C–WR–M, and WR–M/C–WR–M/VDRL) are significant \((P\) being 0.1 per cent., 0.1 per cent., and \(0.5 \times 10^{-4}\) respectively). Despite these relatively small percentage variations, it is possible to demonstrate significant differences; this is explained by the relatively large number of old cases. In the fresh cases, no significant differences can be demonstrated between the various combinations.

In view of the greater specificity of the individual reactions from the combination C–WR–M/VDRL/MR (Table IV) it appears advisable to prefer that combination.

**Discussion**

These preliminary investigations seem to corroborate the opinions of most investigators that cardiolipin antigen is both sensitive and specific.
CARDIOLIPIN ANTIGEN—I

TABLE V

PERCENTAGES OF SENSITIVITY IN VARIOUS COMBINATIONS OF THREE TESTS FOR "FRESH" AND "OLD" SYPHILIS

<table>
<thead>
<tr>
<th>Combinations of Tests</th>
<th>WR-M/VDRL/M</th>
<th>WR-M/C-WR-M/VDRL/M</th>
<th>WR-M/VDRL/M/KR</th>
<th>C-WR-M/VDRL/M/KR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of &quot;old&quot; cases</td>
<td>728</td>
<td>724</td>
<td>663</td>
<td>680</td>
</tr>
<tr>
<td>Percentage sensitivity</td>
<td>96-3</td>
<td>97-1</td>
<td>98-3</td>
<td>96-2</td>
</tr>
<tr>
<td>Number of &quot;fresh&quot; cases</td>
<td>181</td>
<td>181</td>
<td>168</td>
<td>174</td>
</tr>
<tr>
<td>Percentage sensitivity</td>
<td>91-7</td>
<td>91-7</td>
<td>94-6</td>
<td>91-9</td>
</tr>
</tbody>
</table>

Our work has also confirmed the high degree of sensitivity of the WR-M in primary and secondary syphilis as well as in "fresh latent" cases. The inferior sensitivity of C-WR-M compared with WR-M in primary and "fresh latent" cases has also been pointed out. The greater sensitivity of the WR-M in fresh cases has been demonstrated before (Lundbäck, 1949), but he only distinguished between primary and other cases of syphilis. A greater sensitivity of complement-fixation tests with cardiolipin antigen in older cases was found by Andujar and others (1949), but no results were reported for fresh cases.

The missing of a few fresh cases of syphilis through the use of C-WR-M instead of WR-M will be compensated to a certain degree by the fact that the fresh cases are generally diagnosed clinically, and that the sero-reactions merely confirm the significance of chancre, Spirochaeta pallida, and secondary manifestations. The secondary cases will mostly be serologically positive with cardiolipin antigen.*

An extended fixation time in the C-WR-M tests has been examined in a number of sera. Instead of the 2-hour period at 4°C, used in the routine examinations for the antigen-complement-serum mixture, 8 and 24 hours at 4°C have been tried. These experiments are still in progress, and the results will be published later. So far it can be said that, as was to be expected, the sensitivity is appreciably increased by an extended fixation time, but the specificity is somewhat affected by an 8-hour period and appreciably affected by a 24-hour period. Extension of the fixation time is by no means a new idea; Kolmer used 18 hours' fixation at 6 to 8°C (Kolmer and Lynch, 1948).

Quantitative examinations of sera from old cases of syphilis suggest that quantitatively the C-WR-M is somewhat stronger than the WR-M, and positive reactions are occasionally found with C-WR-M when the WR-M tests are negative.

Many non-specific reactions can be avoided by using cardiolipin antigen. The WR-M and C-WR-M have been examined in parallel experiments, and the results will be published later.

In diseases known to produce non-specific reactions, Rein and Bossak (1946) found somewhat greater specificity with cardiolipin, but non-specific reactions still occurred, especially in mononucleosis, malaria, and leprosy. Similar experiences have been reported by Stout (1947), Kent and others (1948), and Stokes and James (1949).

In regard to the specificity of the WR-M, a report of comprehensive investigations was published by Wiingaard (1948), who found that 0-24 per cent. of all blood samples submitted were non-specifically positive in the WR-M and/or KR and MR. This percentage represents about 1,000 non-specific reactions annually out of the 400,000 samples received each year. In order to achieve a better appraisal of the advantages and disadvantages of the WR-M, this test was investigated for the year 1943. The investigation was based on the monthly statistics prepared by the Sero-diagnostic Department of all the positive blood samples which were not already to be found in the files of this Institute. This statistical material was supplemented by details obtained from the Board of Health to which all newly diagnosed cases of syphilis must be notified. Practically all diagnoses of fresh syphilis are thus covered by the statistics, including those with negative blood samples. At the time in question, the incidence of syphilis was very high. The investigation revealed that out of 1,305 definite cases of primary syphilis verified by demonstration of Spirochaeta pallida and/or typical chancre, 5-3 per cent. had the WR-M as the only positive reaction, while the combination of WR-M/KR was positive in 61-4 per cent. of the primary cases. However, 24-4 per cent. of the 340 non-specific
cases recorded in the same year were positive for the WR–M only.
As the incidence of fresh syphilis is now declining sharply to the pre-war level, while sero-diagnostic examinations are being more frequently performed as a routine test, the question arises whether the few undetected positive reactions in patients with fresh syphilis are not offset by the fact that a considerable number of non-specific, positive WR–M reactions (a number which is expected to increase in proportion with the number of samples examined) will be avoided.

Non-specific positive sero-reactions constitute a particular problem in pregnant women, because the risk to the foetus of congenital syphilis does not permit a long period of observation (Krag and Røjel, 1947; Stokes and James, 1949; Idsøe and Vogelsang, 1950).

The slide-test with cardiolipin antigen is a very sensitive and rather specific method requiring little equipment. Isolated, weak-positive reactions will hardly be of any major importance, but should be evaluated in the light of the results from macro-tests.

The MR has again proved to be very sensitive and specific, despite the relatively simple method of preparing the antigen.

The results of the tests with the KR will not be discussed in detail because of the special conditions in connexion with the sensitivity of the antigen used for that test.

Summary

(1) The experiences of previous writers with the cardiolipin antigen are summarized. The technique used in the State Serum Institute for complement-fixture tests is described.

(2) The author’s investigations comprise 1,322 blood samples divided into various groups according to clinical and anamnestic data.

(3) The five following sero-reactions have been compared: Wassermann’s complement fixation as modified by Morch (WR–M); complement fixation with cardiolipin using the same technique as the WR–M (C–WR–M); Kahn’s standard reaction (KR); Meinicke’s clarification test (MR); and a slide test according to the method described by the Venereal Disease Research Laboratory (VDRL).

(4) The WR–M is found to be significantly more sensitive than the C–WR–M in primary and “fresh latent” cases, whereas there is no demonstrable difference in sensitivity between the WR–M and the C–WR–M in secondary and “old” cases. The KR is the least sensitive test, and MR and VDRL are the most sensitive. The Kahn antigen used in these experiments was a little weaker than a standard Kahn antigen received later for comparison.

(5) WR–M is significantly less specific than C–WR–M. MR is the most specific reaction.

(6) By means of tests combined in groups of three, an attempt was made to determine which combination or group of combinations produces the best results. The number of sera examined from “fresh” cases was too small to give any statistically definite result, but the number of sera from “old” syphilitic cases was large enough to provide a basis for comparison.

The most sensitive and promising combinations are WR–M/VDRL/MR and C–WR–M/VDRL/MR, but the statistical evaluation of the difference showed P = 6 per cent., i.e. just above the limit generally applied to a significant difference. It has not been possible, with the material available, to determine which is the most suitable combination.

(7) The potential loss of a few positive, specific sero-reactions in patients with fresh syphilis when using the C–WR–M instead of the WR–M technique is discussed; this loss should be evaluated in the light of the greater specificity of the C–WR–M technique.

(8) The final conclusion is deferred, pending examination of further material.

I am indebted to Dr. G. Rasch for his valuable assistance in the preparation of the statistical material.

References

CARDIOLIPIN ANTIGEN—I


