CONTRIBUTION TO THE STUDY OF THE SEROLOGY OF LEPROSY

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Serological investigations of leprosy began with Eitner, in 1906. Since then a tremendous amount of work has been done for the purpose of finding a laboratory technique suited for the study of immunology of this disease. The phenomena of serum lability, the presence of polyfixing antibodies, and the lack of a culture of the leprosy bacillus have hindered these investigations, so that serology has failed to gain a definite place in that study.

Since 1943 we have carried on a series of investigations in an attempt to demonstrate specific changes in the serum in leprosy. These are reviewed in this paper.

NONSPECIFIC SERUM REACTION

The lability of the sera of leprosy patients was studied with three flocculating systems, based on different principles: the Wolff test, involving differences of pH, the Takata Ara test, employing an unstable colloidal system, and the Kahn test, in which ubiquitous lipids are used. The results obtained, shown in Table 1, confirmed the known phenomena of unspecific lability of leprosy sera (3).

<table>
<thead>
<tr>
<th>Test</th>
<th>Lepromatous</th>
<th>Tuberculous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Wolff</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>Takata Ara</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>Kahn</td>
<td>16</td>
<td>11</td>
</tr>
</tbody>
</table>

MICROFLOCCULATION REACTIONS WITH LEPROMA LIPIDS

To study how leprosy sera react with an antigen composed of the total lipids extracted from leproma tissues with chloroform, we employed an original microflocculation technique (3).
Preparation of the extract.—Lepromatous nodules are boiled in water for 30 minutes, cleaned of epidermis and ground to a paste. During the grinding chloroform is added and pipetted off repeatedly, until it comes clear. The chloroform is evaporated off and replaced with ether, and that is centrifuged to throw down the bacilli. The residue left after evaporating off the ether is weighed and dissolved in 100 times as much 95 per cent alcohol (by volume). After setting for 2 days the clear solution is removed from the sediment to be used as the antigen.

Titration.—Titration is done as with the Kahn antigen. In each of several vials 0.1 ml. is placed and 2, 2.1, 2.2 (etc.) ml. of 0.9 per cent sodium chloride solution is added. From each suspension 0.05 ml. is removed and distributed in the receptacles of a Kline polyconcavity plate, and 0.5 ml. of saline is added to each. After shaking the plate for 4 minutes, the emulsions are examined under the microscope (50x). The dilution which shows only perfectly dispersed minute particles is taken as the titer.

Saline emulsion.—To 0.01 ml. of the antigen in a Kahn vial the necessary amount of saline is added quickly and, after closing with a rubber stopper, the mixture is shaken vigorously for one minute. The emulsion is allowed to stand for 5 minutes before using.

Technique of the test.—The test is made in a hanging-drop slide, using 0.05 ml. of serum heated at 56°C for 10 minutes and 0.05 ml. of the antigen suspension. The slide is agitated by rotation for 4 minutes and set aside at room temperature for at least 10 minutes.

Reading of the results.—The results can be read directly, or with a magnifying glass or the microscope. In the negative reaction the emulsion remains homogenous, presenting microscopically only dispersed fine particles of the antigen lipids. Positive reactions show appreciable flocculation, distinguishable by their aspect and size as weakly, moderately, markedly and strongly positive (1+ to 4+). Flocculations showing microscopically only very small agglomerates are counted as doubtful. Control tests with normal and positive sera should be made.

The results obtained in the first groups of cases tested, comprising 17 lepromatous and 22 tuberculoid, showed that the sera of the former are much more labile than those of the latter; and the percentages of positives—70.6 and 13.6, respectively—were much like those obtained with the nonspecific tests. This seemed to suggest that our test had the same significance as do those systems.

Nevertheless, to ascertain more definitely the sensitivity and specificity of this microflocculation test, we applied it in a larger number of leprosy cases, and to sera from normal persons and contacts and from supposedly nonleprous patients with other diseases. The results, given in Table 2, show a sensitivity of 73.5 per cent for lepromatous cases and 4.8 per cent for tuberculoid cases. Taking all other sera tested—including the 140 serologically positive syphilis cases, of which only 3, or 1.8 per
cent, reacted positively—99.4 per cent were negative, which figure serves as an index of specificity.

### Table 2.—Results of the leproma-lipid microflocculation test in leprosy and various other conditions.

<table>
<thead>
<tr>
<th>Source of serum</th>
<th>Number of cases</th>
<th>Positive</th>
<th>Negative</th>
<th>Per cent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepromatous leprosy</td>
<td>162</td>
<td>119</td>
<td>43</td>
<td>73.5</td>
</tr>
<tr>
<td>Tuberculoid leprosy</td>
<td>62</td>
<td>3</td>
<td>59</td>
<td>4.8</td>
</tr>
<tr>
<td>Leprosy contacts</td>
<td>106</td>
<td>4</td>
<td>102</td>
<td>3.7</td>
</tr>
<tr>
<td>Healthy persons</td>
<td>1,108</td>
<td>2</td>
<td>1,106</td>
<td>0.2</td>
</tr>
<tr>
<td>Syphilis/a</td>
<td>140</td>
<td>3</td>
<td>137</td>
<td>1.8</td>
</tr>
<tr>
<td>Pulmonary tuberculosis</td>
<td>120</td>
<td>1</td>
<td>119</td>
<td>0.8</td>
</tr>
<tr>
<td>Parasitoses/b</td>
<td>65</td>
<td>0</td>
<td>65</td>
<td>0.0</td>
</tr>
<tr>
<td>Other diseases/c</td>
<td>53</td>
<td>0</td>
<td>53</td>
<td>0.0</td>
</tr>
<tr>
<td>Total, nonlepromous sera/d</td>
<td>1,021</td>
<td>10</td>
<td>1,011</td>
<td>99.4</td>
</tr>
</tbody>
</table>

/a Serologically positive cases.
/b Malaria, febrile; American trypanosomiasis; cutaneous trypanosomiasis.
/c Brucellosis, typhoid fever, exanthematous typhus, etc.
/d Not included in this table are 1,021 sera from supposedly healthy persons who were not examined clinically, which all gave negative reactions.

These results demonstrate that our microflocculation test reveals a phenomenon characteristic of leprosy of the lepromatous form. For this reason it should be given a significance different from that of the nonspecific flocculation systems. Furthermore, from the serological point of view it can be considered as a diagnostic test for leprosy, inasmuch as it fulfills the requirements of “diagnostic sensitivity,” i.e., optimum sensitivity with maximum specificity. For this reason we call it the qualitative or diagnostic microflocculation test (4).

### The Quantitative or Dosimetric Test

A study of the different factors which affect the reaction—salt concentration, serum-antigen relationship, etc.—has shown that the conditions of the original technique seem to be the optimum. To make sure that that is so and to eliminate the possibility that it is affected by zone phenomena, and also to determine quantitatively the flocculating capacity of the sera, we designed a technique which we call the quantitative or dosimetric test (5).
### Table 3—Results of the quantitative microagglutination test with serum from leprosy patients.

<table>
<thead>
<tr>
<th>Type of case</th>
<th>Curve</th>
<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepromatous</td>
<td>0; 1; 1; 2; 2; 2; 2; 2; 3; 3; 3; 3</td>
<td>1,024/a</td>
</tr>
<tr>
<td>Lepromatous</td>
<td>1; 1; 2; 3; 3; 4; 4; 4; 4; 4; 3; 3; 3</td>
<td>226</td>
</tr>
<tr>
<td>Lepromatous</td>
<td>1; 1; 2; 3; 3; 3; 3; 3; 2; 2; 1; 1; 0</td>
<td>128</td>
</tr>
<tr>
<td>Lepromatous</td>
<td>3; 3; 4; 4; 4; 4; 3; 2; 1; 1; 0; 0</td>
<td>64</td>
</tr>
<tr>
<td>Lepromatous</td>
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<td>64</td>
</tr>
<tr>
<td>Lepromatous</td>
<td>1; 1; 2; 3; 3; 2; 2; 2; 1; 1; 0; 0</td>
<td>22</td>
</tr>
<tr>
<td>Lepromatous</td>
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<td>22</td>
</tr>
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<td>Lepromatous</td>
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<td>16</td>
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<td>16</td>
</tr>
<tr>
<td>Lepromatous</td>
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<td>16</td>
</tr>
<tr>
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<td>16</td>
</tr>
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</tr>
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<td>8</td>
</tr>
<tr>
<td>Lepromatous</td>
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<td>4</td>
</tr>
<tr>
<td>Lepromatous</td>
<td>0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0</td>
<td>1</td>
</tr>
<tr>
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<td>1</td>
</tr>
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<td>Lepromatous</td>
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<td>0</td>
</tr>
<tr>
<td>Indeterminate</td>
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<tr>
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<td>1</td>
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<td>1</td>
</tr>
<tr>
<td>Indeterminate</td>
<td>0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0</td>
<td>1</td>
</tr>
<tr>
<td>Tuberculoid</td>
<td>0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0</td>
<td>0</td>
</tr>
<tr>
<td>Tuberculoid</td>
<td>0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0</td>
<td>0</td>
</tr>
</tbody>
</table>

/a This titration was extended with four more dilutions than usual, to determine the end point.

/b Nine other tuberculoid cases included in the authors' original table gave this totally negative result.
Antigenic suspension.—This is prepared in the same manner as for the qualitative test.

Serum dilutions.—The inactivated serum is diluted progressively in 8 test tubes, each containing 0.3 ml. of 0.9 per cent saline. An equal amount of serum is added to the first tube and, after thorough mixing, that amount of the dilution is transferred to the second tube, and so on. The dilutions thus obtained range from 1/2 to 1/256.

Technique of the test.—In a 12-place Kline plate, the antigen emulsion is distributed as follows: 0.005, 0.01, and 0.025 ml. in the first three places, and 0.05 ml. in each of the other nine. To each of the first four places 0.05 ml. of pure inactivated serum is added, and to the eight other places 0.05 ml. of each of the series of serum dilutions. The titer concentrations of the antigen-serum mixtures thus obtained are: 1/10, 1/5, 1/2, 1, 2, 2, 4, 8, 16, 32, 64, 128 and 256. The plate is shaken and allowed to stand as before.

Reading of results.—The readings are made by noting the degree of flocculation (1± to 4±) in each cavity, and the titer is indicated according to the denominator of the highest dilution in which flocculation is seen. We use numerical notations instead of “plus” symbols to express the results of the reaction. Thus, for example, a curve may be 01243222100, in which case the titer would be 64. Readings of ± at the right are ignored in determining the titer. The fourth place of the plate, in which the serum-antigen ratio is 1, represents the relation with the qualitative test.

The results obtained in various cases of different types are shown in Table 3. From that it will be seen, for one thing, that the 1:1 serum-antigen ratio employed in the qualitative test is the optimum one, for in it the maximum degree of flocculation is seen in most cases. Zone phenomena can be seen, especially in untreated or deficiently treated advanced lepromatous cases.

In applying this quantitative test there have been seen new and interesting serum phenomena related to the clinical form of the disease (5, 6). A fundamental aspect is that sera from lepromatous cases have the characteristic of flocculating widely to the right and left from the 1:1 serum-antigen ratio, whereas in general those from tuberculoid cases do not flocculate at all.

A graphic representation of the reactions, shown in Text-fig. 1, reveals typical serological curves of the two polar types of leprosy, lepromatous and tuberculoid, and intermediate curves in the form called indeterminate.

For a clearer exposition of the serological curves in relation to the clinical forms, the various types which are obtained are represented schematically in Text-fig. 2, classified as of four groups. Group I pertains to the lepromatous form, groups II and III to the indeterminate one, and group IV to the tuberculoid type.

The curves of groups I and IV are readily distinguishable. The curves relating to groups II and III (indeterminate cases)
offer difficulties of interpretation. In fact, pretuberculoid indeterminate cases (group III) may sometimes show curves like those of the tuberculoid type (Curves i and j), or to those of prelepromatous indeterminate cases (Curve g). Likewise, prelepromatous indeterminate cases (group II) may give curves similar to those of the lepromatous type (Curve d), or to those of the pretuberculoid form (Curve g). Inasmuch as the serological curves of a given case are not immutable, but change according to the evolution of the disease or under the influence of treatment, serial studies are necessary. This point is emphasized later.

TEXT-FIG. 1. Graphic representation of the serological curves of the quantitative microflocculation reaction in characteristic cases of the different clinical forms of leprosy. The vertical broken lines mark the dilution corresponding to the 1:1 serum-antigen ratio of the qualitative reaction.

COMPLEMENT FIXATION TEST WITH LEPROMA LIPIDS ANTIGEN

The antigen used in the microflocculation tests may also be
used in the complement fixation test. This matter, discussed elsewhere (1), will not be gone into here except to say that on

Text-Fig. 2. Schematic curve forms of the quantitative reaction pertaining to the different clinical forms of leprosy, the symbols at the right indicating the intensity of flocculation. Group I, lepromatous curves; group II, lepromatous indeterminate curves; group III, pretuberculoid indeterminate curves; group IV, tuberculoid curves.
the whole the results have been much like those of the flocculation test.

**Physicochemical study of the antigen**

In a preliminary report, Bonatti and Lebron (2) showed that the antigen used in these tests is essentially composed of lipids. They determined several physical constants and experimented with a fractionation procedure, and concluded that it contains neutral fats, fatty acids, cholesterol and lecithin. It probably contains no protein substances, and it does not contain any glucosides.

**Pathogenic considerations**

The characteristics revealed by the flocculation phenomena and the deviation of complement with our leprous lipid antigen have convinced us that they are the results of a biologic reaction of the antigen-antibody type. It was therefore necessary to investigate the antigenic capacity of this antigen, and also the nature of the antibody involved.

**Antigenic capacity of the leproma lipid extract**

The results of this investigation were reported at the Havana Congress (7).

In the experiment the lepromin lipids were suspended in physiological saline and injected subcutaneously into 37 guinea-pigs in different doses and at different intervals. Blood was obtained by heart puncture and the flocculation test was made.

Positive results were obtained with 16 out of the 37 animals, or 43 per cent. They occurred two to three weeks after the first injections, but the positivity did not persist long, only from one to two weeks. Guinea-pigs which had spontaneously turned negative responded positively to a new injection of antigen, after a few hours and for several days. These results demonstrate that the antigen injected into laboratory animals is capable of provoking a special serum condition which is detectable in vitro by the tests under study, and which is attributable to the presence of circulating antibodies.

**Nature of the antibody**

We undertook to ascertain if the antibody in patients' sera that are concerned in our reaction with the lipid antigen are of the same nature as the one responsible for the Rubino reaction (8).
Sera from lepromatous patients, serologically positive to our flocculation and complement deviation reactions, were placed in contact with formolized erythrocytes and with normal cells from sheep, in order to absorb the heterophile and nonspecific antibodies, respectively. After absorption, the quantitative test was made.

The results of this experiment, shown in Table 4, demonstrate that the antibody detectable in vitro is of the specific heterophile antibody type, similar to that involved in the Rubino test. When the serologically positive sera were put in contact with normal sheep's red cells, only the nonspecific antibodies were absorbed and the reactions were still positive; indeed, in most cases they were intensified. In contrast, when the sera were treated with formolized erythrocytes the heterophile antibodies were absorbed and the reactions became negative.

<table>
<thead>
<tr>
<th>Results before absorption</th>
<th>Results after absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal erythrocytes</td>
</tr>
<tr>
<td>3222211000</td>
<td>3221100000</td>
</tr>
<tr>
<td>1111111000</td>
<td>1111111000</td>
</tr>
<tr>
<td>3222111000</td>
<td>3222211000</td>
</tr>
<tr>
<td>1111111000</td>
<td>1111111000</td>
</tr>
<tr>
<td>4432211000</td>
<td>4333334000</td>
</tr>
<tr>
<td>1111111000</td>
<td>2333321100</td>
</tr>
</tbody>
</table>

In another series of 13 sera in which only the qualitative test was performed the results were identical. In still another series of tests made with serologically negative specimens, the results remained negative after absorption.

DISCUSSION

The investigations here reviewed demonstrate that it is possible to reveal changes peculiar to leprous sera by using appropriate techniques. Our experience indicates that there are benefits offered by serology as a laboratory procedure in leprosy. We refer mainly to the microflocculation tests, both qualitative (diagnostic) and quantitative (dosimetric), because they are simple and easy to perform even by nonspecialists.

With regard to the qualitative or diagnostic test, it is so designated because it possesses the requisites of diagnostic sensi-
tivity: optimum sensitivity with maximum specificity. We believe that it may have value as a routine laboratory method in the detection of overlooked cases of leprosy, especially of the lepromatous type, in endemic areas. Its possible value for early diagnosis can only be decided by further investigations, applying it to leprosy contacts over long periods of time.

The use of the quantitative or dosimetric test has brought out interesting serological phenomena with relation to the clinical form of the disease and the status of the infection.

With regard to the clinical form, the graphic representation of the results of this reaction has enabled us to establish different serological curves corresponding to the clinical forms. In frankly lepromatous or tuberculoid cases the curves are easy of interpretation. That is not so with the curves of the indeterminate form, for although they have their own characteristics they may occasionally resemble those of the lepromatous type at the onset or in regression, or else they may assume the characteristics of the tuberculoid type. We believe it probable that further investigations will enable us to establish more clearly the curves pertaining to the indeterminate forms, or perhaps to distinguish only two types of serological curves, namely, tuberculoid and lepromatous. Consequently, we are of the opinion that serology may be considered as a new element in the classification of leprosy cases.

With regard to the evolution of the disease, serial study of the quantitative test offers further reasons for affirming the significance of serology in the problems presented by the leprosy infection. In the first place, the serological curve of a given patient is not immutable; it may vary appreciably in accordance with the clinical development. Weakly positive curves, with slight deviation to the right or left of the 1:1 serum-antigen ratio, pertain to the initial stages of the lepromatous type of leprosy or to the prelepromatous indeterminate form. We have seen such curves acquire the typical lepromatous characteristics, i.e., wide deviation to the right and left of the 1:1 serum-antigen ratio and high degrees of flocculation, when the patients progressed unfavorably. On the other hand, we have also observed the contrary effect, namely, strongly positive serological curves with high titer and wide right and left deviation undergoing modification until the results were doubtful or negative, in cases responding favorably to treatment.

Interesting observations have been made with regard to the evolution of the so-called indeterminate form. Those cases which,
because of their clinical, laboratory and serological characteristics are classified as prelepromatous, and which later change to the lepromatous condition, show corresponding changes in their flocculation curves. On the other hand, pretuberculoid indeterminate cases which have curves of the tuberculoid type and which transform to the clinically tuberculoid type show no changes in the quantitative reaction. These facts lead us to believe that the serological test may have prognostic value with respect to the evolution of the leprosy infection, referring particularly to the indeterminate forms.

For another thing, we believe that a pathogenic interpretation of these serological phenomena may be possible on the basis of the investigations made concerning the antigenic capacity of lipid extract used as an antigen, and of the specific heterophile nature of the circulating antibodies demonstrable in vitro. We have shown that our antigen is capable of provoking experimentally the formation of circulating antibodies, and that these are of a biological nature similar to those demonstrable in vitro in leprosy sera. Considering them as specific heterophile antibodies, our reactions would therefore be of the type of the heterophile reactions.

The following explanation of the formation of these circulating antibodies in the leprosy sera is offered. In consequence of the conflict between the bacillus and the cell there may be produced, within the affected cell, degradation products essentially of lipid nature (lipoproteins?) consisting of a mixture of the lipids of the tissue and the bacilli. These heterologous substances, liberated from degenerated cells, may act as a complete antigen on the antibody-forming apparatus (reticuloendothelial system), resulting in the production of circulating antibodies (specific heterophiles).

This interpretation is in accord with clinical and laboratory observations: the presence of vacuolated cells containing bacilli in the infiltrates agrees with the positive serological reactions; there is also concordance between the intensity of infection (abundance of vacuolated cells) and the frankly lepromatous curves; while the absence of vacuolated infiltrates and of bacilli agrees with the negative curves. Consequently, our microflocculation reaction may indicate the degree of lepromatous infection.

In other words, it may indicate the lack of resistance to the infection, or it may reveal the presence of lepromatous tissues in activity.

Whatever the pathogenic nature of our microreaction, it is
a phenomenon independent of and opposed to that of hyper-
resistency, since we have found that a frankly positive sero-
logical reaction coincides with a condition of anergy to lepromin.
The concordance between the state of the infection and sero-
logical results leads us to believe that serology may be utilized
as a valuable factor in decisions for the granting of conditional
parole to patients with the lepromatous form of leprosy.

SUMMARY

The authors have reviewed a series of investigations, de-
signed to demonstrate specific changes in leprosy sera, which
they have carried out since 1943. To that end they have em-
ployed a lipid extract of leproma tissues as the antigen of micro-
floculation and complement fixation tests. For its simplicity
and ease the former is preferred. Two techniques of that test
have been used, called the qualitative or diagnostic reaction and
the quantitative or dosimetric reaction. The diagnostic test is
thus designated because it possesses the requisites of diagnostic
sensibility; it is used for diagnostic purposes. The quantitative
technique is employed for the interpretation of the clinical form
and the state of the infection. A pathogenetic interpretation of
the serological phenomena observed is given, and the micro-
reaction used is described as one of the specific heterophile
reaction type. It is believed that serology may be of value as a
decisive factor in the granting of conditional parole to lepro-
matous leprosy patients.

RESUMEN

Los autores hacen una reseña de una serie de investigaciones,
que vienen realizando desde el año 1943, tendientes a demostrar
alteraciones específicas en los sueros leprosos. Con tal fin
utilizan un extracto lipídico, extraído de lepromas, como antígeno,
en reacciones de floculación y fijación de complemento. Prefe-
rieren las primeras, por su sencillez y fácil realización. De ellas,
practican dos técnicas, que denominan cualitativa o diagnóstica
y cuantitativa o dosimétrica. La microreacción cualitativa la
denominan así, por que cumple con los requisitos de la sensi-
bilidad diagnóstica; la emplean con fines diagnósticos. La
técnica cuantitativa, la utilizan para la interpretación de forma
clínica y estado de infección leprosa. Dan ha conocer su interpre-
tación patogénica de los fenómenos serológicos observados y
catalogan, las microreacciones que emplean, como del tipo de
las reacciones heterófílas específicas. Además, opinan, que la
serología puede tener valor como elemento de juicio en el alta condicional de enfermos de lepra lepromatosa.

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