

# Demonstration of specific 19S(IgM) antibodies in untreated and treated syphilis

## Comparative studies of the 19S(IgM)-FTA test, the 19S(IgM)-TPHA test, and the solid phase haemadsorption assay

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**SUMMARY** Sera from 408 patients with untreated or treated syphilis were examined by three different tests to demonstrate treponema-specific 19S(IgM) antibodies. Antibody titres in the 19S(IgM)-fluorescent treponemal antibody (FTA) test and the solid phase haemadsorption assay (SPHA) did not correlate. The 19S(IgM)-*Treponema pallidum* haemagglutination assay (TPHA) and the SPHA partly correlated based on the concentration of treponema-specific 19S(IgM) antibodies in the patient's serum. Under experimental conditions antibody titres in the 19S(IgM)-FTA test and the 19S(IgM)-TPHA correlated consistently.

For specificity and sensitivity, the 19S(IgM)-FTA test correlated best with the clinical findings in both untreated and successfully treated patients. Although the 19S(IgM)-TPHA has about the same degree of specificity, the reading of the results is technically more complicated. The specificity of the SPHA was very high. In patients with untreated syphilis, however, the SPHA is adversely affected by a high rate of false non-reactive results, since it consists of two reactions with appreciable differences in sensitivity. Thus, higher sensitivity cannot be expected in the SPHA.

An immunoabsorption technique using an adequate antigen and a specific, enzyme-labelled antiserum might provide an alternative test which is simple to perform, highly specific, and consistently sensitive.

### Introduction

In the last 10 years the demonstration of specific 19S(IgM) antibodies has acquired clinical and diagnostic importance in diseases induced by both viruses and bacteria. The value of 19S(IgM) diagnostic tests in patients with syphilis has been pointed out by several workers.<sup>1-6</sup> In the micro-method of the 19S(IgM)-fluorescent treponemal antibody (FTA) test, described by Müller and Loa<sup>4</sup> and later modified by Müller and Oelerich,<sup>7</sup> treponema-specific 19S(IgM) antibodies in the patient's serum are separated from those of the 7S(IgG) class by gel filtration before testing. This

technique avoids false-reactive as well as false non-reactive results, which can occur in the IgM-FTA-ABS test.<sup>4 6 8 9</sup> The 19S(IgM)-FTA test is an infallible method for detecting patients with untreated syphilis, but it requires expensive technical equipment.

Instead of using indirect immunofluorescence, the fractions of the serum after gel filtration can be investigated quantitatively by the *Treponema pallidum* haemagglutination assay (19S(IgM)-TPHA).<sup>6</sup> If there is any treponema-specific 19S(IgM) activity in the fractions of the first elution peak, this can be recognised in most cases.

Recently, Schmidt<sup>10</sup> reported results obtained in syphilitic sera by a solid-phase haemadsorption assay (SPHA). The test, which is easy to perform, is based on the solid-phase immunosorbent technique (SPIT).<sup>11</sup>

This paper presents the results of comparative investigations of sera from 408 patients with treated

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and untreated syphilis at various stages of the disease using the 19S(IgM)-FTA test, the 19S(IgM)-TPHA, and the SPHA and reports experiments on the specificity and sensitivity of the SPHA technique.

### Materials and methods

#### SYPHILITIC SERA

Sera from 82 patients with untreated primary, secondary, or tertiary syphilis, from 12 patients with reinfections with *T pallidum*, and from 314 patients with adequately treated syphilis were investigated. Nineteen of the treated patients' sera were non-reactive in the 19S(IgM)-FTA test but showed treponema-specific antibodies of the 8S(IgM) type.<sup>12</sup> All specimens were accompanied by a description of the current clinical symptoms or a brief clinical history.

Sera were screened by standard techniques of the TPHA in accordance with the manufacturer's directions (Fujizoki Pharmaceuticals Co Ltd, Tokyo, Japan) and by the FTA-ABS test<sup>13</sup>; all gave positive results. In all sera from untreated patients treponema-specific 19S(IgM) antibodies could be demonstrated by the 19S(IgM)-FTA test. The sera of adequately treated patients were non-reactive in this test. The tests were performed after storage of the sera for a maximum of 24 hours at 4°C.

#### FRACTIONATION OF SERA BY GEL FILTRATION

The micro-method of Ultrogel ACA 34 filtration was performed on all sera. A 0.7-ml quantity of active serum was passed through a 1.5 × 14-cm column with phosphate buffered saline (PBS), pH 7.3, 15 ms conductivity at 22°C. Fractions of 1.3 ml were collected.<sup>7</sup>

#### 19S(IgM)-TPHA TECHNIQUE

After gel filtration of the serum eight fractions (starting with the peak fraction of the first elution maximum) were investigated quantitatively with Fujizoki TPHA reagents. The titre of 19S(IgM) antibodies was calculated in almost the same way as in the 19S(IgM)-FTA test. The highest titre, estimated in the region of 19S(IgM) elution, was multiplied by 6, the dilution factor produced by gel filtration.

#### 19S(IgM)-FTA TECHNIQUE

The method for demonstrating treponema-specific 19S(IgM) antibodies in fractions after gel filtration by indirect immunofluorescence has been described.<sup>7</sup> The Nichols strain of *T pallidum* was used as antigen. An FITC-labelled rabbit anti-human-IgM serum with  $\mu$ -chain specificity (Dako Immunochemicals, Copenhagen, Denmark) was used at a dilution of 1/70.

#### SOLID-PHASE HAEMADSORPTION ASSAY

The coating of the polystyrene microtitre plates, absorption time, temperature, incubation conditions, and the quality of the anti-human-IgM serum are very important preconditions for optimal SPHA results. The conditions used were comparable to those described by Schmidt.<sup>10</sup> Briefly, 1/400-1/800 dilutions of rabbit anti-human-IgM serum ( $\mu$ -chain specific: Dako Immunochemicals, Copenhagen, Denmark) in PBS were used for coating the solid-phase in the cups of polystyrene plates. The plates were not dried before being used but were washed three times with 0.05% Tween 20 PBS. Sensitised and unsensitised sheep erythrocytes from the Fujizoki TPHA kit were used at a dilution of 1/13. Results were read after incubation for about 18 hours at room temperature. For further technical details see Schmidt.<sup>10</sup>

#### ENZYME-LINKED IMMUNOSORBENT ASSAY

##### (ELISA)

##### *Preparation and peroxidase-labelling of soluble treponemal antigen*

Suspensions of *T pallidum* (Nichols strain) from testes of infected rabbits, prepared in PBS and containing no cell detritus, were homogenised by ultra-sonication (Sonicator TM, Model W 185 F; 6 × 45 sec/50W at 4°C). The ultra-sonicate was centrifuged at 2500 × g for 20 minutes. The protein content of the supernatant (soluble *T pallidum* antigen) was estimated. According to the protein concentration the supernatant was labelled with horseradish peroxidase (HRPO, Sigma Chemical Co, St Louis, Mo, USA) by the method of Wilson and Nakane.<sup>14</sup>

##### *Enzyme reaction*

Forty milligrams o-phenylenediamine and 20  $\mu$ l 30% hydrogen peroxide as substrate were dissolved in 100 ml citrate buffer (0.1 mol/l citric acid and 0.2 mol/l Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O), and 100  $\mu$ l per cup were applied to perform a single enzyme reaction. After incubation for five minutes at 20°C in the dark, the reaction was stopped by the addition of 4 mol/l sulphuric acid. The colour intensity was read at 492 nm in a Multiscan (Titertek).

### Results

#### COMPARISON OF ANTIBODY TITRES BY DIFFERENT TESTS

##### *19S(IgM)-FTA test and SPHA*

These two systems for estimating antibody titres are based on different techniques (see above). As expected the results of the 19S(IgM)-FTA test and the SPHA did not correlate. Sera with high antibody

titres in the 19S(IgM)-FTA test may give SPHA results that range between non-reactive and as high as 1/512. Conversely, in our study no patient with typical symptoms of untreated primary, secondary, or tertiary syphilis whose serum was non-reactive in the 19S(IgM)-FTA test was seen.

**19S(IgM)-TPHA and SPHA**

Antibody titres estimated by the 19S(IgM)-TPHA and the SPHA are compared in fig 1. Of interest is the correlation of antibody titres in sera from patients with primary syphilis (and normally very low SPHA titres). Furthermore, the sensitivity of the 19S(IgM)-TPHA was three dilution steps higher than in the SPHA (fig 1).

In patients with secondary or tertiary syphilis, however, the higher the titre of treponema-specific 19S(IgM) antibodies in the serum, the closer the titres become in the two assays. In the lower range of titres the mean correlation is appreciably lowered. In the upper range of titres 19S(IgM)-TPHA and SPHA show the same sensitivity.

False non-reactive results occurred much more frequently in the SPHA, at the lower borderline values, than in the 19S(IgM)-TPHA (fig 1).

**19S(IgM)-TPHA and 19S(IgM)-FTA test**

The antibody titres estimated by the two assays correlated well independently of the stage of untreated syphilis (fig 2). The estimated mean values of correlation show a straight line which is parallel to the ideal correlation of titres.

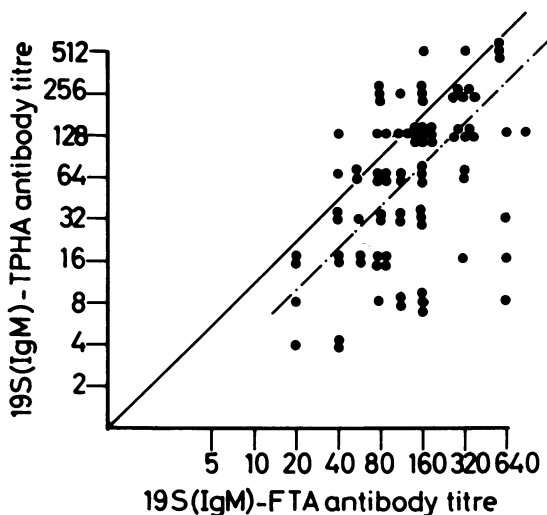


FIG 2 Comparison of 19S(IgM)-TPHA and 19S(IgM)-FTA test antibody titres in patients with untreated primary, secondary, or tertiary syphilis. — — — Mean antibody titre.

**SPECIFICITY AND SENSITIVITY OF THE SPHA**

The results of the SPHA on the sera of patients with untreated primary, secondary, and tertiary syphilis are summarised in table I. The SPHA was less sensitive when the results are compared with those of the 19S(IgM)-FTA test or the 19S(IgM)-TPHA. The rate of false non-reactive SPHA results was very high

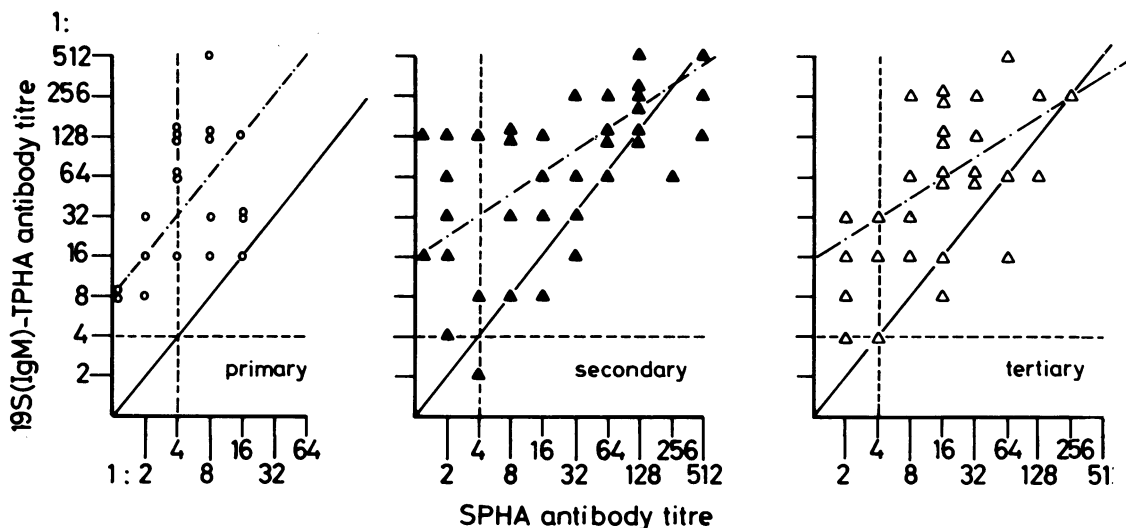


FIG 1 Comparison of 19S(IgM)-TPHA and SPHA antibody titres in patients with untreated primary, secondary, or tertiary syphilis. - - - Borderline value of reaction specificity; — — — mean antibody titre.

TABLE I Solid-phase haemadsorption assay (SPHA) antibody titres in untreated patients with clinical symptoms of primary, secondary, or tertiary syphilis. (Sera that showed competitive inhibition in the IgM-FTA-ABS are not excluded.)

19S(IgM)-FTA reactive sera from untreated patients with:	No of sera tested	SPHA antibody titres					NR	Non-reactive SPHA results (%)
		<8	8	16	32	>32		
Primary syphilis	20	9	5	4	0	0	2	11 (55)
Secondary syphilis	37	8	4	4	4	15	2	10 (27)
Tertiary syphilis	29	7	4	8	4	6	0	7 (24)
Total	86	24	13	16	8	21	4	28 (33)

\*Non-reactive in the serum dilution 1/2 (before addition of sensitised sheep erythrocytes).

in the patients with primary syphilis (55%). About one-third of all the infections in patients with clinical symptoms of untreated syphilis would not have been diagnosed by the SPHA if the antibody titre of 1/8 had been used as a borderline value as described by Schmidt.<sup>10</sup>

All the sera from 296 patients with adequately treated syphilis gave non-reactive results in the 19S(IgM)-FTA test and in the 19S(IgM)-TPHA. None gave an SPHA titre higher than 1/4.

In the sera of 18 patients with adequately treated syphilis no 19S(IgM) but only a treponema-specific 8S(IgM) antibody could be demonstrated. As expected a positive SPHA result was found in 14 of these cases (SPHA titre  $\geq$ 1/8). In one patient the SPHA titre was 1/512 (table II).

In 12 patients reinfection with *T pallidum* was diagnosed clinically. Most of these patients' sera gave only low titres of treponema-specific 19S(IgM) antibodies by the 19S(IgM)-FTA test, but two-thirds of the sera showed false non-reactive SPHA results (table II).

IMMUNOADSORPTION BY THE TPHA AND ELISA  
The 19S(IgM) fraction was separated by gel filtration from the sera of three patients with untreated secondary syphilis. The TPHA titre of treponema-specific 19S(IgM) in the non-enriched eluates was estimated to be between 1/140 and 1/160 (Fig 3:A).

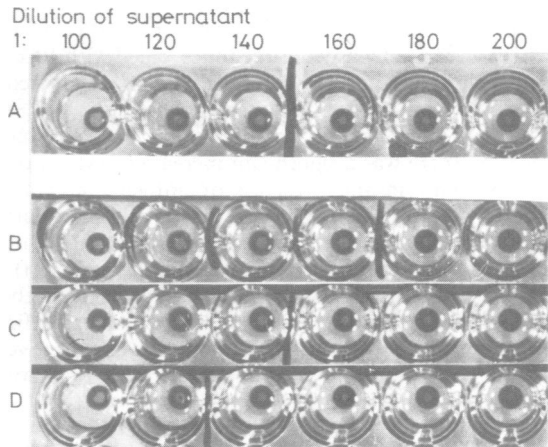


FIG 3 TPHA of treponema-specific 19S(IgM) antibodies before (A) and after (B-D) immunoabsorption on anti-IgM-coated solid-phase.

One hundred microlitres of an undiluted eluate, containing the treponema-specific 19S(IgM) portion of the sera, was put in the cups of polystyrene microtitre plates coated with anti-human-IgM serum (solid-phase). After three hours' incubation at 37°C in a humid chamber the supernatant fluids were

TABLE II SPHA antibody titres in groups of patients with 8S(IgM) antibodies or treponemal reinfections

	No tested	SPHA antibody titres					NR*	False SPHA results (%)
		<8	8	16	32	>32		
19S(IgM)-FTA non-reactive, 8S(IgM) reactive sera from patients with adequately treated syphilis	18	4	2	3	7	2	0	14 (78)†
19S(IgM)-FTA reactive sera from patients with re-infections	12	8	2	0	0	2	0	8 (66)‡

\*Non-reactive in the serum dilution 1/2 (before addition of sensitised sheep erythrocytes).

† False-positive

‡ False-negative

tested for a decrease in the level of treponema-specific 19S(IgM) antibody by the TPHA and ELISA techniques.

In the TPHA the antibody titre was not decreased. In the range of mean error the supernatants still showed a specific antibody titre of between 1/120 and 1/160 with a mean value of 1/140 (fig 3:B-D).

Despite this negative result we suspected that immunoadsorption of treponema-specific 19S(IgM) antibodies from patients' sera to the solid-phase must occur during the first reaction step. This was confirmed as follows: the supernatant fluids of the first reaction step were removed from the cups, which were then washed three times, filled with 100 µl of a soluble peroxidase-labelled *T pallidum* antigen at dilutions of 1/10, 1/25, and 1/50 and incubated at 4°C overnight. After completion of the enzyme reaction there was a significant increase of extinction (at 492 nm) in the presence of antigen-conjugate dilutions of 1/10 and 1/25 compared with the results of 19S(IgM) negative cups (extinction factor  $\geq 2$  of the mean value of negative controls in the same test).

By this experiment we demonstrated qualitatively beyond any doubt the binding of treponema-specific 19S(IgM) antibody to the solid-phase. A quantitative estimate of the extent of immunoadsorption cannot be made by this method.

## Discussion

For evaluating treatment requirements in syphilis the demonstration of treponema-specific 19S(IgM) antibodies has recently aroused scientific and diagnostic interest, especially in cases of latent and late latent infections.<sup>2-5 15-18</sup> The IgM-FTA-ABS test, formerly used for this purpose, has been shown to be unreliable. If the patient's serum contains a rheumatoid factor, as well as treponema-specific 7S(IgG) antibodies, false-reactive results may occur. Also, false non-reactive results are possible if the fixation of the 19S(IgM) antibody to the antigen is partially or completely inhibited by an excess of 7S(IgG) antibodies of the same specificity.<sup>8-10</sup>

Separation of the 19S(IgM) from the 7S(IgG) fraction of serum by gel filtration before testing overcomes these limitations.<sup>1 4-7 18</sup> The 19S(IgM)-FTA test was found to be reliable in most patients who were untreated or who had received only inadequate treatment.<sup>16 18</sup> Unfortunately, this test requires highly developed techniques and can therefore be carried out only in specialised laboratories. Thus, simpler methods with similar specificity and sensitivity were looked for. Schmidt's<sup>10</sup> report of comparable results with the SPHA led us to continue the experiments with the SPHA which we had started some years earlier (unpublished data).

The comparative studies reported here show that the correlations of the results by the various tests do differ. No correlation could be observed between the antibody titres of the 19S(IgM)-FTA test and those of the SPHA. When the titres of the 19S(IgM)-FTA test and the 19S(IgM)-TPHA, however, and those of the 19S(IgM)-TPHA and the SPHA were compared a relationship could be found (figs 1 and 2). In the 19S(IgM)-TPHA and in the SPHA sheep erythrocytes sensitised with fragments of *T pallidum* are used as antigen. In both the 19S(IgM)-FTA test and the 19S(IgM)-TPHA the previously separated 19S(IgM) portion of the serum is tested.

The relationship between the results of the 19S(IgM)-FTA test and the 19S(IgM)-TPHA is clearly important (fig 2). The difference in titres of about one-dilution step results from the test conditions and seems to be negligible. The reading of the titre in the 19S(IgM)-TPHA is difficult, however, in some cases and impossible in others. Therefore, the 19S(IgM)-TPHA cannot be recommended for the routine detection of 19S(IgM) antibodies in syphilis.

In view of the expensive equipment needed for the highly sensitive 19S(IgM)-FTA test and the difficulties in estimating titres in the 19S(IgM)-TPHA (especially in the presence of high-titre 7S(IgG) antibodies of the same specificity), the question arises whether the sensitivity of the SPHA ensures the necessary diagnostic certainty in all syphilitic patients needing treatment. We found a false non-reactive result (titre  $\leq 1/4$ ) in more than half the patients with primary syphilis and in about one-third of the patients with untreated syphilis (table I). In patients reinfected with *T pallidum* the SPHA results were unreliable in more than 75% of cases. Furthermore, the SPHA is unable to distinguish between 19S(IgM) and 8S(IgM) antibodies (table II). These observations contrast with those of Schmidt.<sup>10</sup> On the other hand, the high specificity of the SPHA reported by this author was confirmed in a large number of adequately treated cases.

One reason for the differences in the results may be that Schmidt<sup>10</sup> excluded sera that showed competitive inhibition, whereas these were included in our study. Several technical circumstances which might explain the divergence in the results have been mentioned. A fundamental factor remains to be considered, however: the SPHA consists of two immune reactions, each with an appreciable difference in sensitivity. From the experiments of Engvall and Perlmann<sup>19</sup> a difference in sensitivity between the immunoadsorption and indirect haemadsorption phase of about  $10^3$  can be expected. This fact must influence the test results.

Cantarero *et al*<sup>20</sup> have shown that the binding of

IgG on polystyrene in the solid-phase immunoassay does not depend on the total amount of IgG in the cups. Under normal experimental conditions about 30% of the total IgG will be fixed on the polystyrene. Our experiments agree with these observations and have shown that immunoadsorption of treponema-specific 19S(IgM) antibodies (with titres between 1/120 and 1/140) on the anti-IgM solid-phase cannot be detected by haemadsorption (fig 3) but can be with the more sensitive ELISA technique.

These findings possibly indicate that to obtain a reactive SPHA the percentage of treponema-specific 19S(IgM) antibodies of the total 19S(IgM) in a serum sample must be very high. This is confirmed by our SPHA results in patients with secondary syphilis and by the high proportion of treponema-specific 19S(IgM) antibodies in the total serum 19S(IgM). Further confirmation of this is evident in the negative results for the other groups of untreated patients; if in the case of primary or tertiary syphilis the proportion of treponema-specific 19S(IgM) in the total 19S(IgM) is still, or has become, low, then the anti-IgM of the solid-phase is blocked mostly by treponema-unspecific 19S(IgM). The non-reactive SPHA result seems to be due to the indirect haemadsorption, which is strikingly less sensitive than the first step of the assay.

The demonstration of treponema-specific 19S(IgM) antibodies may assume considerable medical importance in the future. Tests which are simple to perform, highly specific, and consistently sensitive are therefore needed. The results of the present studies show that the SPHA does not fulfil these conditions completely. These may be achieved, however, if the antibody of the patient's serum was bound to a properly antigen-coated solid-phase by immunoreaction and identified by a highly specific, enzyme-labelled antiserum, such as in the ELISA. The application of this technique is under investigation.

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