Microcapsule agglutination test for *Treponema pallidum* antibodies

A new serodiagnostic test for syphilis

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SUMMARY For the serodiagnosis of syphilis a quantitative passive agglutination (MCA-TP) test for antibodies to *Treponema pallidum* was performed with chemically stable microcapsules with no antigenic activity instead of with conventional sheep erythrocytes. The microcapsules were easily sensitised with the antigen of sonicated *Treponema pallidum* by treatment with glutaraldehyde.

Compared with the *Treponema pallidum* haemagglutination test (TPHA) the MCA-TP test was superior for detecting cases of primary syphilis. Furthermore, the decrease in antibody titre during treatment was more evident in this test than in the FTA-ABS or the TPHA tests. The MCA-TP test performed on IgM and IgG gel-filtered fractions of sera from patients with syphilis proved that the sensitised microcapsule antigen reacted sharply with the IgM antibodies specific to syphilis.

Introduction

Among the various serological tests for syphilis using *Treponema pallidum* as antigen, such as the *T pallidum* immobilisation (TPI),¹ the *T pallidum* immune adherence (TPIA),² the *T pallidum* agglutination (TPA),³ the *T pallidum* complement fixation (TPCF),⁴ the Reiter protein complement fixation (RPCF),⁵ the fluorescent treponemal antibody-absorption (FTA-ABS),⁶ and the *T pallidum* haemagglutination (TPHA)^{7,8} tests, the most commonly used for clinical serodiagnosis are the FTA-ABS and the TPHA tests. These tests are also recommended by the World Health Organisation and the Center for Disease Control (Atlanta, Georgia, USA).

As it is the most specific, the FTA-ABS test is regarded as the most reliable confirmatory test. The use of a fluorescence microscope and the technical skill required, however, limit the value of the FTA-ABS test as a routine diagnostic test.

The TPHA test developed by Tomizawa *et al*⁸ has high sensitivity and specificity. This method, modified

by Cox *et al*,⁹ is widely used as a microvolume procedure for the serodiagnosis of syphilis. It is not, however, sensitive enough for the diagnosis of primary syphilis, and both the TPHA and the FTA-ABS tend to remain positive even after antisyphilitic treatment.¹⁰⁻¹² Investigations have aimed at the development of a test which shows the effect of treatment. Changes in specific IgM antibodies after treatment have been reported by various workers.¹³⁻¹⁸ The animal erythrocytes used in the TPHA test may show non-specific agglutination with sera from patients with high titres of heterophile antibodies. Unless lyophilised, these cells rapidly deteriorate.

To overcome such difficulties we have performed passive agglutination tests using chemically stable microcapsules (MC) sensitised with various antigens, for example, *Leptospira* spp¹⁹ and *T pallidum*. The MC used in our study had the following features: (a) an absence of antigenic substance on the particle surface, thus avoiding non-specific reactions; (b) chemical stability; (c) the possibility of mass production with uniform quality; and (d) the possibility of modification of particle characteristics such as particle size, specific gravity, and particle surface properties according to the purpose of the test.

We report the results of the microcapsule agglutination test for detecting antibodies to *T pallidum* (MCA-TP).

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Material and methods

SYPHILITIC SERA

Sera from 30 patients with untreated primary, secondary, late, and congenital syphilis were investigated. Sera were also collected from nine patients in the same group after treatment. Each patient attended the department of dermatology at the University of Tokyo Hospital, which provided us with a brief clinical history. A further 65 sera from patients with syphilis and 100 sera from patients who did not have syphilis were obtained from the same hospital. Before the MCA-TP test was performed sera were screened by the rapid plasma reagin (RPR) circle card test (Hynson, Westcott & Dunning Inc, Baltimore, USA), the TPHA test (Fujizoki Pharmaceutical Co, Tokyo, Japan), and the FTA-ABS test.⁶

FRACTIONATION OF SERA BY GEL FILTRATION

A 0.35 ml sample of serum was fractionated on a Sephacryl S-300 (Pharmacia Fine Chemicals, Sweden) column with a length of 90 cm and a diameter of 1.6 cm. Elution was performed with 0.15 mol/l phosphate-buffered saline (PBS), pH 7.2; 2.5 ml fractions were collected.

Optical absorption at 280 nm of eluted fractions showed three peaks (figs 1 and 2) corresponding to IgM, IgG, and albumin respectively. The separation of IgM from IgG was confirmed by the IgM-FTA-ABS and the IgG-FTA-ABS tests which indicated the presence of class-specific antibodies only. To confirm the presence of antibodies specific for *T pallidum* absorption tests with *T pallidum* were conducted on some IgM fractions which were positive in the MCA-TP test but negative in the FTA-ABS or the TPHA tests.

TPHA AND FTA-ABS TESTS

The TPHA test was performed in accordance with the microvolume procedure modified by Cox *et al.*⁹ The FTA-ABS test was performed with *T pallidum* (Nichols strain) as the antigen and with FITC-labelled anti-human IgM (μ -chain specific) and anti-human IgG (γ -chain specific), both supplied by Dako Immunochemicals (Copenhagen, Denmark).

PREPARATION OF MICROCAPSULE PARTICLES

Polyurea MC

These particles with a red dye inside were prepared as described.¹⁹ Blue, magenta, and fluorescent-coloured urea MC were also prepared by replacing the red dye with an oil-soluble blue dye (Kayaset Blue; Nippon Kayaku Co, Japan), an oil-soluble magenta dye (Sumiplast Red FB; Sumitomo Chemical Co, Japan), and a coumarin-derived fluorescent dye (White Flour Blue; Sumitomo Chemical Co, Japan) respectively.

The specific gravity of the MC and the *T pallidum* antigen-sensitised MC was regulated by changing the mixing ratio of the core materials as shown in table 1.

TABLE 1 Changes in specific gravity of the microcapsules and *T pallidum* microcapsules produced by mixing the ratio of the core materials

Diisopropylnaphthalene (g)	14.6	11.1	7.6	6.6	15.6	5.9
Chlorinated paraffin (g)	10.4	13.9	17.4	18.4	9.4	19.1
Specific gravity of MC	1.07	1.11	1.15	1.16	1.06	1.17

Polyurethane MC

Ethylenediamine-propyleneoxide addition product (0.1 g) was dissolved in a mixture of 11.8 g of diisopropylnaphthalene and 13.2 g of chlorinated paraffin (chlorine content 50%). The resulting solution was cooled with iced water, and 4 g of 50% methyl-ethylketone solution of tolylenediisocyanate-trimethylolpropane addition product (Desmodur-L; Bayer Chemicals) was dissolved in this. The resulting solution was emulsified into 65 g of 5% aqueous solution of polyvinyl alcohol (degree of saponification 88%; degree of polymerisation 500) until an average drop size of 7 μ m was obtained. The emulsion was diluted with 100 g of water and reacted for one hour at 75°C to obtain polyurethane MC.

PREPARATION OF TREPONEMAL ANTIGENS

T pallidum (Nichols strain) was cultured in rabbit testes. The organisms were purified by differential centrifugation and resuspended to about 10⁸ cells per ml in PBS. The organisms were disrupted by sonication (model 5202; Ohtake Works Co, Japan) at 20 kHz for 10 minutes and after the addition of 0.1% sodium azide were stored at 4°C. *Treponema phagedenis* (Reiter strain) was cultured anaerobically for five days at 37°C in a modified Brewer's medium²⁰ supplemented with calf serum at 10% (v/v). The cells were washed three times by centrifugation, adjusted to about 10⁸/ml by the addition of PBS, sonicated, and stored at 4°C as above.

PREPARATION OF SENSITISED MICROCAPSULE ANTIGEN

Various methods for the sensitisation of MC with *T pallidum* were tested; these included the use of glutaraldehyde,²¹ chromium chloride,²² bis-diazotised benzidine,²³ tannic acid,²⁴ and cyanuril chloride.²⁵ The glutaraldehyde method gave the most reproducible and stable sensitisation and was used throughout the study. MC were washed twice with saline, resuspended in PBS to a concentration of 1.5% (v/v), mixed with an equal volume of 0.25% glutaraldehyde at 37°C for one hour, washed twice with saline, and resuspended in twice their volume of PBS. The suspension was mixed with an equal volume

FIG 1 Immunological response of untreated sera from patients at various stages of syphilis after gel filtration in the TPHA (● — — ●) and the MCA-TP (○ — ○) tests. The curve (● — ●) shows the protein absorption of each fraction after gel filtration measured at a wavelength of 280 nm.

of antigen of optimal concentration, incubated with agitation for one hour in a 37°C water bath, and allowed to stand overnight in a refrigerator. The antigen cell concentration for MC sensitisation which gave stable and reproducible agglutination patterns was in the range 2×10^7 to 4×10^7 cells/ml. A concentration exceeding 1×10^8 cells/ml caused non-specific reactions. The sensitised MC suspension was washed twice with 0.2% glycine-saline by centrifugation at 3000 rev/min for 10 minutes and

resuspended to the original volume in PBS containing 3% bovine serum albumin (BSA) and 2% sucrose.

MCA-TP TEST PROCEDURE

The MCA-TP test was performed by the microtitration method using disposable V-type plates. PBS (containing 1% BSA and 1% sucrose) was used for all serum dilutions. The serum specimens were serially diluted twofold with a 0.025 ml microdiluter and the suspension of sensitised MC antigen was

FIG 2 Immunological response in the serum from a patient treated for early secondary syphilis after gel filtration in the TPHA (● — — ●) and the MCA-TP (○ — ○) tests. The curve (● — ●) shows the protein absorption of each fraction after gel filtration measured at a wavelength of 280 nm.

delivered to each serum dilution with a 0.025 ml dropper. The mixtures were shaken well and kept overnight at room temperature, and the agglutination patterns at the bottom were observed the next day. The titre of the specimen was expressed as the reciprocal of the highest serum dilution showing a positive MCA-TP pattern. Human non-syphilitic sera were used as controls.

Results

SELECTION OF MICROCAPSULES

Before sensitisation a test was performed to investigate the use of the MC as carrier for the passive agglutination tests. As wall membrane, polyurea and polyurethane were compared; the former was selected because of its superior stability, although the ability to couple antigen was similar. The MC, ranging in specific gravity from 1.07 to 1.17 and in average particle size from 3 to 40 μm , were prepared. MC with an average size of 7 μm were most suitable because of the ease of observation and their sensitivity in the passive agglutination test using the microtitre method. We considered the incorporation of a dye in the MC core would improve the accuracy of reading of the agglutination pattern without affecting the surface property of the wall. Comparison of blue, red, magenta, colorless, and fluorescent MC showed that MC containing red dye in the core were the most satisfactory for easy pattern reading. The dye, contained inside the MC, did not affect the antigen-antibody reaction taking place on the surface of the MC. The MC containing fluorescent dye gave strong fluorescence under ultraviolet irradiation and allowed slight changes in the agglutination pattern to be identified. Because of the cumbersome test procedure,

which required an ultraviolet source and a darkroom, these were not evaluated further. In view of these different features polyurea membrane MC of specific gravity 1.1, average particle size 7 μm , and containing red dye were used in the test.

EFFECT OF STABILISERS ON MCA-TP TEST

The MCA-TP tests were performed after the addition of dextran, polyvinyl alcohol, ficoll, sucrose, pullulan (a linear polymer of glucose), BSA, or normal rabbit serum, at various concentrations in a range of 1–10%, as a reaction stabiliser to the diluent (0.15 mol/l PBS, pH 7.2) of the test serum. We found that the formation of agglutination patterns was facilitated by the addition of 1% BSA and 1% sucrose. The same antibody titres were obtained with two lots of antigen in tests performed over a six-month period. The pH of the diluent within the range of pH 5.0 to 9.0 did not affect the test results.

SPECIFICITY OF MCA-TP TEST TO TREPONEMA-SPECIFIC ANTIBODIES

To investigate the specificity of the MCA-TP test the syphilitic sera, non-syphilitic sera, anti-cardiolipin rabbit sera, anti-*T. phagedenis* rabbit sera, and human sera from patients with leptospirosis were tested with MC sensitised with *T. pallidum* antigen.

The MCA-TP tests showed antibody titres of 160–10 240 in the sera of 65 patients with syphilis identified as positive in the TPHA, the FTA-ABS, and the RPR tests. On the other hand the MCA-TP test showed antibody titres in the range of 10–40 for 100 non-syphilitic sera identified as negative in the TPHA, the FTA-ABS, and the RPR tests. The same was true of three anti-cardiolipin rabbit sera, two anti-*T. phagedenis* rabbit sera, and 16 human leptospirosis sera. Furthermore, three sera which caused strong non-specific agglutination of unsensitised cells in the TPHA test did not show non-specific agglutination of the unsensitised MC.

To investigate cross-reactivity with anti-*T. phagedenis* antibodies agglutination reactions were carried out with MC sensitised as described for *T. pallidum* and the above sera. The two anti-*T. phagedenis* sera which were homologous with the sensitising antigen showed antibody titres of 5120 and 10 240, but other sera showed agglutination reactions only in the range of 10–40. Consequently, the MCA-TP antigen was considered to be free from cross-reactivity with anti-*T. phagedenis* antibodies.

SENSITIVITY OF MCA-TP TEST WITH SERA FROM PATIENTS WITH DIFFERENT STAGES OF SYPHILIS
The sensitivity of the RPR, the TPHA, the FTA-ABS (γ and μ chain specific) and the MCA-TP test in the detection of antibodies in the sera of nine patients with

primary, 10 with secondary, six with late, and five with congenital syphilis was investigated. The results are summarised in table II. Even when the TPHA test was negative (titre < 80) (cases 1, 2, 3, 5, 7, and 8) the MCA-TP test on sera from patients with primary syphilis detected antibody at titres of 640-5120. On the other hand the MCA-TP test did not show consistent results with sera from patients with secondary, late, and congenital syphilis; the antibody titres were similar to or slightly higher than those obtained with the TPHA test.

TABLE II Antibody titres* in the sera of patients with syphilis

Serum No	Stage of syphilis	Serological tests			
		RPR	FTA-ABS		TPHA MCA-TP
			μ Chain	γ Chain	
1	Primary	N†	5	20	N 1280
2	Primary	16	40	20	N 2560
3	Primary	2	40	20	N 5120
4	Primary	16	40	80	80 10 240
5	Primary	1	5	5	N 1280
6	Primary	16	20	80	80 10 240
7	Primary	8	40	80	N 5120
8	Primary	4	10	20	N 640
9	Primary	32	80	80	320 2560
10	Secondary	64	40	320	160 5120
11	Secondary	8	N	20	80 1280
12	Secondary	32	5	1280	640 2560
13	Secondary	32	10	1280	2560 10 240
14	Secondary	128	N	2560	40 960 5120
15	Secondary	512	N	2560	20 480 40 960
16	Secondary	64	40	2560	20 480 40 960
17	Secondary	8	N	40	2560 5120
18	Secondary	64	N	320	20 480 20 480
19	Secondary	256	N	1280	40 960 40 960
20	Late	128	5	2560	20 480 5120
21	Late	2	5	80	320 640
22	Late	1	N	20	160 160
23	Late	2	N	80	320 160
24	Late	4	N	160	640 640
25	Late	2	N	80	320 160
26	Congenital	16	N	320	2560 1280
27	Congenital	8	N	80	640 1280
28	Congenital	4	N	40	640 1280
29	Congenital	8	N	160	640 640
30	Congenital	1	N	40	160 320

*Expressed as the reciprocal of the endpoint titre

†N (negative) means an antibody titre less than 1, 5, or 80 in the RPR, FTA-ABS, or TPHA test respectively

SENSITIVITY TO TREPONEMA-SPECIFIC IgM ANTIBODIES

The TPHA and the MCA-TP tests were performed on the IgM and IgG fractions obtained by gel filtration on Sephacryl S-300 of sera from patients with various stages of syphilis.

In the early stage of primary syphilis (fig 1a) the TPHA and the IgM-FTA-ABS tests were unable to detect antibodies in the IgM or in the IgG fractions; the MCA-TP test detected antibody at a titre of 16 in the IgM fraction. In the later stages of primary syphilis (fig 1b) both tests showed antibodies in the IgM and IgG

fractions, but the MCA-TP test showed higher titres, particularly in the IgM fractions.

After absorption with *T pallidum* the sera from the two patients with primary syphilis showed that the highest antibody titres in the IgM fractions were reduced to 8 and 16 respectively, indicating the presence of IgM antibodies to *T pallidum* which react with the sensitised MC antigen.

In the sera of patients with early secondary syphilis (fig 1c) the highest antibody titre found in the IgM fractions was 2 in the TPHA but 16 in the MCA-TP test, indicating the greater sensitivity of the MCA-TP test in detecting IgM antibodies. In the later stage of secondary syphilis (fig 1d) the IgM antibodies were still detected by the MCA-TP test but not by the TPHA test.

In the sera from patients with late syphilis (fig 1e) and congenital syphilis (fig 1f) the treponema-specific antibodies were detected only in the IgG fractions, both by the TPHA and the MCA-TP tests.

CHANGES IN MCA-TP TITRES AFTER TREATMENT

The changes in the MCA-TP titres before and after penicillin treatment were observed in two cases of primary syphilis and in seven cases of secondary syphilis (table III). In the FTA-ABS and the TPHA tests the titre after treatment decreased in five and three cases respectively, remained constant in three cases in each test, and increased in one and three cases respectively. On the other hand the MCA-TP test showed a decrease in the antibody titre in eight cases; the decrease in the antibody titres was two-fold in two cases, eight-fold in five cases, and 32-fold in one case. These results corresponded to those in the RPR test, indicating that the MCA-TP test can be useful in judging the effect of anti-syphilitic treatment.

TABLE III Comparative changes in antibody titres in the RPR, FTA-ABS, TPHA and MCA-TP tests in nine patients after treatment of primary and secondary syphilis

Serological tests	Changes in titres after treatment		
	No. of cases decreased	No. of cases unchanged	No. of cases increased
RPR	9	0	0
FTA-ABS (IgG)	5	3	1
TPHA	3	3	3
MCA-TP	8	1	0

To analyse further these results paired sera from patients with late primary syphilis, which after treatment showed a decrease in the antibody titre in the MCA-TP test but not in the TPHA test, were fractionated and tested for changes in IgM and IgG antibody titres (figs 1b and 2).

After treatment the titre of the IgG antibodies detected by the TPHA test was two-fold higher than

that before treatment but IgM antibodies could not be demonstrated. On the other hand the MCA-TP test, showing substantially the same titres of IgG antibodies before and after treatment, showed a sharp decrease in the IgM antibody titre after treatment although the titre was still 4.

Discussion

Unlike the microcapsules used for the controlled release of enzymes or drugs²⁶ already used in the medical field we adopted the microencapsulation technique²⁷ to improve the performance of reaction carrier by changing the colour and its specific gravity. The addition of a dye to the interior of the MC improved the contrast in the reading of the agglutination pattern without affecting the antigen-antibody reaction taking place on the external surface of the MC. The red MC proved easiest for pattern reading, but the fluorescent MC appeared promising for certain applications. The appropriate specific gravity, obtained by suitably selected internal materials, improved the sensitivity of the agglutination reaction with a shortened pattern formation time, which was reduced to about 3-4 hours compared with about 5-6 hours in the conventional method.

The MCA-TP test showed antibody titres of at least 160 in all the syphilitic sera and of 10-40 in the non-syphilitic sera tested. The positive-negative borderline was therefore at the titre of 80 although this will require further confirmation in a larger series.

The *T pallidum*-sensitised MC antigen was non-responsive to the anti-*T phagedenis* rabbit sera and the *T phagedenis*-sensitised MC antigen was non-responsive to the syphilitic sera. Even without the use of absorbent containing *T phagedenis* component, as used in the TPHA test, non-specific reactions were not observed in the MCA-TP test; this indicates the absence of cross-reactivity between both types of antibodies.

Furthermore, three sera which showed non-specific agglutination of unsensitised cells in the TPHA test were presumed to have contained heterophilic antibodies to sheep erythrocytes. As the unsensitised MC were non-reactive with these sera, we concluded that they have no antigens on their surface. This suggests that the synthetic polyurea carrier we used can eliminate the carrier-induced non-specific agglutination which is unavoidable in the passive agglutination reaction when erythrocytes are used as carrier. Consequently, the MCA-TP test is considered to be highly specific in the detection of syphilitic antibodies.

The MCA-TP test proved to be sensitive for detecting antibodies in the sera of patients with

primary syphilis and showed sufficiently high antibody titres (640-5120) even when the TPHA test was negative. A comparison of the reactivity of these two tests on the IgM and IgG fractions obtained by gel filtration of syphilitic sera confirmed that the MCA-TP test was more reactive with IgM antibodies than either the TPHA or the FTA-ABS tests. Although the TPHA and the FTA-ABS tests did not detect antibodies of the IgM and IgG classes in the serum fractions in the early stages of primary syphilis the MCA-TP test demonstrated antibody at a titre of 16 in the IgM fraction. In the sera of patients with late primary syphilis the highest antibody titres in the IgM and IgG fractions were both 8 in the TPHA test but 256 and 32 respectively in the MCA-TP test. This again indicated the high sensitivity of the MCA-TP test in detecting IgM antibodies.

After antisyphilitic treatment the FTA-ABS and the TPHA tests still detect treponemal antibodies. The antibody titres in sera from two cases of primary syphilis and from seven cases of secondary syphilis decreased after treatment in only three of nine cases in the TPHA test but in eight of nine cases in the MCA-TP test. After treatment the TPHA test no longer detected IgM antibodies but showed antibody titres to the IgG antibodies two-fold higher than before treatment. This resulted in the total antibody titre remaining constant. The MCA-TP test showed a substantial decrease in the IgM antibody titres but the IgG titres were similar before and after treatment; this resulted in a decrease in the total antibody titre after treatment. The behaviour of the IgM and IgG antibodies during the course of treatment and the difference in reactivity of the MCA-TP and the TPHA tests to these antibodies will have to be investigated further in a larger series.

The results obtained on fractionated sera seemed, however, to indicate that the MCA-TP was able to detect small amounts of syphilitic IgM antibody that cannot be detected by the FTA-ABS or the TPHA tests. The test is capable of reflecting the change in IgM antibodies in the course of syphilitic treatment, which may permit its use in monitoring the efficacy of treatment.

The differences in the reactivity of the MCA-TP and the TPHA tests seemed to be related to the properties of the carriers and to differences in the antigens used, as had been found in our study of leptospirosis.⁹ This had shown that the insoluble antigens after sonication played a more important role in MC agglutination than the soluble antigen, but again further investigation is needed to clarify this point.

Various workers have pointed out the clinical importance of the detection of treponema-specific IgM antibodies in syphilitic sera.¹³⁻¹⁸ Based on the measurement of treponema-specific antibody in the

Microcapsule agglutination test for *Treponema pallidum* antibodies

IgM fraction of syphilitic sera Müller *et al*^{18 28} reported that the results of the IgM-FTA test coincided well with the clinical diagnosis of untreated and successfully treated patients and that the results of the TPHA test on the IgM fraction had similar characteristics. The MCA-TP test, which has a high specificity for *T pallidum* antibodies and high sensitivity in detecting IgM antibodies, appears to provide better information on the monitoring of treatment and is a useful method for the diagnosis of early syphilis.

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