Reactivity of Recombinant *Treponema Pallidum* (r-Tp) Antigens With Anti-Tp Antibodies in Human Syphilitic Sera Evaluated by ELISA

Katsuya Fujimura, Nobuyuki Ise, Eiichi Ueno, Takeya Hori, Nobuyuki Fujii, and Masahisa Okada*

Central Research Laboratories, Fujirebio, Hachioji, Japan

We evaluated the immunoreactivity of recombinant *Treponema pallidum* (r-Tp) antigens with human sera by indirect enzyme-linked immunoabsorbant assay (ELISA). We expressed antigens with a molecular weight (MW) of 17KDa, 15KDa, 47KDa, and 42KDa, which are believed to be major immunoreactive membrane proteins of Tp cells. The expressed proteins were described by adding the prefix M, S, and G to the corresponding Tp antigens, namely, mature antigens, signal sequence containing antigens, and glutathione s-transferase (GST)-fused antigen in this report. A rather high expression occurred for M47 and S42 proteins in the *Escherichia coli* system, whereas for M15 and M17 proteins, a poor expression was

observed. However, a fairly high expression occurred for G15 and G17. Thus expressed proteins were purified by means of chromatographies to a level of > 95%, and the purified proteins were found to be reactive with TPHA positive serum by Western blotting (WB). An ELISA performed with a serum of 1/1000 dilution using these purified antigens for coating on the solid phase showed that G17 antigen was more effective in detecting syphilis antibodies in human serum than M47, S42, and G15. There was a good consistency between ELISA and TPHA, whereby the cutoff indexes (CI) on ELISA showed a correlation coefficient of 0.7276 in logarithmic TPHA titers. J. Clin. Lab. Anal. 11:315–322, 1997. © 1997 Wiley-Liss, Inc.

Key words: recombinant *Treponema-pallidum* antigen; indirect enzyme-linked immunosorbent assay; immunoreactivity; syphilis antibody; *Treponema* hemagglutination assay; G17 antigen

INTRODUCTION: FORMS OF EXPRESSED R-Tp ANTIGENS

Syphilis is a disease caused by the spirochetal bacterium *Treponema pallidum* (Tp). Clinical diagnostics issues related to syphilis and the latest methods for the detection of syphilis antibodies in human blood by immunoassay, immunoblotting, and polymerase chain reaction (PCR) have been reviewed briefly (1). Among the existing immunological methods, we can distinguish two common confirmatory treponemal tests: the agglutination format such as the T. pallidum hemagglutination assay (TPHA) (2) and the microhemagglutination assay (MHA-Tp), and the immunostaining analysis by fluorescent treponemal antibody adsorption test (FTA-ABS) (3). Although the FTA-ABS is tedious to perform and time-consuming, it is considered to be the most sensitive and specific of all treponemal tests. The ELISA format has been shown to have an excellent sensitivity, comparable to that of the TPHA (3). Enzyme immunoassays can be used not only for the detection of anti Tp-IgG or anti Tp-IgM antibodies, but also for their simultaneous detection using double conjugates (4). Whereas both anti-Tp IgG and anti-Tp IgM tests

can be used for the detection of syphilis, anti-Tp IgM is considered a more important tool for the treatment of active syphilis (4). It was suggested that the Western immunoblot assay may become an alternative to the FTA-ABS assay for routine use in the clinical laboratory (5). Since even highly purified antigens from inoculated Tp may contain a certain amount of contaminating materials such as flagella of Tp, native Tp antigens may cause a nonspecific reaction in the assay of test serum samples, and this may result in lower sensitivity and poor reproducibility. To circumvent these potential problems in immunoassays, researchers have constructed Tp genes for the expression of recombinant antigens in bacterium systems such as *E. coli* and focused on Tp membrane proteins (6–8), which are definitely immunogenic. The major immunoreactive antigens of these membrane proteins have been reported to have a MW of 47, 42, 17, and 15KDa based on WB analy-

*Correspondence to: Masahisa Okada, Central Research Laboratories, Fujirebio, 51 Komiya-cho, Hachioji 192, Japan.

Received 30 January 1997; accepted 29 April 1997.

sis (8–10). The putative amino acid sequences of 47, 42, 17, and 15KDa have been demonstrated (11–17). Here, we describe several forms of expressed r-Tp antigens related to the membrane proteins mentioned above and the detection of anti-Tp antibodies by means of indirect ELISA, and compare the ELISA results with those of the conventional TPHA method.

MATERIALS AND METHODS

Monoclonal antibodies TAB15 to G15, TAB17 to G17, and TAB47 to M47 were prepared in mouse ascites following the conventional method (18) and purified from the ascites by affinity chromatography on Affigel protein A-agarose (Bio-Rad, Hercules, CA) through elution by 0.1 mol/L acetate buffer pH 4.2. Most of the chemicals used are listed below. All other chemicals used were of reagent-grade quality.

Preparation of r-Tp Antigens

We constructed Tp genes expressing three forms of recombinant proteins: mature antigens, antigens containing signal sequences, and antigens fused with GST at the NH₂ terminal of the mature antigen. The amino acid sequences of the antigens containing signal sequences were as follows (12–17):

S15(16):	Met----	Ala	Cys	-----Arg
	1		18	141
M15:		Met	Cys	-----Arg
		17	18	141
G15:	Met---- GST----	Leu	Cys	-----Arg
			18	141
S17(17):	Met----	Leu	Cys	Val-----Lys
	1		22	156
M17:		Met	Val	-----Lys
		22		156
G17:	Met---- GST----	Leu	Cys	Val-----Lys
			22	156
S42(14,15):	Met----	Cys	Ala	Ser-----//---Arg
			22	346
M47:	1		Met	Ser-----//---Arg
			23	346
G47:	Met---- GST----	Leu	Cys	Ala Ser-----//---Arg
			22	346
S47(12,13):	Val-----	Cys	Gly	-----//-----Gln
	1	20		434
M47:		Met	Gly	-----//-----Gln
		20		434
G47:	Met---- GST----	Leu	Cys	Gly -----//-----Gln
		20		434

In this study, the expressed form of the mature antigens was described as M15, M17, M42, M47, in which the NH₂ terminus residues were replaced by methionine (met). For fusion-type antigens, the expressed form was described as G15, G17, G42, and G47, in which GST has the 1–228aa sequence with methionine as amino terminus, and leucine as

carboxyl terminus (19). For antigens containing signal sequences, the expressed form was described as S15, S17, S42, and S47, which correspond to 15, 17, 42, and 47KDa, respectively, as depicted above. *Treponema pallidum* cells (Nichols strain) were extracted from a rabbit testis inoculated for Tp cells by sonication followed by centrifugation. Genomic Tp DNA was prepared from Tp cells by the conventional method described here briefly (20). Tp cells were incubated in the presence of SDS (0.5% final concentration) for 30 min at 37°C, and Proteinase K (final concentration 100 µg/ml, Boehringer Mannheim Biochemicals, Indianapolis, IN) was added, following which the mixture was incubated for 4 hr at 50°C. Finally, the DNA was purified by repeating phenol-chloroform extraction and ethanol precipitation (20). The DNA fragments of 15, 17, 42, 47KDa (11–17) were prepared by PCR, essentially the same method as described elsewhere (21), using genomic DNAs as templates and synthetic oligonucleotides as primers prepared and slightly modified based on the known DNA sequence by Model 381A DNA synthesizer (Applied Biosystems, Foster City, CA). The sequences CATATG, GAATTC, and GGATCC in the primers described below are the cleavage site of the restrictive enzymes NdeI, EcoRI, BamHI, respectively. The sense strand and antisense strand primers exemplified in S-form were:

for S15: 5' of bases to 5'GGTCATATGGTGAAA-AGAGGTTCGGTTCGC-3'
and 5'ACCGAATTCCTGCTAATAATGGCTTCCTT-3';
for S17: 5' of bases to 5'TCTCATATGAAAGGATCTG-TCCGCGCGCTGT3'
and 5'CAGGAATTCCTATTTCTTTGTTTTTTTGAGC-AC3';
for S42: 5' of bases 5'AACCATATGAATGCTCA-TACGCTTGTGTACTC3'
and 5'TACGAATTCTCATCGAGAGGCTCCTTCT-TCTTCGT3';
and for S47: 5' of bases to 5'GTACATATGAAAGTGAA-ATACGCACTACTTTC-3'
and 5'GGCCCCGGATCCTTAAGACACACGGGA-TAGGACAC-3'.

Thus prepared Tp DNAs were inserted into the expression vectors of PW6A or PWG6A. The PWG6A vector was constructed from the PGEMEX1 (Promega, Madison, WI) and the PGEX2T (Pharmacia, Uppsala, Sweden) vectors, which provided a T7 promoter and a regulatory unit of laqIq, respectively. The PW6A vector was prepared by deletion of the gene corresponding to the GST gene from the PWG6A vector. An expression vector constructed as GST fused to 17KDa is exemplified in Figure 1. The resulting vector was designated PWG6A-17, and the other vectors were designated similarly. The *E. coli* strain BL21(DE3) was transformed by each vector containing a Tp gene. The bacterial cells were grown in LB medium (DIFCO, NJ) for 3 hr and cultured for 4 hr in the presence of IPTG. The cells were then harvested and disrupted by sonication with the Ultrasonic Homogenizer Model

GST-17 Expression Plasmid, pWG6A-17

Fig. 1. Schematic gene construction for the expression of recombinant antigen GST-17(G17) expression plasmid. T7^P:T7 promoter, lac^O: lac operator, GST: glutathione s-transferase, 17K: TP 17KDa gene, Amp^r: ampicillin resistant gene, TT: transcription terminator.

US-150T (Nippon Seiki Co., Tokyo, Japan). The lysate suspension was centrifuged at 15,000 rpm with the Himac Centrifuge SCR20B (Hitachi Koki Co., Tokyo, Japan) for 20 min at 4°C. For G17 and G15, the supernatant was applied on DEAE-Sepharose (Whatman DE52, UK) ion exchange chromatography, and the adsorbed fraction was eluted by NaCl gradient; then the elution was applied on the Phenyl-Sepharose Fast Flow (Pharmacia) chromatography, and the protein fractions containing G17 or G15 antigens were eluted with decreasing NaCl gradient. For M47, the purification procedure was essentially the same as for G17, except that the Phenyl Sepharose CL4B was used instead of the Phenyl Sepharose FF. For S42, the supernatant was applied on the Phenyl Sepharose CL4B chromatography, and the adsorbed fraction was eluted by decreasing NaCl gradient and further purified by Q-Sepharose FF ion exchange chromatography.

Determination of Protein Concentrations

The concentration of purified proteins was measured (Micro BCA protein assay; Pierce Chemical, Rockford, IL). The yields of expressed protein in the culture were estimated using lysate suspension with SDS-PAGE analysis. Two volumes of lysate were dissolved in one volume of 5mM of Tris-buffer containing 1% SDS and 1% 2-mercaptoethanol, and boiled at 100°C for 1 min. Then, 10 µl of a mixture of a serial dilution with the same buffer as mentioned above was applied on the SDS-PAGE. The protein concentrations of this mixture were estimated by densitometric analysis (Densitograph AE-6900, ATTO K.K., Tokyo, Japan) of the intensity of the stained band of the gels with coomassie brilliant blue after destain-

ing using essentially the same procedure as described below. In parallel experiments, these protein concentrations were determined in comparison with the density of stained bands of specific amounts of bovine serum albumin (BSA: Oriental Yeast Co., Tokyo, Japan) by densitometric analysis on SDS-PAGE.

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE) (22) and Western Blotting (WB)

Purified antigens were analyzed by SDS-PAGE and WB (23,24) (Fig. 2a,b). For SDS-PAGE, r-Tp antigen samples were given 3% SDS and 2% 2-mercaptoethanol in 5 mM Tris buffer pH 7.4, boiled for 1 min and electrophoresed on 12.5% polyacrylamide gel slabs with discontinuous gradient buffer (22). Gels were calibrated with a standard (Bio-Rad Labs) containing a broad range of molecular weight markers described in the legend of Figure 2. Gels were stained for 30 min at room temperature by 0.2% coomassie brilliant blue R250 in 50% ethanol-10% acetic acid, and destained several times with 30% ethanol-10% acetic acid. For WB, the electrophoretic transfer of proteins from gels to nitrocellulose paper (pore size: 0.45 µm, Schleicher and Schuell, Germany) was performed essentially by the same method as Burnelle's (23,24). Human TPHA positive serum with a titer of 640 was used as primary antibody, and goat antihuman antibodies conjugated with peroxidase (Biosource International, Camarillo, CA) were used as secondary antibody. The nitrocellulose paper, which underwent incubation with human serum and subsequently with the secondary antibody, was washed with PBS

Fig. 2. Purified recombinant antigens expressed in *E. coli*. Panel **a**: SDS-PAGE of recombinant G17, G15, M47, and S42. Lane 1 represents molecular mass standards, lanes 2–5 represent G17, G15, M47, and S42 antigens, respectively. Molecular mass markers in kDa: Aprotinin (7.2kDa), lysozyme (20.4kDa), soybean trypsin (28.3kDa), carbonic anhydrase (34.8kDa), ovalbumin (49.5kDa), bovine serum albumin (79.5kDa), β -galactosidase (115kDa) and myosin (208kDa). All lanes were stained with coomassie brilliant blue. Panel **b**: Western blotting of recombinant G17, G15, M47, and S42 with human TPHA positive serum. Lanes 1–5 were the same as described in (a). Lane 1 was stained with coomassie brilliant blue, the others by WB.

buffer containing 0.05% Tween 20. A solution of 10 mM (2,2-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) ammonium salt) (ABTS) was applied to the paper, and an intense color of catalyzed ABTS with POD was detected. To prepare this solution, 100 mg ABTS were dissolved in 100 ml of 10 mM citric acid and 30 mM disodium phosphate buffer, and subsequently 12 μ l of 30% H₂O₂ were added (the final concentrations of ABTS and H₂O₂ were ~ 2 mM and 1 mM, respectively).

Indirect ELISA

For preparation of ELISA plate, the wells of a plastic microplate (Falcon 3912: Micro Test III Flexible assay plate, Becton Dickinson, San Jose, CA) were coated with G17, G15, M47, or S42 antigens at various concentrations in 10 mM PBS buffer for 2 hr at room temperature. Then, the wells were treated with a 10 mM PBS blocking solution containing 1% skim milk, pH 7.4, for 4 hr at ambient temperature. They were subsequently washed with PBS buffer containing 0.05% Tween 20 (Wako Co., Osaka, Japan) and stored at 4°C until use.

For the procedure for indirect ELISA, the ELISA was carried out by two-step sandwich format. Fifty μ l of test sera were placed in the wells of the plastic microplate and incubated for 1.5 hr at ambient temperature. Then, the wells were washed 3–4 times with 10 mM PBS buffer containing 0.05% Tween 20, and subsequently 50 μ l of POD-conjugate with goat anti-human IgG antibodies (TAGO Immunologicals™, Biosource International) were added and incubated 1.5 hr. After washing the wells with PBS buffer several times, 50 μ l of ABTS/H₂O₂ solution were added. After incubating the plate for 5 min, the reaction was stopped by adding 100 μ l of oxalic acid (Wako), and the absorbance of each well at 405 nm was measured with a plate reader (Titertech Multiskan Plus, Flow Laboratories, VA). Not otherwise stated, the sera used for this study was diluted to 1,000-fold with 10 mM PBS buffer containing 1% skim milk and 0.05% Tween 20, pH 7.4.

For comparison with TPHA, we performed the ELISA using 29 different human serum samples (Boston Biomedica, BBI, West Bridgewater, MA). The assay conditions were the same as for the indirect ELISA method mentioned above, whereby we chose to use 3 μ g/ml for G17, and 10 μ g/ml for G15, M47, and S42 as the coating concentrations of the wells. These concentrations gave near maximum reactivity on ELISA using control serum with a titer of 640 by TPHA assay. If the absorbance of the reaction system was higher than 2, the serum was further diluted to lower absorbance. The absorbance value corresponding to the 1/1,000 dilution was calculated. The mean absorbance values ($\pm$ 1SD) of 29 different healthy human donor sera that showed negative results on the TPHA were as follows: 0.074 ($\pm$ 0.033) for G17, 0.048 ($\pm$ 0.031) for G15, 0.046 ($\pm$ 0.037) for M47, and 0.061 ($\pm$ 0.066) for S42. The mean absorbance values plus 3SD were temporarily taken

as cutoff values. The cutoff values thus obtained were 0.171 for G17, 0.141 for G15, 0.156 for M47, and 0.259 for S42. In this study, the cutoff index (CI) was determined by dividing the absorbance of samples, as measured by ELISA, by the above cutoff values. Dilution tests of serum and monoclonal antibodies were performed using a concentration of 0.5 μ g/ml of M47, G15, and G17 for coating the plates. The mouse monoclonal antibodies against G15, G17 and M47 were prepared in house by the conventional method described above.

For the detection of IgM-class anti-Tp antibodies in human serum, we used goat antihuman IgM antibodies conjugated with POD (TAGO Immunologicals™) instead of POD conjugate with goat antihuman IgG antibodies. Otherwise, the assay procedures and the calculation of cut-off values as well as cutoff index were carried out under the same conditions as for IgG detection.

In this study, the data points indicate the mean value of two experiments.

TPHA Assay

The TPHA assay was performed using a commercial kit following the manufacturer's instructions (Fujirebio, Tokyo, Japan). Titers of 80 and above were judged as positive. For comparison with the CI of ELISA, we took the logarithmic values of TPHA titers; $\pm$ 80 titer was taken as 80 in this study. The ELISA-TPHA correlation coefficient was calculated by least-squares analysis between CI and logarithmic values.

Subjects

Twenty-nine adult sera that showed a positive reaction by rapid plasma reagent (RPR) test performed by the manufacturer (BBI, West Bridgewater, MA) were purchased. No information was available on the clinical condition of these sera. Twenty-nine healthy human adult donor serum samples were used as negative controls for the ELISA assay. The serologically positive serum used to optimize the conditions of the ELISA assay was prepared by pooling all sera from BBI, and the TPHA titer of the resulting serum was 640. Twelve known syphilitic adult sera samples previously tested for VDRL were obtained from Prof. J.K. Maniar (Grant Medical College, Bombay, India). All of the samples that were compared with the ELISA test were TPHA-positive.

RESULTS

Expression of Recombinant Antigens

A poor expression of r-Tp antigens was observed for M15, S15, M17, and M42 Tp genes in the *E. coli* system, judging from CBB staining of the gels on the SDS-PAGE and WB using the pooled TPHA positive human serum. Yet, a rather high expression occurred not only for S17, S42, and S47 genes containing a signal sequence, but also for the mature gene of M47. The highest expression was obtained for G15 and G17

genes in which mature genes were fused with GST genes. However, there was no significant difference in the degree of expression between M47 and G47, and between S42 and G42. The yield of the expressed protein for M47 was 70 mg/L of culture suspension; for S47, S42, S17, G42, and G47 it was ~40 mg/L; and for G17 and G15, it was 210 mg/L.

After expression, the r-Tp antigens were purified from the lysates of cells that were transformed by the expression vectors described in Materials and Methods. The purified G17, G15, S42, and M47 were analyzed by SDS-PAGE (Fig. 2a). The CBB-stained gels showed a single band for all four antigens with a molecular weight of ~44K, 43K, and 48.2K and 47.9KDa for G17, G15, M47, and S42, respectively, and their purity was found to exceed 95% by densitometric analysis (Fig. 2a). A trace band was observed only at the dimer position and was also reactive by WB, especially for G17 (17) and S42 despite the presence of 2-mercaptoethanol (Fig. 2b). All the recombinant antigens showed immunoreactivity with human TPHA positive serum by WB analysis (Fig. 2b).

Optimization for Indirect Microplate ELISA

We optimized the pH and the recombinant antigen concentrations to coat the solid phase for the indirect ELISA. Regardless of the antigen used for coating (such as G17, G15, M47, or S42), no significant change in absorbance was observed at pH 5–11 on the ELISA, whereas at below pH5, absorbance decreased for all antigens. Thus, pH 7.4 was chosen as the pH for antigen coating of the microplate. Figure 3 shows the reactivity of recombinant antigens M47, G17, G15, and S42 with anti-Tp antibodies in human serum. Absorbance increased gradually with increasing antigen concentrations and reached its maximum at ~5 µg/ml for all recombinant antigens (Fig. 3a). When comparing maximum intensity of absorbance, the value of G17 was 7–8 times higher than that of M47, S42, and G15. These results clearly indicate that sig-

nificantly higher immunoreactivity can be attained by using 17KDa fused with GST.

In regard to the negative controls, all four M47, G15, S42, and G17 antigens gave an absorbance value of < 0.05 at a concentration of up to 20 µg/ml of coating antigen using the TPHA negative serum (Fig. 3b). Under similar experimental conditions as for the ELISA of Figure 3, we compared the reactivity between S47 and M47, S42 and G42, as well as M17 and G17. S47 exhibited almost the same reactivity as M47, and a similar reactivity was also observed between S42 and G42 (data not shown). M17 achieved the same absorbance as G17 at a concentration of up to 2 µg/ml, i.e., at a level 10 times higher than G17, indicating that M17 is less reactive than G17 (data not shown). Basically, we believed that r-Tp antigens should be constructed as native antigens. Thus we chose to use r-Tp antigens in their mature form for M47 and also because M47 showed the same immunoreactivity as S47. G15 was chosen because it was the only antigen with sufficient expression. For 17KDa, there was a trace amount of expression of M-form and therefore we chose G17. Furthermore, G17 showed a higher immunoreactivity than M17.

As for S42 and G42, their expression level and immunoreactivity were almost equal, because the absorbance of the negative sera by G42-ELISA due to nonspecific reaction was higher than that by S42-ELISA, and therefore we opted to omit G42 in this study. We thus chose G15, G17, S42, and M47 recombinant proteins as the representative antigens for 15K, 17K, 42K, and 47KDa molecules, respectively, to evaluate human sera by ELISA. The evaluation of assay sensitivity is usually performed using a seroconversion panel. Here, we evaluated the detection sensitivity of ELISA using human TPHA positive sera by comparing the dilution factor of the serum (Fig. 4a). The highest dilution factors obtained at 0.5 absorbance were 4.1×10^{-4} for G17, 7.4×10^{-3} for G15, and 6.5×10^{-3} for M47. It was apparent that for M47 and G15 to have an absorbance equivalent to that of G17 antigen, 16–18 times more serum was needed. When using monoclonal antibodies against recombinant G17, G15 and M47 antigens, significantly strong reactivities with the corresponding antigens on the solid phase were obtained in all cases (Fig. 4b).

Fig. 3. Reactivity of recombinant M47, G17, G15, and S42 antigens with syphilis antibodies on ELISA. Panel (a) is for M47 (■), G17 (●), G15 (▲), and S42 (◆). The coating concentration of ELISA plates are given A 1:1,000 dilution of TPHA positive serum with a titer of 640 was used. Panel (b) was for the same as (a) using TPHA negative serum.

Evaluation of Human Sera Using Recombinant G17, G15, M47, and S42 on Indirect ELISA

Prior to evaluating the reactivity of recombinant antigens with individual serum samples on the ELISA, a TPHA assay was performed using 29 different human serum samples. All 29 samples tested were positive (Table 1) by TPHA. As negative control, 29 healthy donor sera samples were tested and showed no reactivity. Next, an ELISA was performed using G17, G15, M47, or S42 to coat the solid phase and sera diluted to 1:1,000 with PBS buffer (Table 1). For G17, the CIs were higher than for G15, M47, and S42 antigens in all se-

Fig. 4. Effect of serum dilution on the detection of syphilis antibodies by ELISA using recombinant G17, G15 and M47 antigens. Panel (a) represents the absorbance of human TPHA positive serum with a titer of 640 for G17 (●), G15 (▲), and M47 (■). Panel (b) represents the absorbance of monoclonal antibodies (MAb), TAB17 (●), TAB15 (▲), and TAB47 (■) for G17, G15 and M47, respectively.

rum samples tested, and none of the sera showed a CI of < 1.0. However, two samples (nos. 2, 3) for G15, five samples (nos. 1–4, 11) for M47, and three samples (nos. 1, 2, 22) for S42 had a CI of < 1.0. In comparison with a logarithm of TPHA titer, there was a rather good correlation of CI with a

coefficient of 0.7276 for G17 (Fig. 5), but for G15 ($r = 0.5320$), M47 ($r = 0.2664$), and S42 ($r = 0.3937$), the correlation was less significant. Table 2 shows the ELISA test results of known syphilitic status sera. In regard to CI, 10 sera with secondary syphilis and two asymptomatic sera showed essentially the same results as in Table 1. Thus the reactivity of G17 was higher than that of G15 and M47 in all the sera tested.

DISCUSSION

The purpose of this study was to evaluate the feasibility of using recombinant antigens instead of native antigens, which are usually prepared from inoculated rabbit testis for the syphilis antibody detection. Needless to say, avoiding the use of native antigens is good both for production and ethical reasons. We have demonstrated that the quantity of recombinant antigen expressed in the *E. coli* system was dependent on the construction of Tp genes in the vectors used. When using the PW6A vector that contains the 15KDa, 17KDa, or 42KDa Tp gene without signal sequence, namely, mature genes, only a small quantity of M15, M17, and M42 proteins was expressed, whereas a large quantity of M47 protein was expressed despite the absence of a signal sequence. Thus the mature gene is not the reason for the poor expression. Coinciding with our results for G15 and G17, a high expression of recombinant

TABLE 1. CI of Human Sera by ELISA Using Recombinant Antigens G17, G15, M47, and S42

Sample number	TPHA, titer	ELISA, CI			
		G17	G15	M47	S42
1	+/-80	1.7	1.2	0.3	0.1
2	80	2.8	0.2	0.6	0.1
3	80	5.6	0.5	0.4	1.1
4	160	4.3	2.2	0.9	1.8
5	160	4.9	1.0	3.9	1.2
6	320	16.6	4.1	5.0	2.9
7	320	16.3	3.1	2.9	1.2
8	320	34.0	23.6	16.6	17.1
9	320	29.3	2.1	1.1	1.0
10	320	32.2	9.7	6.3	7.0
11	320	35.6	2.4	0.8	1.0
12	640	20.3	4.0	3.1	2.4
13	640	26.1	22.6	10.6	11.5
14	640	27.5	5.0	1.7	1.5
15	640	35.5	5.9	4.3	3.2
16	640	32.0	12.1	15.7	12.0
17	1280	44.9	9.4	5.7	4.8
18	1280	42.6	41.4	28.4	22.2
19	1280	42.4	13.6	7.7	5.2
20	1280	44.2	27.2	10.4	18.7
21	1280	42.5	11.6	7.9	5.5
22	2560	26.4	4.3	1.9	0.6
23	2560	43.1	14.8	2.8	6.3
24	2560	40.0	11.0	1.5	7.2
25	5120	45.1	10.1	6.2	8.9
26	5120	41.4	19.3	7.3	7.5
27	5120	32.0	5.2	2.1	1.4
28	5120	43.4	25.6	20.6	11.7
29	5120	44.7	29.4	1.2	11.7

Fig. 5. Correlation between ELISA and TPHA. The relationship between CIs of G17-ELISA and the logarithm of TPHA titers was calculated by least-squares analysis, and the correlation coefficient is $\gamma = 0.7276$. Data points shown in Table 1 were replotted.

proteins fused to GST had already been observed (17). Based on the MW estimated by SDS-PAGE analysis (Fig. 2a), the size of combined GST and Tp proteins for recombinant antigens G17 and G15 seems reasonable. A similar observation was made for G17 with MW of 42KDa (17). The results of reactivity experiments based on dose response (Fig. 3) and, based on serum dilution (Fig. 4), indicate that G17 has a stronger reactivity with anti-Tp antibodies in human serum than M47, G15, and S42, thus suggesting that either the avidity or the amount of anti-17KDa antibodies in human serum was likely much greater than that of 47KDa, 15KDa and 42KDa.

We evaluated 29 individual adult human serum samples by TPHA assay; all samples tested positive by TPHA. On the ELISA, most of the samples tested positive when recombinant antigens were used, especially G17 whose CI values are higher than those of G15, M47, and S42 antigens. Whereas ELISA resulted in a different signal detection from TPHA,

which showed lattice formation, similar immunoreaction kinetics occurred on both TPHA and ELISA. Thus it would be reasonable to speculate that the reactivity of the recombinant antigen is either identical or superior to that of the native antigen. One might assume that antigens other than G17, G15, M47, and S42 might be needed for the complete detection of syphilis antibodies, but that is unlikely because of the consistency between CI and the titer of TPHA (Fig. 5).

On the TPHA system, both IgG- and IgM-classes of anti-Tp antibodies can be detected because of a homogeneous reaction condition based on particle agglutination. However, on the ELISA, only human IgG antibodies can be detected and, therefore, we used goat antihuman IgM antibodies conjugated with POD (described in Materials and Methods) on a limited number of serum samples. IgM antibody detection results were more or less consistent with those of IgG antibody detection for all three antigens (G17, M47, and G15) (data not shown).

From a practical point of view, although only a small number of samples with clinical status were tested by ELISA with r-Tp antigens, G17 showed the highest CI among G17, M47, and G15 (Table 2). We believe that G17 antigen, although it contains GST, was highly reactive to anti-Tp antibodies and was highly expressed by the recombinant technique, and thus was quite useful as an antigen for the detection of Tp antibodies in human sera by ELISA test. Although the reactivity of M47 or G15 was lower with Tp antibodies, we think that not only may these two proteins play an important role in the detection of primary syphilis (9), but they may also turn out to be highly reactive in other clinical stages. In any case, the recombinant antigens presented here may also be useful for other assay methods such as TPHA.

ACKNOWLEDGMENTS

We express our gratitude to Professor J.K. Maniar for providing us syphilitic patient sera, as well as to Ms. Yumi Fujita for her invaluable contribution regarding the TPHA assay, and to Mrs. Junko Tanno and Ms. Caroline Galan for their assistance in editing this work.

REFERENCES

1. Norgard MV: Clinical and diagnostic issues of acquired and congenital syphilis encompassed in the current syphilis epidemic. *Current Opin Infect Dis* 6:9–16, 1993.
2. Miller JN: Value and limitations of nontreponemal and treponemal tests in the laboratory diagnosis of syphilis. *Clin Obstet Gynecol* 18:191–203, 1975.
3. Lefevre J-C, Bertrand M-A, Bauriaud R: Evaluation of Captia enzyme immunoassays for detection of immunoglobulins G and M to *Treponema pallidum* in syphilis. *J Clin Microbiol* 28:1704–1707, 1990.
4. Farsy CE, Hunter EH, Larsen SA, Cerny EH: Double-conjugate enzyme-linked immunosorbent assay for immunoglobulins G and M against *Treponema pallidum*. *J Clin Microbiol* 20:1109–1113, 1984.
5. Byrne RE, Laska S, Bell M, Larson D, Phillips J, Todd J: Evaluation of *Treponema pallidum* western immunoblot assay as a confirmatory test for syphilis. *J Clin Microbiol* 30:115–122, 1992.

TABLE 2. CI of Human Syphilitic Sera by ELISA Using Recombinant Antigens G17, G15, and M47

Sample number	ELISA, CI			Clinical status ^a
	G17	G15	M47	
1	74.0	33.3	46.1	SS
2	4.7	0.9	2.1	SS
3	31.7	4.4	2.4	SS
4	52.4	3.2	6.1	AS
5	74.8	4.1	1.9	AS
6	5.6	0.4	5.4	SS
7	85.5	40.7	49.5	SS
8	7.1	2.0	1.2	SS
9	65.6	43.6	45.9	SS
10	76.0	23.9	50.1	SS
11	79.6	31.5	27.0	SS
12	83.2	31.4	39.4	SS

^aSS: secondary syphilis; AS: asymptomatic.

6. Radolf JD, Norgard MV, Schulz WW: Outer membrane ultrastructure explains the limited antigenicity of virulent *Treponema pallidum*. *Proc Natl Acad Sci* 86:2051–2055, 1989.
7. Cox DL, Chang P, McDowall AW, Radolf JD: The outer membrane, not a coat of host proteins, limits antigenicity of virulent *Treponema pallidum*. *Infect Immun* 60:1076–1083, 1992.
8. Radolf JD, Chamberlain NR, Clausell A, Norgard MV: Identification and localization of integral membrane proteins of virulent *Treponema pallidum* subsp. *pallidum* by phase partitioning with the nonionic detergent Triton x-114. *Infect Immun* 56:490–498, 1988.
9. Hanff PA, Fehniger TE, Miller JN, Lovett MA: Humoral immune response in human syphilis to polypeptides of *Treponema pallidum*. *J Immunol* 129:1287–1293, 1982.
10. Hensel U, Wellensiek H-J, Bhakidi S: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting as a serological tool in the diagnosis of syphilitic infections. *J Clin Microbiol* 21:82–87, 1985.
11. Norgard MV, Chamberlain NR, Swancutt MA, Goldberg MS: Cloning and expression of the major 47-kilodalton surface immunogen of *Treponema pallidum* in *Escherichia coli*. *Infect Immun* 54:500–506, 1986.
12. Hsu P-L, Chamberlain NR, Orth K, Moomaw CR, Zhang L-Q, Slaughter CA, et al.: Sequence analysis of the 47-kilodalton major integral membrane immunogen of *Treponema pallidum*. *Infect Immun* 57:196–203, 1989.
13. Weigel LM, Brandt ME, Norgard MV: Analysis of the N-terminal region of the 47-kilodalton integral membrane lipoprotein of *Treponema pallidum*. *Infect Immun* 60:1568–1576, 1992.
14. Hansen EB, Pederson PE, Schouls LM, Severin E, Embden JDA: Genetic characterization and partial sequence determination of a *Treponema pallidum* operon expressing two immunogenic membrane proteins in *Escherichia coli*. *J Bacteriol* 162:1227–1237, 1985.
15. Schouls LM, Ljsselmuiden OT, Weel J, Embden JDA: Overproduction and purification of *Treponema pallidum* recombinant-DNA-derived proteins TmpA and TmpB and their potential use in serodiagnosis of syphilis. *Infect Immun* 57:2612–2623, 1989.
16. Purcell BK, Swancutt MA, Radolf JD: Lipid modification of the 15-kilodalton major membrane immunogen of *Treponema pallidum*. *Mol Microbiol* 4:1371–1379, 1990.
17. Akins DR, Purcell BK, Mitra MM, Norgard MV, Radolf JD: Lipid modification of the 17-kilodalton membrane immunogen of *Treponema pallidum* determines macrophage activation as well as amphiphilicity. *Infect Immun* 61:1202–1210, 1993.
18. Kohler G, Milestein C: Continuous cultures of fused cells secreting antibodies of predefined specificity. *Nature* 256:495–497, 1975.
19. Smith DB, Johnson KS: Characterization of lipid-modified immunogenic proteins of *Treponema pallidum* expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67:31–40, 1988.
20. Champion CI, Miller JN, Lovett MA, Blanco DR: Cloning, sequencing and expression of two class B endoflagellar genes of *Treponema pallidum* subsp. *pallidum* encoding the 34.5- and 31.0-Kilodalton proteins. *Infect Immun* 58:1697–1704, 1990.
21. Frohman MA, Dush MK, Martin GR: Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc Natl Acad Sci USA* 85:8998–9002, 1988.
22. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970.
23. Burnette WN: Western blotting: Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analyt Biochem* 112:195–203, 1981.
24. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354, 1979.