SCRIPTA MEDICA (BRNO) – 74 (5): 353–360, *November 2001*

WESTERN BLOT DETERMINATION OF IgG AVIDITY IN PRIMARY AND SECONDARY SYPHILIS

WOZNICOVÁ V., VOTAVA M.

Department of Microbiology, Faculty of Medicine, Masaryk University, Brno

„He who knows syphilis, knows medicine.“
Sir Wiliam Osler

A b s t r a c t

In this study, we analysed IgG avidity to p 15.5 kD, p 17 kD and p 47 kD antigens by a new avidity assay termed avidity western blot. The determination was performed in two groups. Group I consisted of 27 patients with primary and secondary syphilis and group II comprised 10 subjects with latent syphilis. We found that low avidity antibodies were directed against the p 17 kD and p 47 kD antigens in group I and against the p 47 kD antigen in group II. No low-avidity IgG antibodies were detected by ELISA, a routine method for avidity assessment.

K e y w o r d s

Low-avidity IgG, Syphilis, Western blot

INTRODUCTION

During the early stage of any infection, low avidity antibodies are produced. Avidity, the term used for the binding strength of antibodies and antigens, depends on the variable ability of different B-lymphocyte clones to recognise an antigen. In the first weeks of infection, when the amount of antigen is relatively high, many B-lymphocyte clones participate in contacting antigens of the infectious agent. However, a relatively small number of them are able to bind the antigen strongly.

In the course of infection, the offer of an antigen relatively decreases and, in the competition for the antigen, only B-lymphocyte clones with high-affinity receptors win because they are able to bind the antigen more successfully than the other cells. This phenomenon leads to an increase in IgG avidity during the course of infection. This fact is widely used to diagnose the acute phase of many infections (e.g. toxoplasmosis, CMV infection, rubella, etc.) (*1, 2*).

The serological examination of IgG avidity is performed by modified ELISA or by immunofluorescence. In the routine ELISA method, treatment with 6M or 8M urea is used instead of a common washing solution. Each determination is carried out in the usual way and also with the use of urea in the first washing step.

Urea disrupts the binding of low-avidity IgG to the antigen and, therefore, if the sample contains low-avidity IgG, its optical density in the well with urea will be significantly lower (3).

Although changes in IgG avidity are a common phenomenon and avidity analysis provides a useful tool for staging infections generally, the usefulness of this method is limited. Surprisingly, no low avidity IgG antibodies have yet been detected in syphilis, while in the course of another spirochetal infection, Lyme borreliosis, low avidity IgG have been demonstrated (*4, 5*).

Primary syphilis involves chancre and regional lymphadenitis, secondary lues usually involves generalised rash, mucous patches and condylomata lata. These two stages last about 12 to 20 weeks and then syphilis becomes latent. During the first two years, the disease is called early syphilis (*6*).

In order to learn more about the avidity of antisyphilitic IgG in general and about IgG against separate treponemal proteins in particular, we designed an approach to characterise immunoglobulins G directed against the treponemal proteins p15.5 kD, p17 kD and p47 kD, which are considered to be the most specific antigens of *T. pallidum* (*7, 8, 9*). The procedure was carried out with the use of an avidity modification of western blot analysis (*10*).

MATERIALS AND METHODS

Samples

A total of 37 serum samples were divided into two groups. Group I consisted of 27 samples from the patients who suffered from primary or secondary syphilis and had a positive rapid reagin reaction (RRR) and IgM positivity in ELISA. Group II comprised 10 samples from subjects with latent syphilis. This group included 5 samples taken from cases with early latent and 5 from cases with late latent syphilis. None of them was either RRR or IgM positive.

The samples were collected from patients admitted to dermatovenerological wards of hospitals in Moravia or to the General Hospital of the Faculty of Medicine, Charles University in Prague.

ELISA method

A Captia SelectSyph-G (Trinity Biotech, Ireland) kit was used. Samples were diluted to achieve the cut off value. The test was performed in two modifications. In one, the manufacturer's protocol was followed, in the other, the standard solution in the first washing step was replaced by a treatment with 8M urea for 5 min that was repeated three times. The avidity index (AI), expressed as a percentage, was calculated as the ratio of the value obtained in the well with urea to that in the well without it.

Western blot procedure

Western blot strips manufactured by MarDx (Carlsbad, USA) were used. Two procedures were applied to each serum sample. One blot was performed according to the manufacturer's instructions, the other was modified in that the standard solution in the first washing step was replaced with 8M urea. Each strip was carefully washed three times for 5 min on a rocking shaker. The position and intensity of 15.5 kD, 17 kD and 47 kD bands were determined, as described below.

Western blot band position and intensity analysis

The position of the developed band was determined by comparison with the positive control evaluation pattern, which is the part of each set. The western blots were scanned and saved as *.tif

Mean: 100.47
Std Dev: 15.42
Median: 98
Pixels: 6600
Level:
Count:
Percentile:

Fig. 1:
Example of a histogram generated for p17 kD in a patient with primary syphilis. The western blot analysis did not involve urea treatment.

Mean: 193.91
Std Dev: 19.88
Median: 190
Pixels: 6600
Level:
Count:
Percentile:

Fig. 2:
Example of a histogram generated for p17 kD in the same patient with primary syphilis. The western blot analysis involved urea treatment.

files. The intensity of bands was evaluated using the Adobe Photoshop 3.0 software (Adobe Systems Incorporated) by which histograms of pixels at the sites of bands were generated *(Figs 1, 2)*. The intensity of bands with and without urea treatment, expressed as the position of medians on a 255-point scale (0, the darkest; 255, the lightest) was compared. The statistical significance of differences for each antigen was tested by the Wilcoxon test (Statistica for Windows, Statesoft, Inc.).

RESULTS

Serological findings in both tested groups are summarised in *Table 1*. Out of 27 group I patients, 24 (almost 89 %) were IgM positive; of three IgM negative patients, two were RRR positive and one patient remained negative in both tests. This patient was our earliest case because he was diagnosed at 10 days of the history of chancre; he had positive values only in the western blot test.

As for the IgG avidity, none of the samples was found to contain low-avidity antibodies according to the generally accepted criterion, i.e., avidity index value less than 30 % (*Table 2).*

Statistically significant changes after 8M urea treatment were revealed in two of three bands in group I and in one band in group II *(Table 3).* In group II, the finding in the 47 kD band was only borderline ($P=0.043$) while, in group I, the findings in 17 kD and 47 kD bands were highly significant ($P<0.001$).

DISCUSSION

Avidity analysis is an important part of the diagnostic procedure in toxoplasmosis and other infections which carry a risk of congenital transmission (2). Avidity analysis provides a useful tool in staging these infections and in predicting potential foetal damage. The staging in syphilis is not a diagnostic problem because, in pregnant patients, the risk for the foetus is always taken into consideration. This fact may explain the scarcity of information on antisyphilitic IgG avidity in the relevant literature.

The routinely used serological techniques have not been efficient enough to reveal low-avidity IgG antibodies in syphilis. This is because they usually enable us to determine only the total binding strength of a serum to the complex natural antigen (in syphilis this is Nichols' strain of *T. pallidum*) or to a mixture of recombinant antigens. In order to learn more about avidity to individual proteins, the ELISA technique has recently been modified to be used in western blot analysis.

The lack of evidence about the production of low-avidity IgG during syphilis is related to the fact that, so far, ELISA has been the method of choice, although it is not suitable for this purpose. Our results support this opinion, because all our samples tested by ELISA were characterised by an avidity index higher than 50 %, even though 11 samples were taken from patients who had primary syphilitic chancre. It means that their infections did not last more than 90 days (ulcus durum usually appears within 3 weeks of sexual intercourse). In other infections, 90-days is a period during which low-avidity IgG antibodies are detected.

Table 1
Serological findings in the tested groups

Stage	Number of samples	Titre of anticardiolipin antibodies	Number of IgM positive samples
Primary or secondary syphilis	27	1:2 – 1:64	24 (89 %)
Latent syphilis	10	< 1:2	0

Table 2
Range of avidity indexes determined by ELISA in various stages of syphilis

Stage	Number of samples	Range of AI* (%)
Primary or secondary syphilis	27	57 – 98
Latent syphilis	10	84 – 100

* AI, avidity index determined by ELISA

Table 3
Statistical significance of changes in 15.5 kD, 17 kD and 47 kD bands after treatment with urea

Stage	Significant changes in bands		
	15.5 kD	17 kD	47 kD
Primary or secondary syphilis	No	Yes	Yes
Latent syphilis	No	No	Yes

Differences were considered significant when $P < 0.05$

The probable reason for this finding is that the syphilis spirochete outer membrane has special features. *T. pallidum* contains a relatively small number of integral membrane proteins (100-fold less than the outer membranes of typical gram-negative bacteria), a fact that may permit the organism to evade the human immune response. This bacterium also has a high content of plasma membrane-associated lipoproteins which are not membrane-spanning but membrane-anchored (*11, 12*). The outer membrane proteins react poorly with syphilitic sera, as determined by conventional immunoblotting (*13*).

We assume that the low avidity of these highly specific proteins can not be demonstrated by ELISA because its „antibody background“, which involves reactions to a wide range of antigens (both genus specific and unspecific proteins), blurs the response of low-avidity antibodies to highly specific treponemal antigens. Our findings of low-avidity IgG antibodies, made by western blot analysis directed against highly specific proteins, confirm this statement.

Interestingly, both group I (primary and secondary syphilis) and group II (latent disease) had no low-avidity antibodies to the protein 15.5 kD but showed low-avidity IgG against the p 47 kD. Although the p 15.5 kD has been thought to be important for protective immunity, some new findings do not support this role. (14) The tested groups differed mainly in IgG avidity directed against the p 17 kD.

The application of avidity assays to the diagnosis of syphilis and the analysis of band intensity by western blot (15) together with the use of exact statistical evaluation should be the objectives of further clinical and laboratory research.

If we take Sir William Osler's statement for granted, we should admit that what we know about medicine is still not enough.

Acknowledgement

We thank Marcela Dražďáková, M.D., for her kind support and for providing many important serum samples.

Woznicová V., Votava M.

WESTERNBLOT PŘI STANOVENÍ AVIDITY IgG U PRIMÁRNÍ A SEKUNDÁRNÍ LUES

Souhrn

Analyzovali jsme aviditu IgG vůči treponemovým proteinům 15.5 kD, 17 kD a 47 kD novou metodou – aviditním westernblotem. Stanovení bylo provedeno ve dvou skupinách pacientů. První tvořilo 27 pacientů s primární a sekundární lues, druhá obsahovala 10 osob s lues latens. Zjistili jsme, že v první skupině byly nízkoavidní protilátky namířeny proti p 17 kD a p 47 kD a ve druhé vůči p 47 kD. Obvyklou metodou stanovení avidity IgG, ELISA, nebyly nízkoavidní protilátky zachyceny.

REFERENCES

1. *Bodeus M, Feyder S, Goubau P*. Avidity of IgG antibodies distinguishes primary from non-primary cytomegalovirus infection in pregnant women. Clin Diagn Virol 1998; 9: 9–16.
2. *Cozon GJ, Ferrandiz J, Nebhi H, Wallon M, Peyron F***.** Estimation of the avidity of immunoglobulin G for routine diagnosis of chronic *Toxoplasma gondii* infection in pregnant women. Eur J Clin Microbiol Infect Dis 1998; 17: 32–36.
3. *Hedman K, Lappalainen M, Seppaia I, Makela O*. Recent primary toxoplasma infection indicated by a low avidity of specific IgG. J Infect Dis 1989; 159: 736–740.
4. *Borkhardt HL, Zielinski S*. Influence of cardiolipin antibodies on the binding of treponemal specific antibodies in the fluorescence treponemal antibody absorption test and the *Treponema pallidum* immobilisation test. J Med Microbiol 1997; 46 :965–972.
5. *Guerineau AL, Dhote R, Christiann F, Rayet P, Assous MV.* Differentiation between early and late complicated Lyme borreliosis by specific IgG avidity. Lancet 1999; 354: 1096–1097.
6. *Young H*. Syphilis: New diagnostic directions. Int J STD 1992; 3: 391–413.
7. *Gerber A, Krell S, Morenz J*. Recombinant *Treponema pallidum* antigens in syphilis serology. Immunobiol 1997; 196: 535–549.
8. *Weigel LM, Brandt ME, Norgard MV*. Analysis of the N-terminal region of the 47-kilodalton integral membrane lipoprotein of *Treponema pallidum*. Infect Immun 1992; 60: 1568–1556.
9. *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 1992; 60: 1076–1083.
10. *Marcolino PT, Silva DAO, Leser PG, Camargo ME, Mineo JR*. Molecular markers in acute and chronic phases of human toxoplasmosis: Determination of immunoglobulin G avidity by western blotting. Clin Diagn Lab Immunol 2000; 7: 384–389.
11. *Fraser CM, Norris CJ, Weinstock GM et al*. Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. Science 1998; 281: 375–388.
12. *Blanco DR, Miller JN, Lovett MA*. Surface antigens of the syphilis spirochete and their potential as virulence determinants. Emerg Infect Dis 1997; 3: 11–20.
13. *Radolf JD, Robinson EJ, Bourell KW et al.* Characterization of outer membranes isolated from *Treponema pallidum*, the syphilis spirochete. Infect Immun 1995; 63: 4244–4252.
14. *Centurion-Lara A, Arrol T, Castillo R et al*. Conservation of the 15-kilodalton lipoprotein among *Treponema pallidum* subspecies and strains and other pathogenic treponemes: genetic and antigenic analyses. Infect Immun 1997; 65: 1440–1444.
15. *Kowal K, Weinstein A***.** Western blot band intensity analysis. Application to the diagnosis of Lyme arthritis. Arthritis Rheum 1994; 37: 1206–1211.