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The diagnosis of toxoplasmosis using IgG avidity

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SUMMARY

Current methods to establish the duration of toxoplasma infection in pregnant women and for the diagnosis of toxoplasmosis in the neonate or HIV infected patient have significant limitations. We assessed the precision of a commercial ELISA for the detection of toxoplasma specific IgG and adapted the assay to measure avidity using an elution agent washing step. The sensitivity and specificity of the ELISA were 100 and 75% respectively and optimal measurement of avidity was achieved using 6 M urea as the elution agent.

Toxoplasma lymphadenopathy of less than 3 months duration was associated with low avidity specific IgG but some discordant findings were recorded. Serial measurement of IgG avidity assisted the distinction between actively produced antibody in infants with congenital toxoplasmosis and passively acquired antibody of maternal origin in uninfected babies. There was no significant difference between avidity levels in HIV infected patients with or without cerebral toxoplasmosis.

INTRODUCTION

Toxoplasma infection of immunocompetent individuals is usually asymptomatic or associated with a mild, self-limiting illness with or without lymphadenopathy. Severe, life-threatening disease can follow toxoplasma infection in the immunocompromised including the foetus or HIV infected person [1]. Congenital toxoplasmosis may result when maternal infection is acquired during the pregnancy although rare cases associated with pre-conception infection have been reported in both immunocompetent [2] and immunocompromised women [3]. The risk of maternal–foetal transmission of infection is dependent on the gestational age at the time of maternal exposure to the parasite. Consequently, accurate dating of the duration of maternal toxoplasmosis is required in order to assess the risk of subsequent congenital infection. Unfortunately most maternal infections are not recognized due to lack of symptoms or the non-specific nature of the illness. The presence of detectable IgM or IgA in the mother's blood is associated with recently acquired infection but the persistence of production of these immunoglobulins is variable and can be prolonged. Levels of specific IgG rise quickly after infection is established, reaching a peak within 2 months followed by a slow decline over many years [4].

Investigation of suspected congenital toxoplasmosis in the post-natal period is equally problematic. Even the most sensitive of assays will not detect specific IgM

in all cases so that serial assessment of toxoplasma specific IgG during the first year of life is required to establish the status of the child [5]. Cerebral toxoplasmosis associated with HIV infection most often follows reactivation of the patient's chronic, previously quiescent infection. Such reactivations are not associated with quantitative changes in specific IgG production nor the detection of specific IgM and the diagnosis may not be confirmed until a trial of therapy is undertaken [6].

Due to these limitations, improved serological assays are required for the investigation of toxoplasma infection. It has been shown that the strength of the bond between antibody and individual epitopes increases with the duration of infection [7]. Measurement of the avidity of the antibody/antigen interaction has been applied to viral infections including rubella [8] and Puumala virus [9]. Recently avidity maturation has been demonstrated in patients infected with toxoplasma [10, 11]. As commercial IgG assays are increasingly used for the investigation of suspected toxoplasma infection we elected to study the adaption of one such assay, the Enzygnost IgG ELISA (Hoechst–Behring) for the measurement of antibody avidity. The clinical application of antibody avidity measurement to the dating of toxoplasma infection, investigation of suspected congenital infection in the neonate and diagnosis of reactivated disease in association with AIDS was assessed.

METHODS

Toxoplasma specific IgG ELISA (Enzygnost–Behring)

In this assay, specific IgG in test sera binds to toxoplasma antigen fixed to a solid phase and is detected by rabbit anti-human γ chain FAB fragment conjugated with peroxidase and a chromogenic substrate. The assay was performed according to the manufacturer's instructions. Briefly 10 μl of each test serum, together with negative and positive controls, were added to 200 μl of sample buffer, mixed and then 20 μl transferred to a 200 μl volume of peroxidase buffer in a microtitre well pre-coated with toxoplasma antigen. The plate was covered with foil and incubated in a moist atmosphere for 60 min at 37 °C. Each well was washed three times with phosphate buffer containing Tween 20 and excess fluid drained. A 100 μl volume of anti-human IgG conjugate was added to each well, which was then re-sealed and incubated for a further 60 min at 37 °C. After further washing 100 μl of chromogen substrate was dispensed into each well and incubated for 30 min at room temperature. The reaction was stopped by adding 100 μl of 0·5 M sulphuric acid and the absorbance recorded at 450 nm. The mean absorbance value of the negative controls ($\bar{A}$ neg) was calculated. Positive results were defined as samples with an absorbance value above $\bar{A}$ neg + 0·025 A and the quantitative findings were calculated by comparison with the control positive serum supplied in the kit.

Evaluation of the IgG ELISA

IgG ELISA findings were compared to results of the dye test and latex agglutination test [12]. The specificity of the ELISA was assessed by testing 88 sera without toxoplasma specific antibody (dye test < 4 iu, latex agglutination

test titre < 16). A further 2 jaundiced, 2 lipaemic and 6 haemolysed toxoplasma IgG negative sera were processed as well as 3 sera containing anti-nuclear factor, 5 with cytomegalovirus specific IgG and 3 with rheumatoid factor. Five dye test negative sera were exposed to excess heat inactivation at 56 °C for 3 h before being tested by the IgG ELISA. Ten samples previously found to produce false positive latex agglutination results (dye test < 4 iu, latex agglutination test titre $\geqslant 64$) were assessed. The sensitivity of the ELISA was investigated by testing 270 dye test positive ($\geqslant 4$ iu) sera and the correlation between quantitative results of the two assays was studied by testing 50 sera found to contain 4–8000 iu. Potential false negative reactions were evaluated by freeze thawing 5 dye test positive sera 5 times and then testing using the ELISA. Reproducibility of the IgG ELISA was defined by testing 8 sera representing a range of dye test values (< 4 iu–8000 iu) on 4 separate occasions and calculation of the coefficient of variation.

Adaptation of the IgG ELISA for the measurement of antibody avidity

Toxoplasma specific avidity was measured using the 'bind and break' method and the calculation of an avidity index [8]. Various concentrations of the hydrogen-bond disrupting agent, urea and diethylamine, were used to elute the IgG from the immobilized antigen. Duplicate microtitre plates were washed in 2 M, 4 M, 6 M or 8 M urea solution and a further plate was washed using 0·035 M diethylamine solution. A 250 μl volume of the elution agent was added to each respective well following the incubation of test sera in antigen-coated microtitre trays. After 5 min exposure to the elution agent at room temperature, the plates were washed and processed as described previously. Absorbance readings from plates washed in elution agent (EA) or phosphate buffer (PB) were used to calculate an avidity index (AI) where

$$\text{AI} = \frac{\text{absorbance after EA wash}}{\text{absorbance after PB wash}} \times 100\,\%$$

Discrimination between low and high avidity antibody was evaluated by testing 11 acute sera from patients known to have toxoplasma associated lymphadenopathy for 3 months or less and 25 sera from patients shown to possess toxoplasma specific IgG for at least 12 months by repeated serotesting. All 36 sera were tested using each concentration of urea or DEA in the wash solution.

Clinical application of antibody avidity measurement

The relationship between antibody avidity and time since the acquisition of infection was assessed by testing 29 sera from patients with lymphadenopathy of known duration and toxoplasma specific IgM detected. Toxoplasma specific IgM was measured using a μ-chain capture ELISA [13] and the immunosorbent agglutination assay ISAGA [14].

A total of 5 babies with congenital toxoplasmosis (confirmed by the persistence of specific antibody at 12 months of age) and 5 babies shown not to be infected were studied. All the babies were delivered to mothers with toxoplasma specific IgG and IgM detected during the pregnancy. Two or more samples of sera obtained from each child were examined.

The role of antibody avidity measurement in the diagnosis of cerebral

toxoplasmosis was assessed by testing 12 patients with AIDS and active toxoplasma infection and 12 patient with AIDS and quiescent toxoplasma infection. Cerebral toxoplasmosis was diagnosed on the basis of a compatible clinical and radiological presentation and subsequent response to specific therapy [6].

RESULTS

IgG ELISA

The results of assessing 88 dye test/latex agglutination test negative and 270 positive serum samples using the IgG ELISA are presented in Table 1. The sensitivity of the IgG ELISA was 100% and there were no false negative reactions following repeated freeze thaw cycles. The specificity of the IgG ELISA was 76%, positive predictive value 93% and negative predictive value 100%. False positive ELISA reactions were not associated with excessive heat inactivation, false positive latex agglutination reactions nor the physical appearance of the sample. Non-specific IgG ELISA reactions were found to be associated with alternative immunoglobulin factors as 1 of 3 samples with anti-nuclear factor, 3 of 5 with cytomegalovirus specific IgG and 1 of 3 containing rheumatoid factor produced false IgG ELISA results. A comparison of quantitative IgG ELISA and dye test findings is shown in Fig. 1 as a result difference/mean plot to display relative agreement. The mean difference (d) and standard deviation of the differences (s) for results of dye test and IgG ELISA were calculated. The mean difference indicates the bias between the two tests and was found to be $-0{\cdot}16$. Hence IgG ELISA gave results, on average, slightly greater than the dye test. The standard deviation of the differences was found to be 2·5 indicating the variability of results between the dye test and IgG ELISA. The limits of agreement ($d \pm 2S$) were $-5{\cdot}16$; $+4{\cdot}84$. The coefficient of variation of the IgG ELISA was found to be 3·4%.

IgG avidity measurement

When 2 M or 4 M urea was used as an elution agent there was no clear distinction between the avidity indices calculated for sera from patients acutely infected with toxoplasma and those with chronic infection. The use of 8 M urea or diethylamine as an elution agent resulted in partial distinction between acutely infected sera (low avidity index) and chronic infected sera (high avidity index). However, there was considerable overlap between the groups. The clearest separation of the avidity index between acute and chronic toxoplasmosis was found when using 6 M urea as the elution agent (Fig. 2). All 11 sera from acutely infected patients were associated with an avidity index $< 45\%$ while 24 of 25 sera from patients with chronic toxoplasmosis were found to have an avidity index $> 50\%$ (Fig. 3). On the basis of these findings 6 M urea was adopted as the elution agent in all further investigations.

The 29 sera taken from patients with lymphadenopathy of known duration had all had toxoplasma specific IgM detected by the ISAGA and 24 tested positive using the ELISA. All samples taken from patients with lymphadenopathy present for 3 months or more were found to contain toxoplasma specific IgG of high avidity ($AI > 50\%$). Of 14 samples taken from patients with lymphadenopathy for less than 3 months, 8 had toxoplasma specific IgG of low avidity ($AI < 45\%$)

Table 1. *Quantitative comparison of IgG ELISA with dye test and latex agglutination findings*

IgG ELISA	Dye test/ agglutination test: Positive	Negative
Positive	270	21
Negative	0	67

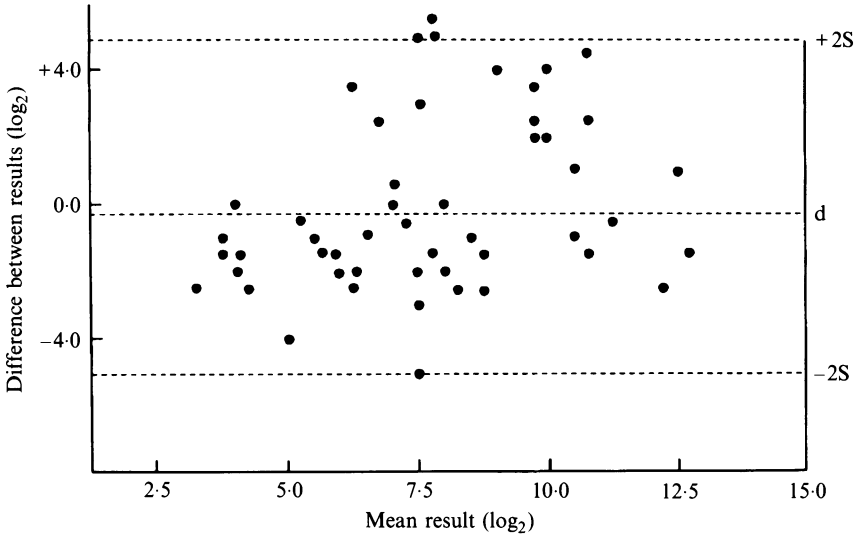

Fig. 1. Graph showing correlation between dye test and IgG ELISA findings. The mean result for each specimen was calculated by addition of the quantitative dye test and IgG ELISA results (each expressed as a logarithm to the base 2) and division by a factor of two. The difference between results for each specimen was found by subtracting the IgG ELISA result ($\log_2$) from the dye test result ($\log_2$). Quantitative dye test and IgG ELISA results were recorded in international units.

and 6 had high avidity (AI > 50%). A comparison of duration of lymphadenopathy with avidity of toxoplasma specific IgG is given in Fig. 4. Combining the findings from all patients with lymphadenopathy of known duration and chronic infection defined by long-standing dye test positivity, the precision of the IgG avidity assay for the determination of duration of infection was as follows: sensitivity 76%; specificity 96%; positive predictive value 95%; negative predictive value 80%.

Results of serial IgG avidity measurement in congenitally infected and uninfected infants are given in Table 2. The avidity of toxoplasma specific IgG detected in uninfected infants did not show significant change with increasing age in any of the 5 patients studied (cases 6–10). Conversely 2 of the 5 infants with congenital infection (cases 1 and 4) showed a marked decrease in the avidity of the IgG during the first year of life. Two further infants (cases 2 and 3) were shown to have a less marked decline in avidity. The remaining congenitally infected child (case 5) was first investigated when aged 2 years and the IgG avidity showed a progressive increase over the following 6 months.

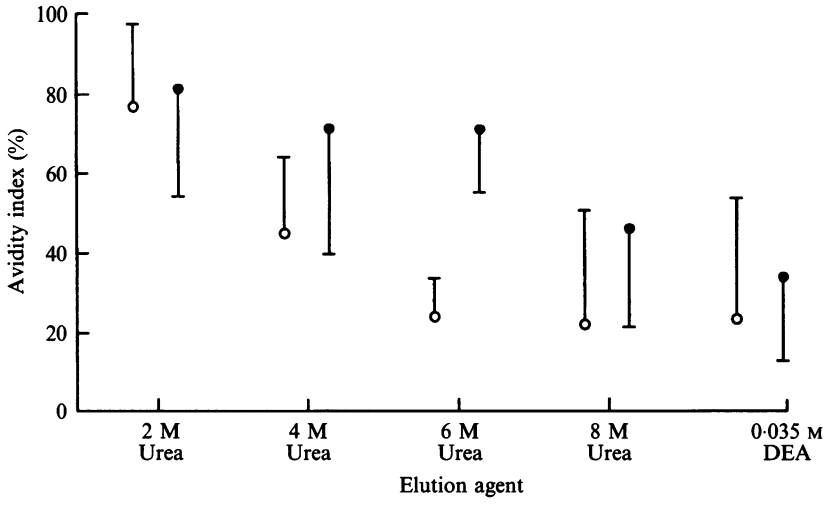

Fig. 2. Avidity index of toxoplasma specific IgG in acute and chronic infection: comparison of elution agents. ○, Mean AI in acute infection; ●, mean AI in chronic infection; ⊥, standard deviation.

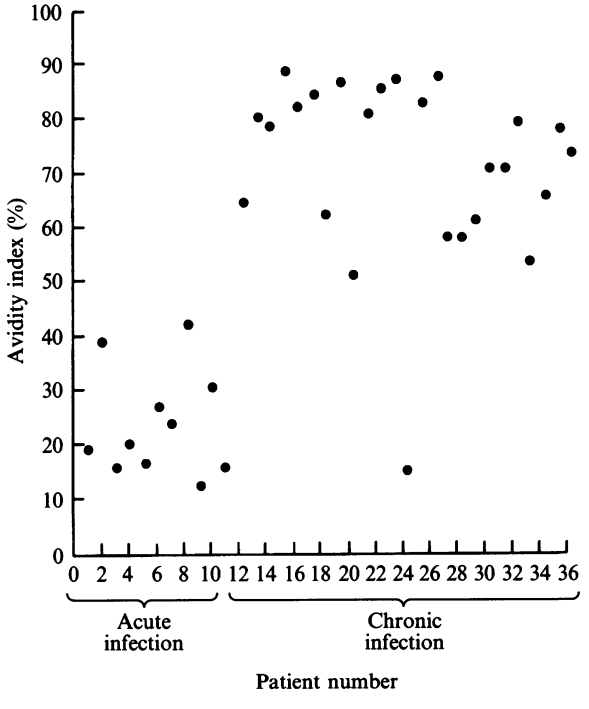

Fig. 3. Measurement of IgG avidity in acute and chronic toxoplasma infection using a 6 M elution agent.

All 12 patients with AIDS and cerebral toxoplasmosis were found to have toxoplasma specific IgG of high avidity (AI > 50%) as did all 12 patients with AIDS and quiescent toxoplasma infection. There was no significant difference in the quantitative avidity indices between the two groups.

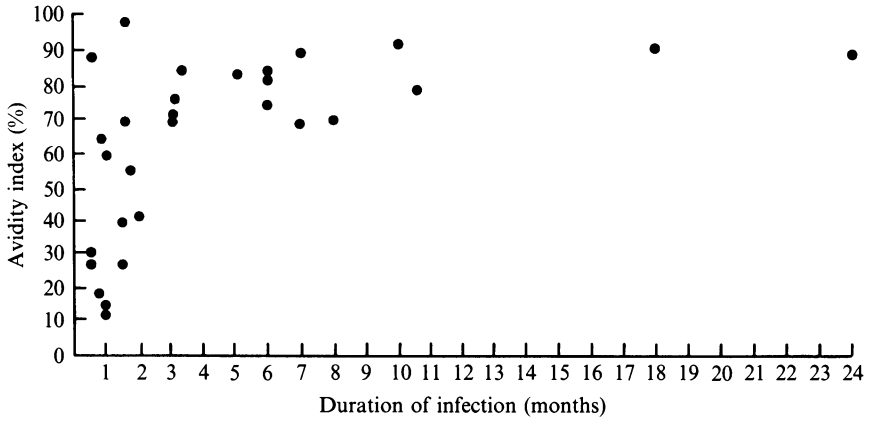

Fig. 4. Comparison of IgG avidity with duration of infection.

Table 2. *Serial IgG avidity measurement in children with congenital toxoplasmosis and uninfected infants*

Congenital toxoplasmosis			Uninfected		
Case	Age (months)	Avidity index (%)	Case	Age months	Avidity index (%)
1	0	63	6	0	53
	1	63		5	61
	5	58			
	7	42	7	0	74
	10	37		9	73
2	4	88	8	0	57
	9	69		3	69
3	1	85	9	0	91
	2	76		3	99
4	0	84	10	0	73
	3	20		3	65
5	24	15			
	26	42			
	30	91			

DISCUSSION

The increased numbers of serological tests for toxoplasmosis performed in routine laboratories has been accompanied by the adoption of ELISA techniques but few of these assays have been accurately assessed in comparison to reference tests. Our results show that IgG ELISA has reduced specificity but optimal sensitivity. Consequently this assay is suited for use as a screening assay as significant positive samples will not be overlooked and false positive reactions can be identified by additional tests. We compared findings from the dye test and IgG ELISA by plotting the mean result against result differences of each pair. This approach is less familiar than conventional comparison of direct titres and calculation of correlation coefficients but it has the advantage of producing additional information regarding the agreement of results and the nature of any differences [15]. Typically the IgG ELISA produces a higher result than the quantitative dye test. This difference reflects the different antigen profiles presented in each assay

for antibody detection. The standard deviation of the difference between dye test and IgG ELISA results is greater than that reported for the latex agglutination test or the direct agglutination test [12]. Consequently quantitative IgG ELISA results show greater variation from the reference assay than other commercial tests. This variation may be reduced by altering the antigen preparation incorporated in the ELISA.

Having established the precision of a commercial ELISA we were able to adapt this assay to measure IgG avidity and achieved optimal discrimination between high and low avidity using 6 M urea as an elution agent. Other workers have found 6 M urea to be a suitable elution agent when measuring avidity of toxoplasma specific IgG [10, 11, 16] although a higher concentration of urea [9] and diethylamine [7] has been used successfully with other pathogens. Antibody avidity can be measured by recording ELISA findings for a series of dilutions of a specimen of serum with and without the use of an elution agent in the performance of the assay. Two dilution curves are obtained for each serum sample and the difference in reading at 50% maximum absorbance value [11] or the serum dilutions at a pre-defined level of absorbance [10] can be used to calculate avidity as a function of the 'curve shift'. The advantage of the method used in the current study was that only a single dilution of the specimen was assayed with and without elution agent; the amount of reagents and technical time required to perform the test was reduced. A direct comparison between values obtained using 'curve shift' and single dilution avidity index measurement for toxoplasma specific IgG has not been performed. Low avidity antibody to rubella has been detected with greater sensitivity using a 'curve shift' method compared to an avidity index technique [17]. When the level of specific antibody is relatively low, avidity measurement by the 'curve shift' method may be preferred. However, the enhanced sensitivity of this approach results in prolonged detection of low avidity antibody. Consequently, for the identification of recent infection, measurement of antibody avidity by curve shift produces results of high sensitivity but lower specificity whereas the avidity index findings are highly specific but of lower sensitivity.

We have confirmed the findings of others showing acute and chronic toxoplasma infection can be separated by the presence of low and high avidity IgG respectively [10, 11, 16] and shown that the avidity of toxoplasma specific IgG increases for 3 months after infection before reaching a plateau. Although the maturation of toxoplasma specific IgG avidity has not been studied extensively a similar time course has been observed in one study of toxoplasmosis [18] and with other infectious disease [9]. Although most cases of toxoplasma infection were associated with the expected level of IgG avidity according to the duration of infection, discordant findings were recorded. Acute infection with high avidity and chronic infection with low avidity was documented. These findings may reflect in part the limitation of using lymphadenopathy as a marker of toxoplasma infection or the variation in the individual's immune response to infection. A low avidity result is a good indicator of infection of less than 3 months duration. However, a high avidity result is less reliable as an indicator of chronic infection. IgG avidity findings cannot be regarded as entirely reliable for dating the duration of toxoplasma infection and the results of this assay should be interpreted in parallel

with other tests such as measurement of specific IgG and IgM levels [19]. Prospective studies of the value of IgG avidity in predicting the outcome of maternal toxoplasmosis are required. Initial findings suggest IgG avidity measurement represents a precise method for the verification of acute maternal toxoplasmosis [18].

All children born to mothers with acute toxoplasmosis associated with the pregnancy will have detectable specific IgG due to passive transfer of antibody across the placenta. As the absence of IgM and the failure to isolate the parasite from the placenta or neonatal blood does not exclude congenital infection, serial investigations of the child are performed at 2-month intervals, measuring the level of toxoplasma specific IgG in the infant's blood [5]. In all cases the initial level of IgG falls as passively acquired antibody of maternal origin is removed from the circulation. If the child has not been infected, the decline of IgG continues until all antibody is lost. Conversely, if the child has congenital toxoplasmosis, the initial decline ceases and specific IgG levels become stable or rise during the first 12 months of life as the child's immune system reacts to the infection. Passively acquired antibody would be expected to have the avidity of the maternal IgG at delivery and to remain constant while the quantitative level of IgG falls. All the non-infected infants studied showed constant IgG avidity. Actively produced antibody will initially be of low avidity and show a progressive increase in the avidity index as the immune response matures. The congenitally infected child will have passively acquired maternal antibody of constant avidity in addition to actively produced antibody which shows a progressively increasing avidity index in early life. Two of the congenitally infected infants studied showed a significant reduction of avidity of IgG. This may reflect the removal of maternal IgG of relatively high avidity and a progressive replacement with actively produced low avidity antibody. Passively acquired antibody will usually be completely removed from the child's circulation by the age of 12 months. In one patient (case 5) with congenital infection the maturation of the child's own immune response may have resulted in measurement of specific IgG of increasing avidity. Our results suggest that serial measurement of IgG avidity may assist the discrimination between active production of antibody with congenital toxoplasmosis and passively acquired antibody of maternal origin associated with the uninfected child. Further studies involving a larger number of cases and controls are required to confirm these proposals but similar patterns of IgG avidity maturation have been observed in infants with and without congenital HIV infection [20].

The majority of cases of cerebral toxoplasmosis with AIDS in the UK are associated with secondary reactivation of the patient's chronic, previously quiescent toxoplasma infection [6]. Our findings indicate that maturation of specific IgG results in the production of high avidity antibody and that reactivation of infection in cerebral toxoplasmosis does not affect this process. In the minority of cases a cerebral toxoplasmosis resulting from primary infection, low avidity IgG is detected, reflecting the early immune response to the parasite.

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